Universidade Nova de Lisboa

Instituto de Higiene e Medicina Tropical

Canine leishmaniasis: the role of hepatocytes and Kupffer cells during *Leishmania infantum* infection

Maria Armanda Viana Rodrigues

DISSERTATION IN SUPPORT OF A CANDIDATURE FOR A DOCTORATE DEGREE IN BIOMEDICAL SCIENCES, SPECIALIZATION IN CELLULAR AND MOLECULAR BIOLOGY, BY UNIVERSIDADE NOVA DE LISBOA, INSTITUTO DE HIGIENE E MEDICINA TROPICAL.

(MARCH, 2016)

Universidade Nova de Lisboa

Instituto de Higiene e Medicina Tropical

Canine leishmaniasis: the role of hepatocytes and Kupffer cells during *Leishmania infantum* infection

Author: Maria Armanda Viana Rodrigues
Degree in Cellular Biology and Biotechnology (FCUL)
Master in Cellular Biology and Biotechnology (FCUL)

Supervisor: Prof. Dr. Gabriela Santos-Gomes (IHMT/UNL)

Co-supervisors: Prof. Dr. Isabel Pereira da Fonseca (FMV/ULisboa)

Prof. Dr. Joana Miranda (FF/ULisboa)

Dissertation in support of a candidature for a Doctorate degree in Biomedical Sciences, specialization in Cellular and Molecular Biology, by Universidade Nova de Lisboa, Instituto de Higiene e Medicina Tropical, under supervision of Prof. Dr. Gabriela Santos-Gomes.

Financial support by PhD scholarship SFRH/BD/73386/2010 and projects PTDC/CVT/113121/2009 and PTDC/CVT/118566/2010, funded by the Portuguese Foundation for Science and Technology (FCT) with co-participation of the European Union Fund (FEDER).

Communications and publications performed in dissertation context

Papers in preparation

Rodrigues A, Santos-Mateus D, Alexandre-Pires G, Valério-Bolas A, Rafael-Fernandes M, Alves-Azevedo R, Ligeiro D, Pereira M, Nunes T, Pereira da Fonseca I, Santos-Gomes G. Canine hepatocytes activate innate immune receptors against *Leishmania infantum.*

Rodrigues A, Alexandre-Pires G, Santos-Mateus D, Ligeiro D, Valério-Bolas A, Martins C, Rafael-Fernandes M, Pereira M, Pereira da Fonseca I, Santos-Gomes G. Innate immune receptors of canine Kupffer cells sense *Leishmania infantum* differently from blood monocytes.

Rodrigues A, Santos-Mateus D, Valério-Bolas A, Claro M, Martins C, Alexandre-Pires G, Santos-Gomes G. Analysis of cell memory in liver of mice infected with *Leishmania infantum.*

National scientific meetings

Poster presentation

Rodrigues A, Alexandre-Pires G, Santos-Mateus D, Aires-Pereira M, Valério-Bolas A, Rafael-Fernandes M, Silva-Pedrosa R, Pereira da Fonseca I, Santos-Gomes G. A exposição a *Leishmania infantum* causa alterações metabólicas nos hepatócitos caninos. V Jornadas Científicas do Instituto de Higiene e Medicina Tropical. Lisbon, Portugal, 12th December 2014.

Claro M, Rodrigues A, Santos-Mateus D, Valério-Bolas A, Martins C, Alexandre-Pires G, Santos-Gomes G. Analysis of cell memory in liver of mice infected with *Leishmania infantum*. Fundação Astrazeneca Innovate Competition, iMed 5.0 Conference. Rectory of Universidade NOVA de Lisboa, 12-13 October 2013.

Rodrigues A, Santos-Mateus D, Valério-Bolas A, Pereira M, Alexandre-Pires G, Pereira-da-Fonseca I, Santos-Gomes G. Isolation of primary dog hepatocytes. III Jornadas Científicas do Instituto de Higiene e Medicina Tropical, Lisbon 14th December 2012.

Oral presentation

Rodrigues A, Alexandre-Pires G, Santos-Mateus D, Pereira MA, Valério-Bolas A, Rafael-Fernandes M, Marques C, Pereira da Fonseca I, Santos-Gomes G. Impacto na função metabólica e na resposta imunitária de hepatócitos de cão expostos a *Leishmania infantum.* IV Jornadas Cientificas do Instituto de Higiene e Medicina Tropical, Lisbon 11th December 2013.

International scientific meetings

Poster presentation

Rodrigues A, Alexandre-Pires G, Santos-Mateus D, Ligeiro D, Valério-Bolas A, Rafael-Fernandes M, Pereira M, Pereira da Fonseca I, Santos-Gomes G. 3D canine hepatocyte culture system modeling the *Leishmania infantum* liver infection. XXIV International Symposium on Morphological Sciences, Istanbul, Turkey, 2-6 September, 2015. Anatomy, Vol. 9 / Suppl 2 S132.

[http://www.anatomy.org.tr/issue/2015s2/pdf/04.pdf]

Rodrigues A, Alexandre-Pires G, Santos-Mateus D, Ligeiro D, Valério-Bolas A, Martins C, Rafael-Fernandes M, Pereira M, Pereira da Fonseca I, Santos-Gomes G. Innate immune receptors of canine Kupffer cells sense *Leishmania infantum*. 4th European Congress of Immunology, Vienna, Austria, September 6-9, 2015. Abstract Book pp 528.

[http://www.eci-vienna2015.org/images/docs/ECI2015_Abstract-Book-v2.pdf]

Rodrigues A, Alexandre-Pires G, Santos-Mateus D, Valério-Bolas A, Rafael-Fernandes M, Aires-Pereira M, Pereira da Fonseca I, Santos-Gomes G. Impact on cell metabolism of dog hepatocytes when exposed to *Leishmania infantum*. Congress of the European Association of Veterinary Anatomists. Cluj-Napoca, Romania, July 23-26, 2014. Anatomia, Histologia, Embryologia, Journal of Veterinary Medicine C, 43, Supl. 1: pp. 80-81.

[http://onlinelibrary.wiley.com/doi/10.1111/ahe.12127/epdf]

Rodrigues A, Alexandre-Pires G, Santos-Mateus D, Marques, C. Valério-Bolas A, Rafael-Fernandes M, Fonseca I, Santos-Gomes G. Impact on the metabolic function and immune activity of dog hepatocytes when exposed to *Leishmania infantum*. EMBO young scientists' forum. Lisbon 15-16 July, 2013. Instituto de Medicina Molecular (IMM).

Oral presentation

Rodrigues A, Santos-Mateus D, Alexandre-Pires G, Valério-Bolas A, Rafael-Fernandes M, Alves-Azevedo R, Ligeiro D, Pereira M, Nunes T, Pereira da Fonseca I, Santos-Gomes, G. *Leishmania infantum* modulates the metabolism of dog hepatocyte. XIX Congreso de la Sociedad Espanola de Parasitología, II Encuentro Internacional de Parasitólogos de Espanha, Francia, Italia y Portugal. Vitoria-Gasteiz, Spain, 23-25 July 2015. Abstract book pp H5.

[http://www.congresoparasitologia.org/info/H.pdf]

Rodrigues A, Teixeira F, Martins C, Santos-Mateus D, Carvalho S, Alexandre-Pires G, Lopes R, Gaspar M, Cruz E, Tomás A, Santos-Gomes G. Zoonotic visceral leishmaniasis - New insights in the dynamics of cellular memory. WorldLeish 5, Porto de Galinhas, Brazil. 2013. Abstract book O182.

Near this Spot

are deposited the Remains of one

who possessed Beauty without Vanity,

Strength without Insolence,

Courage without Ferocity,

and all the virtues of Man without his Vices.

This praise, which would be unmeaning Flattery

if inscribed over human Ashes,

is but a just tribute to the Memory of

Boatswain, a Dog

Lord Byron, 1808

Dedicated to the most important persons in my life:

Tiago and my Parents,

and to the treasured memory of my Grandmother.

Acknowledgements

To Professor Gabriela Santos-Gomes, from Instituto de Higiene e Medicina Tropical (IHMT), Universidade Nova de Lisboa (UNL), for receiving me at the laboratory (some years ago) and for the magnificent scientific guidance, for encouraging me to take this adventure and accomplish this project. Thank you for being more than just my thesis supervisor, thank you for being a friend. You are a great scientist and an amazing person. Thank you for the revision of the thesis, encouragement when it was needed and for ever being present throughout this journey, with a scientific advice or just to talk. Thank you for the good experiences of teaching on the other side of the Atlantic, those were also great adventures.

To Professor Isabel Fonseca, from Faculdade de Medicina Veterinária, Universidade Técnica de Lisboa, for having accepted to be co-supervisor of the present work. Thank you for being available whenever it was needed and for the revision of the present thesis.

To Researcher Joana Miranda, from Faculdade de Farmácia da Universidade de Lisboa (FFUL), Research institute for medicines (iMed), for having accepted to participate in this project and for maintaining a friendship and always being available for any advice or just to talk.

To Professor Graça Pires, from Faculdade de Medicina Veterinária, Universidade Técnica de Lisboa, for accepted to be part of this project. Thank you for your good humor, patience, and never-ending availability to embrace our new crazy ideas. Without your assistance this project would not be the same. Thank you for performing the flow cytometry analysis, fluorescent confocal microscopy and scan electron microscopy photos that were so important for this work.

To Researcher Fátima Nogueira, from IHMT, UNL and Researcher Manuela Colla Carvalheiro, from iMed, FFUL, for accepted to be my tutorial commission. Thank you for your availability to read my work reports and attend the meetings.

My thanks to Professor Lenea Campino for allowing the utilization of fluorescence microscope.

Many thanks to Professor Marcelo Silva for the utilization of the inverted microscope and plate reader whenever I needed and my thanks to Professor Celso Cunha for sharing his laboratory equipment with me when it was most needed. These demonstrate the spirit of cooperation of IHMT.

To Professor Ana Tomás, from Instituto Biologia Molecular e Celular (IBMC), for providing the GFP-*L. infantum* transformed parasites and recombinant proteins used in this study. Thank you for your important collaboration.

To Researcher Luiz Filipe Passero and Researcher João Lago, from Faculdade de Medicina da Universidade de Sao Paulo, Brazil, for providing the ursolic acid, tested in the present work for its leishmanicidal activity. Thank you for your important collaboration.

To Professor Bartira Rossi Bergmann, from Instituto de Biofísica Carlos Chagas Filho, UFRJ, Rio de Janeiro, Brazil, for providing the Chalcone-8 and Quercetin tested in the present work for its leishmanicidal activity. Thank you for this important collaboration.

To Dr. Pedro Faísca, from Universidade Lusófona, for your availability to process the mice histological preparations. My thanks also to technician Susana Dias for the patience explaining all the process and protocols involved.

To Dr. Sância Ramos, director of Anatomia Patológica do Centro Hospitalar Ocidental E.P.E – Hospital Santa Cruz, for the availability demonstrated when help was asked. To technician Armandina Manuel for the patience in explaining and teaching all about histological cuts and coloration, and also for the interest demonstrated in the present work.

To Researcher Dário Ligeiro from IPST - Instituto Português do Sangue e da Transplantação - Centro do Sangue e da Transplantação de Lisboa, for the collaboration on obtaining the confocal fluorescence microscopy images. Thank you for the availability demonstrated when help was asked.

To Researcher Telmo Nunes from Faculty of Sciences, Microscopy Center, Universidade de Lisboa, for preparation of samples for scan electron microscopy. Thank you for the fruitful collaboration

To Researcher Dinora Lopes and technicians Victor Santos and Rosário Tito from the animal facilities of IHMT. My thanks for the immense collaboration in mice inoculation and treatment.

To Portuguese Foundation for Science and Technology (FCT), for funding my PhD scholarship SFRH/BD/73386/2010.

To my "half-brain" David Santos-Mateus. Thank you for being present since the first "liver-day" to the last one. You are a great scientific and bad-tempered mind! Thank you for being such a friend. My "gigantic" thanks also to Ana Valerio-Bolas, Mariana Rafael-Fernandes, Débora Almeida, Mafalda Claro, Ana Rita Pedrosa, Joana Cavaco Silva and Maria de Aires - my "companion in situation". It was your presence, your aid, your friendship that helping me to overcome the bad lad-days. Thank you for always help me in the "liver-days". Thank you for all the scientific and less scientific discussions. Thank you for all the good times in the lab, the "family" lunches and all coffee-times. I know that this lab, during these four years had very special people and now I have very special friends for life.

My thanks also to Cátia Marques and Marcos Santos, for performing the analysis for the dogs used in the present study and always respond to my questions and doubts.

To Cíntia Philipon for your friendship and for many interesting talks. Thank you for loaning the compounds.

To Jéssica de Jesus, thank you for your friendship, joy, and help with the RT-PCR. It was a very important help in a stressful time.

To Sara Ventura and Raquel Azevedo, those who were not frightened by RNA extraction in "massive" scale. Your help was priceless in the "final run".

To Pedro Ruas and João Tabanez, for keeping a good lab ambience and always be helpful. To Áurea Gabriela and Joana Monteiro for your friendship and precious help with the microscope scales.

To my parents for all their love and care. For their presence in everything in my life and for helping me be who I am and be where I am now. To my grandmother. You always will be an example for me with your strength and good advices.

And at last, to Tiago. Thank you for your love and patience. Thank you for pick me up at IHMT so many times at "evil" hours and for cooking and waiting me to have dinner. Thank you for making me feel so special. Thank you for your support in everything in my life. Thank you for your interest in biology and for have accepted the challenge of painting my cells. Love is everything.

Resumo

Estudo da resposta imunitária exibida pelo fígado de cão infetado por *Leishmania infantum*

Maria Armanda Viana Rodrigues

PALAVRAS-CHAVE: Leishmaniose canina; Hepatócitos; Células de Kupffer; Imunidade inata; Memória celular.

A leishmaniose canina (LCan) é uma doença zoonótica causada por *Leishmania infantum*, um parasita protozoário transmitido por flébotomos. O fígado constitui um órgão vital e de grande importância metabólica, sendo também um alvo preferencial para *L. infantum*. Apesar do complexo metabolismo, o fígado apresenta mecanismos efetores que podem contribuir para a direta eliminação do parasita de forma ainda não completamente compreendida. Assim, este estudo tem por objetivo analisar o papel dos hepatócitos e das células de Kupffer (CK) na infeção por *L. infantum*. A resposta imune orquestrada pelo cão contra *L. infantum* é constituída por um largo espetro de respostas de imunidade inata e adquirida. Estudos em cães infetados demonstraram que a imunidade ao parasita é órgão-específica. Para analisar a interação entre *L. infantum* e os hepatócitos, no presente estudo, hepatócitos e CK foram isolados de cães saudáveis e cultivadas em dois sistemas de cultura diferentes. O sistema 2D consiste na clássica cultura em placa e o sistema 3D tira partido da tendência dos hepatócitos para agregar e permite às células recriar a organização natural existente no órgão. *L. infantum* apresentou um claro tropismo para os hepatócitos, aderindo fortemente à membrana celular. No entanto, não é claro se o parasita entrou nos hepatócitos. Estas interações levaram a uma ativação da imunidade inata, aumentando a expressão génica dos receptores NOD1 e NOD2, bem como TLR2, em sinergia com a diferenciação de um perfil misto de citocinas anti- e pro-inflamatórias, que conduziu à diminuição de atividade das CYPs450. Três novos compostos com reconhecida atividade anti-leishmanial (ácido ursólico, chalcona-8 e quercetina) foram testados e comparados com o fármaco classicamente utilizado no tratamento da leishmaniose. Os compostos experimentais apresentaram boa atividade leishmanicida e não afetaram a viabilidade dos hepatócitos, diminuíram a inflamação celular e restauraram a atividade das CTPs450, havendo ainda a hipótese de serem metabolizados em parte pelas referidas enzimas. As CK mostraram-se permissivas a amastigotas e promastigotas e elevaram a expressão de NOD1 e TLR2 na presença do parasita. No entanto, *L. infantum* é capaz de regular a resposta imunitária nas CK, evitando a ação de citocinas anti e pró-inflamatórias e do stress oxidativo, aumentando, por outro, lado a síntese de ureia. A adição de antimoniato de meglumina permitiu às CK recuperar a ativação e expressão de recetores de imunidade inata e produção de citocinas. A co-cultura de CK infetadas com hepatócitos revelou que as células em conjunto foram capazes de ativar a imunidade inata. Os hepatócitos evidenciaram, assim, um papel interessante na orquestração de uma resposta imune sinergética com as CK contra *L. infantum*. Foram

ainda observadas diferenças entre a resposta imune exibida por macrófagos do sangue (MØ) e CK infetados por *L. infantum*. A análise da memória linfocitária hepática, realizada em modelo murino de leishmaniose visceral, demonstrou que o tratamento com antimoniato de meglumina elevou os níveis de memória e o número de células efetoras, melhorando a resposta imunitária do fígado, salientando o seu papel como órgão imunitário. As proteínas recombinantes de *L. infantum*, *Li*rCyp1 e *Li*rSOD foram reconhecidas pelas células de memória de animais infetados e tratados, indiciando que estas proteínas podem vir a ser incorporadas numa vacina terapêutica ou profilática.

Abstract

Canine leishmaniasis: the role of hepatocytes and Kupffer cells during *Leishmania infantum* infection

Maria Armanda Viana Rodrigues

KEYWORDS: Canine leishmaniasis; Hepatocytes; Kupffer cells; Innate immunity; Cellular memory

Canine leishmaniasis (CanL) is a zoonotic disease caused by *Leishmania infantum*, a protozoan parasite transmitted by sand flies. The liver is a vital and major metabolic organ which also constitutes a *L. infantum* preferential target. Despite the complex metabolism, liver possesses effector mechanisms that can contribute to direct elimination of *Leishmania* in a not yet defined way. Thus, this study aimed to assess the role of hepatocytes and Kupffer cells (KC) in the canine liver infected by *L. infantum*. A wide spectrum of innate and acquired immune responses is orchestrated by the canine host when facing *L. infantum* infection. CanL infection studies have indicated that parasite immunity is organ-specific. In the present study, canine hepatocytes and KCs were isolated from healthy stray dogs and two different culture systems were used to analyze the interaction of *L. infantum* with hepatocytes. The 2D-culture system consists in the classical plate culture and the 3D-culture system takes advantage of the natural aggregation properties of hepatocytes and allows cells to recreate some of the natural tissue architecture. *L. infantum* exhibited evident tropism to hepatocytes and strongly attached to the cells in both cultures system. However, it was not clear if parasites entered the hepatocytes. These interactions lead to activation of innate immunity by increasing the gene expression of NOD1 and NOD2 receptors in synergy with the increase in TLR2 expression. TNF-α cytokine expression was increased creating a pro-inflammatory cellular microenvironment and, as consequence, decreasing the CYP450 activity. This downregulation of CYPs may increase toxicity of clinical drugs in some cases. Three new potential leishmanicidal drugs were tested, ursolic acid, chalcone-8 and quercetin in comparison to the standard meglumine antimoniate. Experimental drugs revealed good leishmanicidal activity and did not reduce hepatocyte viability. The addition of leishmanicidal drugs decreased the inflammation and restored the CYP450 activity, which were also probable involved in the metabolization of the tested compounds. Regarding KCs, these cells were permissive to *L. infantum* amastigotes and promastigotes. KCs increased expression of NOD1 and TLR2 innate immune receptors. Even so, the parasite regulates KC immune response, avoiding the generation of pro- and anti-inflammatory cytokines and the oxidative burst, increasing the synthesis of urea. The addition of meglumine antimoniate allowed KC to activate, increasing the expression of innate immune receptors and of pro-inflammatory cytokines, breaking the silencing imposed by the parasite. The addition of *L. infantum* infected KCs to canine hepatocytes in a co-culture, was able to activate cells innate immunity. Hepatocyte seemed to have an interesting role in orchestrating a synergistic immune response against *L. infantum* parasites. Major differences were also found between the immune response exhibited by blood derivate macrophages (MØ) and liver KC, when facing

infection by *L. infantum*. The assessment of the liver lymphocyte memory performed in the murine model of visceral leishmaniasis demonstrated that the treatment with antimoniate meglumine increases the levels of memory and effector T cells, enhancing liver immune response. The liver has higher levels of immune memory T cells, evidencing its role as an immunological organ. The recombinant proteins *Li*rCyp1 and *Li*rSOD, are strongly recognized by memory cells from infected and treated mice, indicating these proteins might be used in a prophylactic or therapeutic vaccine formulation.

Table of Contents

List of Figures

List of Tables

Abbreviation list

2D	Bidimensional
3D	Tridimensional
4-MU	4-Methylumbelliferone
ABC	Adenosine triphosphate (ATP)-Binding Cassette Transporters
ACL	Anthroponotic cutaneous leishmaniasis
Ag	Antigen
AgT	Total antigen
ALD	Alcoholic liver disease
APC	Antigen presenting cells
AROD	Alkoxyresorufin O-dealkylation
AVL	Anthroponotic visceral leishmaniasis
BROD	7-benzyloxyresorufin O-dealkylation
CanL	Canine leishmaniasis
cDNA	Complementary deoxyribonucleic acid
CH-8	Chalcone 8
CL	Cutaneous leishmaniasis
CR	Complement receptor
CSA	Crude soluble antigen
C_T	Threshold cycle
CVBD	Canine vector borne diseases
CYP450	Cytochrome P450
DAT	Direct agglutination test
DCs	Dendritic cells
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dsDNA	Double strand DNA
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EROD	7-ethoxyresorufin O-dealkylation

FBS	Fetal bovine serum
GFP	Green fluorescent protein
GIPLs	Glycosylinositol phospholipids
GSH1	Gamma-glutamylcysteine synthetase
HAART	Highly active antiretroviral therapy
HIV	Human immunodeficiency virus
HSC	Hepatic stellate cells (liver)
IFA	Indirect fluorescent antibody
IL	Interleukin
IFN	Interferon
iNOS	Inducible nitric oxide synthase
IPTG	Isopropyl β-D-1-thiogalactopyranoside
KCs	Kupffer cells
LAT	Latex agglutination test
LB	Luria broth
LBP	Lipid-binding proteins
LCL	Localized cutaneous leishmaniasis
LirAMP	*Leishmania infantum* recombinant aminoproteinase
LirCyP1	*Leishmania infantum* recombinant cyclophilin A
LirSOD	*Leishmania infantum* recombinant superoxide dismutase
LPG	Lipophosphoglycan
LPS	Lipopolysaccharide
LSEC	Sinusoidal endothelial cells (liver)
MAC	Membrane attack complex
MCL	Mucocutaneous leishmaniasis
MCS	Multiple cloning site
MDR	Multidrug resistance
MDR1	P-glycoprotein MDR1
MgA	Meglumine antimoniate
MHC I	Major histocompatibility complex I
MHC II	Major histocompatibility complex II
MØ	Macrophage

MR	Mannose-fucose receptor
mRNA	Messenger ribonucleic acid
MROD	7-methoxyresorufin O-dealkylation
MRPA	ABC-thiol transporter MRPA
MZ	Marginal zone (spleen)
MZMs	Marginal zone macrophages (spleen)
NAFLD	Non-alcoholic fatty liver disease
NK	Natural killer cell
NKT	Natural killer T cell
NO	Nitric oxide
NOD	Nucleotide-binding oligomerization domain
PALS	Periarteriolar lymphoid sheaths (spleen)
PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PKDL	Post-kala-azar dermal leishmaniasis
PROD	7-pentoxyresorufin O-dealkylation
PRR	Pattern recognition receptor
PSG	Promastigote secretory gel
PTR1	Pteridine reductase
QC	Quercetin
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT	Reverse transcriptase
Sb	Sodium stibogluconate
SCHN	Schneider's Drosophila media
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SOC	Super optimal broth with catabolite repression
TAE	Tris base, acetic acid and EDTA buffer
T_{AN}	Annealing temperature
T_{CM}	Central memory T cells

T_E	Effector T cells
T_{EM}	Effector memory T cells
TGF-β	Transforming growth factor beta
Th	T helper
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
UGT	Uridine 5'-diphospho-glucuronosyltransferase
URA	Ursolic acid
VL	Visceral leishmaniasis
VLDL	Very-low density proteins
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
ZVL	Zoonotic visceral leishmaniasis

1 General Introduction

1.1. Leishmaniasis

Leishmaniases constitutes a group of diseases caused by an intracellular protozoan of the genus *Leishmania* that parasites a large range of mammalian hosts including humans, dogs and rodents. Leishmaniasis is caused by more than 20 *Leishmania* species that can be transmitted to humans by the bite of infected female sand flies.

Traditionally, wild canids and especially the domestic dog are the hosts responsible for the persistence of the disease and the source of infection for the vector. The domestic dogs are good reservoirs of the parasite, as they have high parasite burdens in the skin and a chronic and long lasting disease (João *et al.*, 2006). In humans, leishmaniasis covers a large spectrum of clinical symptoms, ranging from self-healing cutaneous infections (CL) to visceral leishmaniasis (VL) also known as kala-azar, which can be fatal if not treated. The outcome of infection in humans and dogs depends on both the parasite species involved and its virulence and on the host immune response (Goto and Lindoso, 2004). The need to better understand the immune response generated against *Leishmania* infection, the development of better anti-leishmanial drugs and of complementary strategies of treatment and, the development of an affordable vaccine are the main goals set for leishmaniasis research.

1.2. Biology of *Leishmania*

1.2.1. Leishmania morphology and life cycle

Leishmania ssp. are unicellular digenetic parasites, whose life cycle involves two hosts, a vertebrate and an invertebrate. During life cycle, *Leishmania* has two basic body forms: the amastigote, an intracellular form that occurs in the vertebrate host and the promastigote, an extracellular flagellated/mobile form in the sand fly (*Phlebotomus* ssp. and *Lutzomyia* ssp.) vector. Amastigotes are taken up from the blood of an infected vertebrate host when the female sand fly gets a blood meal. During digestion in the sand fly gut, amastigotes are released to extracellular environment and differentiate into

promastigote form. In the gut of the female sand fly, promastigotes attach to the midgut-hindgut epithelium microvilli and divide by binary fission.

Promastigotes possess a number of single-copy organelles with defined sub-cellular locations. Morphologically, presents an elongated form of about 10-30 μm long and 2.5-5 μm width depending on the species, a central nucleus, Golgi apparatus, mitochondria, which incorporates the kinetoplast, a region of highly condensed chromatin, the basal body and a long external flagellum that emerges from the anterior part of the cell, via the flagellar pocket, and confers motility (Figure 1A and B). After initial ingestion of blood meal from the host (Figure 2), promastigotes development in the sand fly takes 8-20 days and, followed by multiplication in the midgut-hindgut, they move forward to the pharynx where they produce a partial or complete blockage of the sucking apparatus (Dhingra and Satapathy, 2014).

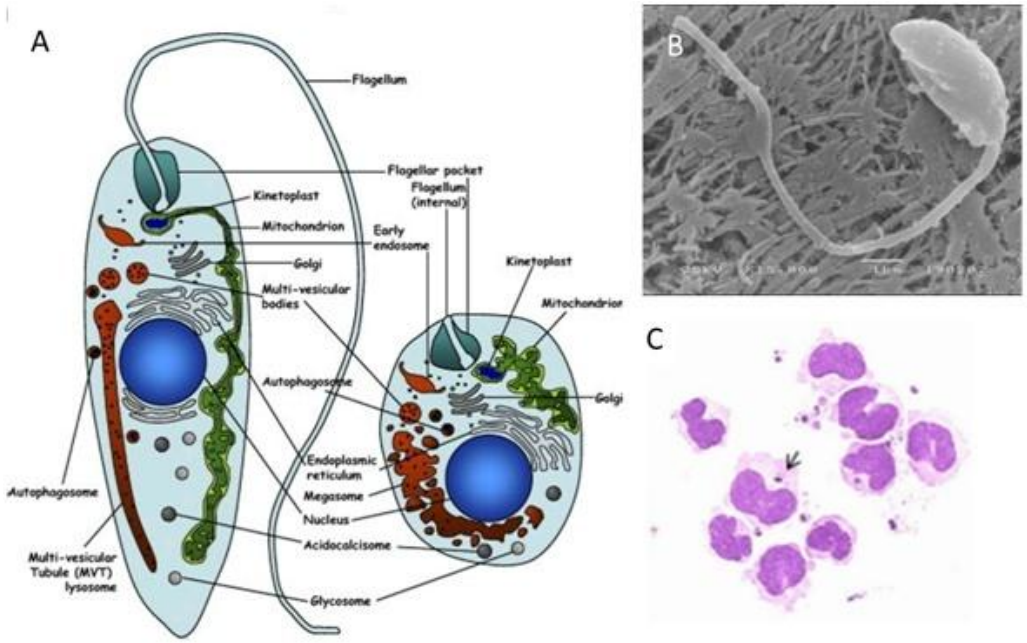


Figure 1: *Leishmania* ssp. morphological forms.
A) Schematic representation of the main intracellular organelles of *Leishmania* promastigote (left) or amastigote (right) forms. The flagellar pocket marks the anterior end of the cell (adapted from Besteiro *et al*., 2007). B) Electron scanning of *Leishmania infantum* cultured promastigote. C) Intracellular amastigotes (black arrow) under optical microscopy inside a macrophage (magnification 400x).

Due to the impairment of sucking mechanism, to feed again the sand fly needs to bite the vertebrate host and regurgitating metacyclic promastigotes that are introduced into the bite wound allowing the natural transmission of *Leishmania* to occur. Metacycle promastigote forms are resistant to complement attack and quickly invade

local phagocytes, subsequently transforming into amastigotes inside the host cells (Cupolillo *et al.*, 1995). Although the deposition of promastigotes in the vertebrate dermis includes sand fly saliva, the salivary glands are not usually infected as in the case of the African trypanosomes in the tsetse fly or the *Plasmodium* in the mosquito.

The amastigote (meaning "without a flagellum") is the intracellular non-motile form in the vertebrate host. Although the amastigote is not really devoid of a flagellum, it is merely that the flagellum does not protrude beyond the cell surface and cannot be seen by light microscopy (Figure 1A and C). Intracellular amastigote exhibits a round or oval shape of around 2-6 μm of diameter. This morphological form also presents a nucleus, the Golgi apparatus and mitochondria with the kinetoplast. Inside the parasitophorous vacuole of the host cell amastigote form replicates by longitudinal binary fission. Promastigotes can be grown *in vitro* at 24-27 ºC in several axenic culture mediums, such as NNN medium, which is constituted by a solid phase of blood agar and a liquid phase containing a physiologic salt solution, or in different available liquid media (e.g., Scheneider insect drosophila medium, RPMI, D-MEM, Glasgow minimal essential medium) sera supplemented. Amastigotes usually are grown *in vitro* in cultured cells and can also be grown in axenic liquid media at 37 ºC under special conditions.

In cutaneous leishmaniasis (CL), the parasite stays in the skin, causing lesions that can ulcerate or evaluate to spontaneous cure, depending on the species of *Leishmania* and on the immune response of the host. The mechanisms which determine the dissemination of the parasite from the initial site of infection to mucosal tissues and cartilages, as in the case of mucocutaneous leishmaniasis (MCL) or to internal organs (e.g. spleen, liver and bone marrow), as in visceral leishmaniasis (VL) are not yet clear (Murray *et al.*, 2002). Organ-specific immunity has been described in various experimental VL studies in mouse and dog models (Santos-Gomes *et al.*, 2002; Alexandre-Pires *et al.*, 2010).

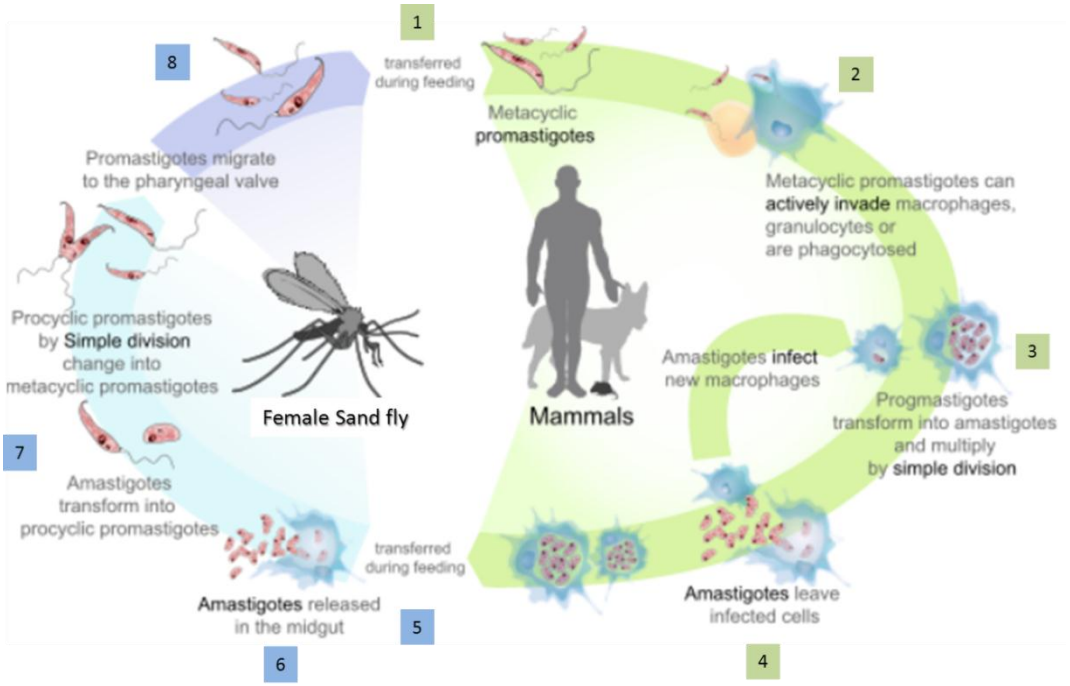


Figure 2: *Leishmania* ssp. life cycle.
Transmission of *Leishmania* occurs during the blood meal of phlebotomine female sand flies (1), which introduces metacyclic infective promastigotes into the vertebrate host dermis. Parasites are phagocyted by macrophages (2) and in the interior vacuoles transformed into amastigotes (3). Amastigotes replicate inside the cells (4) and infected macrophages may be retained on the skin (CL and MCL) or migrate to the inner organs, visceralizing the infection (VL). During another blood meal, the phlebotomine female ingest the infected macrophages (5) and in the midgut the amastigote leaves the cells (6) and matures to virulent and infective promastigotes (7) ready to be injected in the next mammal host (8). Image released into the public domain by Mariana Ruiz Villarreal and adapted

1.2.1.1. *Leishmania* in the vector: co-evolution of parasite and vector

Phlebotomine sand flies are dipteran insects belonging to the family *Psychodidae* and approximately 700 species have been described to date. Of these, evidence of *Leishmania* vectorial capacity has been demonstrated for about 30 species. Currently there are two groups of *Leishmania* parasites that are classified into different subgenera, according with the part of the sand fly gut that are colonized by the parasite. Indeed, the original division of mammal infective *Leishmania* into the subgenera *Leishmania* and *Viannia* by Lainson and Shaw (1977) was largely based on this character, a separation which has been subsequently confirmed by DNA sequence based phylogenetic analyses (Croan *et al.*, 1997; Noyes *et al.*, 2002). Female sand flies (*Phlebotomus* spp. in the Old World, *Lutzomyia* spp. in the New World) acquire *Leishmania* parasites when feed on an infected mammal host. Sand flies are Telmophages, meaning they insert their saw-like mouthparts into the skin and agitate

them to produce a small wound into which the blood flows from superficial capillaries. It is this damaged tissue that activates and attracts skin macrophages into the blood pool, enabling their subsequent uptake by the sand fly. The change in environmental conditions, from the mammalian host to the sand flies midgut, mainly characterized by a decrease in temperature and an increase in pH, triggers the parasite transformation in the motile promastigote form in the vector (Bates and Rogers, 2004). In the vector, the amastigote give rise to the procyclic promastigote, the first promastigote stage (Figure 3). This is a slow motile and active replication form that promotes a fast and exponential increase in parasite numbers.

In *Leishmania* subgenus, the initial parasite phase is confined by the peritrophic matrix, a chitin and protein mesh secreted by the midgut epithelium that encloses the blood meal, while digesting it (Secundino *et al.*, 2005). After a few days, the nectomonad parasites begin to differentiate into the elongate, strongly motile promastigotes. These are migratory forms that accumulate at the anterior end of the peritrophic matrix and break out of the blood meal from the action of a parasite secreted chitinase (Shakarian and Dwyer, 2000). Then, the parasites moves towards the anterior midgut, most of them attaching to the microvilli of the midgut epithelium, were replicate actively. Parasites that result from the replicative process reach the stomodeal valve that guards the junction between foregut and midgut. The existence of this intermediate phase of promastigote form is a mark of a true vector, since the parasite persists beyond the blood meal, avoiding expulsion during defecation. The ability to attach is an important property of *Leishmania* promastigotes (Sacks and Kamhawi, 2001). Lipophosphoglycan (LPG), the major parasite surface glycoconjugate, binds to galectin on the sand fly gut epithelium of some species, establishing a key parasite-vector interaction, such as *Leishmania major* in *Phlebotomus papatasi* (Pimenta *et al.*, 1992). However, more recent findings indicate that non-LPG mediated attachment can also be used by some *Leishmania* species (Myskova *et al.*, 2007). Once reached the stomodeal valve, the nectomonad promastigotes transform into leptomonad promastigotes, shorter forms that continue to replicate. These are responsible for the secretion of promastigote secretory gel (PSG), which plays a key role in transmission. Nectomonad/leptomonad promastigotes attach themselves to the cuticle lined surface of the valve and differentiate into haptomonad promastigotes (Killick-Kendrick *et al.*,

1974). This form of attachment is mechanistically different to that seen in the midgut epithelium and is mediated by the expansion of the flagellar tip into hemidesmosome-like structures (Wakid and Bates, 2004). Finally, some of the leptomonads promastigotes differentiate into metacyclic promastigotes, the mammal infective stage. These parasites are deposited in the skin of a new mammalian host when the sand fly gets another blood meal, leading to the transmission of the parasite.

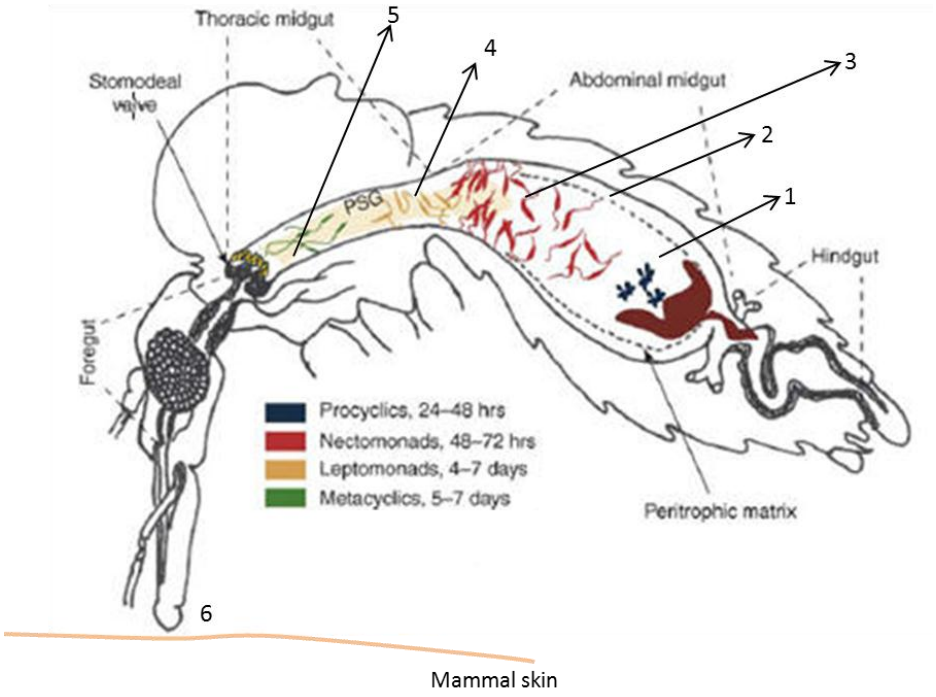


Figure 3: *Leishmania* (*Leishmania*) inside the sand flies vector.
1) Amastigotes ingested with the blood meal are transformed into weakly motile procyclic promastigotes; 2) Four hours after the blood meal, the blood is enclosed in the peritrophic matrix made of chitin and proteins secreted by the midgut, which protects the promastigotes from the enzymatic activity. 3) Within 48-72 h the peritrophic matrix is degraded, releasing the elongated and highly motile nectomonad promastigote that migrates to the anterior midgut. 4) The nectomonads transform into leptomonad promastigotes and release the promastigote secretory gel. This gel fills the anterior midgut into the foregut. 5) At the foregut the non-infectious procyclic promastigotes transform into the infective metacyclic promastigotes. 6) The sand flies regurgitate and expel the metacyclic promastigotes when taking a new blood meal in a mammal. (Adapted from http://dna.kdna.ucla.edu/parasite_course-old/leish_files/subchapters/morphology%20and%20life%20cycle.htm)

Parasites of the subgenus *Viannia* are only found in the New World and therefore all the vectors are of *Lutzomyia* (*Lu.*) spp. The initial parasite events are similar to those described for the subgenus *Leishmania*. Amastigotes are taken up and transformed into the replicative procyclic promastigotes. However, the majority of the parasites can be found in the pyloric region of the hindgut (Nieves and Pimenta, 2000),

a defining feature of this subgenus. After the hindgut phase, there is a forward migration followed by parasite accumulation at the anterior midgut, PSG secretion and differentiation of metacyclic promastigotes. The detailed kinetics of this anterior migration has not been fully explored. Although the route is slightly different and the parasite stages involved have not been well defined the final development goal is similar in both subgenus, resulting in an effective capacity of infect new host and propagate the parasite (Bates, 2007).

It is also of interest to look at the fitness cost of the fly vector by sustaining the development of infective *Leishmania* and by transmitting the infection. For example, the deviation of nutrient resources to parasite growth associated with the considerable distension of the gut by the gel-like plug produced by the parasite, damaging the stomodeal valves have the potential for causing injuries to sand flies. Roger and Bates (2007) examined the effect of infection on the longevity of *L. longipalpis*. Both *L. mexicana* and *L. infantum* infections were found to significantly reduce longevity under normal laboratory conditions (average of 11 days of life for non-infected controls and of 9 days for both *L. mexicana* and *L. infantum* infected sand flies. Although little is known regarding the survival of flies in nature, it is recognized that some respects of the natural environment can be more stressful and hazardous than the conditions experienced in laboratory insectaries. Curiously, *Leishmania* infections do not significantly impact on immediate reproductive fitness of the sand fly. An interesting balance occurs in the *Leishmania*-sand fly relationship. In fact, more aggressive and quickly developing infections will lead to increased accumulation of PSG by metacyclic promastigotes, thereby increasing the probability of transmission. On the other hand, this fast developing infection will also exert a greater fitness cost on the vector, thus reducing the probability of transmission. Thus, any alteration of feeding behavior by the parasite will independently increase the probability of transmission and this will be a clear selective advantage. Such behavior might include factors encouraging the sand fly to find a host and begin feeding and those leading the sand fly to prolong the feed or re-feed. Therefore, the timing of vector feeding with respect to parasite development is crucial, since successful transmission of parasites is dependent on vector survival. The vectors should survive long enough to assure the complete development of the parasite, reaching the infective stage (Rogers and Bates, 2007).

Thus *Leishmania* transmission results of a combination of several factors: (i) the PSG physical blockage of the stomodeal valve, ensuring enhanced regurgitation of infective forms, (ii) the subsequent exacerbation of infection in the mammalian host by the PSG action in association with the components of vector saliva as adjuvant and, (iii) the manipulation of feeding behavior according with the presence of infective forms available for transmission. All together, the above elicited issues highlight the close co-evolution of *Leishmania* parasites and their own sand fly vectors (Rogers *et al.*, 2002; Rogers and Bates, 2007).

1.2.2. Parasite virulence factors

During the blood meal, the insect vector deposits metacyclic promastigotes in the skin of the host. These promastigotes, the virulent form of *Leishmania,* are able to initiate an infection. Once in the dermis, the parasites are exposed to new hostile environments, such as the extra-cellular matrix (ECM) of connective tissue or the innate immune system and must interact with a variety of obstacles, to establish the infection within macrophage phagolysosomes. It is widely accepted that the distinct leishmaniasis clinical manifestations are a consequence of the interaction of the parasite (depending on parasite species) and the host's immune system.

Some parasite components are characterized as virulence factors, enabling the parasite to invade and establish infection in the mammalian host, contributing to *Leishmania* pathogenesis. The most known and described virulence factors from *Leishmania* include glycoinositol phospholipids (GIPL), lipophosphoglycan (LPG), proteophosphoglycan (PPG), the 11 kDa kinetoplastid membrane protein (KMP-11), several proteinases and metalloproteinases. Although the exact impact of these *Leishmania* components on the mammalian host's immune system is not yet totally clarified there are evidences that these components modulate the interactions between the parasite and the host immune cells, favoring infection outcome (Silva-Almeida *et al.*, 2012). GIPL were reported to help *Leishmania (L.) major* to survive inside macrophages by inhibiting nitric oxide synthase and protein kinase C (Zufferey *et al.*, 2013). LPG is a macrophage ligand that is directly involved in the initial stages of the infection. Assays carried out with a mutant strain of *L. major* deficient in the gene *lpg1* (*lpg1*$^{-}$) showed that the mutant parasites appear to attenuate virulence during the

infection of murine macrophages, despite presenting no other phenotypical changes. The *lpg1*$^{-}$ promastigotes were highly susceptible to the complement system and to the oxidants produced by host cells. In addition, these parasites lost their ability to inhibit phagolysosome fusion, leading to their own destruction inside macrophages. It has also been reported that *Lmajorlpg*2 null mutants (*lpg2*$^{-}$) were unable to survive in sand flies or in mammalian host cells. These highly altered parasites lacked all the phosphoglycans, including LPG and proteophosphoglycans (Svárovská *et al.*, 2010). Interestingly, *Leishmania* LPG has been shown to inhibit the nuclear translocation of NF-κB in monocytes, leading to a subsequent diminution of IL-12 production (Argueta-Donohué *et al.*, 2008) and can also influence the host's early immune responses by modulating the activity of dendritic cells, inhibiting antigen presentation and promoting an early IL-4 release (Liu *et al.*, 2009). All together, these actions promote the parasite establishment and survival.

Proteophosphoglycans are highly glycosylated polypeptides with functions not entirely clear. It is speculated that its long chain that covers the plasma membrane of the parasite could play some role in binding to macrophage receptors. Within macrophages, the secretion of modified PPG by parasites appears to contribute to the maintenance of the parasitophorous vacuole (Peters *et al.*, 1997).

KMP-11 is a hydrophobic protein that has been described to be associated with LPG. This protein presents immune regulatory properties and is able to induce the expression of IL-10 in cells of human patients with cutaneous and mucocutaneous leishmaniases (Carvalho *et al.*, 2005). However, the mechanism that supports this regulation remains unclear.

Proteases are also important virulence factors, as they are enzymes that hydrolyze peptide bonds and have the potential to degrade peptides and proteins. As so, they are potentially involved in a broad range of biological functions, including the infection process. Proteases are usually classified based on the catalytic domain. They can be serine-, threonine-, aspartyl-, metallo- or cysteine-proteases. Among these, only the aspartyl-, metallo- and cysteine-protease classes have been extensively studied in *Leishmania* species (Silva-Almeida *et al.*, 2012). There are several parasite proteases known to be involved in pathogenesis and playing important roles in parasite invasion

and migration through host tissues, degradation of immune related proteins, immune evasion and activation of inflammation (McKerrow *et al.*, 2006). The most known protease is the gp63, a metalloproteinase that covers the body surface of *Leishmania*. This protease belongs to the metzincin class (peptidase family M8) and is abundantly expressed on the surface of *Leishmania* ssp. and related to other trypanosomatid protozoan (Etges *et al.*, 1986). Gp63 has a very important biological role, associated with the parasite protection against the action of enzymes of the vector midgut and from the phagolysosomes of host macrophages. Additionally, when inside the mammalian host gp63 is required to assure the resistance of promastigotes to lysis mediated by the complement, as the presence of this active protease greatly reduced the fixation of the terminal complement components on parasite surface by increasing the conversion of C3b to its inactive form, the C3bi (Yao *et al.*, 2003).

Produced by the leptomonad promastigotes, a virulence factor highly important in the establishment of infection on the mammalian host is the PSG, mainly constituted by a high molecular weight glycoprotein called filamentous proteophosphoglycan (fPPG) (Ilg *et al.*, 1999). The identification of PSG in the sand flies brings a new recognition of the blocked fly hypothesis. The gel-forming properties of fPPG could fill and distend the anterior midgut of the sand fly, extending through the stomodeal valve into the foregut. Although fPPG is clearly the major component of PSG and a critical element of transmission and disease exacerbation, other products secreted by the parasite or by the sand fly present in the plug could have as yet undescribed biological effects. When co-inoculated with metacyclic promastigotes has been shown that PSG cause disease exacerbation, leading to an enhancement of pathology and of a parasite load increase (Bates and Rogers, 2004). It should also be noted that the PSG plug obstructing the anterior midgut is packed with promastigotes. Essentially, the plug is constituted by a pellet of promastigotes embedded in PSG. When observed *in situ* by microscopy, PSG plug has little evidence of life, as the cells appear immobile. However, when dissected out into culture medium, the PSG readily dissolves and releases promastigotes that regain their motility. The majority of these parasites are leptomonad promastigotes, one of the main pieces of evidence that this stage is responsible for the secretion of PSG. The other life cycle stages associated with the plug

are metacyclic promastigotes. Interestingly, these are mainly located at the poles of the plug, at ideal positions for transmission.

Some of the virulence factors are intrinsic to the parasite forms, but it is widely discussed that also the components of the vector can be directly linked with the virulence of the parasite. Vector's saliva is a potent pharmacological active fluid that directly affects the hemostasis, release of inflammatory mediators and cell immune activity of vertebrate hosts. These actions are crucial for a successful blood meal and the modifications on host physiology caused by pharmacologically active molecules may favor the transmission of *Leishmania* parasites that colonize the digestive tract of the sand fly (Andrade *et al.*, 2007). Sand fly saliva is a well-established disease exacerbation factor. To achieve a successful blood meal, sand flies must overcome host's hemostasis system, which includes blood coagulation cascade, vasoconstriction mediators, fibrinolysis and platelet aggregation. Besides hemostasis, sand flies must also evade the host's innate and acquired immune responses. To overcome these obstacles, sand flies develop a salivary secretion with an array of potent pharmacological components, such as anticoagulants, anti-platelet, vasodilators and, notably, immunomodulatory and anti-inflammatory molecules (Kamhawi, 2000). These biological active mediators with redundant and synergize activities generate a proper environment for a successful blood feeding and also for parasite establishment. Most of the existing knowledge concerning resistance and susceptibility to *Leishmania* infection was built based on experimental models. Titus and Ribeiro (1988) first demonstrated that saliva from *Lu. longipalpis* enhanced *L. major* infection when the parasite was co-inoculated with the sand fly salivary gland sonicate (SGS) in mice. Although not in its natural vector, the addition of san fly saliva enhanced lesion size and raised parasite burden in the lesions. Similar findings were further reported with other *Leishmania* species (Andrade *et al.*, 2007).

In murine models of leishmaniasis, resistance to infection is associated with the expression of interferon (IFN)-γ and interleukin (IL) -12 driving a $CD4^+$ Th1 immune response, while susceptibility is related to production of IL-4 and the development of a $CD4^+$Th2 response. Saliva from *L. longipalpis* seems to drive, by an unknown mechanism, the host immune response to a Th2 type, less effective in terms of parasite clearance, allowing parasite persistence. In endemic areas where potential host is highly

exposed to sand fly bites and saliva inoculation, the salivary components also seem to display an immune response in the host, leading to antibody production and cell mediated immune activation. However, the specific role of antibodies against sand fly saliva in the outcome of *Leishmania* infection is not fully known. Experimental studies, together with clinical data, suggest a potential transmission blocking mechanism. BALB⁄c mice repeatedly exposed to *L. longipalpis* bits produced high levels of anti-salivary antibodies, with predominance of the IgG1 subclass (Belkaid *et al.*, 1988). Total serum IgG from these animals recognized predominantly bands of 45, 44 and 16 kDa, displaying some similarity to human anti-saliva humoral responses, that recognize most of the bands. These proteins were also the major targets of the human antibody response in an endemic area (Barral *et al.*, 2000). As these proteins are widely recognized, they are natural candidates to be used as markers of exposure to *L. longipalpis* bits. Besides antibody production, an intense cellular infiltrate of neutrophils, eosinophils and macrophages at the site of sand fly bites also occurs. Moreover, mice pre-exposure to *P. papatasi* SGS or immunized with SP15, a protein present in *P. papatasi* saliva, were able to block the establishment of *L. major* infection (Andrade *et al.*, 2007). Also in humans, the response generated by sand fly saliva seems to exert a direct effect on human antigen presenting cells. *L. longipalpis* SGS inhibit the production of IL-10 and tumor necrosis factor (TNF)-α, but induce IL-6, IL-8 and IL-12p40 release by lipopolysaccharide-stimulated monocytes and dendritic cells. Besides cytokine production, sand fly saliva also interfered with the expression of monocytes/macrophages co-stimulatory molecules. *In vitro,* the saliva effect on human immune cells can be reverted by incubation with anti-SGS antibodies, revealing the importance of a specific humoral response for the neutralization of sand fly saliva harmful effects on the human immune system (Costa *et al.*, 2004).

The combination of experimental data indicates that the components of sand fly saliva can be used in a combined vaccine with *Leishmania* antigens to enhance protection. Furthermore, as sand flies feed on many other animals besides humans, immune approaches that target specific salivary components may enable protection against leishmaniasis in different vertebrate species, as in the case of CanL.

1.2.3. Evolution and taxonomy of *Leishmania*

Leishmania infections in humans have a long history, dating back as far as the first century AD. Evidencies of leishmaniasis have been found in mummies in anchient Egipt and in upper Nubia (Albert *et al.*, 2006). Pre-Incan pottery from Ecuador and Peru displayed skin lesions and facial deformities that are typical of cutaneous and mucocutaneous leishmaniases. Incan texts from the 15th and 16th centuries and accounts from Spanish conquistadors noted the presence of skin lesions on agricultural workers. These ulcers, similar to leprosy lesions were named "white leprosy", "Andean sickness" or "valley sickness." Detailed descriptions of the "Balkh sore" were written by Arab physicians in the 10th century. Indian physicians also described "kala-azar" or "black fever" disease now known as visceral leishmaniasis. The disease became known as leishmaniasis after William Leishman, a Glaswegian doctor serving with the British Army in India. In 1901, Leishman developed one of the earliest stains of *Leishmania*. In Dum Dum (India), Leishman discovered ovoid bodies in the spleen of a British soldier who was experiencing high fever, anemia, muscular atrophy and swelling of the spleen. Leishman described this illness as "dum dum fever" and published his findings in 1903. Charles Donovan also recognized these symptoms in other kala-azar patients and published his discovery a few weeks after Leishman. After Donovan described the oval bodies using Leishman's stain, these forms became known as Leishman-Donovan bodies and officially this parasite turn out to be known as *Leishmania donovani* (Bari, 2006). By linking this protozoan with kala-azar, Leishman and Donovan had discovered the genus, *Leishmania*.

Leishmania organisms have been assigned to different species primarily based on clinical, biological, geographical and epidemiological criteria and, later, immunological, biochemical and genomic data. Hierarchical taxonomic schemes have been proposed using the subgenus, complex of species and species. The concept of species complexes for grouping the *Leishmania* species initially proposed based on biological and biochemical characteristics has been revised and modified (Schönian *et al.*, 2010). In order to access the true risk associated with leishmaniasis, it is of the highest importance to know the distribution of *Leishmania* species and the evolutionary

relations between them. As a morphological distinction of *Leishmania* species is impractical, DNA-based criteria are now used to identify the different species.

Leishmania belongs to the phylum Kinetoplastida, which lacks a fossil record. Molecular studies have shown that kinetoplastids are probably related with the euglenids (Dooijes *et al.*, 2000). Kinetoplastids and euglenids are included in the eukaryote super group *Excavata*, whose fossil register point out for their emergence during the Ordovician period (Roger and Hug, 2006). Since *Leishmania* is a digenetic parasite it is difficult to consider the emergence of the current genus *Leishmania* before the appearance of adequate hosts, in particular the vector. The first fossil member of the genus *Leishmania,* designed as *Paleoleishmania proterus,* a trypanosomatid associated with a blood-filled female sand fly in Cretaceous Burmese amber and described by Poinar and Poinar (2004). This fossil dates from the Early Cretaceous and brought new insights regarding transmission. The discovery of a sand fly larvae, which develop in habitats containing free living flagellates with trypanosomatid characteristics raise the hypothesis that these flagellates were ingested by the sand fly larvae and probably multiplied inside them. Once in an adult sand fly, the flagellates could be transmitted to a vertebrate, thus establishing a continuing cycle between vectors and vertebrates. These events took place before the appearance of placental mammals during the Paleocene. It was after this period that *Phlebotomus,* the current vector of *Leishmania,* appeared.

The evolution of *Leishmania* has been explained by two major hypotheses related to a Neotropical or Palaearctic origin (Figure 4). Regardless its origin, the dissemination of *Leishmania* followed the migration of vectors and vertebrate hosts (Perrotey *et al.*, 2005). The definitive host of primitive *Leishmania* may have been reptiles or ancient mammals. Mainly based on the published gene sequence phylogenies and in the form of a number of rooted phylogenetic trees which place Neotropical species at the root and Palaearctic at the crown of the tree, Harry Noyes in 1998 has renewed the hypothesis of a Neotropical origin for the genus *Leishmania.* According to this hypothesis, *Sauroleishmania* subsequently evolved from mammalian parasites in the Palaearctic regions (Noyes *et al.*, 2000). Additionally, the earliest fossil of sand flies was found at the south of the Tethys Sea, in Lebanon and was from the Cretaceous period, thus lived in Gondwana at 120 million years ago. Before this, Phlebotomine sand flies probably had lived for a long time in Pangaea from where separate lineages

could have developed in the Neotropics, such as the Old World sand flies species (Noyes *et al.*, 2000). However, this hypothesis was contested by Kerr (2000) which defended a Palaearctic origin for *Leishmania.* From biochemical and molecular evidences, Kerr argues that the phylogenetic trees indicating that subgenus *Viannia* and the genus *Endotrypanum* are ancestral to subgenus *Leishmania* are un-rooted and based on the assumption of a constant evolutionary rate, which led to the underestimation of *Sauroleishmania* and *Leishmania* antiquity. This approach suggests that *Sauroleishmania* was originated in Cretaceous reptiles of the Palaearctic region. As reptiles declined the phlebotomine ancestral species adapted to feeding on emerging rodents rather than reptiles. *Leishmania,* along with its phlebotomine vector and a rodent reservoir may have evolved in the Palaearctic and at the end of the Eocene dispersed to the Nearctic. The fossil record of sand flies, although very scarce, provides evidence for the separation of *Phlebotomus* and *Lutzomyia* during the Oligocene, coinciding with a break in the land connection of the Palaearctic with the Nearctic. After the formation of the Panamanian Land Bridge, about 3 million years ago, the dissemination of sigmodontine rodents, *Leishmania* and *Lutzomyia* accelerated rapidly. A variety of mammals of South American origin, such as echimyid rodents, porcupines, sloths and opossums, lacking prior exposure to *Leishmania*, may have been particularly susceptible to the parasite. Furthermore, an adaptation to new mammalian hosts in response to climatic changes might have greatly accelerated the rate of speciation of *Leishmania*. A third *Leishmania* phylogeny hypothesis based on the major divisions within the genus is discussed by Momen and Cupolillo (2000). In this hypothesis a Neotropic origin is recalled for the subgenus *Viannia* and *Paraleishmania,* while an African origin is proposed for the subgenus *Leishmania* and possibly also to *Sauroleishmania*. The current distribution of *Leishmania* in the New World regions is explained as the product of multiple introductions of *Leishmania* parasites into Neotropics. Altogether, molecular trees, fossil records, historical events and biogeographical, entomological and ecological data need yet to be consolidated, supporting one hypothesis regarding the origin of *Leishmania* and the resulting human disease (Tuon *et al.*, 2008).

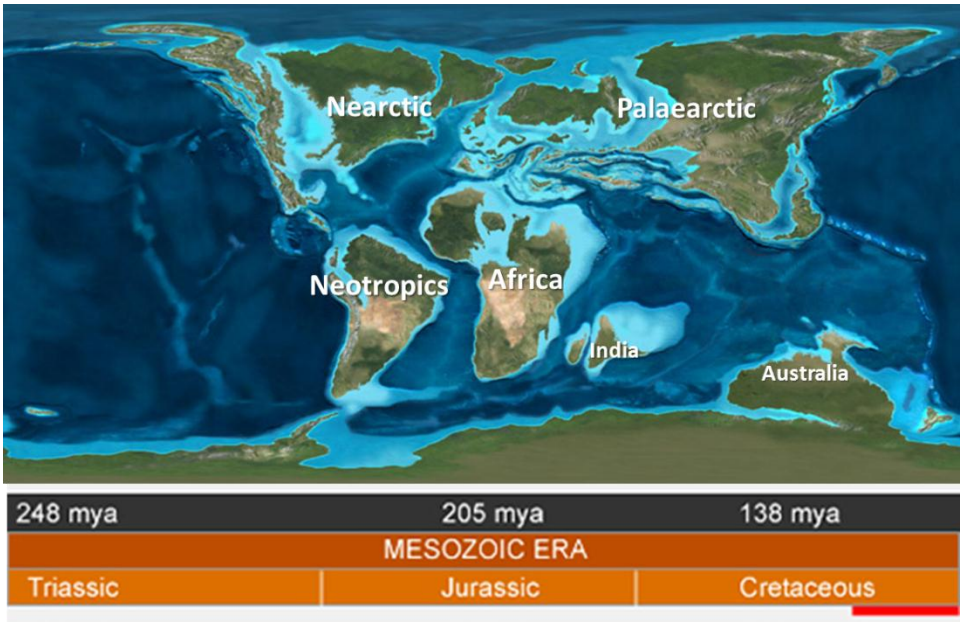


Figure 4: Map of the world in the late Cretaceous.
Red bar shows the isolation of the Neotropics from the Palaearctic. The land bridge of Panama did not reconnect the two continents until 3 million years ago in the Pliocene. Light blue indicate shallow seas that covered large parts of the Palaearctic and Nearctic during the Mesozoic and Cenozoic. (Adapted from Ron Blakey, NAU Geology, in http://cpgeosystems.com/globaltext2.html)

The most accepted standard procedure for the characterization and identification of strains of *Leishmania* is the multilocus enzyme electrophoresis (MLEE). This technique was firstly used in 1990 by Rioux and co-workers to classifying the *Leishmania* genus. However, the disadvantages of MLEE, as it is a very demanding and time consuming technique and has a moderated discriminatory power, have restricted its usage, being substituted by other methodologies for species discrimination using molecular markers. The development of new molecular techniques provided additional information for phylogenetic analyses. Several molecular markers have been described and used to investigate phylogenetic relationships within the entire genus. Some of these markers are: the internal transcribed spacer (ITS) 1 and 2 of the ribosomal DNA array (Cupolillo *et al.*, 1995, Dávila and Momen, 2000, Berzunza-Cruz *et al.*, 2002, Kuhls *et al.*, 2005, Villinski *et al.*, 2008), the repetitive DNA sequence (Piarroux *et al.*, 1995), the gene for the catalytic polypeptide of DNA polymerase α (Croan *et al.*, 1997), the gene encoding the largest subunit of RNA polymerase II (Croan and Ellis, 1996), the cytochrome oxidase II gene (Ibrahim and Barker, 2001), the glycoprotein 63 gene (Mauricio *et al.*, 2007), cysteine protease B genes (Hide *et al.*, 2008), the mini-exon (Sukmee *et al.*, 2008), 7SL RNA (Zelazny *et al.*, 2005), the cytochrome B gene

(Luyo-Acero *et al.*, 2004; Asato *et al.*, 2009). Also, multilocus microsatellite typing (MLMT) fragment analysis, coding and noncoding DNA sequences of nuclear or mitochondrial origin, random amplified polymorphic DNA (RAPD) and multilocus sequencing typing (MLST), which targets conserved genes, have been used (Lukes *et al.*, 2007). Most of these studies include only species of either the New or Old World, or isolates from one particular geographic region. Although, many molecular methods have been introduced to unravel the *Leishmania* taxonomy, defining *Leishmania* species or accepting all of the described species is still controversial. The application of numerical taxonomy and cladistics techniques for the analysis of electrophoretic data results in the current classification system (Table 1).

At present, about 30 species of *Leishmania* that infect mammals have been described. Species of both subgenera *Leishmania* and *Viannia* can be pathogenic to humans and produce diverse clinical manifestations. The species within the subgenera were established by MLEE (Rioux, *et al.*, 1990). The validity of the taxonomic classification scheme has been questioned several times, and the debate centers on the species status of *L. (V.) panamensis, L. (V.) peruviana, L. (L.) infantum, L. (L.) chagasi, L. (L.) archibaldi, L. (L.) garnhami, L. (L.) pifanoi* and *L. (V.) lainsoni* (Bañuls *et al.*, 2007). Cupolillo *et al.* (2000), by analogy with a similar division in *Trypanosoma,* proposed the separation of the genus *Leishmania* into two divisions, *Euleishmania* and *Paraleishmania*. The *Euleishmania* comprised the subgenera *Leishmania* and *Viannia* as described by Lainson and Shaw (1977) and the *Paraleishmania* included *L. hertigi, L. deanei, L. colombiensis, L. equatorensis, L. herreri* and the *Endotrypanum* strains. Using molecular criteria, the *Paraleishmania* can also be further sub-divided: one group formed by parasites of hystricomorph rodents (porcupines) which includes *L. hertigi* and *L. deanei* and the other group constituted by the remaining species which are principally parasites of sloths. *Endotrypanum* are unique among *Trypanosomatidae,* as they are able to infect erythrocytes in the mammalian host. Strains of *Endotrypanum* currently maintained in laboratory collections form a polyphyletic group within the *Paraleishmania*. Currently there are only two *Endotrypanum* species described, *E. schaudinni* and *E. monterogeii*. These species are included in *Paraleishmania* from which they are currently indistinguishable and the name *Endotrypanumis is* reserved for the true intraerythrocytic parasite of sloths (Momen and Cupolillo 2000). Also the

taxonomic position and phylogenetic relationship of lizard infecting *Leishmania* remains uncertain, as a number of lizards infecting parasites previously classified as *Leishmania* but apparently undergoing peripylarian development in sand flies, have been placed into the genus *Sauroleishmania*.

Table 1: Simplified classification of *Leishmania* species.
The genus *Leishmania* consists of the subgenera *Leishmania*, *Viannia* and *Sauroleishmania*, the latter corresponding to lizard parasites. These subgenera match the *Euleishmania* section. Species not classified in any of these subgenera were included in the *Paraleishmania* section. Table based on MLEE and according to Cupolillo *et al.* (2000), Schonian *et al.* (2010) and Laison, (2010). OW – Old World; NW – New World.

Division	Subgenus	Complex	Mammal species	Lizard species
Euleishmania	*Leishmania*	*L. donovani*	*L. donovani* (OW) *L. archibaldi* (OW) *L. infantum* (OW) [syn. *L. chagasi* (NW)]	
		L. tropica (OW)	*L. tropica* *L. killicki* *L. aethiopica*	
		L. major (OW)	*L. major* *L. gerbilli* *L. arabica* *L. turanica*	
		L. mexicana (NW)	*L. mexicana* (also *L. pifanoi)* *L. amazonensis* (syn. *L. garnhami)* *L. aristidesi* *L. venezuelensis* *L. forattinii* *L. enriettii*	
	Viannia	*L. braziliensis (NW)*	*L. braziliensis* *L. peruviana*	
		L. guyanensis (NW)	*L. Guyanensis* *L. panamensis* *L. shawi* *L. naiffi* *L. lainsoni* *L. lindenbergi* *L. utingensis*	
	Sauroleishmania			*L. tarentolae* *L. adleri*
Paraleishmania			*L. colombiensis* *L. equatorensis* *L. hertigi* *L. herreri* *L. deanei*	

1.2.4. Distribution and epidemiology

Leishmaniases are geographically widespread diseases with potential to increase their incidence worldwide. The intensification of travel and population migration, such as immunological naive populations in endemic areas and the movement of infected people into non-endemic countries may contribute to increasing leishmaniasis incidence worldwide. Global warming and other environmental factors may also be held into account as potential sources of increasing incidence (Lukes *et al.*, 2007; Aagaard-Hanse *et al.*, 2010). Leishmaniases are prevalent in 98 countries on the five continents. Approximately 1.3 million new cases occur annually, of which 300 000 are VL, mainly occurring in Bangladesh, Brazil, Ethiopia, India, Nepal, South Sudan and Sudan. Moreover, one million of CL are reported by Afghanistan, Algeria, Brazil, Colombia, the Islamic Republic of Iran, Pakistan, Peru, Saudi Arabia, the Syrian Arab Republic and Tunisia, and of MCL are occurring in Brazil, Peru and in the Plurinational State of Bolivia (WHO, 2015) (Figure 5). Although, as reporting is mandatory in only 33 out of 98 affected countries, the true dimension of leishmaniasis remains unknown.

Apart from the absence of consensus about the distinctiveness of some *Leishmania* species that causes disease in humans, a situation well described by the impasse in the classification of New World *L. chagasi* or the classification of *L. archibaldi* and *L. killicki*. Now is widely accepted that *L. chagasi* is a synonym of *L. infantum*, but still considered by some Latin American authors as two distinct species. Similar controversy is found in the classification of *L. archibaldi* and *L. killicki* as distinct from the closely related species *L. donovani* and *L. tropica*, respectively. From the 15 *Leishmania* species known to cause disease in humans 13 have a zoonotic nature (Table 2). In this case the reservoir of the parasite is sylvatic animals with habitats where humans are accidental hosts. Only two species of *Leishmania* are considered as having an exclusive or predominant anthroponotic transmission pattern, *L. donovani* (including *L. archibaldi*) and *L. tropica* (including *L. killicki*), meaning that it is mainly transmitted between people, who act as the reservoir hosts. Even for those species a presence of an animal reservoir has been indicated in several endemic settings. Several other *Leishmania* species have been found in animals but not in humans: *Leishmania gerbilli*, *Leishmania turanica* and *Leishmania arabica* from Old World rodents,

Leishmania equatoriensis from arboreal mammals in Ecuador and *Leishmania* sp. from red kangaroo (Gramiccia and Gradoni, 2005).

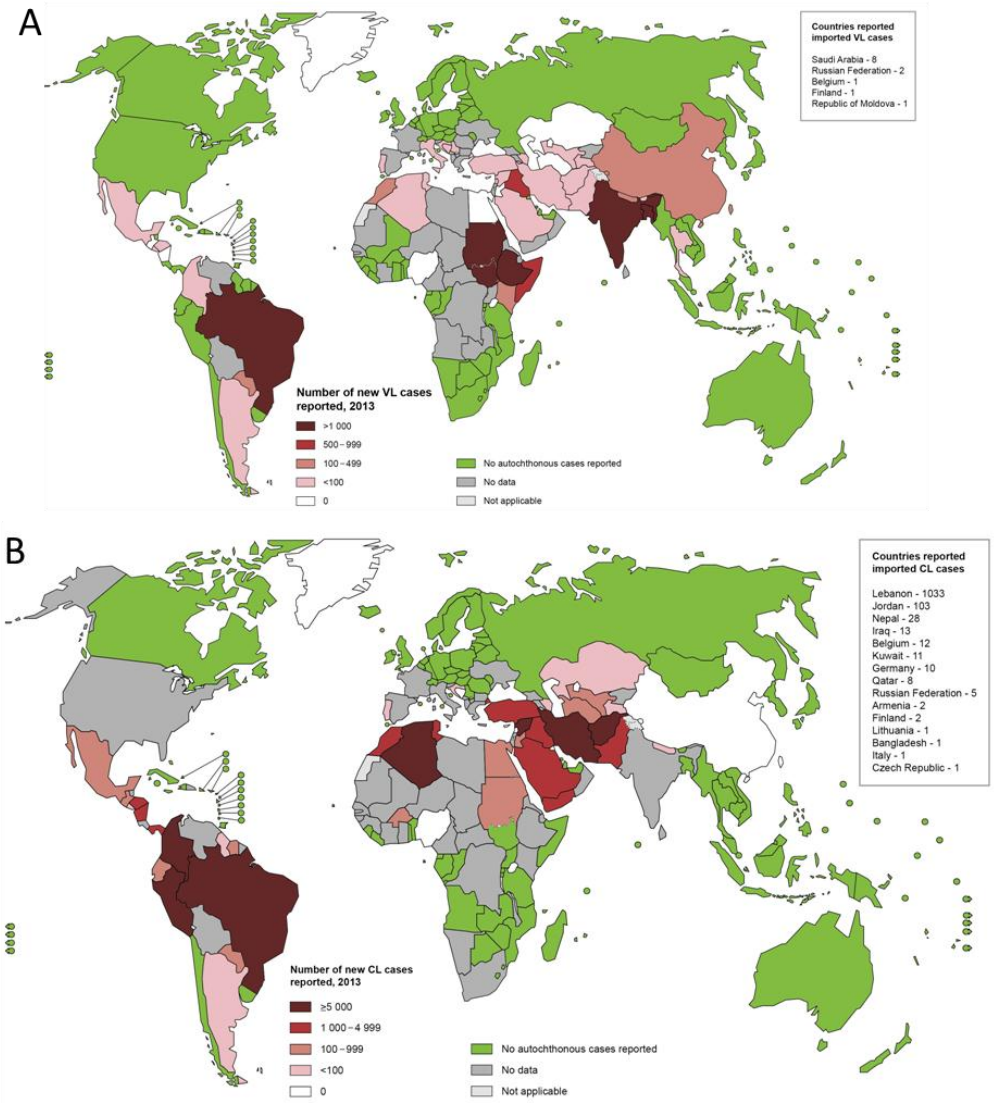


Figure 5: Leishmaniasis global distribution.
A) Global distribution of visceral leishmaniasis (VL); B) Global distribution of cutaneous leishmaniasis (CL) (WHO, 2013). Images available at:
http://gamapserver.who.int/mapLibrary/Files/Maps/Leishmaniasis_2013_VL.pngand
http://gamapserver.who.int/mapLibrary/Files/Maps/Leishmaniasis_2013_CL.png

Zoonotic visceral leishmaniasis (ZVL) is the most widespread zoonotic leishmaniasis caused by a single parasite species, *L. infantum*. It is a life threatening disease, as the parasite multiplies in the macrophages of the reticuloendothelial system, resulting in generalized signs and symptoms like fever, splenomegaly and pancytopenia. This disease occurs in the Mediterranean basin, several countries of Central and South America (especially in Brazil) and also in Asia. Sporadic cases of localized cutaneous

leishmaniasis (LCL) due to dermotropic *L. infantum* variants are also found in the same endemic areas. The vector in the New World is *L. longipalpis* but in the Old World several species are involved, mainly belonging to the genus *Phlebotomus* (*P. perniciosus, P. ariasi, P. neglectus* and others). In ZVL can be distinguished two types of reservoirs, the domestic reservoir mainly constituted by the domestic dog and the sylvatic reservoir mainly constituted by foxes, jackals and wolves. In the Mediterranean basin *L. infantum* asymptomatic infections are common in healthy populations and clinical disease is associated with age, being children under 2 years the most affected (Šiško-Kraljević *et al.*, 2013). Malnutrition and immunosuppression (e.g. HIV co-infection, immunosuppressive drugs and immunocompetence decline) are other risk factors of disease development (Gramiccia and Gradoni, 2005).

Cutaneous leishmaniasis (CL), also considered generally a zoonotic disease, is found in Africa, Asia and in most Latin American countries, caused by different *Leishmania* species. Depending on parasite species and of the competence of the host immune system, the clinical features of CL are localized nodules, ulcerative lesions that can self-healing multiple lesions or even disfiguring lesions. In the Old World, *L. major, L. aethiopica* and dermotropic *L. infantum* variants are the agents for CL. In the New World, there are several species causing CL namely: *L. braziliensis, L. guyanensis, L. lainsoni, L. naiffi, L. panamensis, L. peruviana, L. shawi, L. mexicana, L. amazonensis, L. venezuelensis* (Gramiccia and Gradoni, 2005). Rarely these infections may evolve toward a diffuse CL [*L. aethiopica* (OW), *L. ganhami* (NW) and *L. pifanoi* (NW)] with multiple, not ulcerating nodules distributed over large areas of the skin, or to MCL [*L. braziliensis* (NW) and *L. panamensis* (NW)] that progresses to severe ulceration of oronasopharyngeal mucosa and cartilages, causing disfiguring lesions.

Zoonotic cutaneous leishmaniasis (ZCL) is mainly caused by *L. major* and *L. aethiopica* and occurs in most part of the Central Asia, Middle East and North Africa. The transmission of the parasite through the vector is maintained by wild rodent (gerbil colonies). The risk of ZCL may be increased when agricultural projects are implemented and irrigation systems extended. These types of man-made ecological changes are accompanied by the intrusion of large numbers of non-immune immigrants in areas where leishmaniasis sylvatic cycle occurs (Dawit *et al.*, 2013).

Table 2: Co-relation of zoonotic *Leishmania* species, human disease, geographical distribution and the main reservoir
(Adapted from Gramiccia and Gradoni, 2005).

Leishmania species	Disease in humans	Geographical distribution	Main reservoir host
L. (L) infantum (Syn. *L. chagasi)*	Visceral leishmaniasis; Localised cutaneous leishmaniasis	Mediterranean basin; Middle East and Central Asia to Pakistan; China; Central and South America	Dog
L. (L) major	Localised cutaneous leishmaniasis	North Africa, Middle East and Central Asia; Subsaharian Africa and Sahel belt	Gerbillidae rodents various rodents
L. (L) aethiopica	Localised cutaneous leishmaniasis; Diffuse cutaneous leishmaniasis	Ethiopia, Kenya	Rock hyraxes
L. (L) mexicana	Localised cutaneous leishmaniasis	Central America	Various forest rodents
L. (L) amazonensis (Syn. *L.ganhami* and *L. pifanoi*	Localised cutaneous leishmaniasis; Diffuse cutaneous leishmaniasis	South America, north of the Amazon	Various forest rodents
L. (L) venezuelensis	Localised cutaneous leishmaniasis	Venezuela	unknown
L. (V) braziliensis	Localised cutaneous leishmaniasis; Mucocutaneous leishmaniasis	South America, Central America and Mexico	Numerous rain forest mammals (suspected)
L. (V) guyanensis	Localised cutaneous leishmaniasis	Guyanas, Brazil	Sloths
L. (V) lainsoni	Localised cutaneous leishmaniasis	Brazil, Bolivia, Peru	Rodents
L. (V) naiffi	Localised cutaneous leishmaniasis	Brazil, French Guyana, Ecuador, Peru	Armadillos
L. (V) panamensis	Localised cutaneous leishmaniasis; Mucocutaneous leishmaniasis	Central America, Colombia, Ecuador	Sloths
L. (V) peruviana	Localised cutaenous leishmaniasis	Peruvian Andes	Dog
L. (V) shawi	Localised cutaneous leishmaniasis	Brazil	Arboreal mammals (suspected)

Despite this widespread geographic distribution and the involvement of different rodent hosts, *L. major* appears genetically uniform by MLEE typing, even though that is a phonogram constructed by Rioux and coworkers (1990) shows some geographical subdivision. In the New World, CL are originally considered sylvatic zoonoses. Several species of *Leishmania* host's have shown a remarkable potential to adapt to environmental changes introduced by man and can show a synanthropic distribution, as seen in tropical rain forest, secondary forests or peri-urban areas. Urban leishmaniasis has been identified as an increasing problem in Latin American (Mott *et al.,* 1990). Zoonotic forms of the disease are influenced by human activities, mainly the migration from rural to urban areas, deforestation, environmental changes and ecological modifications that might have important consequences in the distribution of vectors and reservoirs and of the respective population size (Gil *et al.*, 2010). CL, once a typical rural disease, is spreading into urban and peri-urban areas of countries worldwide. Temporary population movement due to conflicts and famine can contribute to the generation of new epidemic foci of disease. Non-immune populations migrated into endemic areas and on their return imported the parasite into a previously non-endemic area. Other factors contributing to new foci of LV are the high prevalence of malnutrition and the lack of access to diagnostic as well as treatment facilities (Collin *et*

al., 2004). Displaced populations exposed to loss of assets and subsequent famine may face extremely high mortality.

Anthroponotic leishmaniasis may also occur in high densely populated urban settings, as in Afghanistan, Islamic Republic of Iran, Morocco and Syrian Arab Republic. *L. tropica* is the mainly anthroponotic species in the old world responsible for the anthroponotic cutaneous leishmaniasis (ACL) (Reithinger *et al.*, 2003). *L. donovani* is the major agent for the anthroponotic cutaneous leishmaniasis (ACL) in the old world and infects people of all ages. Also, anthroponotic visceral leishmaniasis (AVL) should be susceptible to elimination with rapid case detection and treatment combined with local vector control. However, one of the most important interventions may well be the socio economic development. *L. donovani* is only found in the Old World where is notoriously associated with AVL of rural poor populations living in the northeast of the Indian subcontinent and with AVL of displaced persons in East Africa (Ready, 2014).

Post-kala-azar dermal leishmaniasis (PKDL) may occur after recovery in some cases of VL caused by *L. donovani.* This syndrome is characterized by a maculopapular, macular or nodular rash around the mouth, which spreads for the entire body. In Africa, where PKLD is common, the disease usually occurs within 6 months of after VL treatment. PKDL patients are thought to be a reservoir for transmission of VL and may be difficult to treat, especially in some patients in East Africa who suffer from a severe form of PKDL. In South Asia, this syndrome is relatively rare, may occur several years after VL has been cured and required prolonged treatment. In India, PKLD is seen in 1-3% of successfully treated cases of VL. PKDL is characterized by lesions rich in parasites, which favors anthroponotic transmission in Africa as well as in India (Musa *et al.*, 2013).

In Brazil, Colombia and Venezuela, migration, urbanization and deforestation largely contributed to ZVL increases as a public health problem (WHO, 2002). In Brazil, a country that accounts for more than 90% of all ZVL cases reported worldwide, has registered an increase in the incidence of the disease in the last 40 years. During this period, a massive migration occurred from the rural areas in large cities. Migrants settled in the surroundings of the main cities, building at the edges of the cities, closing of forested area. This may have established a link between the urban areas and the

sylvatic cycles, bringing humans closer to vector breeding sites and facilitating vector adjustment to peridomestic habitats. Furthermore, the presence of dogs in the households may amplify the cycle of infection and disease (Costa *et al.*, 1990; Costa *et al.*, 1995).

In Europe there are only two transmission cycles with proven long-term endemism: ZVL and CL both caused by *L. infantum* throughout the Mediterranean region and, ACL caused by *L. tropica* now occurring sporadically in Greece (Ready, 2010). Also in Europe climatic changes, as global warming, maybe in the origin of *L. infantum* expansion to northern countries, where normally the vector could not survive to transmit the parasite due to the long duration of the lower temperatures. In Italy, a study made by Capelli and co-workers in 2004 indicates a spread of CanL, to non-endemic areas in the northern part of the country. These studies indicate that the parasite *L. infantum* is advancing, as well as its vector, into non-endemic areas, which poses a problem to veterinarian and public health authorities. There are also evidences indicating a northward expansion of the parasite, as in Germany and in France, where a novel CanL focus has been identified outside the traditional leishmaniasis endemic range (Bogdan *et al.*, 2001). The domestic dog is the only reservoir host of major veterinary importance and, in Europe, there is a large market for prophylactic drugs and treatment of CanL also caused by *L. infantum*. Domestic cats might be secondary reservoir hosts of *L. infantum* in southern Europe, because they are experimentally infectious to sand flies and natural infections can be associated with feline retroviruses (Maroli *et al.*, 2007).

Also HIV infection appears to increase susceptibility to VL affecting its epidemiology. In places where the access to antiretroviral therapy is limited, the prevalence of VL is rising. In northern Ethiopia, the rate of co-infected patients increased from 19% during 1998–1999 to 34% during 2006–2007 (Alvar *et al.*, 2008; WHO, 2013). Starting from the early 1990s, the impact of *Leishmania*-HIV co-infections was recognized as an alarming problem by international health authorities (Desjeux, 1996). Cases were reported from 35 countries world-wide, but most have been recorded in southwestern Europe namely France, Italy, Portugal (Campino *et al.*, 1994) and Spain, showing a deadly overlapping between HIV and ZVL. The strong influence of the HIV-associated immunosuppression on the virulence of *L. infantum*

infection did not occur for other agents of zoonotic leishmaniasis, such as *L. major* in tropical Africa (Guiguemdé *et al.*, 2003) or New World CL agents. However, by the end of the 1990s, several reports indicated that the *L. infantum*/HIV epidemic peak was over, fact that was attributable to the introduction of the highly active antiretroviral therapy (HAART). Currently, very few HIV-infected individuals with clinical VL are annually recorded in southern Europe. Records are mainly from AIDS patients unresponsive to HAART. Analogously, the feared *Leishmania*-HIV epidemic in endemic countries of Latin America such as Brazil, which was expected because of the increasing overlap of the two infections, has not occurred thanks to the free and universal distribution of antiretroviral drugs. At the same time the prevalence of both HIV and *L. infantum* infections have remained unchanged or even increased in overlap areas. Because the current benefic effect of HAART on the outcome of VL disease could be reversed by the emergence of HIV drug resistance, current surveillance activities on leishmanial infections in asymptomatic HIV-infected individuals should be continued (Gramiccia and Gradoni, 2005). On the other hand, VL is being reported with increasing frequency in transplant recipients, with several cases reported thus far. The most likely pathogenetic mechanisms of VL in transplant patients are either primary infection acquired by immunosuppressed subjects traveling in regions of endemicity or reactivation of a preexisting asymptomatic infection in the recipient, while acquisition through either an infected graft or blood transfusion probably represents an uncommon situation (Biglino *et al.*, 2009). Nevertheless, the risk of transfusion-transmitted leishmaniasis should not be completed dismissed as demonstrated in a study by Luz *et al.*, (1997), where a significantly higher seroprevalence was observed among multiply-transfused patients than among voluntary blood donors in regions of high endemicity, pointing out the need to control the blood transfusion donor and receptors.

As leishmaniasis is distributed through the world and with potential to increase, the development of new therapeutic targets and drugs and the development of affordable and effective vaccine, are the major challenges of controlling the dissemination of these parasite diseases. However, in parallel this has to be accompanied by the improvement of sanitary conditions on precarious population settlements.

1.2.5. Control strategies

1.2.5.1. Diagnosis

The clinical diagnosis of leishmaniasis is complex because its clinical features are common to others frequently occurring diseases in the same geographic area, such as malaria, typhoid and tuberculosis in case of VL or leprosy, fungal or bacterial infections, tuberculosis or syphilis in the case of CL and MCL, among many other diseases. Also, many of these diseases can be present along with VL, in cases of co-infection.

The natural sequestration of the parasite in the spleen, liver, bone marrow or lymph nodes difficult parasite isolation and further complicates the diagnostic. Laboratory diagnosis of leishmaniasis can be made by the following procedures: (i) demonstration of parasite presence in tissues of relevance by light microscopy with the examination of the stained specimen, followed by *in vitro* culture, or animal inoculation; (ii) detection of parasite DNA in tissue samples; (iii) immunodiagnosis by detection of parasite antigen in tissue, blood or urine samples or (iv) by specific detection of antileishmanial antibodies (immunoglobulins) (Sundar and Rai, 2002).

The commonly used method for VL diagnosis has been the identification of parasites in splenic or bone marrow aspirate. The confirmed presence of parasites in aspirate biopsy specimens or in the peripheral blood buffy coat attests for a positive diagnosis. However, the procedure to obtain a spleen aspirate may be life threatening due to the risk of internal hemorrhage. Besides the Giemsa and Leishman stain, *Leishmania* can also be detected in tissue samples using fluorescent antibodies specific to the surface receptors of the parasite. Fluorescein isothiocyanate isomer or rhodamide B isothiocyanate conjugated antiserum is usually used for this purpose. Fluorescent monoclonal antibodies are also used for identification of the parasite specie. Culture of parasites can improve the sensitivity of detection, but *Leishmania* culture in routine clinical practice is time consuming and an expensive procedure. However, cultures are required for an accurate diagnosis of the infection, as a supplement to other methods or to provide a diagnosis when routine methods have failed.

Leishmania strains can be maintained as promastigotes in artificial culture medium. The culture media used may be monophasic (Schneider's insect medium, M199, Glasgow medium, RPMI or Grace's insect medium) or diphasic (Novy-McNeal-Nicolle medium and Evans' modified Tobie's medium). Culture tubes are inoculated with bone marrow, lymph node or splenic aspirate and incubated at a temperature between 22º and 27°C. The tubes are followed weekly, during 4 weeks, by microscopy to detect promastigotes presence. After this period are discarded as negative. If promastigotes are present, they are maintained by weekly passage to fresh medium. Blood can also be used to isolate the parasite, from the cellular deposit after a centrifugation, but the method is slow and takes longer. In areas of endemicity of the disease, recognition of *Leishmania* species is not usually performed. However, identification of an organism to the species level is epidemiologically helpful and is also important in the treatment and prognosis of travelers, which usually are highly susceptible to the parasite and tend to develop unusual manifestations of the disease (Boelaert *et al.*, 2004). Due to the limitations inherent to the classic techniques used for detection of parasites and sometimes dependent on their isolation, new approaches for parasite detection have been attempted since the early 1980s.

The development of PCR has provided a powerful approach to the application of molecular biology techniques to the diagnosis of leishmaniasis. There are different target sequences used, which include ribosomal RNA genes, kinetoplast DNA (kDNA), miniexon-derived RNA (medRNA) and the β-tubulina gene region. Species level identification can be achieved by analysis of amplified kDNA using primers from conserved regions of different *Leishmania* species kDNA minicircles (Singh *et al.*, 1999). PCR analysis could be used to detect the presence of *Leishmania* in spleen, liver, lymph node and bone marrow aspirates, whole blood and buffy coat of suspected VL patients. Several other PCR based techniques had been developed in order to create more specific techniques, with potential to identify the parasite species and to be performed with whole blood from potential patients. A PCR–ELISA technique was developed and is based on the PCR amplification and labeling of target amplicons and their hybridization with target specific probes, capture of labeled hybrids into a microplate or tube and subsequent detection by immunoassay. The generated amplicons are usually labeled with digoxigenin molecules and are easy to detect using

anti-digoxigenin antibodies. This PCR-ELISA technique presents a high sensitivity inVL diagnosis using peripheral blood samples (Singh and Sivakumar, 2003). More recently, an evaluation of an oligochromatography-PCR for diagnosis of VL, CL and PKDL, showed a high sensitivity (> 95%) on lymph, blood and bone marrow samples from confirmed VL patients (Saad *et al.*, 2010). Another interesting approach is a rapid fluorogenic PCR technique. Wortmann *et al.*, (2001) used a fluorescent DNA probe for a conserved rRNA gene that is amplified using flanking primers with great sensitivity and specificity demonstrated in clinical samples. The real-time PCR has the advantage of being quantitative, which could be useful in the follow up of treatment, allowing the assessment of the parasite burden (Bossolasco *et al.*, 2003; Mary *et al.*, 2004). PCR could also prove to be an important tool in assessing the success of VL treatment. Studies using PCR analyzed VL patients who tested negative by PCR with lymph node tissue, none relapsed or developed PKDL. However, more than half of patients who tested positive by PCR with lymph node tissue either relapsed from VL or developed PKDL after apparent cure of disease following supervised treatment. Though, the role of PCR as defining technique to describe the patient status or the prognosis is still debated, as a substantial number of patients, who tested positive by PCR, after apparent cure, did not relapse or develop PKDL. These results elucidate the limitation of PCR in deciding the end point of treatment. The PCR positivity observed in these patients may be due to the presence of non-viable parasites. Similarly, PCR analysis performed on healthy inhabitants of endemic areas may result positive (Elmahallawy *et al.*, 2014), which may lead to the erroneous conclusion that they suffer from VL. In these cases, a combination of techniques may be helpful in defining the status of these patients, using direct agglutination test (DAT), which shows low titers in healthy endemic controls and PCR. However, PCR techniques remain complex and expensive and, in most leishmaniasis endemic countries, they are restricted to a few hospitals and research centers.

Several studies have demonstrated the presence of leishmanial antigens in the urine of VL patients. The antigen is detected quite early during the infection and the results of animal experiments suggest that the amount of detectable antigen tends to decline rapidly following therapy. It has been developed a latex agglutination test (KAtex, Kalon Biological, UK) for detecting leishmanial antigen in urine of patients

with VL has showing high specificity from 82% to 100%, but moderate sensitivity. This method is nowadays useful in the diagnosis of disease in cases where there is a deficient antibody production as in immunocompromised patients. In summary, the latex agglutination test is simple, easy to perform, inexpensive, rapid and can be used as a screening test. Because it promises to be a test of cure in populations of developing areas, efforts have been made to improve the performance of this technique (Srivastava *et al.*, 2011).

The human body makes an attempt to fight against VL by producing the highest levels of antibodies. This is due to polyclonal activation of the B cells, resulting in marked elevation of immunoglobulin M (IgM) and IgG against various nonspecific proteins. The consistent presence of high levels of antibodies against parasite antigens can simplify VL diagnosis. Several serological techniques are based on detection of these antibodies. Current serological tests are based on four principal formats: indirect fluorescent antibody (IFA), enzyme-linked immunosorbent assay (ELISA), western blot and direct agglutination test (DAT). The sensitivity depends upon the assay and its methodology, but the specificity depends on the antigen rather than the serological format used. IFA shows acceptable sensitivity (87%–100%) and specificity (77%–100%) (Boelaert *et al.*, 2004). Promastigote forms should be the antigen of choice for VL diagnosis of by the IFA because they minimize cross-reactivity with trypanosomal sera. However, the need for a sophisticate fluorescence microscope leaves the IFA test to the reference laboratories. DAT is based on direct specific agglutination of *Leishmania* promastigotes by serum anti-*Leishmania* antibodies. Trypsinized coomassie-stained promastigotes can be used either as a suspension or in freeze-dried form that can be stored at room temperature for at least two years, facilitating its use in the field (Oskam *et al.*, 1999). Although DAT showed a high degree of repeatability within the centers, its reproducibility across the centers was quite weak (WHO, 2014). Moreover, difficult field conditions, time consuming incubations, the fragility of aqueous antigen, the lack of cold chain and batch-to-batch variations in the antigen, along with the non-standardization of test readings, have severely limited its applicability in regions of endemicity. Two DAT procedures are available, the EasyDAT described in 2003 with the same sensitivity, specificity and durability as the traditional DAT antigen method, offering the additional advantage of cost reduction and

standardization (Gómez-Ochoa *et al.*, 2003) and the fast agglutination screening test (FAST) developed by Schoone *et al.*, (2001) with an incubation time reduced to only 3 h, mainly indicated for screening large populations. However, anti-*Leishmania* antibodies may persist for several years after treatment, leading to positive DAT titers in cured patients. Although DAT results should be carefully analyzed DAT remains the serological test of choice as well as the first antibody detection test for VL used in the field. The latex agglutination test (LAT) is one of the recently developed rapid diagnostic tests for detection of anti-*Leishmania* antibodies against the A2 protein derived from the amastigote form, as well as those against crude antigens derived from promastigotes of an Iranian strain of *L. infantum*. Both antigens were selected using dot blotting of positive and negative pooled sera and used to sensitize 0.9-µm latex beads. Tests were carried out in serum of human patients with VL (confirmed with parasitological isolation), health controls and patients with other non-leishmaniasis disease. Canine serum was also tested from dogs with confirmed CanL and healthy. Compared with the controls, human sera from DAT-confirmed patients yielded a sensitivity of 88.4% and specificity of 93.5% on A2-LAT (amastigote). A good degree of agreement was found between A2-LAT and DAT, with the advantage of LAT being a test much faster to complete (Ghatei *et al.*, 2009; Akhoundi *et al.*, 2013).

ELISA has been used as a potential serodiagnostic tool for almost all infectious diseases, including leishmaniasis. The technique is highly sensitive, but its specificity depends upon the antigen used. Several ELISA have been devolved using different antigens. The commonly used antigen is a crude soluble antigen (CSA). It is prepared by repeated freezing and thawing of a suspension of promastigotes in phosphate-buffered saline, followed by centrifugation. The supernatant is used as soluble antigen and is used to coat ELISA plates. The sensitivity of CSA-ELISA ranges from 80% to 100% and specificity from 84% to 95% (Ryan *et al.*, 2002). However, cross-reactivity with sera from patients with tuberculosis, trypanosomiasis and toxoplasmosis has been reported (Bray, 1976; Smrkovski and Larson, 1977). A cloned of a conserved portion of a *L. chagasi* kinesin-related protein used as a recombinant antigen (rK39) has been reported to be highly reactive to sera from human VL and CanL when run in an ELISA format (Burns *et al.*, 1993). Using this recombinant antigen, 99% specificity and sensitivity were reported in immunocompetent patients

with clinical VL. Because of the conditions prevailing in areas of endemicity, any sophisticated method cannot be employed on a wider scale. Other recombinant hydrophilic *L. chagasi* antigens rk9 and rk26 have been tested, leading to an increase in the list of available antigens for serodiagnosis of VL (Mohapatra *et al.*, 2010). Moreover, heat shock proteins HPS70 or histones proteins H2A, H2B, H3 and H4 may have potential use for VL serodiagnosis. Furthermore, when lipid-binding proteins (LBP) were used as antigens have shown high sensitivity and absence of cross-reactions with sera of patients with other diseases (Brito *et al.*, 2000). A rapid test, based on rK39 antigen, has been developed in the form of a ready-to-use immunochromatographic strip test. The recombinant antigen is immobilized on a nitrocellulose band membrane. However filed testing results show that the sensitivity of the test is not constant, as it can change with the geographical area of the test. These differences in sensitivity may be due to differences in the antibody responses observed in different ethnic groups and so it can be useful as preliminary diagnostic tool, but a confirmatory diagnosis is needed in the positive cases. It has been noted that all antibody detection tests share the same drawbacks; the antibodies remain positive for many months after the patient has been cured and do not differentiate between current and past infection. In endemic regions, asymptomatic infected people can also be positive in these tests (Elmahallawy *et al.*, 2014).

Immunoblotting, also known as western blot, can also be used to identify LV positive patients. For this type of testing, promastigotes are cultured, lysed and the proteins are separated on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins are electrotransferred onto a nitrocellulose membrane and incubated with the patient serum. The western blot technique provides detailed antibody responses to various leishmanial antigens (Brito *et al.*, 2000; Santos-Gomes *et al.*, 2000) and has been found to be more sensitive than the IFA test and ELISA, in particular for HIV patients with VL, but is time consuming and requires specialized equipment and technicians, thus limiting its use to research laboratories and not in diagnose.

Diagnosis confirmation of CL and MCL is usually performed in the laboratory by direct identification of amastigotes in Giemsa-stained lesion smears of biopsies, scrapings, or impression smears. Amastigotes are observed as round or oval bodies, 2–4

μm in diameter, with the characteristic nucleus and kinetoplast. As for CL diagnosis, different procedures are used to isolate the parasite from the lesions. This can be achieved by scraping the lesion, after application of anesthetic in order to collect material to microscope slides to stain and examine under optical microscopy. A tissue biopsy is also used to collect tissue for processing to microscope observation or for PCR detection. An aspirative biopsy performed with a needle in a syringe with phosphate saline buffer may also be used to isolate the parasite, as this exudate can be used to initiate a culture. Both tissue biopsy and needle aspirate can lead to the parasite species identification. Seroimmunological tests for detection of specific *anti-Leishmania* antibodies, as described above, can also be performed. However, these methods are not commonly used because antibodies tend to be undetectable or presented in low titers due to poor humoral response (Amaral *et al.*, 2000; Romero *et al.*, 2005). Although parasite isolation by culture and parasite DNA detection by PCR are sensitive methods are not currently used in developing countries.

In case of CanL it is also necessary to have tools that allow a correct diagnosis. Diagnosis of CanL is based on the presence of clinical signs together with positive specific antibody assay. Infection can be confirmed by demonstration the presence of amastigotes on stained slides or in cultures of biopsy aspirates of the bone marrow or lymph nodes or, even of healthy skin biopsy specimens. Diagnostic antibody tests available for veterinarian use include IFA, DAT and ELISA. These tests vary in sensitivity and specificity, and although they detected a specific humoral immune response, they do not prove or rule out active infection. These assays may give false-positive reactions with sera of dogs imported from endemic areas that have been vaccinated against *Leishmania* ssp. and in dogs infected by *Trypanosoma cruzi*, another protozoan that sometimes infects dogs in the Americas. However, the rK39 dipstick immunoassay does not cross-react with *T. cruzi* or *Babesia*. PCR and real time PCR assays are also available at few veterinary diagnostic laboratories and can be helpful in confirming infection in individual patients and as part of an overall control program (Solano-Gallego *et al.*, 2011).

1.2.5.2. Treatment

Leishmaniases are considered by the World Health Organization (WHO) one of the main neglected diseases in the world, affecting primarily the poor population in underdevelopment countries with great social-economic impact. However, the commercial interest from pharmaceutical companies in developing new compounds to treat leishmaniasis and other tropical neglect diseases is limited. These limitations arise from the fact that the treatments have to be affordable, to ensure their access from the affected poor populations.

None of the currently available drugs can be considered ideal due to several factors such as considerable toxicity, severe adverse reactions, long treatment duration or high costs. On the other hand, our understanding of *Leishmania* biology has not yet been translated into effective new drugs, with only a few new alternative drugs emerging in the past years (Menezes *et al.*, 2015).

Antimony compounds constitute the first line of treatment for all forms of leishmaniasis for more than 70 years. However, only in recent years its chemical structure and mechanism of action has been defined. Also in the recent years, a large-scale increase in clinical resistance to pentavalent antimonials has been reported, mainly in India, where 65% of patients treated for the first time failing to respond to treatment (Murray, 2005). The second line of drugs includes pentamidine and amphoterin B, but severe side effects combined with high costs, limit their use. Miltefosine (hexadecylphosphocholine), originally developed as an anticancer agent, is now approved and used as an oral drug for the VL treatment However, problems with costs and long half-life are being raised.

CL was usually treated with local therapy. At the end of the 19th century in Tashkent (Uzbekistan), pure lactic acid was applied to the lesions to cauterize it. Other cauterizing agents, including copper sulfate, old battery acid, plant extracts and heating of the lesions, were also applied (Olliaro and Bryceson, 1993). Antimony has been used as a drug for several centuries, but the modern use of it began only in 1905 when Plimmer and Thompson showed the activities of sodium and potassium tartrate against trypanosomes. This discovery leads to the subsequent use of this drug in the treatment of human trypanosomiasis in Africa. Then, the use of trivalent antimony was spread to

countries affected by leishmaniasis. However, later on this drug was found to be highly toxic as well as very unstable in a tropical climate, evidencing a suboptimal treatment in terms of clinical, resistance and relapses. The development of a less toxic pentavalent antimonial led to the synthesis of antimony gluconate (Solustibosan) and sodium stibogluconate (Pentostam) (Menezes *et al.*, 2015). Now a days the most commonly used organic compounds of antimony (Sb) are sodium stibogluconate and meglumine antimoniate. Pentavalent antimonial has been the most frequently used drug for VL and CL treatment despite their variable effectiveness. Antimonials can cause serious adverse effects, as it can be accumulated in tissues, causing severe cardiotoxicity, pancreatitis and nephrotoxicity that require hospitalization and close monitoring of patients. The adverse effects and the lengthy treatment period lead to treatment noncompliance and abandonment, favoring the emergence of resistant *Leishmania* in India. However, antimonial still are used as the first-line treatment in the endemic areas world-wide (Haldar *et al.*, 2011). Sensitivity of different *Leishmania* species to antimonials varies differently. For example Navin *et al.*, (1992) published a study were *L. brazilensis* showed to be more sensitive to sodium stibogluconate (Pentostam) than *L. mexicana*, highlighting the importance of a specific approach in the treatment of leishmaniasis. Resistance is associated with host factors such as decreased drug uptake, increased efflux mechanism, reduced concentration inside the parasite, inhibition of drug activation, inactivation of active drug and gene amplification. The role of ATP-Binding Cassette Transporters (ABC) transporters, such as P-glycoprotein and multidrug resistance (MDR)-related protein, in drug resistance was proven in laboratory isolates of *Leishmania* ssp. (Haldar *et al.*, 2011). However, its role in the field isolates is still not clear.

Amphotericin B is an antifungal agent extracted from the filamentous bacteria *Streptomyces nodusus*. It is used as the second line drug in patients with leishmaniasis. Amphotericin B formulations, such as lipid complex, colloidal form or the liposomal form, were developed to reduce adverse effects caused by the pure amphotericin B solution and improve pharmacokinetics and bioavailability. Despite proven less toxic, these alternative formulations of amphotericin B have limitations for use in developing countries: they have high costs and are unstable at higher temperatures, requiring cooling chain to be maintained. Although amphotericin B has been proven as a

successful therapeutic agent for patients with leishmaniasis, there are few reports indicating the emergence of resistance. Despite the mechanism of resistance of amphotericin B is not yet clear a resistance increase could be expected because of the high frequency of its use, mainly in India (Mohapatra, 2014). More recently, Purkait and coworkers (2012) demonstrated the generation of amphotericin B resistant parasites associated with a molecular mechanism that leads to changes in membrane composition, conducting to a decreased in amphotericin B binding and reduced uptake by the parasite. Also MDR1 gene in the ABC transporters of promastigotes seems to be responsible for the drug efflux mechanism and subsequently leads to resistance.

Pentamidine, other alternative drug currently used for leishmaniasis treatment, is highly toxic and triggers important adverse effects, such as diabetes mellitus, severe hypoglycemia, hypotension, myocarditis, renal toxicity and can, ultimately, cause death. Nowadays, due to the appearance of resistance cases, high toxicity and low efficacy, this drug is less used (Singh *et al.*, 2012a). Currently, pentamidine are mainly used in combined therapeutic protocols.

Paromomycin is a broad spectrum antibiotic extracted from the bacteria *Streptomyces rimosus* subsp. *paromomycinus*. It is active against several parasites and constitutes an alternative drug for leishmaniasis treatment. Low cost, fewer side-effects than other drugs, better efficacy and shorter duration of administration make this drug popular and thought to be a candidate for the first line therapy for VL patients. However, parenteral formulations still cause serious adverse reactions, including nephrotoxicity and hepatotoxicity and it is likely the acquisition of resistance in monotherapy. To combat these problems, newer formulations like paromomycin loaded with albumin microsphere and paromomycin with liposome formulation have been developed exhibiting better results (Wiwanitkit, 2012, Mohapatra, 2014).

Miltefosine (hexadecylphosphocholine) is the first oral drug for VL treatment and exhibits a high cure rate (Sundar *et al.*, 2002). It also had shown good results against antimony resistant VL and PKDL cases and in India, it is proposed to be the first-line drug in the kala-azar elimination program. The mechanism of action of this drug is not yet fully understood and because of anthroponotic transmission in the Indian subcontinent, the misuse of the drug and its longer half-life are the two factors that may

lead to a rapid acquisition of resistance to this drug (Bhandari *et al.*, 2012). More recently, there are reports of miltefosine resistant cases from Nepal (Pandey *et al.*, 2012). The proposed mechanism of *Leishmania* resistance to miltefosine was inactivation of genes responsible for drug uptake, but several other factors may be also responsible for the mechanism of resistance of miltefosine apart from point mutation.

Depending on the country, there are various protocols for CanL treatment, but the combination of antileishmanial drugs like meglumine antimoniate together with allopurinol, a well-known leishmaniostatical drug, is the most frequently chosen protocol. The duration of the treatment depends on the severity of the disease, individual tolerance to drugs and side effects, as well as clinical response. As in humans, there are also several side effects when these drugs are used in dogs. Meglumine antimoniate can induce nephrotoxicity and miltefosine can give origin to gastrointestinal problems. For each dog, it is important to determine CanL clinical stage before starting treatment in order to apply the most adequate treatment, potentiating a better prognosis. Nevertheless, parasitological cure is rarely achieved and relapses are common. Once leishmaniasis is diagnosed, dogs should have a close follow up after the first month of treatment, including a thorough physical examination, serum biochemical analysis and urinalysis. Subsequently, every six months, the patient should be reevaluated and serology against *Leishmania* repeated. If available, real-time PCR evaluation should also be done. Dogs in a more advanced disease stage should be evaluated every 1–2 months during treatment with particular emphasis on the analysis of functional parameters of the most susceptible organs, like kidneys and liver (Kaszak *et al.*, 2015).

The current available treatments for leishmaniasis present several challenges as they can have high toxicity and be used for a long term, causing severe adverse reactions. This often leads to treatment, abandonment and failure. In addition, many current antileishmanial drugs do not completely eliminate the parasites from infected individuals, leading to future relapses. Alternative protocols, such as the rational combination of drugs to combat the resistance developed in monotherapy, or combinations of immunotherapy and antileishmanial drugs are now being developed, as they can reduce individual doses, treatment duration and adverse effects (Menezes *et al.*, 2015). Recently, a trial was carried out in East Africa for the treatment of VL,

comparing a lower dose of paromomycin in association with sodium stibogluconate during 17 consecutive days and, with sodium stibogluconate in monotherapy for 30 consecutive days. The combination of paromomycin and sodium stibogluconate showed efficacy and safety similar to sodium stibogluconate, suggesting that this drug combination is suitability for VL treatment in East Africa, as it reduces the time needed for treatment (Musa *et al.*, 2012). Other studies are being conducted in order to evaluate the safety and efficiency of several antileishmanial drug combinations. Controlled release systems, such as liposomes and nanoparticles, provide a greater efficacy and safety, as drugs are more target driven, reducing the dose and adverse reactions of conventional formulations. Another strategy to improve antileishmanial treatment is the identification of new targets both in parasites and in host cells. Potential new targets for drugs have been identified in molecular and biochemical studies and some are being validated. Studies designed to better understand the biology of host-parasite interactions would facilitate the design of more effective drugs against *Leishmania* infection (Menezes *et al.*, 2015).

1.2.5.3. Reservoir and vector control

Leishmaniases are emerging diseases caused by a parasite with ability to adapt to changing environments, spreading into new geographical regions. Constant epidemics and the military importance of the disease, along with the lack of a human vaccine or chemoprophylaxis, have increased the interest in the biology and control of phlebotomine sand flies and also driven attention to the reservoirs, in particular to the VL peridomestic reservoir, the dog. The importance of CanL as a possible measure of human disease risk was demonstrated in Brazil, where a rise in the number of human cases was preceded by an increase in CanL prevalence (Miró *et al.*, 2008).

Primary available strategy to control the dissemination of leishmaniasis includes control of reservoir populations and vectors. A study by Kassi *et al.*, (2008) demonstrated that in order to reduce CL risk significantly a reservoir population should be eliminated in a 500 m radius of a protected area. However, the imposition of this kind of measures is questionable and other alternatives are proposed to control the vector population. The interventions selected would then depend on the incidence of disease in that particular area, the population being targeted, the reservoir, the particular

vector, the environment, the acceptability of the intervention and the availability of funds. Most of adult sand fly control techniques can be placed in the following categories: (i) application of long-lasting insecticides to surfaces, often interior walls, to either kill or repel sand flies or as space-sprays that kill the sand flies within a treated space, (ii) barriers and treated netting/clothing and use of topical repellents on the skin. Of these techniques, residual spraying of houses and animal shelters is probably the most useful and the most used, with several insecticides available for use. A study performed by Tatreault *et al.*, (2001) tested the susceptibility of four species of sand flies from the Middle East (*P. bergeroti, P. langeroni, P. papatasi* and *P. sergenti*) to various insecticides. This study indicated that DDT (an organochlorine) and malathion (an organophosphate) exhibited less sand fly-specific toxicity than the pyrethroid insecticides, including resmethrin and cyfluthrin. DDT was the first insecticide used on a large scale for control of sand flies. It was applied in large amounts in India, the former Soviet Union, Brazil and China, causing some reduction in sand fly populations. However, biological questions as whether the reduction in the number of insects has a direct impact on disease risk and on insect biodiversity were raised (Lane, 1991). The use of residual sprays is limited by several environmental factors, including high summer temperatures, strong radiation and the accumulation of dust. These conditions may reduce insecticide toxicity or efficacy. In Brazil, the interior and exterior applications of deltamethrin were accomplished by a significant reduction of sand fly populations inside the house, but the exterior spraying were ineffective. The long-term effect on morbidity due to leishmaniasis, however, was not assessed. Deltamethrin application in other geographic areas often had similar results (Falcão *et al.*, 1991).

In some ways, phlebotomine vector control is similar to that performed for malaria vectors. The exception is the larval control, as most larval control techniques used against mosquitoes, are inappropriate for sand fly because the aqueous habitat of the mosquito larva is different from the highly organic soil required by the sand fly immature stages. However, the use of *Bacillus sphaericus* for sand fly larval control is possible. In this innovative technique, bait-fed adult sand flies were used to carry the bacteria control agent to larval habitats in animal burrows, resulting in larval mortality (Robert *et al.*, 1997). Adult sand fly and mosquito vectors share characteristics that make control for one similar to the control for the other. In fact, some researchers

believe that a reason for leishmaniasis resurgence is a reduction in the use of insecticides for malaria control (Lane, 1991). Reductions in leishmaniasis were also noted with antimalarial programs that relied on interior residual sprays in Italy, Iran, Bangladesh and Peru (Alexander and Maroli, 2003).

Other alternative insecticides are the space sprays which do not leave significant residues in the habitat and have also been part of some sand fly control programs. These sprays can be used in the interior or exterior and are aimed at the insects present in the environment at spraying-time (usually the flying insects). Although the efficacy of this measure is still debated, as sand flies are very small insects and the insecticides used are short-lived, interior fogs are still a part of some vector control programs and can provide temporary relief from insect bites. It is also important to notice that the repellency and contact irritancy of space sprays makes more important protecting humans from exposure to vectors than is the lethal effect of the insecticide. A new class of chemicals with repellent properties, including neem oil, citronella, linalool and geraniol, has also been tested against sand flies showing diverse levels of effectiveness (Muller *et al.*, 2008). The inconsistent results obtained from interior residual sprays and the continued development of insect resistance to the insecticides of choice, including DDT, increase the importance of the use of insecticide-treated nets (ITN) as a measure of sand fly control. The ITN can provide considerable protection against the local indoor feeding sand flies. ITN are very attractive as they can be effective, relatively cheap and sustainable. In addition, the pyrethroid insecticides used to treat the nets have relatively low mammalian toxicity and good insecticide activity (Alexander and Maroli, 2003). Nevertheless, the results of large-scale ITN programs have been inconsistent as they are dependent on individual behave. Trials in Iran and Syria had disappointing results. However, in Sudan a significant VL reduction was observed following the mass distribution of fine-mesh ITN (Ritmeijer *et al.*, 2007).

Although certain wild animal species may be involved in ZVL epidemiology, domestic dogs appear to be the principal reservoir host of *L. infantum* throughout the world. Much of the focus on ZVL control is currently placed on these animals, particularly in ZVL screening, vaccination and protection with collars impregnated with insecticides such as deltamethrin. Killick-Kendrick *et al.*, (1997) were the first

demonstrating that such collars (Scalibor® Protector Bands) exerted a potent repellent and anti-feeding effect on *P. perniciosus*, killing up to 60% of the insects within 2 h of exposure. Collars also killed about 50% of sand flies exposed to protected dogs during the same period. Based on laboratory evaluations, it has been suggested that, at least in the Mediterranean and Middle East sub-regions, this measure could be expected to protect dogs from most sand fly bites, retaining a protective and killing effect for a complete biting season (Killick-Kendrick *et al.*, 1997). It has also been suggested that given the long-term effect of collars (up to 34 weeks), supplying them to the majority of dogs in a *L. infantum* focus would reduce contact between vectors and reservoirs sufficient to diminish the risk of infection for both dogs and humans.

In the absence of a human vaccine, disease prevention will continue to focus on vector control, amplified by other measures such as reservoir culling (measure yet not proved to be efficacious in the reduction of leishmaniasis incidence), serological screening and personal protection. The adaptability of the parasites and the vectors has permitted the disease to spread into new environments, including suburban and urban environments. In order to address the continued spread of this disease, increased efforts in the fields of urban entomology, civil engineering, disease surveillance and tropical urban ecology are required (Claborn, 2010). More focalized measures may require increased community participation and education in preventative measures against leishmaniasis. Improved information on sand fly biting behavior, resting and breeding sites would make delivery of existing compounds more efficient, resulting in lower costs of interventions, higher efficacy and fewer detrimental effects to the environment (Alexander and Maroli, 2003).

1.2.5.4. Vaccines

The possibility of achieving a vaccine for leishmaniasis is encouraged by the well described fact that most individuals, who have been infected with *Leishmania* and recover from the infection, become resistant to later re-infection. Also, the ancient practice of leishmanization can be seen as further proof of biological feasibility of vaccine development, at least for CL. Widely practiced in the Middle East and Central Asia, individuals, mainly the girls, was deliberately exposed to the parasite in a similar process to vaccination, using thorns or sharp instruments to introduce live parasites and

artificially simulate a cutaneous infection, in an unexposed site on the body to prevent the risk of facial lesions through natural infection. Leishmanization have been empirically developed and were in use in various countries (former Soviet Union, Central Asia, Israel and Iran), remaining the only group of human leishmaniasis vaccines to show real efficacy (Nagill and Kaur, 2011; Beaumier *et al.*, 2013). However, issues relating to the biological safety of such live vaccine were raised, mainly about dose control of and strain used as well as the difficulty of getting a standardized vaccine product. Together with the occurrence of persistent lesions, lead to the limitation of its use.

Despite different *Leishmania* species causing a range of clinical manifestations, genomic analysis indicates a large degree of sequence homology between species, suggesting it may be possible to generate broadly effective vaccines against different clinical diseases. Up to this time, several strategies have been followed in an attempt to mimic the effect of leishmanization in a safer way. Whole-killed (autoclaved) *Leishmania* promastigotes were also tested as vaccines against CL and VL. Killed *L. major* parasite as vaccines against CL with BCG as adjuvant, was tested in Iran and showed that this approach was considered safe with no evidence of an exacerbating response following natural infection. However, low in immunogenicity was reported being considered multiple doses or other adjuvants applications to increase its immunogenicity and achieve the goal of reduce the incidence of CL (Momeni *et al.*, 1999). Similar trials were conducted in Colombia with *L. amazonensis* with positive results. The administration of killed parasites was able to significantly increase *Leishmania*-antigen lymphocyte proliferation (Vélez *et al.*, 2000). However, concerns remain regarding the feasibility of developing killed, whole-parasite vaccines, including the variation in results obtained from different fields and clinical trial sites in the past and potential difficulties in producing such a product to good clinical manufacturing standards.

Various attenuated parasites have also been tested in animal models. These parasites are generally taken up by host cells in a similar way to virulent parasites and persist for some time without replicating. This allows the host immune system to respond to the parasite antigens. Radio-attenuated and biochemically altered parasites

without adjuvant have proven to confer good protection in mice and hamsters (Rivier *et al.*, 1999). Although concerns regarding back conversion to virulence make the latter option questionable for human use. Genetically modified *Leishmania* parasites lacking essential virulence genes, such as dyhydrofolate reductase, biopterin reductase or cysteine proteases, may overcome this problem and could produce attractive vaccine candidates against leishmaniasis (Veras *et al.*, 1999, Kumar and Engwerda, 2014). Development of drug-sensitive *Leishmania* mutants to be used as vaccines alone or with adjuvant has been proposed as a mechanism to induce anti-leishmanial immunity (Muyombwe *et al.*, 1997). Based on the large degree of sequence homology between species, it has also been proposed the use of non-human pathogenic *Leishmania* species like *L. tarantolae*, which can stimulate protection against virulent *L. donovani* strains (Breton *et al.*, 2005). However, the main problem with using killed or attenuated parasites are the concerns relating to safety and feasibility for large-scale use in the field.

The technological possibility to create recombinant proteins, conjugated to some degree of conservation between *Leishmania* species lead to several vaccine proposals either alone or combined with adjuvant or with bacteria/recombinant virus as a delivery vehicle. There have been significant efforts in recent time to identify recombinant antigens that can protect against *Leishmania* infection in experimental models. Some of these antigens include kinetoplastid membrane protein-11, sterol 24-c-methyltranferase, amastigote specific protein A2, cysteine proteinase B, promastigote surface antigen 2, nucleoside hydrolase and surface expressed glycoprotein gp63. Although most of these recombinant antigens have been tested in animal models for their immunogenicity and protective efficacy only a few have progressed to clinical trials in non-human primates, dogs or in preclinical human studies (Kumar *et al.*, 2010; Singh *et al.*, 2012b; Kumar and Engwerda, 2014). In recent years, liposomes have been developed as a delivery system for vaccine and as potent adjuvant. The gp63 *Leishmania* vaccine was analyzed in new studies with a liposomal formulation. It was authenticated that distearoyl-phosphatidylcholine (DSPC) liposome used with the immunodominant gp63 of *L. donovani* promastigotes functions as adjuvant and were able to promote significant protection against progressive VL in susceptible BALB/c mice. The follow up of the study demonstrated that the control of disease progression and parasitic burden in mice

vaccinated with gp63 in cationic DSPC liposomes was promoted IFN-γ production and down regulation of IL-4. In addition, $CD8^+$ T cell response was elicited by liposomic gp63, pointing to a long term protection (Bhowmick *et al.*, 2008). The first candidate of this type of vaccines to make it into phase I and II clinical trials was LEISH-F1 from The Infectious Disease Research Institute (IDRI, USA). Recombinant *Leishmania* polyprotein LEISH-F1 (formerly known as Leish-111f) is constituted by three recombinant proteins derived from *L. major* and conserved across various *Leishmania* species, including *L. donovani*, *L. chagasi* and *L. braziliensis*. The three proteins included in the vaccine are: *Leishmania* elongation initiation factor (LeIF), thiol-specific antioxidant (TSA) and *Leishmania major* stress-inducible protein 1 (LmSTI1) (Chakravarty *et al.*, 2011). LEISH-F1 was administrated with an adjuvant monophosphoryl lipid A (MPL) plus squalene (SE) in the final vaccine formulation LEISH-F1+MPL-SE. Phase I clinical trials indicate that the vaccine is immunogenic, safe and well tolerated in populations with and without leishmaniasis history. Additionally, LEISH-F1 vaccine also has demonstrated some therapeutic significance in patients with MCL (Nascimento *et al.*, 2010). Leish-111F/MPL-SE defined vaccine progressed to human phase-I and phase-II clinical trials in healthy volunteers from United States and Colombia were demonstrated safety and immunogenicity in healthy volunteers. Trials of the vaccine in subjects with CL in Brazil (Nascimento *et al.*, 2010) and ML in Peru (Llanos-Cuentas *et al.*, 2010) also showed that it was likewise well-tolerated. The last clinical trial showed that the LEISH-F1+MPL-SE vaccine is safe and immunogenic in healthy subjects with and without history of previous infection with *Leishmania donovani* in India (Chakravarty *et al.*, 2011). With the great preliminary successes of the LEISH-F1 vaccine, IDRI redesigned the vaccine taking their new construct Leish-110f (LEISH-F2) through phase I and phase II clinical trial. The new Leish-110f includes a construct without the histidine tag on the N-terminus, as well as the replacement of Lys274 with Gln, in an effort to overcome possible regulatory concerns and to facilitate the manufacturing process. Leish-110f was administrated with adjuvant natural (MPL-SE) or synthetic (EM005) agonists of Toll-like receptor 4 (Bertholet *et al.*, 2009). Positive findings related to immunogenicity indicate an immune response sufficient to control parasite multiplication upon primary infection and prevented disease. A newer LEISH-F3 vaccine, for use against VL, is in a

phase I trial of healthy adult volunteers in the USA. The LEISH-F3 vaccine is a fusion polypeptide made by linking in tandem two *Leishmania* proteins: residues 1-314 of the *Leishmania infantum/donovani* nonspecific nucleoside hydrolase (NH) protein and residues 2-353 of *Leishmania infantum* sterol 24-c-methyltransferase (SMT) protein (WHO, 2014). The LEISH-F3 vaccine is given with GLA-SE, a novel TLR-4-based adjuvant, and is compared to administration of LEISH-F3-MPL-SE adjuvant or just the vaccine. A second phase I trial is prewired to take place in India (https://clinicaltrials.gov/ct2/show/NCT01751048).

It is also believed that salivary proteins of sand fly vectors (*Phlebotomus* and *Lutzomyia* ssp.) have immunomodulatory properties, which may be good vaccine candidates. Salivary proteins, such as PpSP15, maxadilan, LJM17, LJM19 and LJM143, have been reported as potent immunogens, inducing lymphocytic infiltration with up-regulation of IFN-γ and IL-12 (Collin *et al.*, 2009). However, this protection might be only transient. A study demonstrated that this protection is time-dependent and limited to a short-term exposure to sand flies immediately before infection. These results explained the persistence of leishmaniasis in endemic areas and should be taken into account when designing anti-*Leishmania* vaccines based on sand fly saliva (Rohousová *et al.*, 2011).

DNA vaccines to prevent leishmaniasis are also undergoing development and testing. This approach has several advantages, such as low costs of production, stability of materials, sustained expression of relevant antigens and efficient generation of effector and memory immune responses. Immunization with naked DNA is a relatively new approach in which single or multiple genes encoding various antigens are cloned into a mammalian expression vector. The administration through simple intramuscular injection and multiple plasmids can be combined to yield multivalent vaccines. The mechanism by which DNA vaccination generates potent immune responses appears to be induced by the inherent adjuvant activity of non-methylated DNA sequences that stimulate innate responses, then by replication within the host leading to the *in situ* expression of the encoded proteins (Duthie and Reed, 2014). Several studies have examined the use of experimental DNA vaccines against several *Leishmania* species. Mice vaccination with DNA encoding gp63 or LACK, antigens already identified as highly immunogenic, elicited antigen-specific Th1 responses and reduced lesion

development when challenged with *L. major* (Gurunathan *et al.*, 1997; Walker *et al.*, 1998). A significant advance was described in a study by Guha *et al.* (2013) where the target antigen was the hemoglobin receptor (HbR) of the parasite. HbR is the receptor for hemoglobin endocytosis and also has hexokinase activity. It is expressed on the cell surface of the parasite and is conserved among different species. Using a DNA vaccine approach and tested it in an experimental model of VL the authors showed that HbR-DNA vaccination of mice stimulated the production of antigen-specific IgG2a antibodies and promoted the generation of antigen-specific T-cell responses that were able to produce multiple Th1-related cytokines simultaneously. In hamster model, vaccination with HbR-DNA protected animals from virulent *L. donovani* challenge, allowing them to survive the infection. As the results were obtained in the absence of adjuvant they are very encouraging. However, DNA vaccines have shown great promise in animal models, but have not yet proven their utility in humans. There have been no clinical trials beyond phase II to test DNA vaccines in humans. The major challenge for DNA vaccine candidates remains the demonstration of safety and efficacy in humans in both clinical trials and field settings (Kumar and Engwerda, 2014).

Another strategy for the development of a vaccine for VL is known as heterologous DNA-prime protein-boost (HPB) and has been used with several antigens such as ORFF, cysteine proteinases or gp63. These had also shown some success but are yet to reach the level of clinical trials. Ramiro *et al.* (2003) observed 60% protection in dogs immunized with a plasmid carrying the gene for the LACK antigen from *Leishmania infantum* (LACK-DNA), followed by a second immunization with the recombinant vaccinia virus (rVV) containing the same gene (rVV-LACK), followed by *L. infantum* challenge. Since the immune response in a canine model differs significantly from murine and human hosts Dondji *et al.* (2005) and Tewary *et al.* (2005) conducted similar studies using the murine model for VL and found comparable levels of protection. The success of vaccine development depends upon understanding the immunobiology of pathogen/host interactions, selection of appropriate vaccine candidates and choosing the right adjuvant or delivery vehicle. In addition, the vaccine must be able to generate long-lasting immunity, be efficient and safe as well as affordable. The development of a vaccine against leishmaniasis has been slow, but the

increasing knowledge gained in recent years is opening the way for renewed efforts to create and test new vaccines for preventing and or treat leishmaniasis.

The development of an effective vaccine for CanL represents a cost-effective tool for interrupting the transmission cycle and controlling ZVL in the human populations. Canines, particularly domestic dogs, are the main reservoir for VL species and are considered the main source of zoonotic transmission to humans. As previously discussed, treatment of CanL has low efficacy with drugs successfully used in human VL treatment, rarely achieving a cure state (Baneth and Shaw, 2002). Prevention has been, so far, the best way to prevent CanL transmission with the use of insecticide impregnated collars in domestic dogs to reduce the risk of contracting CanL. The culling of seropositive dogs has long been recommended in Brazil, but had not led to a reduction in the number of human VL cases and may be of limited value (Courtenay *et al.*, 2002). Consequently, the development of vaccines against CanL is an attractive approach to controlling infection in dogs, reducing the parasite reservoir and consequently diminishing the risk of parasite transmission to human populations. Immunological characterization of CanL reveals cellular and humoral immune responses similar to human infection, including immune deregulation and increased IL-10, associated with disease manifestation and progression. Disease resistance is linked to strong Th1 immune responses, including IFN-γ expression. Therefore, like in human VL vaccine, an effective CanL vaccine needs to induce strong and long lasting cell mediated immunity. Adjuvant choice must be carefully considered for CanL interventions, as live BCG is not an appropriate adjuvant for commercial application due to safety problems in dogs and the identification of appropriate and effective adjuvants are essential for safe and effective CanL vaccines (Poot *et al.*, 2009). In addition to adjuvant and also similar to the research to obtain a human VL vaccine, sand fly salivary components are being considered for inclusion in CanL vaccine. Evaluation of a killed *Leishmania* vaccine containing sand fly saliva extract indicated that the vaccine is highly immunogenic and provided support for further development of saliva components as candidates for incorporation in an anti-VL vaccine (Giunchetti *et al.*, 2007; Giunchetti *et al.*, 2008). This is supported by vaccination studies using the hamster VL model, showing that the salivary protein LJM19 was able to protect hamsters from fatal infection with *L. infantum* (Gomes *et al.*, 2008). Currently, there are

two commercially available CanVL vaccines, Leishmune® and Leish-tec® in Brazil. But the search for new vaccines continues under development, including recombinant antigen vaccines and both live and killed whole-cell vaccines. The Leishmune® vaccine produced by Fort Dodge Animal Health was the first commercially licensed vaccine for CanL. This vaccine consists of the fucose mannose ligand (FML) isolated from *L. donovani* plus a saponin adjuvant. It corresponds to a surface antigen present throughout the parasite life cycle, and antibodies raised in vaccinated dogs prevented the binding of procyclic promastigotes to the sand fly midgut (Saraiva *et al.*, 2006). This vaccine induced a significant and strong protective effect during phase III trials in dogs living in ZVL endemic area in Brazil, with a protective efficacy as high as 80%. This protection lasted up to 3.5 years following vaccination, indicating the induction of a long-lasting immunity (da Silva *et al.*, 2000; Borja-Cabrera *et al.*, 2002). Since Leishmune® vaccinated dogs showed a complete absence of parasites, they are noninfectious and this contributes to the breakdown of the zoonotic transmission cycle. During phase III trials of Leishmune® there was a concomitant reduction in human ZVL cases in districts where dogs were vaccinated, indicating that Leishmune® vaccination interrupts parasite transmission to humans (Silva *et al.*, 2000). A decrease in the incidence of human and canine visceral leishmaniasis was registered in endemic areas of Brazil after dog's vaccination (Palatnik-de-Sousa *et al.*, 2009). Thus, Leishmune® acts as a transmission blocking vaccine by clearing parasites from the animal reservoir and preventing the survival of the parasite in the sand fly vector. Currently, this prophylactic vaccine is recommended for healthy, non-infected dogs. Nevertheless, studies performed showed that Leishmune® is also effective as a therapeutic vaccine for naturally infected dogs, particularly when given in combination with leishmanicidal drugs (Borja-Cabrera *et al.*, 2004). An amastigote specific antigen, the recombinant A2 antigen (rA2) constitutes the base of the vaccine Leish-Tec®. This vaccine applied with saponin as adjuvant could induce type 1 immune response. The first clinical trial performed showed that vaccinated animals produced significantly increased levels of total IgG and IgG2 anti-A2 antibodies and remained negative in conventional leishmaniasis serodiagnostic methods. A significantly increased of IFN-γ and low IL-10 levels were detected in vaccinated animals before and after challenge, as compared to control animals. Immunization with rA2 was immunogenic and conferred partial protection to dogs,

allowing the serological differentiation between vaccinated and infected animals, an important requirement for a CanL vaccine (Fernandes *et al.*, 2008). Leish-Tec® is now commercially available and there are no significant differences in the rates of seroconversion, clinical signs or parasite transmission to the vector between dogs immunized with Leishmune® or Leish-Tec® (Fernandes *et al.*, 2014). Together, these studies indicate that rA2 is an important CanL vaccine and future studies should report the impact of this intervention on both canine and human VL infections. As many of the clinical and immunological features of CanL are similar to those observed in human VL, experimental challenge in dogs represents a useful system for evaluating the efficacy of vaccine candidates. The Leish-111f + MPL-SE vaccine, discussed above, is a leading vaccine candidate for human VL that also has shown therapeutic efficacy in CanL trials (Trigo *et al.*, 2010). A third CanL vaccine has been recently licensed in Europe, the CaniLeish®, from Virbac Animal Health. This vaccine is constituted by excreted/secreted antigens purified from the culture supernatant of *L. infantum* promastigotes (LiESAP) with muramyl dipeptide (MDP) as adjuvant (LiESP/QA-21). In the field conducted trials, dogs were kept outdoors, exposed to sand flies in an endemic region of the south of France. Vaccinated dogs' antibody reactivity to the antigen by immunofluorescence antibody (IFA) test showed a strong reaction after two seasons of sand flies activity. Moreover, strong vaccine efficacy (92%) was seen based on leishmanial DNA and parasite detection in bone marrow aspirate. Besides the *in vitro* study of T cell immune response showed high IFN-γ production in vaccinated dogs in comparison to the control ones (Moreno *et al.*, 2014). Other recombinant proteins are also being tested for a potential CanL vaccine and also the use of live attenuated parasite vaccines continues under exploration. However, the elimination of human VL will be difficult to achieve in the presence of persisting animal reservoirs and veterinary intervention is an important tool for reducing the global burden of this disease. The identification of measurable and reliable biomarkers of immunogenicity and protection induced by CanL vaccines may also be informative for human VL vaccine efforts.

1.3. Immunology of leishmaniasis

Understanding the immunological events that result in failure or successful control of the infection by *Leishmania* parasites is fundamental for the design and evaluation of vaccines and therapies against leishmaniasis, both in humans, canine or experimental models of the disease. Control of *Leishmania* infection and disease progression has long been associated with generation of T helper (Th) 1 and Th2 responses respectively. Although the Th1/Th2 paradigm is valid in several aspects, Th2 polarization has never explained the severity of human disease and a number of other T cell subsets, including the generation of a regulatory T and Th17 cell response is not yet completed understood. Thus, all efforts to better understand the complex mechanisms behind the immune response generated by the host to *Leishmania* infection are of high importance, as it can lead to better strategies to control the infection.

1.3.1. Host-parasite interaction in canine leishmaniasis

Wild canines and domestic dogs are the main reservoirs of ZVL caused by *L. infantum* in the Mediterranean area, Middle-East, Asian countries and Latin America. The role of dogs as the main reservoir of ZVL has led to an increased interest in studying the immune response and finding *Leishmania* antigens implicated in protective cellular immunity. CanL is a multisystemic disease with variable clinical signs. Lymphadenomegaly, splenomegaly and hepatomegaly, weight loss, skin ulcers, alopecia, onychogryphosis, among other symptoms can be detected in *Leishmania* infected dogs. Typically it is possible to identify the presence of *Leishmania* amastigotes inside macrophages in the dog's skin, spleen and liver, in a granulomatous inflammatory formation. A fraction of infected dogs develops symptomatic infection resulting in death, while others remain asymptomatic or develop one or more mild symptoms, classified as oligosymptomatic (Ciaramella *et al.*, 1997). There are several evidences pointing to the importance of dog's genetics in susceptibility or resistance to CanL. Studies performed on genetics of human VL have identified several genes involved in disease susceptibility. In the endemic area of VL in Sudan, where the infection is caused by *L. donovani,* 90% of people involved in the study showed immunological evidence of the infection, but only 30% developed human VL. Candidate gene to VL resistance revealed linkage to a polymorphism in the natural

resistance associated macrophage protein 1 (*NRAMP1*) gene, later named *Slc11a1* gene (Bucheton *et al.*, 2003). The *Slc11a1* gene encodes a proton divalent cation antiporter with a role in regulating macrophage function, including upregulation of chemokine and cytokine genes, such as TNF and interkeukin-1β (IL-1β), as well as induction of nitric oxide synthase which are linked to anti-leishmanial immune mechanisms (Blackwell *et al.*, 2001). Epidemiological studies also suggested a role for intrinsic factors in CanL resistance. Two genes have been identified and implicated in susceptibility to the disease. Quinnell *et al.* (2003) performed a study in a cohort of Brazilian mongrel dogs naturally infected with *L. infantum* to investigate the genetic variation of class II molecules of major histocompatibility complex (MHC II), termed 'the dog leukocyte antigen (DLA) system'. It has detected a significant association between the presence of the DLA–DRB1*01502 allele and susceptibility to CanL, indicating that DLA-DRB1 locus plays a major role in determining susceptibility. In another study performed in Spain, haplotypes of the canine *Slc11a1* gene were found associated with CanL susceptibility (Altet *et al.*, 2002). Single nucleotide polymorphism (SNP) mutations in the promoter region of *Slc11a1* gene were also described and associated with susceptibility to human VL and CanL. The presence of haplotype TAG-8-141 has been related to the boxers' predisposing to CanL (Sanchez-Robert *et al.*, 2005). Despite these findings associate genetics with susceptibility to CanL, the individual dog's natural resistance to *Leishmania* infection is probably determined by a more complex multigenic puzzle. Nevertheless, susceptibility to developing the disease is also influenced by non-genetic extrinsic factors such as nutritional status, concomitant infections and parasitism, parasite virulence and previous exposure to CanL (Baneth *et al.*, 2008).

A wide spectrum of innate and acquired immune responses is orchestrated by the canine host when facing a *L. infantum* infection. The two opposite poles of this spectrum are the protective immunity mediated by T cell activation and the susceptibility to the disease marked by the non-protective humoral immune response. It has long been known that dogs that remaining asymptomatic, exhibiting some resistance to infection progression were able to activate cell mediated immune response, presenting lower antibody titers to leishmanial antigens. However, symptomatic or susceptible animals failed to activate a cell mediated immune response, both *in vitro*

and *in vivo*, showing considerably higher serum antibodies to leishmanial antigens, which are not immunoprotective. It is also widely accepted that macrophages play a central role in controlling *Leishmania* infection. The main effector mechanism involved in the protective immune response of dogs infected with *L. infantum* is the activation of macrophages by IFN-γ, IL-2 and TNF-α to kill intracellular amastigotes via the L-arginine nitric oxide (NO) pathway. The pathway directing the production of this effector molecule has been identified as the main principal leishmanicidal mechanism present in macrophages and capable of intracellular killing of *Leishmania* amastigotes (Holzmuller *et al.*, 2006). The emergence of the Th1/Th2 paradigm as opposing cytokine responses in the control of *Leishmania* infections was established by observations on experimental *L. major* infections of resistant mice. These mice develop a T cell response dominated by a $CD4^+$ Th1 phenotype characterized by IFN-γ secretion, while the susceptible mice developed a dominant response with activation of $CD4^+$ Th2 phenotype characterized by production of IL-4 and IL-5. Correlation between a polarized immune response and the infection outcome led to the concept that the balance of Th1/ Th2 immune response determines disease progression or by the contrary, infection control (Scott *et al.*, 1988). Further studies on the immune response generated by resistant C57BL/6 and C3H/HeN mice or susceptible BALB/c mice to *L. major* infection revealed two distinct patterns. Infection of C3H/HeN mice was dominated by IL-12 production and $CD4^+$ Th1 immune response accompanied by high levels of IFN-γ secreted by natural killer (NK) cells and no IL-4 (Scharton and Scott, 1993). In contrast, progressive disease in susceptible BALB/c mice was characterized by the early IL-4 production, absence of IL-12 and a polarized Th2 response. Confirmation that the early IL-4 synthesis drives this Th2 response came from experiments performed in BALB/c mice genetically engineered to be IL-4 deficient and in mice treated with anti-IL-4 antibody, demonstrating that without IL-4 secretion these mice can heal *L. major* infection (Kopf *et al.*, 1996). Julia *et al.* (1996) demonstrated that the cellular origin of IL-4 in BALB/c mice is confined to an oligoclonal $CD4^+$ T cell population with a Vβ4Vα8 T cell receptor which recognizes the *Leishmania* homologue of the receptor for activated C kinase (LACK). Several other experiments lead to the conclusion of the critical importance of IL-12 in mediating a Th1 response and, therefore the resistance to *Leishmania* infection. IL-12 depletion increases

susceptibility of naturally resistant mice and, by the contrary, the conversion of susceptible BALB/c mice to a resistant phenotype is achieved after treatment with IL-12 (Heinzel *et al.*, 1993). It is now known that dendritic cells are the source of IL-12 production, but the antigens responsible for triggering an IL-12 response and the exact mechanisms are not defined. Among other cytokines, the predominant expansion of IFN-γ producing-Th1 cells, which induces the synthesis of nitric oxide directed by the induced nitric oxide synthetase (iNOS), activates the microbicidal function of parasitized macrophages. In resistant C57BL/6 mice infected with a low dose of *L. major*, a clear role for $CD8^+$ T cells in primary immunity is defined. Nevertheless, parasites persist even in resistant mice. Using this low-dose model, it was demonstrated that IL-10 played an essential role in parasite persistence. The sterile parasite cure was only achieved in IL-10 deficient and IL-4/IL-10 double deficient mice, demonstrating the requirement for IL-10 in establishing latency (Belkaid *et al.*, 2001). A key study determined that an endogenous, naturally occurring population of $CD4^+CD25^+$ regulatory T cells (Treg) expressing high CTLA-4 is the source of IL-10, controlling *L. major* persistence and immunity in C57BL/6 mice (Belkaid *et al.*, 2002). Treg constitute 5–10% of $CD4^+$ T cells in normal mice and humans, developing in the thymus where, following high-affinity recognition of self-peptides, they up regulate the transcription factor FoxP3 and the expression of the cell surface marker CD25, essential for their survival and a constitutive marker of Treg in the periphery. Two distinct sub-populations of Treg have been described, naturally occurring Treg involved in the maintenance of peripheral tolerance and antigen-specific T regulatory cells (Tr1) that encounter pathogen derived foreign antigen in the periphery (Roberts, 2006).

Th1/Th2 dichotomy is well characterized in cutaneous leishmaniasis and in the murine model. In contrast, humans VL, CanL and experimentally infected rodents do not show a clear Th1/Th2 dichotomy pattern. Particularly in dogs, infection outcome is highly variable. It is now widely accepted that protective immunity against *Leishmania* parasites is mediated by $CD4^+$ Th1 cell immune response. The balance between *L. infantum* induced mixed Th1 and Th2 responses accounts for parasite replication control, disease progression or clinical cure (Baneth *et al.*, 2008) (Figure 6).

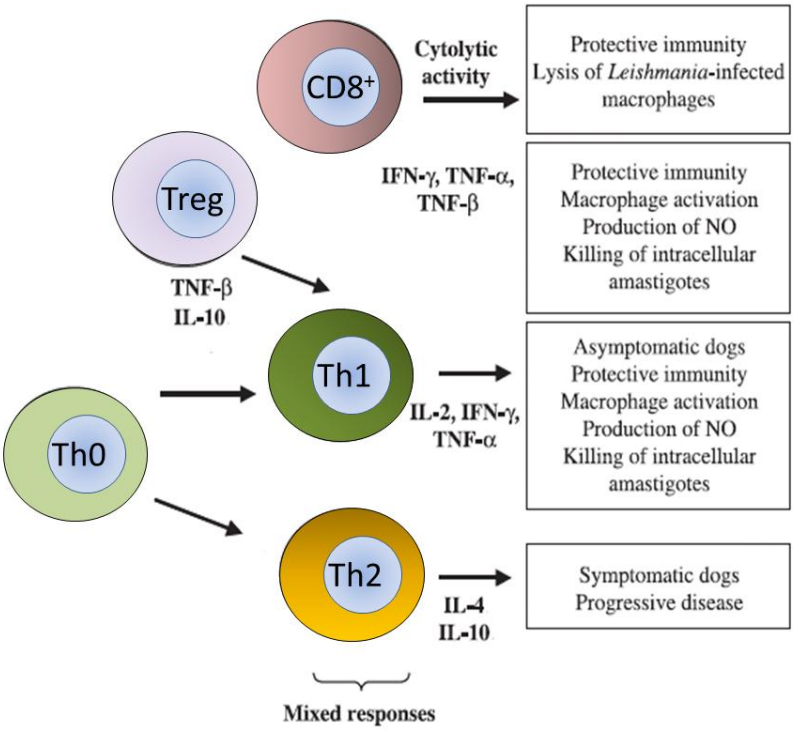


Figure 6: Schematic representation of the immune response generated by dogs to *L. infantum* infection.

Resistance to CanL has been associated with the differentiation of IFN-γ, IL-2 and TNF-α producing Th1 cells, leading to the activation of macrophages and NO production to kill intracellular amastigotes. Th2 response may lead to the polyclonal proliferation of B cells and the production of non-protective antibodies, leading to disease progression. Mix Th1/Th2 immune responses may also occur. T regulatory (Treg) cells constitute a natural occurring population of T cells with regulatory properties (Adapted from Barbiéri, 2006).

Define the profile of cytokines expressed in CanL is a complex task because the broad spectrum of clinical manifestations, the different tissues analyzed and the differences between experimental and natural infection associated with the limited number of studies reported. Longitudinal experimental CanL infection studies have mostly indicated a mixed Th1 and Th2 pattern. In a follow up study Santos-Gomes *et al.* (2002) characterized the cytokine profile of *L. infantum* experimentally infected dogs. The animals showed an initial asymptomatic phase with an absence of or low expression of cytokines during a period of silent establishment of the parasite followed by a short-term production of Th1-type cytokines before the appearance of clinical

signs. In a third phase, animals presented clinical signs and reduced expression of cytokines. During these three phases, animals maintained specific humoral immune responses, immunosuppression of specific lymphocyte proliferation and the capacity to transmit the parasite, since its presence can be detected in the skin. The cellular basis and mechanisms for the development of T cell unresponsiveness in CanL are not fully understood. The majority of infected dogs are likely to develop specific cell mediated immunity expressed as proliferation of lymphocytes stimulated *in vitro* by *Leishmania* antigen or *in vivo* by a positive skin test early in infection. However, as the disease progresses in susceptible dogs, these responses diminish. During a longitudinal follow-up of experimentally infected dogs, blood parasite load and *Leishmania* specific cell mediated immunity were shown to be inversely correlated (Rodríguez-Cortés, *et al.*, 2007). It has been postulated that cell mediated immunity unresponsiveness in progressive disease is due to the decrease in peripheral $CD4^+$ T cell numbers or the decreasing expression of co-stimulatory molecules, accompanied by the unresponsiveness of the remains cells and unprotective humoral responses which allow the dissemination and multiplication of *L. infantum* in different tissues. CanL is frequently associated with a marked humoral response, with production of high antibody titers, which is not protective and points to failure in the control of infection. The levels of *Leishmania* specific immunoglobulins of symptomatic dogs are greater than those found in infected, but asymptomatic dogs and a clear association was shown between these levels, the clinical status and tissue parasite density (Reis *et al.*, 2006). The levels of canine IgG1 and IgG2 subclasses have been extensively studied in an attempt to establish a correlation between the subclass levels, the type of Th immune response and the clinical outcome of infection. A direct correlation between high levels of IgG1 anti-*Leishmania* antibody and the appearance of clinical symptoms was demonstrated in *L. infantum* infected dogs, while IgG2 antibodies were associated with asymptomatic infection (Nieto, *et al.*, 1999). However, these results were not corroborated by other studies in which dogs with positive DTH skin reactions exhibited a polymorphic humoral immune response ranging from seronegative to positive levels of IgG1 or IgG2 (Bourdoiseau, *et al.*, 1997). Studies evaluating IgG1 and IgG2 using polyclonal antibodies frequently have reached conflicting outcomes, possibly because of a lack of specificity of commercial reagents. Furthermore, studies employing

monoclonal antibodies to detect canine IgG1 and IgG2 have shown a steady increase in the production of both subclasses during natural and experimental CanL without indicating a practical use for these levels (Fujiwara, *et al.*, 2005). Apart from the direct action of cytokines and immunoglobulins, the involvement of $CD8^+$ lymphocytes in resistance to canine VL, has also been studied. These lymphocytes were detected in asymptomatic dogs experimentally infected with *L. infantum*, but not in symptomatic animals, suggesting that direct lysis of infected macrophages by cytotoxic T lymphocytes represents an additional effector mechanism in resistance to CanL (Pinelli *et al.*, 1995). In dogs naturally infected with *L. infantum*, a reduction in both $CD4^+$ and $CD8^+$ populations was observed. These populations were partially restored after drug treatment (Bourdoiseau *et al.*, 1997).

For many years, it was thought that $CD4^+$ T helper cells existed as a dichotomy of lineages Th1 and Th2, differentiated by cytokine expression patterns. However, recently it became apparent that the T helper cell population was not limited to these two subsets. Although it has long been appreciated that IL-17 (also known as IL-17A) production by T cells is required for protection against some pathogens, it was demonstrated that IL-17A was produced by a unique subset of T helper cells. Subsequently, it was definitively shown that T cells could differentiate into IL-17-producing cells *in vitro* and *in vivo* independently of Th1 or Th2 cell development thereby establishing Th17 cells as a unique T helper cell lineage. Functionally, Th17 cells play a role in host defense against extracellular pathogens in mediating the recruitment of neutrophils and macrophages to infected tissues. Moreover, it has become evident that aberrant regulation of Th17 cells may play a significant role in the pathogenesis of multiple inflammatory and autoimmune disorders (Korn *et al.*, 2009). IL-22 is also produced by Th17 cells and is particularly involved in immunity in epithelial and mucosal surfaces. IL-17 and IL-22 are pro-inflammatory cytokines that play a protective role against intracellular parasites, such as *Leishmania* (Nascimento *et al.*, 2015). However, the role of IL-17 and IL-22 during *Leishmania* infection remains controversial. In order to preserve host immunological balance (immune homeostasis), T regulatory lymphocytes (Tregs) have an important role in suppressing the immune response in human leishmaniasis (Rai *et al.*, 2012), in murine leishmaniasis (Rodrigues *et al.*, 2009) and, probably in CanL (Silva *et al.*, 2014). Tregs

are characterized by the expression of *CD4*, *CD25* and of the highly conserved transcription factor *Forkhead box P3* (Foxp3), serving a pivotal role in stabilizing their regulatory properties. However, Th17 cytokine and FoxP3 have been poorly studied in CanL and not in the visceral organs, requiring further investigations (Hosein *et al.*, 2015).

It is now widely accepted that in dogs, CanL resistance is associated with the differentiation of IFN-γ, IL-2 and TNF-α producing Th1 cell subset leading to the activation of macrophages and NO production to kill intracellular amastigotes. This profile has been seen in dogs that were observed after a successful chemotherapy for *L. infantum* infection, as well in canine macrophage cell line infected with *L. infantum* after incubation with IFN-γ, TNF-α and IL-2, or in macrophages from dogs immunized with killed *L. infantum* promastigotes. On the other hand, susceptibility is associated with high anti-*Leishmania* antibody titers, which are not immunoprotective and also an unresponsive T cell population. Our understanding of canine immunity has expanded and we now know that the immune response generated by the dog against *Leishmania* infection is a result of a complex interaction between the parasite virulence and the silencing imposing effect with genetics and immune status of the host. This complex interaction may lead to mix immune responses with no defined pattern.

1.3.2. Innate immunity in leishmaniasis

The mammalian immune system fights infection through the cooperation of two connected systems, the innate and adaptive immune systems. Adaptive immunity detects an invading agent through recognition of peptide antigens, using antigen receptors expressed on the surface of B and T cells. The recognition of an antigen by its receptor triggers clonal expansion and further development of antigen-specific response. Innate immunity was considered to be a less complex and flexible system until the mid 1990s when Toll receptors were discovered in *Drosophila* species and its mammalian homologues, the Toll-like receptors (TLR) were found to mediate recognition of pathogens by the innate immune system. These findings led to the discovery of pattern recognition receptors (PRR) and their roles in regulation and coordination of the immune response. PRR recognize and respond to evolutionarily conserved microbe specific molecular patterns, the so called pathogen associated molecular patterns

(PAMP) and, therefore, play a critical role in host defense towards pathogenic organisms. The major innate immune cells that respond to *Leishmania* infection are neutrophils and macrophages. These cells are equipped with several PRR that allow them to screen the environment for invading pathogens. Several studies have demonstrated that the expression and activation of PRR in innate immune cells serve as an essential link between the innate and adaptive immune systems. Adaptive immunity is controlled by PRR induced signals at multiple checkpoints, dictating the initiation of a response, the type of response, the magnitude and duration of the response and the production of long term memory. There are several classes of PRR, the membrane bound TLR and C-type lectin receptors (CLR), cytoplasmic PRR that include NOD-like receptors (NLR), AIM2-like receptors, RIG-I-like receptors and several cytoplasmic DNA sensors (Gurung and Kanneganti, 2015). PRR can instruct the adaptive immune system on when and how to respond to a particular infection. This has been demonstrated in studies on PRR, in particular for TLR, which revealed that innate recognition of pathogens results not only in the expression of innate response genes directed to control or eliminate infectious agent, but also in the expression of chemokines and cytokines that promote and shape the subsequent immune reaction.

One of the classical functions attributed to neutrophils is their capacity to phagocytose and kill microorganisms. However, *Leishmania* take advantage of this property and neutrophils provide a transient safe shelter for the parasites, prior to their entry into macrophages where they will replicate. To survival inside neutrophils, the parasite has developed several protective mechanisms including the prevention of the activation of an oxidative burst, thus avoiding the generation of highly toxic reactive oxygen species (ROS) (Laufs *et al.*, 2002) and the ability to be targeted to non-lytic compartments of neutrophils, as reported for *L. donovani* (Gueirard *et al.*, 2008). Neutrophils have a short lifespan and become rapidly apoptotic, leading to their phagocytosis by macrophages. However, following infection by *Leishmania*, their lifespan can be increased to several days as in the case of human neutrophils *in vitro* infected with *L. major* that increased their lifespan to two days, inhibiting the processing of procaspases in the infected cells (van Zandbergen *et al.*, 2004). In addition to their phagocytic function, neutrophils contribute to the initiation of inflammation, a process which is recognized as essential in launching immunity. The

importance of neutrophils as decision shapers in the development of an immune response is recent and they have long been considered as short lived and non-dividing cells of poor interest. However, this view is changing and neutrophils are currently appearing not only as key components of the inflammatory response, but also as cells that display important immunoregulatory roles in different microbial infections (Charmoy *et al.*, 2010). The continuation of immunological studies will improve the knowledge about the cross-talks between neutrophils and other cells immune and non-immune, that leads to the eventual resolution of the lesion, or on the contrary to unimpaired progression of the infection. In this case, factors related to the sand fly vector also need to be re-considered. Peters *et al.* (2009) showed that sand fly inoculation maintains a localized, prolonged neutrophilic response which is different from that observed after needle injection of the parasite. This neutrophilic response impaired protection given to the mice by vaccination with killed *Leishmania* and challenged by the bite of infected sand flies, while such mice exhibited resistance against challenge infection induced through needle inoculation of live parasites. Removal of the neutrophils promoted resistance against sand fly-induced infection, further pointing to the importance of neutrophils as a decisive parameter in shaping the outcome of infection by *Leishmania* parasites.

One of the major problems with *Leishmania* infections is the ability of these parasites to evade and subvert host immune responses. These qualities allow these parasites to persist and establish chronic infections. When *Leishmania* parasites are inoculated by the sand fly, the first barrier that this parasite faces is the complement system. This system is constituted by a group of more than 30 soluble proteins of the plasma that are involved in rapidly clear pathogens from the body. The complement cascade can be activated by three possible ways: classical, lectins and alternative pathway. Antibodies binding to the pathogen initiate the classical pathway, whereas the binding of mannose binding lectin and ficolins activates lectin pathway. In contrast, alternative pathway does not require antibody or lectin binding and is directly activated by the pathogens. All of these pathways result in common activation of C3 convertase that cleaves C3 to generate C3b. C3b deposition onto the pathogen surface induces the deposition of C5b-C6-C7-C8-C9 (C5b-9 membrane attack complex, MAC) complex that promotes lysis of the target pathogen. In addition, C3b acts as an opsonin and

promotes phagocytosis of the pathogen by neutrophils and macrophages. In the case of *Leishmania* infection, the alternative pathway that is highly effective in the clearance of *Leishmania* parasites is activated (Hoover *et al.*, 1985). In the case of normal human serum, parasite clearance was shown to be antibody or C4 independent, requiring the formation of the C5b-9 MAC complex (Mosser and Edelson, 1985). Activation of the classical pathway via IgM anti-*Leishmania* antibodies causes promastigotes agglutinate and eventually parasite killing (Navin *et al.*, 1989). A tremendous selective pressure to survive to the action of complement-mediated killing has resulted in the evolution of several immune evasion strategies that are employed by *Leishmania*. The two major virulence factors of *Leishmania* are the glycocalyx component lipophosphoglycan (LPG) and the metalloprotease gp63. The glycocalyx (the glycoconjugate surface coat) acting as the interface between the parasite and the external environment is essential to the parasite survival as proven by several studies with parasites lacking the glycocalyx or presenting genetic deletions associated with the glycocalyx. In the first case, parasites are highly susceptible to complement killing (Späth *et al.*, 2003a; Yao *et al.*, 2013). In the second, although gp63 depleted *L. major* parasites exhibit a normal growth curve in culture medium and in the sand fly gut they are highly susceptible to complement killing (Joshi *et al.*, 2002). More remarkably, *L. major* parasites deficient in either LPG ($Lpg1^{-/-}$) or all phosphoglycans ($Lpg2^{-/-}$) present attenuate virulence and therefore reduced ability to infect mice (Späth *et al.*, 2000; Späth *et al.*, 2003b). Furthermore, LPG deficient *L. major* also fails to survive within the sand fly's gut, demonstrating their important role in parasite survival (Sacks *et al.*, 2000). In metacyclic promastigotes, LPG is essential for resistance to elimination via complement system activation. LPG binds to C3b, initiating the assembly of C5b-9 complex. However, MAC does not reach the *Leishmania* membrane and is spontaneously vanished as soluble C5b-9 complex (Puentes *et al.*, 1990). In a similar way, gp63 facilitates C3 deposition on *Leishmania* surface, but inactivates C3b (iC3b), inhibiting the formation of subsequent C5b-9 MAC complex (Brittingham *et al.*, 1995). All of the evasion mechanisms lead to LPG and gp63 facilitating C3b opsonization, which promotes phagocytosis through C3b receptor (CD11b) of neutrophils and macrophages, facilitating the entry of viable parasite in target cells. In addition, both LPG and gp63 can directly bind to cell surface receptors such as mannose and fibronectin receptor and

promote their uptake. Thus, LPG and gp63 mediate the parasite entry into the host cells, protecting *Leishmania* from the deadly activity of complement cascade (Gurung and Kanneganti, 2015). This is supported by the study performed in CD11b receptor deficient-mice, which are more resistant to *Leishmania* infection (Carter *et al.*, 2009) further substantiating the importance of CD11b in disease perpetuation. While rapid *Leishmania* phagocytosis via CD11b cell receptor is required for parasite persistence within the host, FcγR-dependent *Leishmania* phagocytosis is required for parasite clearance and disease protection (Woelbing *et al.*, 2006). These studies highlight the constant evolutionary battle between the mammalian host and *Leishmania* to assure its survival.

As for PRR, it was the identification of mammals TLR that made immunologists aware that innate immunity plays an important role in the detection and control of invading pathogens. TLR seem to be sufficient to control adaptive immunity at all checkpoints, leading to adaptive responses characterized by Th1 induction, immunoglobulin production, $CD8^+$ T cell stimulation that culminates in/assure protection from re-infection (Palm and Medzhitov, 2009). So far, it has been identified 10 functional TLR in humans and 12 TLR in mice, being TLR1–TLR9 conserved in both species. The members of TLR family are expressed on cell membranes of both innate immune cells (such as dendritic cells, neutrophils, macrophages, natural killer cells), adaptive immune cells (T and B lymphocytes) and non-immune cells (epithelial and endothelial cells), triggering the synthesis and secretion of cytokines and activation of other host defense programs that are necessary for innate or adaptive immune responses. TLR are type I trans-membrane glycoproteins consisting of an extracellular leucine-rich repeat (LRR) domain that mediate the recognition of PAMP, a trans-membrane domain and an intracellular Toll-interleukin-1 (IL-1) receptor (TIR) domain required for downstream signal transduction. These receptors are predominantly expressed in tissues involved in immune functions, such as the spleen and liver or the peripheral blood leukocytes, as well as those exposed to the external environment such as lungs and gastrointestinal tract. TLR are largely divided into two subgroups depending on their cellular localization and respective PAMP ligands. One group is composed of TLR1, TLR2, TLR4, TLR5, TLR6 and TLR11 that are expressed on cell surfaces and recognizes mainly microbial membrane components such as lipids,

lipoproteins and proteins. The other group includes TLR3, TLR7, TLR8 and TLR9 that are exclusively expressed in the membranes of intracellular vesicles, such as the endoplasmic reticulum (ER), endosomes, lysosomes and endolysosomes, recognizing microbial nucleic acids (Figure 7). The cellular localization of TLR is thought to be important for ligand accessibility, the maintenance of tolerance to self-molecules such as nucleic acids and also for downstream signal transduction (Akira *et al.*, 2006). TLR signaling pathways were intensively studied after the discovery of the TIR domain–containing adaptor molecule MyD88 (myeloid differentiation primary response gene 88). The subsequent identification of additional TIR domain–containing adaptors has shown that individual TLR selectively recruit distinct adaptor molecules, providing specific immune responses accordingly with the infecting agent.

Mice deficient in MyD88 is highly susceptible to *L. major* infections. Wild type C57BL/6 mice infected with *L. major* develop footpad lesions that heal by themselves after several weeks post infection. However, C57BL/6 mice deficient in MyD88 develop non-healing footpad lesions that progressively worsen over the course of infection. The disease progression in the MyD88$^{-/-}$ mice following *L. major* infection is comparable to the infection evolution in highly susceptible BALB/c mice (de Veer *et al.*, 2003; Debus *et al.*, 2003) and can be reverted by treatment with recombinant IL-12 (Muraille *et al.*, 2003). MyD88 dependent pathways appear essential for the development of the protective IL-12 mediated Th1 response against *L. major*. In absence of MyD88 protein, infected mice develop a non-protective Th2 response. LPG of several *Leishmania* ssp. induces mitogen-activated protein kinase and induces the production of IL-12 and TNF-α in a MyD88dependent process, identifying LPG as a potential ligand. *In vitro* studies carried out by *L. donovani* infected macrophages suggested that both TLR2 and TLR3 are involved in the immune responses, specifically signaling the production of TNF-α and NO, important for protective immunity against *Leishmania* (Flandin *et al.*, 2006). Furthermore, transfection studies (293T cells transfected with different TLR) demonstrated that TLR2 is LPG receptor (de Veer *et al.*, 2003). More recently, it was shown that anti-TLR2 antibody in the presence of LPG could inhibit nuclear factor (NF)-κB signaling events, confirming LPG as the direct ligand of TLR2 (Srivastav *et al.*, 2012). However, LPG-deficient *L. donovani* (Lpg1$^{-/-}$) still activates macrophage in a TLR2 dependent manner suggesting TLR2 can also

recognize other phosphoglycan moieties on *Leishmania*. Interestingly, $Tlr2^{-/-}$ mice are resistant to *Leishmania* infection as a result of increased IL-12 production (Guerra *et al.*, 2010). Altogether, these results indicate that *Leishmania* LPG utilizes TLR2 to evade host-immune response.

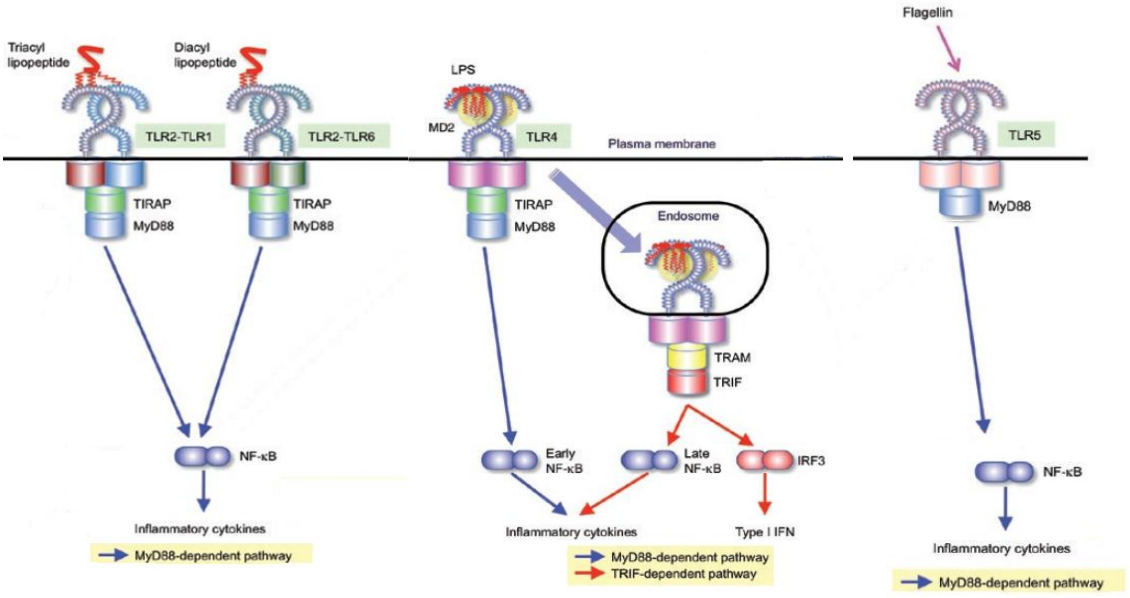


Figure 7: Pattern Recognition Receptors (PRR).
A) TLR2-TLR1 and TLR2-TLR6 heterodimers recognize triacylated and diacylated lipopeptides, respectively. TLR2-TLR1 and TLR2-TLR6 induce NF-κB activation through recruitment of TIRAP and MyD88 adaptors. B) TLR4 complexed with MD2 protein recognizes lipopolysaccharides (LPS). The generation of a multimeric receptor constituted by two copies of the TLR4-MD2-LPS complex signals the early-phase activation of NF-κB by recruiting the TIR domain–containing adaptors TIRAP and MyD88 (MyD88-dependent pathway). The TLR4-MD2-LPS complex is internalized and triggers signal transduction by recruiting TRAM and TIR-domain-containing adapter-inducing interferon-β (TRIF), which directs the expression of interferon regulatory factor 3 (IRF3) giving rise to the late-phase of NF-κB and the induction of type I interferon (TRIF-dependent pathway). Both early- and late-phase activation of NF-κB is required for the induction of inflammatory cytokines. C) TLR5 recognizes flagellin and activates NF-κB through MyD88 (Adapted from Kawai and Akira, 2010).

Based on their ability to induce effector proteins, such as reactive oxygen species and IL-12, *L. donovani* glycoproteins and glycosphingophospholipids have been suggested as potential ligands for TLR4 (Karmakar *et al.*, 2012). $Tlr4^{-/-}$ deficient mice are highly susceptible to *Leishmania* infection and show increased parasite load, indicating that TLR4 contributes to the effective control of *Leishmania* infection *in vivo*, through NO production (Kropf *et al.*, 2004a; Kropf *et al.*, 2004b). Interestingly, these $Tlr4^{-/-}$ mice are able to ultimately clear the parasitic infection, suggesting possible roles for other TLR as well. Indeed, TLR9 has emerged as another important sensor involved

in immunity against *Leishmania* infections. TLR9 recognizes *Leishmania* DNA, increasing IL-12 production and promoting target cell lysis by NK cell (Liese *et al.*, 2007, Schleicher *et al.*, 2007). Moreover, $Tlr9^{-/-}$ mice show increased susceptibility to *Leishmania* infection, presenting increased footpad lesion and high levels of antileishmanial antibody titers (Liese *et al.*, 2007). Similar to $Tlr4^{-/-}$ mice, the disease in $Tlr9^{-/-}$ mice eventually resolves over time. These independent studies highlight the TLR redundancy that is involved in providing protection against *Leishmania* spp. infection. Some *in vitro* studies have insinuated a possible role for TLR3 in immunity against *Leishmania* (Flandin *et al.*, 2006), but the *in vivo* studies with $Tlr3^{-/-}$ mice were not yet being performed and probably will give interesting results, as TLR3 has a MyD88-independent activation. Although TLR3 requires its adaptor TRIF for signaling it has also been suggested to play redundant roles with MyD88 during *in vitro Leishmania* infections (Gallego *et al.*, 2011). Schamber-Reis *et al.* (2013) showed that during *Leishmania* infection, nucleic acid sensing by multiple TLR is a major resistance factor, indicating the redundant and the essential role of nucleic acid sensing TLR3, TLR7 and TLR9, in inducing the IL-12 production and development of a Th1 response and resistance to *L. major* infection. TLR4 and TLR9 play an important role in immunity to recognize and respond against *Leishmania*. Interestingly, *Leishmania* hijacks TLR2, which evolved similarly as TLR4 and TLR9 to respond against *Leishmania*. Indeed, recognition of *Leishmania* LPG seems to promote parasite growth by inhibiting IL-12 production. Thus, during *L. donovani* infection blockade of TLR2 signaling by anti-TLR2 antibody provides significant protection by reducing the parasite burden (Murray *et al.*, 2013). *L. donovani* induces a robust expression of A20 (a deubiquitinating enzyme). A20 blocks TRAF6 Lys63 ubiquitination and signaling through TLR2 (Srivastav *et al.*, 2012). Moreover, *Leishmania* LPG signaling through TLR2 inhibits TLR9 expression and anti-*Leishmanial* responses (Srivastava *et al.*, 2013). *Leishmania* also utilizes several other host proteins to regulate TLR signaling and control the immune response in the host. A study performed by Gupta *et al.* (2014) shows that *L. donovani* inhibits TRAF3 degradation, which is an important step required for TLR4 signaling. In addition, *L. amazonensis* negatively regulates TLR4 signaling by degrading intracellular proteins such as STAT1/STAT2, ERK1/2 and IRF1 (Xin *et al.*, 2008), while *L. major* inhibitor of serine peptidase 2 has been revealed to

inhibit TLR4 activation through neutrophil elastase (Faria *et al.*, 2011). Toll-like receptors serve as the first line defense for innate immune cells and are critically important for protection against *Leishmania* infections. Therefore, *Leishmania* has evolved to evade and manipulate the immune system, overcoming these immune responses to survive within the host.

While TLR guard the extracellular environment and endosomal compartments, cytoplasmic PRR patrol the danger in the cytoplasm. The cytosolic PRR can be separated into two classes based on their mechanism of activation. The first class of cytosolic PRR, which includes the nucleotide binding oligomerization domain containing (NOD)-like receptor (NLR) family members NOD1 and NOD2, the retinoic acid inducible gene I (RIG-I)-like helicases (RLH) and, presumably, the IFN stimulator DNA (ISD) sensor, directly detects cytosolic PAMP and activates several signaling cascades. The second class is constituted by members of the NLR family that are involved in the formation and activation of large, multimeric protein complexes, referred to as inflammasomes, controlling the activation of caspase-1 and the secretion of caspase-1-dependent cytokines such as IL-1β (Meylan, *et al.*, 2006; Palm and Medzhitov, 2009). The role of cytosolic PRR in controlling adaptive immunity is just beginning to emerge. Cytosolic PRR, unlike transmembrane PRR, can distinguish between intracellular and extracellular infections and may even distinguish pathogenic from harmless, commensal microorganisms and danger associated molecular patterns. The NLR proteins are a family of 22 mammalian genes that have diverse functions in innate immunity and inflammation. However, one of the most important discoveries relating to NLR has been the discovery of inflammasomes. NLRP1b, NLRP3 and NLRC4 have been shown to form a multimeric protein complex along with common adaptor molecule, containing a CARD domain and cysteine protease caspase-1called the inflammasomes. Inflammasomes are known to act as cytosolic sensors and regulate cytokine secretion and cell death pathways. The family shares common structural motifs: a central nucleotide-binding domain (NACHT), a C-terminal leucine-rich-repeat (LRR) domain that has been implicated in ligand sensing and an N-terminal effector domain that is either a CARD (caspase-1 activation and recruitment), BIR (baculovirus inhibitor of apoptosis protein repeat) domain or a PYD (pyrin) domain. The NLRP group members contain a PYD, the NAIP include a BIR domain and the NLRC group

proteins contain a CARD domain (Clay *et al.*, 2014). The formation of multiprotein inflammasome complexes constitutes a major pro-inflammatory pathway of the innate immune system. Signals that trigger activation are unique to each of the NLR proteins. Upon activation by the appropriate ligand, NLR is activated and cleave the precursors of IL-1β and IL-18 into their mature secreted forms. The release of these cytokines leads to further inflammatory cascades in the host. Other NLR (NOD1, NOD2) are known to regulate inflammatory immune responses, whereas the molecular functions of many NLR proteins have yet to be discovered. Inflammasomes are central for cleavage and activation of pyrogenic cytokines, IL-1β and IL-18. NLR and inflammasomes are linked to several autoimmune and inflammatory diseases as well as to infections (Davis *et al.*, 2014). However, the knowledge about the roles of NLR in immunity against *Leishmania* infections is just beginning to be explored in models of the many species and varieties of leishmaniasis. Several NLR, including some of the non-inflammasome-activating NLR, play crucial roles in the host adaptive response to pathogens, regulating cellular migration, goblet cell-induced mucous secretion and negative regulation of inflammatory pathways. There has been one report indicating that at least one species of *Leishmania* is capable of activating the NLRP3 inflammasome in macrophages *in vitro* and, *in vivo*, there are evidences that NLRP3 inflammasomes influence the course of murine cutaneous or visceral disease. Knockout mice lacking NLRP3, ASC or caspase-1 infected with *L. amazonensis* developed enlarged lesion size and augmented parasite burden compared to wild type control mice, evidencing that NLRP3 and inflammasome components limit the intracellular growth of *L. amazonensis* in macrophages. Although controversial in some points, *in vitro* data demonstrated that IL-1β released by macrophages infected with *L. amazonensis*, *L. braziliensis* and *L. mexicana* require the expression of NLRP3, ASC and caspase-1. (Fettelschoss *et al.*, 2011; Lima-Junior *et al.*, 2013). Overall, these experiments suggest a protective role for the NLRP3 inflammasome against *Leishmania* parasite load, increasing host resistance to a high parasite load. But also there are evidences of conflict, suggesting that inflammasome assembly or IL-1β could actually exacerbate the lesions, stressing that the underlying mechanisms need further exploration. Regarding the non-inflammasome NLR, little is known about any role for NOD1/2 proteins in leishmaniasis disease, despite their importance in inflammation and in developing adaptive immunity,

highlighting the possible importance of these pathways. The first report of mammalian NLR sensing cytoplasmic microbial PAMP refers to NOD1 and NOD2, which have one and two N-terminal CARD effector domains, respectively and recognize peptidoglycan fragments (Figure 8).

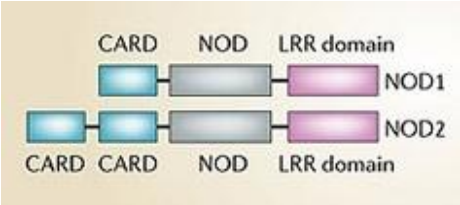


Figure 8: Non-inflammasome NLR, NOD1 and NOD2.
These cytosolic sensors of pathogens and danger-associated molecules have one (NOD1) or two (NOD2) N-terminal CARD effector domains and recognize peptidoglycan fragments (Adapted from Strober *et al.*, 2006).

NOD1 recognizes the peptide γ-d-glutamyl-meso-diaminopimelic acid and chiefly acts as a sensor for gram-negative bacteria, whereas NOD2 recognizes muramyl dipeptide (MDP) and represents a general sensor for most bacteria. Ligand recognition by NOD induces the recruitment of CARD-containing serine/threonine kinase RICK (also called RIP2) to the receptor via CARD-CARD interactions, which eventually leads to NF-κB activation (Inohara *et al.*, 2005; Lee and Kim, 2007). Although several potential regulators of NF-κB activity in the NOD-dependent pathways (TAK1, TRIP-6, GRIM-19 and ERBIN) have been identified (Werts *et al.*, 2006) their mode of action and the *in vivo* physiological importance remain to be determined. Recent experiments have shown that specific activation of NOD1 in the absence of ligands for other PRR is sufficient to induce Th2 and B cell response. NOD1 stimulation was able to potentiate Th1, Th2 and Th17 responses initiated in cooperation with TLR ligands either during immunization or infection with *Helicobacter pylori*. It is thus suggested that when acting coordinated with TLR ligands, NOD1 potentiates and may shape adaptive immunity. Because NOD1 stimulation potentiates all Th responses it is unclear whether this sensor is involved in controlling the type of response induced (Fritz *et al.*, 2007).

1.3.3. Organ specific immune response in leishmaniasis

Visceral leishmaniasis (VL) is caused by the intracellular parasites *Leishmania donovani* and *Leishmania infantum*. In the VL mouse model, there is a distinct organ-specific pattern of parasite growth during the disease establishment. In humans

and genetically susceptible mice, the liver, the spleen and the bone marrow are major sites of parasite growth and pathology. Although there is some evidence regarding the immune response of target organs against *Leishmania* parasites from murine models, little is known of the specific immune responses occurring in these organs in both human and canine leishmaniasis.

One remarkable aspect of murine VL is the unlike growth rate of parasites in infected organs. Infection in the liver is characterized by a rapid increase in the parasite burden in the first 4 weeks of infection followed by clearance of the parasite within 6–8 weeks. This self-curing mechanism in the liver is attributed to the development granulomatous response Th1 dominated immune environment (Stern *et al.*, 1988) characterized by high IFN-γ production by $CD4^+$ T cells. Despite these local responses, the parasite is able to disseminate and produce symptomatic VL. In contrast, infection in the spleen has serious consequences demonstrated by increased parasite burden, disruption of splenic microarchitecture and impaired immune responses resulting in the infection persistence (Ato *et al.*, 2002). Although the exact mechanism by which the parasite establishes chronic infections in the spleen still remains obscure it is now becoming evident that the parasite targets and alters the functions of the host immune system for evasion. Some of the altered mechanisms include suppression of host protective Th1 responses, generation of defective $CD8^+$ T cells and inhibition of dendritic cell (DC) function (Ato *et al.*, 2006). In addition to modifying DC and T-cell function, the parasite also modulates B-cell function for its survival. Furthermore, by directly interacting with different cell subsets, the parasite also generates an immunosuppressive environment by inducing IL-10 production thus favoring its survival in the host.

Ahmed *et al.*, (2003) addressed the question of parasite tropism, using mice infected intradermally with *L. infantum* or *L. donovani*. Parasites were found to persiste in spleen and draining lymph nodes and were initially cleared from liver and skin. This course of infection appears to be representative of subclinical canine and human VL. The tissue site-specific parasite clearance or persistence appears to reflect different local immune responses. This indicates that parasites do not accommodate in the first organ they encounter after intravenous inoculation, evidencing a pattern of infection. Thus, the liver apparently serves as site for initial parasite expansion and the spleen may serve as

a safer organ for the long term persistence of the visceralizing *Leishmania* ssp. Resolution of disease in the livers of mice infected with *L. donovani* or *L. infantum* correlates with the local formation of granulomas. Amastigotes are first observed in Kupffer cells, ensuring parasite survival (Figure 9A). Early parasite replication in the liver and in the spleen is associated with low IFN-γ and IL-12-producing cells (Wilson *et al.*, 2005). Furthermore, infection dynamics are similar in wild type and IL-12p40 knockout C57BL/6 mice, suggesting that IL-12 mediated Th1 response does not influence disease progression until a posterior phase of infection (Wilson *et al.*, 2002).

Resolution of liver infection is attributed to the development of a Th1 dominated granulomatous response, characterized by high IFN-γ production mainly due to $CD4^+$ T cells. This response is initiated by IL-12 secreted-DCs (Gorak *et al.*, 1998). IL-12 is an essential cytokine in the development of protective immunity against *L. donovani*. IL-12 blocking reduced both IFN-γ production and granuloma formation in the liver of infected mice (Murray, 1997) and, by the contrary, supplementation with exogenous IL-12 early decreased liver parasite burden (Murray and Hariprashad, 1995). $CD8^+$ T cells also have an essential role in the hepatic parasite clearance. Indeed, depletion of $CD8^+$ T cells inhibits granulomatous response, resulting in disease exacerbation (Bankoti and Stäger, 2012). In addition to the adaptive T cell response, TNF production and expression of inducible nitric oxide synthase (iNOS) by macrophages help parasite elimination. Indeed, granulomas are poorly formed in the immunodeficient murine model and in humans with progressive visceral leishmaniasis, which do not develop mature granulomas. In VL, hepatic resistance is also attributed to the generation of reactive nitrogen and oxygen intermediates, both playing a non-negligible role in restraining of parasite growth during the early stages of infection (Murray and Nathan, 1999). These might in turn be related to the T cell dependent recruitment of monocytes at the early phase of infection. At a later stage of infection, regulation of iNOS plays an important role in NO generation, pointing to T cell dependent macrophage activation (Cervia *et al.*, 1993).

During a challenge, there is a fast generation of granulomas enriched in $CD8^+$ cells, underlying the importance of $CD8^+$ cells for the development of long term immunity to VL, a finding also observed during *L. major* infection (Müller, 1992). The livers of asymptomatic dogs showed an effective immunity with well-organized

granulomas able to isolate and restring parasite spreading in an immune environment of activated effector T cells, dendritic cells (DCs) and central memory cells. In contrast, liver of symptomatic dogs showed a non-organized and ineffective infiltrate of T cells and heavily parasitized Kupffer cells (Sanchez *et al.*, 2004). Furthermore, the highest proportion of activated effector T cells was also observed in the liver of asymptomatic dogs, correlating with an effective immune response against the parasite. Interestingly, many naive T cells were observed in the liver of symptomatic dogs. Apparently, central memory T cells sensitized against *L. infantum* may migrate to peripheral tissues, providing protection at these vulnerable sites. In contrast, naive T cells migrate almost exclusively to lymphoid organs, which are designed for receiving migrating cells and antigen sampling (Mackay, *et al.*, 1990; Mackay, *et al.*, 1992). Therefore, it is possible that the high proportion of naive T cells found in the liver of symptomatic dogs correspond to a resident mixed T cell subset associated with tolerance or nonspecific activation similar to that described in mice (O'Farrelly *et al.*, 1999). Nevertheless, the role of naive T cells in the liver of infected dogs remains to be defined. Establishment and functioning of liver granulomas in dog is not yet fully characterized as in VL murine model.

In the spleen, where amastigotes can persist throughout the life of the animal, parasite replication begins later and remains at a low level. Spleen and bone marrow are sites of chronic inflammation, characterized by parasite persistence. Chronic spleen infection is associated with splenomegaly and changes in the splenic microarchitecture (Ato *et al.*, 2002; Stanley and Engwerda, 2007) which have consequences on the development of a specific immune response. Paradoxically, pro-inflammatory and inhibitory cytokines are co-expressed in splenic cultures (Wilson *et al.*, 1996). Although IL-10, IL-12, IFN-γ and TGF-β are detected by immunohistochemistry in the spleens of *L. donovani* infected BALB/c mice, IL-4 remained absent (Melby *et al.*, 2001). Typically, following an encounter with the antigen, DCs migrate from the marginal zone (MZ) of the spleen to the T cell rich area known as periarteriolar lymphoid sheaths (PALS) (Figure 9B-C). At PALS DCs interact with T cells, resulting in the induction of an antigen-specific T cell response (Gorak *et al.*, 1998).

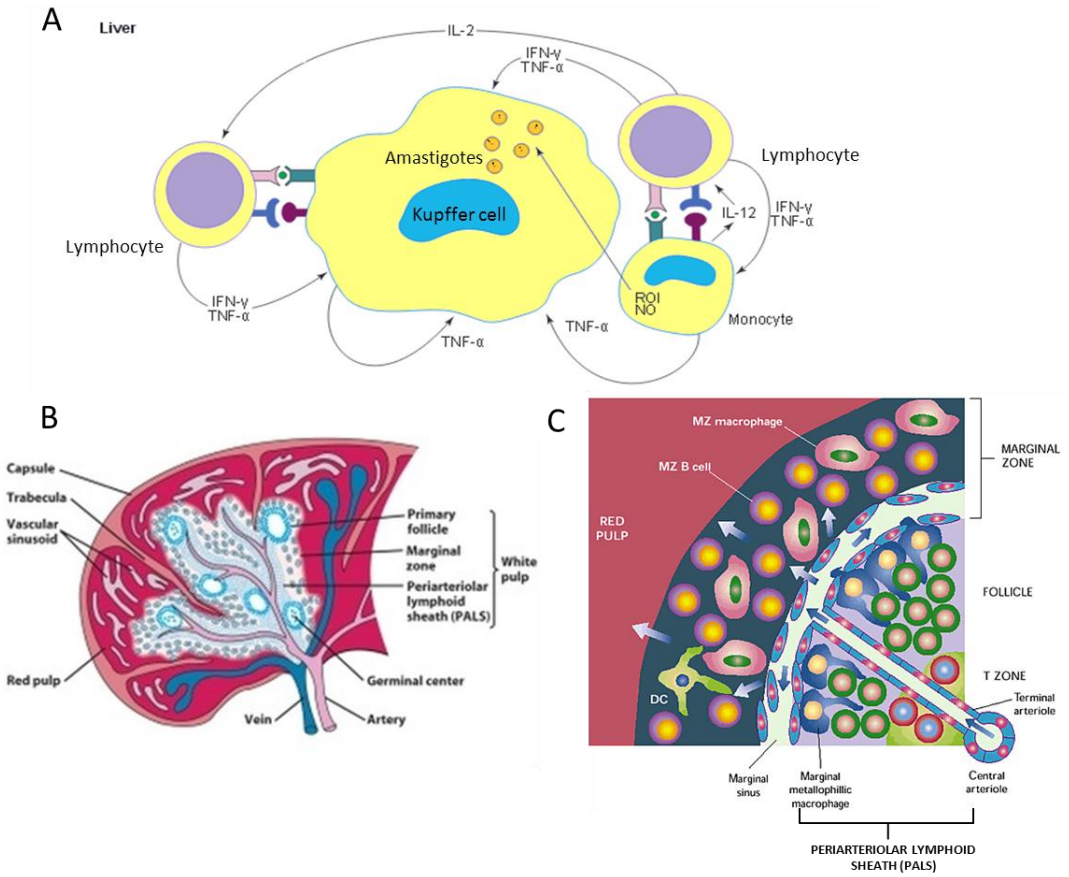


Figure 9: Organ-specific immune response in VL.
A) The ability of the liver to resolve infection is dependent on the formation of inflammatory foci around infected Kupffer cells, consisting of lymphocytes and monocytes, which allow fusion of infected cells and killing of amastigotes by infiltrating activated blood monocytes. B) Schematic representation of a mammalian spleen highlighting the localization of the periarteriolar lymphoid sheaths (PALS) and marginal zone in the white pulp. C) Schematic magnification of a follicle with the distribution of DCs and macrophages in the marginal zone. These cells migrate to the rich T cell area of the periarteriolar lymphoid sheaths (PALS) presenting *Leishmania* antigens, giving origin to an immune reaction. IFN-γ, interferon-γ; IL-12, interleukin 12; IL-2, interleukin 2; NO, nitric oxide; ROI, reactive oxygen intermediates; TNF-α, tumor necrosis factor α; DC, Dendritic cells; MZ, marginal zone. (Adapted from Cyster, 2000; Engwerda and Kaye, 2000 and http://bodterms.weebly.com/pals.html)

During chronic *L. donovani* infection, increase production of TNF by macrophages causes the disruption of splenic MZ (Engwerda *et al.*, 2002). As a consequence of this disruption, DCs and naive T-cells fail to migrate to the PALS, which may result in diminished T cells priming. This impaired migratory capacity also directs the down regulation of important co-stimulatory molecules and the increase expression of DCs inhibitory molecules (Josh *et al.*, 2009), leading to the hypothesis that DC functions are diminished at later stages of infection. This hypothesis is supported by the study conducted by Stäger *et al.*, (2006), where bone marrow-derived DCs LPS-activated were adoptive transferred into mice at a later *L. donovani*, infection

stage resulting in a significant reduction of the splenic parasite burden and possibly in the induction of a protective T cell response.

Splenomegaly is also associated with destruction of the follicular network and the fibroblastic reticular network of DCs (Smelt *et al.*, 1997). In the spleen, the immune responses of symptomatic and asymptomatic dogs were very similar. However, the selective accumulation of $CD44^+$ and $CD4^+$ T cells in the spleen of asymptomatic dogs is highly suggestive of an efficient cellular response, involving the recruitment of parasite-specific $CD4^+$ T cells meant to migrate to target organs (Sanchez *et al.*, 2004).

Culture of spleen cells during *L. infantum* infection showed that there are antigen specific lymphocytes producing IFN-γ even at the peak of liver infection (Murray *et al.*, 2002). IFN-γ stimulates macrophages to produce nitric oxide, a final effector molecule necessary for intracellular parasite elimination. Therefore, it is not immediately apparent why parasites survive in the spleen.

The answer may lay in studies of the trafficking of amastigotes and DCs within the spleen. During a normal antigen specific response in the spleen, DCs migrate from the marginal zone to PALS, a zone containing mainly T cells. At PALS, DCs interact with T cells and initiate antigen specific response. Chemokines CCL21 and CCL19 induce migration of mature DCs, expressing CCR7 at surface (Balazs *et al.*, 2002). During the first few hours of *L. donovani* infection, parasites are localized in marginal zone macrophages (MZMs) of the splenic white pulp, which is lacking mature DCs and T cells. After a week, parasites are found in the red pulp, a macrophage rich area, and DCs failed to move to PALS marginal zone where the antigen-specific response occurs. The cause of this anergy are associated with DCs failing in express surface CCR7 due to increased levels of IL-10 and TNF-α that mediates a decrease of CCL21 and CCL19 release from PALS' cells. The consequence of the mis-localization of parasite antigen-expressing DCs is the failure to interact and activate a T cell specific response. Late in *L. donovani* infection, the splenic architecture is disrupted, losing MZMs expressing normal amounts of TNF-α. Progressive destruction of follicular DCs and the eventual loss of germinal centers occurred (Balazs *et al.*, 2002; Bankoti and Stäger, 2012). This pattern differs from chronic *L. major* infection, causing CL, in which dendritic cells localize normally. In addition, these changes could affect the reactivity of

B cells and, consequently compromise antibody production, which are known to be deregulated during VL (Ronet *et al.*, 2008).

1.3.3.1. Liver structure and immunology

The liver is the largest organ in the mammalian body and it performs a remarkable number of tasks that support the function of other organs and impacts all physiologic systems. The main essential functions of the liver are related to protein synthesis and metabolism, including the metabolism of amino acids, carbohydrates, lipids and vitamins. Also the degradation of toxins, xenobiotics and waste products are essential functions performed by the liver. Nevertheless, the liver is also responsible for several immunological functions as the removal of pathogens and exogenous antigens from the systemic circulation. The anatomic position of the liver and its distinctive vasculature accounts for its unique ability to continuously exchange immunological information.

To accomplish its multiple tasks, the liver is constituted by diverse cell populations sub-divided in parenchymal and non-parenchymal cells (Figure 10).

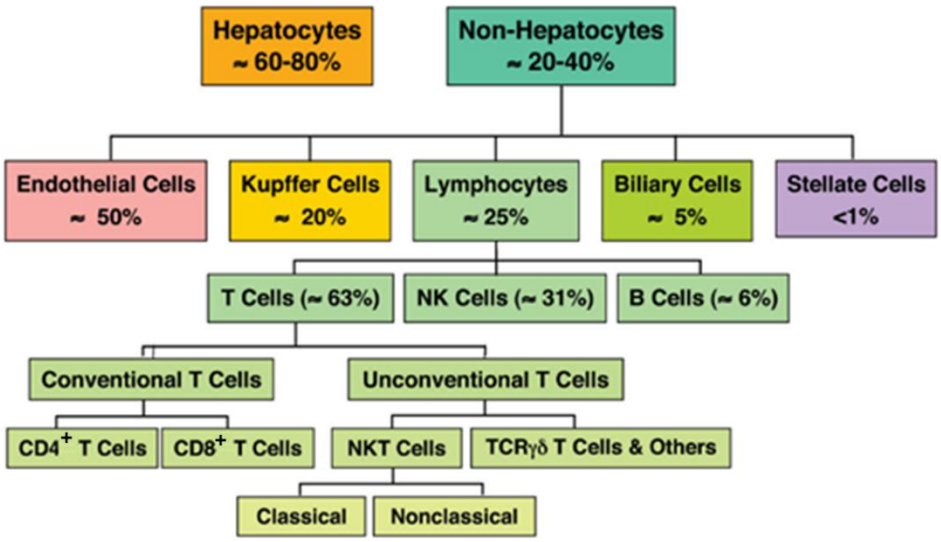


Figure 10: Liver cell constitution.
Numbers indicate cell frequency estimated for a healthy human liver (Adapted from Racanelli and Rehermann, 2006).

Most of the liver volume is occupied by parenchymal cells (hepatocytes) that occupy approximately 78–80% of the total hepatic tissue. Non-parenchymal cells

correspond barely to 5–6% of the total organ (Blouin *et al.*, 1977). The non-parenchymal cells consist of diverse types of cells, including liver sinusoidal endothelial cells (LSEC), Kupffer cells (KC) and hepatic stellate cells (HSC). The liver can be considered to have two separate anatomic areas, the parenchyma and the portal tracts. Structurally, the liver can be further subdivided into five systems comprising the vascular system, the hepatic lobule, the hepatic sinusoidal system, the biliary system and the stroma. Each of these systems directly or indirectly plays an important role in the homeostasis of the innate and adaptive immune system.

Liver structural organization has profound implications in its immune functions (Figure 11). The blood supply depends on: a conventional arterial system from the abdominal aorta that supports predominantly bile ducts and other tissues in the portal tracts, and of two venous systems that includes a minor system from the arterial plexus within portal tracts (peribiliary plexus) and a major system from the splanchnic organs (Racanelli and Rehermann, 2006). Blood enters the hepatic parenchyma mainly via terminal portal vessels and then passes through a network of liver sinusoids, leaving the parenchyma via the central hepatic veins (Figure 11B). About 30% of the total blood passes through the liver every minute, carrying peripheral blood lymphocytes, dendritic cells and potential harmful microbes or antigens (Sheth and Bankey, 2001). Due to the small diameter of sinusoids, minimal increases in systemic venous pressure and perturbations of sinusoidal flow result in stasis, which lengthens the contact between lymphocytes and antigen presenting cells and promotes lymphocyte extravasations. Blood rich in antigens flowing through the liver sinusoids is actively screened by a complex network of conventional and non-conventional antigen presenting cells (APC) (Thomson and Knolle, 2010). Lymphocyte extravasations is further facilitated by fenestrations in the monolayer of sinusoidal endothelial cells (LSEC) that give lymphocyte access to the space of Disse via cytoplasmic extensions and allow interaction with the underlying extracellular matrix, stellate cells and hepatocytes (Wisse, 1970).

The role of the liver as the main metabolic organ increases the rate of exposure to newly produced potential antigens and enhances the inherited risk of over activation of components of the immune system with potentially harmful consequences for individual homeostasis. Thus, the immune system developed mechanisms to promote

tolerance, but is also able to trigger a responsive state at any given time (Kita *et al.*, 2001).

Hepatocytes constitute the majority of the hepatic cells and although the primary roles of these cells are of metabolic nature, such as protein production and toxin neutralization, these parenchymal cells of the liver can also detect pathogens and aid the host immune response. Hepatocytes express innate immune receptors and in many cases have been demonstrated that these cells recognize pathogen associated ligands and display an innate immune response. Zhang *et al.* (2009) reported that TLR3 and TLR4 activation lead the attenuation of viral replication inside hepatocytes and the secretion of inflammatory cytokines such as IFN-α and IFN-γ.

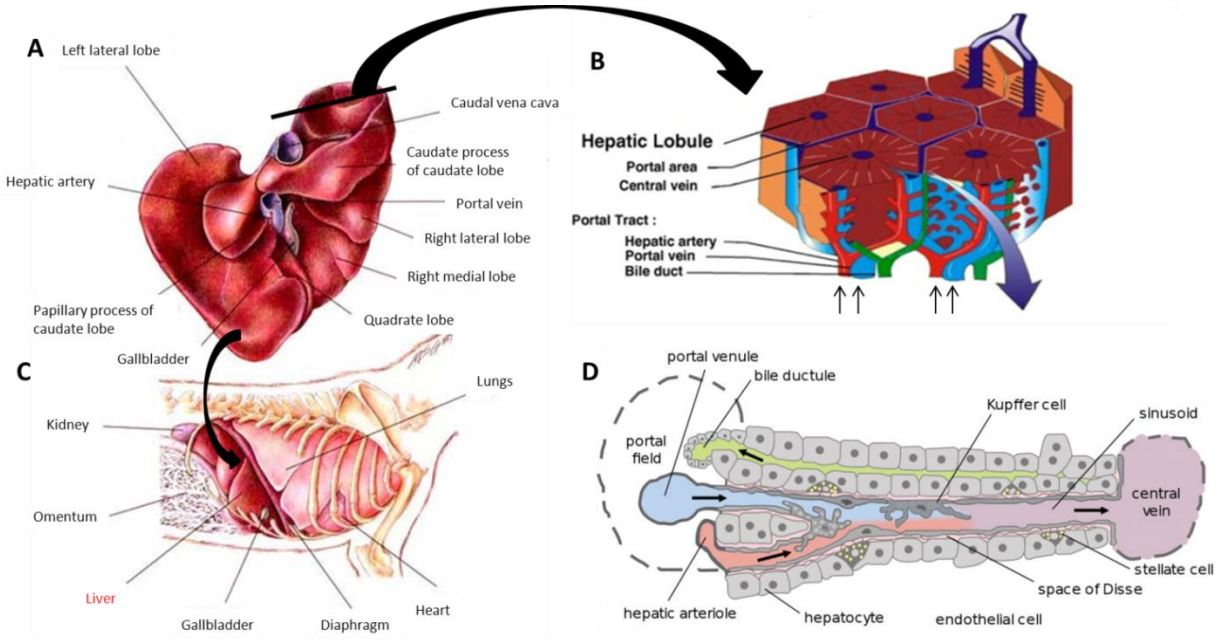


Figure 11: The canine liver, as model for the mammal liver.
A) Morphology of the canine liver; B) Longitudinal cut of canine liver. The arrow indicates the flow of the blood from the portal tract to the central vein. C) Localization of the liver in the canine body surrounded by the adjacent organs. D) Magnification of a hepatic lobule. The arrow indicates the flow of the blood from the portal tract to the central vein. Notice the localization in the space of Disse of Kupffer cells and stellate cells (Adapted from http://www.hillspet.com/dog-care/dog-disease-liver-disease.html, Frevert *et al.*, 2005 and https://bio.mox.polimi.it/nanomedicine/perfusion-characteristics-liver-tissue/).

In addition to their role in innate immunity, hepatocytes have also been implicated in the initiation of the adaptive immune response. Hepatocytes express MHCI and CD1 on the cell surface allowing them to present antigens to both T and natural killer T cells (NKT). However, the lack of expression of co-stimulatory molecules and CD40 skews this process towards tolerance rather than activation (Li and

Tian, 2013). Under inflammatory conditions, some hepatocytes can be induced to express MHCII (Franco *et al.*, 1988). Apparently, MHCII-expressing hepatocytes induce Th2 cell differentiation of uncommitted $CD4^+$ T cells and annul the ability of previously differentiated Th1 to secrete IFN-γ, even in the presence of pro-inflammatory microbial signals. The suppression of Th1 responses by hepatocytes is associated with poor expression levels of Th1-promoting Delta-like Notch ligands. These processes lead to chronic infections (Wiegard *et al.*, 2007).

Hepatocytes can also induce antigen-specific Tregs cells, which prevent autoimmune diseases (Lüth *et al.*, 2008) and regulates NK cell via NKG2A-Qa-1b receptor, resulting in increased IL-10 and decreased IFN-γ production by NK cells (Jinushi *et al.*, 2007). When encountering type I IFN-secreting NKT cells, hepatocytes can stimulate IL-10-expressing $CD8^+$ T cells, which also exhibit regulatory function. Electron microscopy has revealed that hepatocytes and naive T cells physically interact in an ICAM-1–and MHC-dependent fashion (Warren *et al.*, 2006). Taken together, these studies provide strong evidence of a role for hepatocytes in the activation of T cells, however the ability of hepatocyte-mediated activation of T cells remains, given the presence of other APC (LSEC, KCs and DC) in the liver microenvironment.

Hepatocytes are also responsible for the production of most acute-phase proteins and complement components. These two soluble elements of the innate immune system represent an evolutionarily conserved first line of defense against pathogens. Acute-phase proteins such as C-reactive protein can bind to specific microbial surfaces and alert the immune system to the presence of these pathogens. Likewise, components of the complement system such as C3 protein and the mannose-binding lectin directly bind microbial surfaces, triggering the immune system (Sarma and Ward, 2011). Through the production of these critical innate immune proteins, the hepatocyte can extend its role in pathogen detection far beyond the anatomical limits of the liver.

KC are resident stationary cells located in the vasculature are adherent to LSEC and directly exposed to the contents of blood circulating through the liver tissue (Figure 11D). This contrasts with other monocytic and macrophage cell populations located in diverse/different tissues that actively crawl through the tissue searching for pathogens. KC express an array of scavenger receptors, TLR, complement receptors and antibody

receptors, molecules that allow KC to detect, bind and internalize pathogens. Expressing MHC I, MHC II and costimulatory molecules needed for T cell activation, KC are important APC. Furthermore, these receptors drive in part the activation of KC, which leads to production of cytokines and chemokines and allows KC to function as immune sentinels, alerting other components of the immune system to the presence of harmful microbes (Bilzer *et al.*, 2006). Although capable of activating T cells under basal conditions KC are poor activators of the adaptive immune response, preventing reactions to common gut-derived antigen present in the portal circulation. Following TLR activation or when in the presence of inflammatory cytokines, KC can be converted from normally tolerogenic cells into potent APC capable of robust activation of T cells. KC are also extremely effective in activating the invariant NKT (iNKT) cells that live and patrol the sinusoids of the liver, quickly controlling a potential infection (Jenne and Kubes, 2013).

Hepatic stellate cells (HSC), also designated Ito cells, are star-shaped cells that reside in the space of Disse, between hepatocytes and LSEC comprise a minor fraction (5–8%) of total liver cells. Under normal conditions, HSC have a central role in vitamin A and lipid storage, but in a chronic liver damage scenario, HSC transdifferentiate into a fibrinogenic pro-liferating cell leading to liver fibrosis. In addition to their well defined roles in lipid storage and fibrinogenesis, evidences also suggest that HSC act as immune sentinels in the liver (Winau *et al.*, 2008). HSC express molecules required for antigen presentation (MHC I, MHC II, CD80 and CD86) and fluorescent microscopy studies have revealed direct contact between these cells and lymphocytes, supporting the hypothesis that HSC can directly activate naive lymphocytes (Muhanna *et al.*, 2007). However, the ability of HSC to directly activate lymphocytes has been questioned given their low abundance of MHC and of costimulatory molecules. Analysis of the expression of key molecules required for antigen presentation (MHC II, CD80, CD86) performed in fresh isolated HSC did not detect those molecules, but low abundance of others (CD1d) molecules were identified (Ichikawa, *et al.*, 2011). Although, some of these molecules were upregulated after cell culture in the presence of pro-inflammatory cytokines, it appears that under basal conditions HSC do not express the key molecules needed for antigen presentation to lymphocytes. The role suggested for HSC, as regulatory bystanders, promoting Tregs and suppressing Th17

differentiation might represent key players in the mechanism that drives liver-induced tolerance. As a result, these cells may be important in disease progression and in the damage resolution of the liver.

In addition to LSEC, KC and HSC, the liver also contains several populations of dendritic cells (DC). Tissue-resident DCs are present in most tissues with considerable functional and phenotypic heterogeneity amongst DC populations. DC appears to enter the liver predominantly via the portal blood supply, maturing as they transit from the portal circulation to the central vein. DCs are sparsely distributed through the liver and immunohistochemical studies of patient liver biopsies, indicate that they are primarily found in the portal regions and occasionally in the parenchyma (Rahman and Aloman, 2013). Liver DC can internalize antigens derived from the blood and transport these antigens to a regional lymph node. Under steady state conditions, liver DCs in both mice and humans can be divided into two major functional classes: classical DCs (cDCs) and plasmacytoid DCs (pDCs). cDCs express high levels of MHCII and function as highly-efficient professional antigen presenting cells. In contrast, pDCs express relatively lower levels of MHCII and have a relatively limited capacity to capture and present tissue antigens and instead function as major producers of type I IFNs in response to nucleic acids in the setting of viral infection (Pillarisetty *et al.*, 2004). Normally poor activators of T cell immunity, liver DC, under the proper conditions, can be transformed into potent APC by IL-10 blocking or by activation through pathogen-associated molecules

Despite the DC subpopulations mentioned above, another cell population that appears to have characteristics of both DC and NK cells has been identified in the mouse liver (Pillarisetty *et al.*, 2005). NK-DC express both NK cell markers (NK1.1) and DC markers (CD11c) and are present in several tissues but are enriched among the $CD11c^{+}$liver fraction. NK-DC can both directly lyse target tumor cells and efficiently present antigen, activating naive T cells. Additionally, NK-DC can produce large quantities of cytokines, such as IFN-γ, further potentiating the cytotoxic immune response.

Hepatic leukocyte populations differ from those of other tissues in a number of interesting ways. Their distribution is unique: one-third to one-half of leukocytes are

NK cells, making of the liver threefold more rich in these cells than blood (Racanelli and Rehermann, 2006). These innate leukocytes can respond to a large variety of cell surface ligands expressed by damaged or infected cells, making the NK cell a critical sentinel in the detection of viral and intracellular bacterial infections in the liver (Notas *et al.*, 2009). After activation, NK cells release cytotoxic granules containing perforin and granzyme in a cell-directed way. In response to stimulation, these cells also release a large amount of cytokines, such as IFN-γ, and by doing so, shape and direct the immune response and also modulate MHC expression of hepatocytes and HSC (Crispe, 2009).

Phenotypically, nearly 50% lymphocytes express the T cell receptor (TCR) and also there is an enrichment of $CD8^+$ T cells in the liver. Typically, in the blood, $CD4^+$ T cells outnumber $CD8^+$ T cells, but in the liver this ratio is reversed. The liver also contains the largest population of γδT cells in the body (Abo *et al.*, 2000). Although these cells are classically described as innate T cells, the specific function of γδT cells in the liver is not known. The individual γδT cell is often polyreactive, able to bind to several different ligands, foreign and self, in both a MHC-dependent and MHC-independent manner (Vantourout and Hayday, 2013). These characteristics differentiate γδT cells from conventional T cells, which typically recognize a single peptide complex with MHCII. Another distinct characteristic of liver resident lymphocytes is that nearly all of the $CD3^+$ cells in the liver (both αβ and γδ) express intermediate amounts of TCR (Iiai *et al.*, 1992). This clearly distinguishes these cells from thymus-derived peripheral T cells that express abundant levels of TCR. This TCR intermediate cell subset uniformly expresses IL-2Rβ (CD122) and comprises a mix of $NK1.1^-$T cells and $NK1.1^+$ NKT cells (Watanabe *et al.*, 1995). NKT cells are important and potent immunomodulatory population in the liver. These cells express a restricted TCR repertoire and respond to lipid antigens when presented in the context of the MHC-I-like molecule CD1d (Brennan *et al.*, 2013). Like the other types of leukocytes discussed above, the liver is enriched with these NKT cells, when compared to other tissues and NKT cells perform an important role as immune modulators. NKT cells are able of both inflammatory and anti-inflammatory responses, producing a wide array of cytokines that depending on the specific nature of the activating signal can include IFN-γ, IL-4 and IL-17 (Matsuda *et al.*, 2008). As a result, NKT cells have great

potential to shape the host immune response, but two additional characteristics of NKT cells demonstrate the critical importance of this population for the immune surveillance. NKT cells are the only liver-resident lymphocyte to actively patrol the liver vasculature in search of pathogens and are activated by a diverse array of signals that includes pathogens cytokine stimulation, self lipids and even neuromodulatory signals. In the last case, NKT cells of mouse liver have been shown to respond directly to a noradrenergic neurotransmitter released in response to brain ischemic stroke. These cells produced anti-inflammatory cytokines that induced a state of immune suppression in the mouse, reducing inflammation and therefore protecting the brain. However, this immune suppression state makes the host more susceptible to systemic bacterial infections (Wong *et al.*, 2011).

The delicate balance between immunity and tolerance in the liver, results directly from the complex interactions between the various resident immune cells and peripheral leukocyte populations. Under basal conditions, many liver resident cells (LSEC, KC and DC) have a critical role, maintaining a state of immune unresponsiveness, accomplished, in part, by the low expression of MHC and the absence of costimulatory molecules. Furthermore, the direct cell-cell contact between LSEC and liver DC under the continual exposition to pathogen-derived molecules from the gut induces the expression of anti-inflammatory molecules, reinforcing the state of immune tolerance. However, given an appropriate stimulation a robust immune response can be generated in the liver. Introduction of live bacteria, viral infection or the presence of elevated quantities of PAMP drives a rapid and robust inflammatory response involving the recruitment of large numbers of neutrophils and platelets to the liver. The anatomical features, blood supply, diverse network of cells and the broad array of receptors enable the liver to act as a crucial, frontline immune sentinel.

1.4. References

Aagaard-Hansen J, Nombela N, Alvar J. 2010. Population movement: a key factor in the epidemiology of neglected tropical diseases. Trop Med Int Health. 15(11):1281-8.

Abo T, Kawamura T, Watanabe H. 2000. Physiological responses of extrathymic T cells in the liver. Immunol Rev. 174:135-49.

Ahmed S, Colmenares M, Soong L, Goldsmith-Pestana K, Munstermann L, Molina R, McMahon-Pratt D. 2003. Intradermal infection model for pathogenesis and vaccine studies of murine visceral leishmaniasis. Infect Immun. 71(1):401-10.

Akhoundi B, Mohebali M, Shojaee S, Jalali M, Kazemi B, Bandehpour M, Keshavarz H, Edrissian GH, Eslami MB, Malekafzali H, Kouchaki A. 2013. Rapid detection of human and canine visceral leishmaniasis: assessment of a latex agglutination test based on the A2 antigen from amastigote forms of *Leishmania infantum*. Exp Parasitol. 133(3):307-13. doi: 10.1016/j.exppara.2012.12.002.

Akira S, Uematsu S, Takeuchi O. 2006. Pathogen recognition and innate immunity. Cell 124, 783-801.

Alexander B, Maroli M. 2003. Control of phlebotomine sandflies. Med Vet Entomol. 17(1):1-18.

Alexandre-Pires G1, de Brito MT, Algueró C, Martins C, Rodrigues OR, da Fonseca IP, Santos-Gomes G. 2010. Canine leishmaniasis. Immunophenotypic profile of leukocytes in different compartments of symptomatic, asymptomatic and treated dogs. Vet Immunol Immunopathol. 137(3-4):275-83. doi: 10.1016/j.vetimm.2010.06.007.

Altet L, Francino O, Solano-Gallego L, Renier C, Sánchez A. 2002. Mapping and sequencing of the canine NRAMP1 gene and identification of mutations in leishmaniasis-susceptible dogs. Infect Immun. 70(6):2763-71.

Alvar J, Aparicio P, Aseffa A, Den Boer M, Cañavate C, Dedet J, Gradoni L, Ter Horst R, López-Vélez R, Moreno J. 2008. The relationship between leishmaniasis and AIDS: the second 10 years. Clin Microbiol Rev. 21(2):334-59. doi: 10.1128/CMR.00061-07.

Amaral V, Pirmez C Goncalves A, Ferreira V, Grimaldi G. 2000. Cell populations in lesions of cutaneous leishmaniasis of *Leishmania amazonensis* – infected rhesus macaques, *Macaca mulatto*. Mem Inst Oswaldo Cruz 95: 209-216.

Andrade B, de Oliveira CI Brodskyn C, Barral A, Barral-Netto M. 2007. Role of sand fly saliva in human and experimental leishmaniasis: current insights. Scand J Immunol. 66(2-3):122-7.

Argueta-Donohué J, Carrillo N, Valdés-Reyes L, Zentella A, Aguirre-García M, Becker I, Gutiérrez-Kobeh L. 2008. *Leishmania mexicana*: participation of NF-kappaB in the differential production of IL-12 in dendritic cells andmonocytes induced by lipophosphoglycan (LPG). Exp Parasitol. 120(1):1-9.

Asato Y, Oshiro M, Myint C, Yamamoto Y, Kato H, Marco J, Mimori T, Gomez EA, Hashiguchi Y, Uezato H. 2009. Phylogenic analysis of the genus *Leishmania* by cytochrome b gene sequencing. Exp Parasitol. 121(4):352-61. doi: 10.1016/j.exppara.2008.12.013.

Ato M, Maroof A, Zubairi S, Nakano H, Kakiuchi T, Kaye P. 2006. Loss of dendritic cell migration and impaired resistance to *Leishmania donovani* infection in mice deficient in CCL19 and CCL21. Journal of Immunology. 176:9,5486–5493.

Ato M, Stäger S, Engwerda C, Kaye P. 2002. Defective CCR7 expression on dendritic cells contributes to the development of visceral leishmaniasis. Nature Immunology. 3:12, 1185–1191.

BalazsM, Martin F, Zhou T, Kearney J. 2002. Blood dendritic cells interact with splenic marginal zone B cells to initiate T-independent immune responses. Immunity, vol. 17, no. 3, pp. 341–352.

Baneth G, Shaw S. 2002. Chemotherapy of canine leishmaniosis. Vet Parasitol. 106(4):315-24.

Baneth G, Koutinas A, Solano-Gallego L, Bourdeau P, Ferrer L. 2008. Canine leishmaniosis - new concepts and insights on an expanding zoonosis: part one. Trends Parasitol. 24(7):324-30. doi: 10.1016/j.pt.2008.04.001.

Bankoti R, Stäger S. 2012. Differential Regulation of the Immune Response in the Spleen and Liver of Mice Infected with *Leishmania donovani*. J Trop Med. 2012:639304. doi: 10.1155/2012/639304.

Bañuls A, Hide M, Prugnolle F. 2007. *Leishmania* and the leishmaniases: a parasite genetic update and advances in taxonomy, epidemiology and pathogenicity in humans. Adv Parasitol. 64:1-109.

Barbiéri C. 2006. Immunology of canine leishmaniasis. Parasite Immunol. 28(7):329-37.

Bari A. 2006. Chronology of cutaneous leishmaniasis: An overview of the history of the disease. Journal of Pakistan Association of Dermatologists. 16:24-27.

Barral A, Honda E, Caldas A, Costa J, Vinhas V, Rowton E, Valenzuela J, Charlab R, Barral-Netto M, Ribeiro J. 2000. Human immune response to sand fly salivary gland antigens: a useful epidemiological marker? Am J Trop Med Hyg. 62(6):740-5.

Bates P, Rogers M. 2004. New insights into the developmental biology and transmission mechanisms of *Leishmania*. Curr Mol Med. 4(6):601-9.

Bates P. 2007. Transmission of *Leishmania* metacyclic promastigotes by phlebotomine sand flies. Int J Parasitol. 37(10):1097-106.

Beaumier C, Gillespie P, Hotez P, Bottazzi M. 2013. New vaccines for neglected parasitic diseases and dengue. Transl Res. 162(3):144-55. doi: 10.1016/j.trsl.2013.03.006.

Belkaid Y, Hoffmann K, Mendez S, Kamhawi S, Udey M, Wynn T, Sacks D. 2001. The role of interleukin (IL)-10 in the persistence of *Leishmania major* in the skin after healing and the therapeutic potential of anti-IL-10 receptor antibody for sterile cure. J Exp Med. 194(10):1497-506.

Belkaid Y, Kamhawi S, Modi G, Valenzuela J, Noben-Trauth N, Rowton E, Ribeiro J, Sacks D. 1998. Development of a natural model of cutaneous leishmaniasis: powerful effects of vector saliva and saliva preexposure on the long-term outcome of *Leishmania major* infection in the mouse ear dermis. J Exp Med. 188(10):1941-53.

Belkaid Y, Piccirillo C, Mendez S, Shevach E, Sacks D. 2002. $CD4^{+}CD25^{+}$ regulatory T cells control *Leishmania major* persistence and immunity. Nature. 420(6915):502-7.

Bertholet S, Goto Y, Carter L, Bhatia A, Howard R, Carter D, Coler R, Vedvick T, Reed S. 2009. Optimized subunit vaccine protects against experimental leishmaniasis. Vaccine. 27(50):7036-45. doi: 10.1016/j.vaccine.2009.09.066.

Berzunza-Cruz M, Cabrera N, Crippa-Rossi M, Sosa Cabrera T, Pérez-Montfort R, Becker I. 2002. Polymorphism analysis of the internal transcribed spacer and small subunit of ribosomal RNA genes of *Leishmania mexicana*. Parasitol Res. 88(10):918-25.

Besteiro S, Williams R, Coombs G, Mottram J. 2007. Protein turnover and differentiation in *Leishmania*. Int J Parasitol. 37(10):1063-75.

Bhandari V, Kulshrestha A, Deep D, Stark O, Prajapati V, Ramesh V, Sundar S, Schonian G, Dujardin J, Salotra P. 2012. Drug susceptibility in *Leishmania* isolates following miltefosine treatment in cases of visceral leishmaniasis and post kala-azar dermal leishmaniasis. PLoS Negl Trop Dis. 6(5):e1657. doi: 10.1371/journal.pntd.0001657.

Bhowmick S and Ali N. 2008. Recent developments in leishmaniasis vaccine delivery systems. Expert Opin Drug Deliv. 5(7):789-803. doi: 10.1517/17425247.5.7.789.

Bhowmick S, Ravindran R, Ali N. 2008. gp63 in stable cationic liposomes confers sustained vaccine immunity to susceptible BALB/c mice infected with *Leishmania donovani*. Infect Immun. 76(3):1003-15. doi: 10.1128/IAI.00611-07.

Biglino A, Bolla C, Concialdi E, Trisciuoglio A, Romano A, Ferroglio E. 2009. Asymptomatic *Leishmania infantum* Infection in an Area of Northwestern Italy (Piedmont Region) Where Such Infections Are Traditionally Nonendemic. J Clin Microbiol. 48(1):131–136. doi: 10.1128/JCM.00416-09

Bilzer M, Roggel F, Gerbes A. 2006. Role of Kupffer cells in host defense and liver disease. Liver Int. 26(10):1175-86.

Blackwell J, Goswami T, Evans C, Sibthorpe D, Papo N, White J, Searle S, Miller EN, Peacock C, Mohammed H, Ibrahim M. 2001. SLC11A1 (formerly NRAMP1) and disease resistance. Cell Microbiol. 3(12):773-84.

Blouin A, Bolender R, Weibel E. 1977. Distribution of organelles and membranes between hepatocytes and non-hepatocytes in the rat liver parenchyma. A stereological study. J Cell Biol. 72:441–455.

Boelaert M, Rijal S, Regmi S, Singh R, Karki B, Jacquet D, Chappuis F, Campino L, Desjeux P, Le Ray D, Koirala S, Van der Stuyft P. 2004. A comparative study of the effectiveness of diagnostic tests for visceral leishmaniasis. Am J Trop Med Hyg 70: 72-77.

Bogdan C, Schönian G, Bañuls A, Hide M, Pratlong F, Lorenz E, Röllinghoff M, Mertens R. 2001. Visceral leishmaniasis in a German child who had never entered a known endemic area: case report and review of the literature. Clin Infect Dis. 32(2):302-6.

Borja-Cabrera G, Correia Pontes N, da Silva V, Paraguai de Souza E, Santos W, Gomes E, Luz K, Palatnik M, Palatnik de Sousa C. 2002. Long lasting protection against canine kala-azar using the FML-QuilA saponin vaccine in an endemic area of Brazil (São Gonçalo do Amarante, RN). Vaccine. 20(27-28):3277-84.

Borja-Cabrera G, Cruz Mendes A, Paraguai de Souza E, Hashimoto Okada L, de A Trivellato F, Kawasaki J, Costa A, Reis A, Genaro O, Batista L, Palatnik M, Palatnik-de-Sousa C. 2004. Effective immunotherapy against canine visceral leishmaniasis with the FML-vaccine. Vaccine. 22(17-18):2234-43.

Bossolasco S, Gaiera S, Olchini D, Gulletta M, Martello L, Bestetti A, Bossi L, Germagnoli L, Lazzarin A, Uberti-Foppa C, Cinque C. 2003. Real-time PCR assay for clinical management of human immunodeficiency virus-infected patients with visceral leishmaniasis. J Clin Microbiol 41: 5080-5084.

Bourdoiseau G, Bonnefont C, Hoareau E, Boehringer C, Stolle T, Chabanne L. 1997. Specific IgG1 and IgG2 antibody and lymphocyte subset levels in naturally *Leishmania infantum*-infected treated and untreated dogs. Vet Immunol Immunopathol. 59(1-2):21-30.

Bray R. 1976. Immunodiagnosis of leishmaniasis. In Cohen S, Sadun EH, editors. Immunology of parasitic infections. Oxford: Blackwell. 65-76.

Brennan P, Brigl M, Brenner M. 2013. Invariant natural killer T cells: an innate activation scheme linked to diverse effector functions. Nat Rev Immunol. 13(2):101-17. doi: 10.1038/nri3369.

Breton M, Tremblay M, Ouellette M, Papadopoulou B. 2005. Live nonpathogenic parasitic vector as a candidate vaccine against visceral leishmaniasis. Infect Immun. 73(10):6372-82.

Brito M, Mendonca M, Gomes Y, Jardim M, Abath F. 2000. Identification of potentially diagnostic *Leishmania braziliensis* antigens in human cutaneous leishmaniasis by immunoblot analysis. Clin Diagn Lab Immunol. 7:318-321.

Brittingham A, Morrison C, McMaster W, McGwire B, Chang K, Mosser D. 1995. Role of the *Leishmania* surface protease gp63 in complement fixation, cell adhesion, and resistance to complement-mediated lysis. J Immunol. 155(6):3102-11.

Bucheton B, Abel L, Kheir M, Mirgani A, El-Safi S, Chevillard C, Dessein A. 2003. Genetic control of visceral leishmaniasis in a Sudanese population: candidate gene testing indicates a linkage to the NRAMP1 region. Genes Immun. 4(2):104-9.

Burns J, Shreffler W, Benson D, Ghalib H, Badaro R, Reed S. 1993. Molecular characterization of a kinesin-related antigen of *Leishmania chagasi* that detects specific antibody in both African and American visceral leishmaniasis. Proc Natl Acad Sci USA. 90:775-790.

Campino L, Santos-Gomes G, Pratlong F, Dedet JP, Abranches P. 1994. HIV-*Leishmania* co-infection in Portugal: isolation of *Leishmania infantum* MON-24. Trans R Soc Trop Med Hyg. 88(4):394.

Capelli G, Baldelli R, Ferroglio E, Genchi C, Gradoni L, Gramiccia M, Maroli M, Mortarino M, Pietrobelli M, Rossi L, Ruggiero M. 2004. Monitoring of canine leishmaniasis innorthern Italy: an update from a scientific network. Parassitologia. 46, 193–197.

Carter C, Whitcomb J, Campbell J, Mukbel R, McDowell M. 2009. Complement receptor 3 deficiency influences lesion progression during *Leishmania major* infection in BALB/c mice. Infect Immun. 77(12):5668-75. doi: 10.1128/IAI.00802-08.

Carvalho L, Passos S, Dutra W, Soto M, Alonso C, Gollob K, Carvalho E, Ribeiro de Jesus A. 2005. Effect of LACK and KMP11 on IFN-gamma production by peripheral blood mononuclear cells from cutaneous and mucosal leishmaniasis patients. Scand J Immunol. 61(4):337 – 342.

Cervia J, Rosen H, Murray H. 1993. Effector role of blood monocytes in experimental visceral leishmaniasis. Infection and Immunity. 61: 4, 1330–1333.

Chakravarty J, Kumar S, Trivedi S, Rai VK, Singh A, Ashman JA, Laughlin E, Coler R, Kahn S, Beckmann A, Cowgill K, Reed S, Sundar S, Piazza F. 2011. A clinical trial to evaluate the safety and immunogenicity of the LEISH-F1+MPL-SE vaccine for use in the prevention of visceral leishmaniasis. Vaccine. 29(19):3531-7. doi: 10.1016/j.vaccine.2011.02.096.

Charmoy M, Auderset F, Allenbach C, Tacchini-Cottier F. 2010. The Prominent Role of Neutrophils during the Initial Phase of Infection by *Leishmania* Parasites. Journal of Biomedicine and Biotechnology. 2010:719361. doi: 10.1155/2010/719361.

Ciaramella P, Oliva G, Luna R, Gradoni L, Ambrosio R, Cortese L, Scalone A, Persechino A. 1997. A retrospective clinical study of canine leishmaniasis in 150 dogs naturally infected by *Leishmania infantum*. Vet Rec. 141(21):539-43.

Claborn D. 2010. The biology and control of leishmaniasis vectors. J Glob Infect Dis. 2(2):127-34. doi: 10.4103/0974-777X.62866.

Clay GM, Sutterwala FS, Wilson ME. 2014. NLR proteins and parasitic disease. 59(1-3):142-52. doi: 10.1007/s12026-014-8544-x.

Collin N, Gomes R, Teixeira C, Cheng L, Laughinghouse A, Ward J, Elnaiem D, Fischer L, Valenzuela J, Kamhawi S. 2009. Sand fly salivary proteins induce strong cellular

immunity in a natural reservoir of visceral leishmaniasis with adverse consequences for *Leishmania*. PLoS Pathog. 5(5):e1000441. doi: 10.1371/journal.ppat.1000441.

Collin S, Davidson R, Ritmeijer K, Keus K, Melaku Y, Kipngetich S, Davies C. 2004. Conflict and kala-azar: determinants of adverse outcomes of kala-azar among patients in southern Sudan. Clin Infect Dis. 38(5):612-9.

Costa C, Pereira H, Araújo M. 1990. Visceral leishmaniasis epidemic in the State of Piauí, Brazil, 1980-1986. Rev Saude Publica. 24(5):361-72.

Costa D, Favali C, Clarêncio J, Afonso L, Conceição V, Miranda J, Titus R, Valenzuela J, Barral-Netto M, Barral A, Brodskyn CI. 2004. *Lutzomyia longipalpis* salivary gland homogenate impairs cytokine production and costimulatory molecule expression on human monocytes and dendritic cells. Infect Immun. 72(3):1298-305.

Costa J, Viana G, Saldanha A, Nascimento M, Alvim A, Burattini M, Silva A. 1995. Visceral leishmaniasis in the State of Maranhão, Brazil: evolution of an epidemic. Cad Saude Publica. 11(2):321-4.

Courtenay O, Quinnell R, Garcez L, Shaw J, Dye C. 2002. Infectiousness in a cohort of brazilian dogs: why culling fails to control visceral leishmaniasis in areas of high transmission. J Infect Dis. 186(9):1314-20.

Crispe I. 2009. The liver as a lymphoid organ. Annu. Rev. Immunol. 27, 147–163.

Croan D, Ellis J. 1996. Phylogenetic relationships between *Leishmania*, *Viannia* and *Sauroleishmania* inferred from comparison of a variable domain within the RNA polymerase II largest subunit gene. Mol Biochem Parasitol. 79(1):97-102.

Croan D, Morrison D, Ellis J. 1997. Evolution of the genus *Leishmania* revealed by comparison of DNA and RNA polymerase gene sequences. Mol Biochem Parasitol. 89(2):149-59.

Cupolillo E, Grimaldi Júnior G, Momen H, Beverley S. 1995. Intergenic region typing (IRT): a rapid molecular approach to the characterization and evolution of *Leishmania*. Mol Biochem Parasitol. 73(1-2):145-55.

Cupolillo E, Medina-Acosta E, Noyes H, Momen H, Grimaldi G. 2000. A revised classification for *Leishmania* and *Endotrypanum*. Parasitol Today. 16(4):142-4.

Cyster J. 2000. B cells on the front line. Nature Immunology. 1(1):9-10. doi: 10.1038/76859.

da Silva V, Borja-Cabrera G, Correia Pontes N, de Souza E, Luz K, Palatnik M, Palatnik de Sousa C. 2000. A phase III trial of efficacy of the FML-vaccine against canine kala-azar in an endemic area of Brazil (São Gonçalo do Amaranto, RN). Vaccine. 19(9-10):1082-92.

Dávila A, Momen H. 2000. Internal-transcribed-spacer (ITS) sequences used to explore phylogenetic relationships within *Leishmania*. Ann Trop Med Parasitol. 94(6):651-4.

Davis B, Philipson C, Hontecillas R, Eden K, Bassaganya-Riera J, Allen I. 2014. Emerging significance of NLRs in inflammatory bowel disease. Inflamm Bowel Dis. 20(12):2412-32. doi: 10.1097/MIB.0000000000000151.

Dawit G, Girma Z, Simenew K. 2013. A Review on Biology, Epidemiology and Public Health Significance of Leishmaniasis. J Bacteriol Parasitol. 4:166. doi: 10.4172/2155-9597.1000166.

de Menezes J, Guedes C, Petersen A, Fraga D, Veras P. 2015. Advances in Development of New Treatment for Leishmaniasis. Biomed Res Int. 815023. doi: 10.1155/2015/815023.

de Veer M, Curtis J, Baldwin T, DiDonato J, Sexton A, McConville M, Handman E, Schofield L. 2003. MyD88 is essential for clearance of *Leishmania major*: possible role for lipophosphoglycan and Toll-like receptor 2 signaling. Eur J Immunol. 33(10):2822-31.

Debus A, Gläsner J, Röllinghoff M, Gessner A. 2003. High levels of susceptibility and T helper 2 response in MyD88-deficient mice infected with *Leishmania major* are interleukin-4 dependent. Infect Immun. 71(12):7215-8.

Desjeux P. 1996. Leishmaniasis. Public health aspects and control. Clin Dermatol. 14(5):417-23.

Dhingra N, Satapathy S. 2014. Altercating Leishmaniasis: Cultivating Improved Vaccines. American Journal of Infectious Diseases and Microbiology. 2(1):11-16. doi: 10.12691/ajidm-2-1-3

Dondji B, Pérez-Jimenez E, Goldsmith-Pestana K, Esteban M, McMahon-Pratt D. 2005. Heterologous prime-boost vaccination with the LACK antigen protects against murine visceral leishmaniasis. Infect Immun. 73(8):5286-9.

Dooijes D, Chaves I, Kieft R, Dirks-Mulder A, Martin W, Borst P. 2000. Base J originally found in kinetoplastida is also a minor constituent of nuclear DNA of *Euglena gracilis*. Nucleic Acids Res. 28(16):3017-21.

Duthie M, Reed S. 2014. The Emergence of Defined Subunit Vaccines for the Prevention of Leishmaniasis. Curr Trop Med Rep. 1:154–162.

Elmahallawy E, Sampedro Martinez A, Rodriguez-Granger J, Hoyos-Mallecot Y, Agil A, Navarro Mari JM, Gutierrez Fernandez J. 2014. Diagnosis of leishmaniasis. J Infect Dev Ctries. 8(8):961-72. doi: 10.3855/jidc.4310.

Engwerda C, Ato M, Cotterell S, Mynott T, Tschannerl A, Gorak-Stolinska P, Kaye P. 2002. A role for tumor necrosis factor-alpha in remodeling the splenic marginal zone during *Leishmania donovani* infection. Am J Pathol. 161(2):429-37.

Engwerda C, Kaye P. 2000. Organ-specific immune responses associated with infectious disease. Immunol Today. 21(2):73-8.

Etges R, Bouvier J, Bordier C. 1986. The major surface protein of *Leishmania* promastigotes is a protease. J Biol Chem. 261(20):9098-9101.

Falcão A, Falcão A, Pinto C, Gontijo C, Falqueto A. 1991. Effect of deltamethrin spraying on the sandfly populations in a focus of American cutaneous leishmaniasis. Mem Inst Oswaldo Cruz. 86(4):399-404.

Faria M, Reis F, Azevedo-Pereira R, Morrison L, Mottram J, Lima A. 2011. *Leishmania* inhibitor of serine peptidase 2 prevents TLR4 activation by neutrophil elastase promoting parasite survival in murine macrophages. J Immunol. 186(1):411-22. doi: 10.4049/jimmunol.1002175.

Fernandes A, Costa M, Coelho E, Michalick M, de Freitas E, Melo M, Luiz Tafuri W, Resende M, Hermont V, Abrantes F, Gazzinelli R. 2008. Protective immunity against challenge with *Leishmania (Leishmania) chagasi* in beagle dogs vaccinated with recombinant A2 protein. Vaccine. 26(46):5888-95. doi: 10.1016/j.vaccine.2008.05.095.

Fernandes C, Junior J, de Jesus C, Souza B, Larangeira D, Fraga D, Tavares Veras P, Barrouin-Melo S. 2014. Comparison of two commercial vaccines against visceral leishmaniasis in dogs from endemic areas: IgG, and subclasses, parasitism, and parasite transmission by xenodiagnosis. Vaccine. 32(11):1287-95. doi: 10.1016/j.vaccine.2013.12.046.

Fettelschoss A, Kistowska M, LeibundGut-Landmann S, Beer H, Johansen P, Senti G, Contassot E, Bachmann M, French L, Oxenius A, Kündig T. 2011. Inflammasome activation and IL-1β target IL-1α for secretion as opposed to surface expression. Proc Natl Acad Sci U S A. 108(44):18055-60. doi: 10.1073/pnas.1109176108.

Flandin J, Chano F, Descoteaux A. 2006. RNA interference reveals a role for TLR2 and TLR3 in the recognition of *Leishmania donovani* promastigotes by interferon-gamma-primed macrophages. Eur J Immunol. 36(2):411-20.

Franco A, Barnaba V, Natali P, Balsano C, Musca A, Balsano F. 1988. Expression of class I and class II major histocompatibility complex antigens on human hepatocytes. Hepatology. 8(3):449-54.

Frevert U, Engelmann S, Zougbédé S, Stange J, Ng B, Matuschewski K, Liebes L, Yee H. 2005. Intravital observation of *Plasmodium berghei* sporozoite infection of the liver. PLoS Biol. 3(6):e192.

Fritz J, Bourhis L, Sellge L, Magalhaes G, Fsihi J, Kufer H, Collins T, Viala c, Ferrero J, Girardin R, Philpott D. 2007. Nod1-mediated innate immune recognition of peptidoglycan contributes to the onset of adaptive immunity. Immunity. 26(4):445-59.

Fujiwara R, Vale A, França da Silva J, da Costa R, Quetz Jda S, Martins Filho O, Reis A, Corrêa Oliveira R, Machado-Coelho G, Bueno L, Bethony J, Frank G, Nascimento E, Genaro O, Mayrink W, Reed S, Campos-Neto A. 2005. Immunogenicity in dogs of three recombinant antigens (TSA, LeIF and LmSTI1) potential vaccine candidates for canine visceral leishmaniasis. Vet Res. 36(5-6):827-38.

Gallego C, Golenbock D, Gomez M, Saravia N. 2011. Toll-like receptors participate in macrophage activation and intracellular control of *Leishmania (Viannia) panamensis*. Infect Immun. 79(7):2871-9. doi: 10.1128/IAI.01388-10.

Ghatei M, Hatam G, Hossini M, Sarkari B. 2009. Performance of latex agglutination test (KAtex) in diagnosis of visceral leishmaniasis in Iran. Iran J Immunol. 6:202-227.

Giunchetti R, Corrêa-Oliveira R, Martins-Filho O, Teixeira-Carvalho A, Roatt B, de Oliveira Aguiar-Soares R, de Souza J, das Dores Moreira N, Malaquias L, Mota e Castro L, de Lana M, Reis A. 2007. Immunogenicity of a killed *Leishmania* vaccine with saponin adjuvant in dogs. Vaccine. 25(44):7674-86.

Giunchetti R, Corrêa-Oliveira R, Martins-Filho O, Teixeira-Carvalho A, Roatt B, de Oliveira Aguiar-Soares R, Coura-Vital W, de Abreu R, Malaquias L, Gontijo N, Brodskyn C, de Oliveira C, Costa D, de Lana M, Reis A. 2008. A killed *Leishmania* vaccine with sand fly saliva extract and saponin adjuvant displays immunogenicity in dogs. Vaccine. 26(5):623-38. doi: 10.1016/j.vaccine.2007.11.057.

Gomes R, Teixeira C, Teixeira M, Oliveira F, Menezes M, Silva C, de Oliveira C, Miranda J, Elnaiem D, Kamhawi S, Valenzuela J, Brodskyn C. 2008. Immunity to a salivary protein of a sand fly vector protects against the fatal outcome of visceral leishmaniasis in a hamster model. Proc Natl Acad Sci U S A. 105(22):7845-50. doi: 10.1073/pnas.0712153105.

Gómez-Ochoa P, Castillo J, Lucientes J, Gascón M, Zarate J, Arbea J, Larraga V, Rodriguez C. 2003. Modified direct agglutination test for simplified serologic diagnosis of leishmaniasis. Clin Diagn Lab Immunol. 10:967-968.

Gorak P, Engwerda C, Kaye P. 1998. Dendritic cells, but not macrophages, produce IL-12 immediately following *Leishmania donovani* infection. European Journal of Immunology. 28(2):687-95.

Goto H, Lindoso J. 2004. Immunity and immunosuppression in experimental visceral leishmaniasis. Braz J Med Biol Res. 37(4):615-23.

Gramiccia M, Gradoni L. 2005. The current status of zoonotic leishmaniases and approaches to disease control. Int J Parasitol. 35(11-12):1169-80.

Gueirard P1, Laplante A, Rondeau C, Milon G, Desjardins M. 2008. Trafficking of *Leishmania donovani* promastigotes in non-lytic compartments in neutrophils enables the subsequent transfer of parasites to macrophages. Cell Microbiol. 10(1):100-11.

Guerra C, Silva R, Carvalho L, Calabrese K, Bozza P, Côrte-Real S. 2010. Histopathological analysis of initial cellular response in TLR-2 deficient mice experimentally infected by *Leishmania (L.) amazonensis*. Int J Exp Pathol. 91(5):451-9. doi: 10.1111/j.1365-2613.2010.00717.x.

Guha R, Gupta D, Rastogi R, Vikram R, Krishnamurthy G, Bimal S, Roy S, Mukhopadhyay A. 2013. Vaccination with *Leishmania* hemoglobin receptor-encoding DNA protects against visceral leishmaniasis. Sci Transl Med. 5(202):202ra121. doi: 10.1126/scitranslmed.3006406.

Guiguemdé R, Sawadogo O, Bories C, Traore KL, Nezien D, Nikiema L, Pratlong F, Marty P, Houin R, Deniau M. 2003. *Leishmania major* and HIV co-infection in Burkina Faso. Trans R Soc Trop Med Hyg. 97(2):168-9.

Gupta P, Giri J, Srivastav S, Chande A, Mukhopadhyaya R, Das P, Ukil A. 2014. *Leishmania donovani* targets tumor necrosis factor receptor-associated factor (TRAF) 3 for impairing TLR4-mediated host response. FASEB J. 28(4):1756-68. doi: 10.1096/fj.13-238428.

Gurunathan S, Sacks D, Brown DR, Reiner S, Charest H, Glaichenhaus N, Seder R. 1997. Vaccination with DNA encoding the immunodominant LACK parasite antigen confers protective immunity to mice infected with *Leishmania major*. J Exp Med. 186(7):1137-47.

Gurung P, Kanneganti T. 2015. Innate immunity against *Leishmania* infections. Cell Microbiol. 17(9):1286-94. doi: 10.1111/cmi.12484.

Haldar A, Sen P, Roy S. 2011. Use of antimony in the treatment of leishmaniasis: current status and future directions. Mol Biol Int. 2011:571242. doi: 10.4061/2011/571242.

Heinzel F, Schoenhaut D, Rerko R, Rosser L, Gately M. 1993. Recombinant interleukin 12 cures mice infected with *Leishmania major*. J Exp Med. 177(5):1505-9.

Hide M, Bañuls A. 2008. Polymorphisms of cpb multicopy genes in the *Leishmania (Leishmania*) *donovani* complex. Trans R Soc Trop Med Hyg. 102(2):105-6.

Holzmuller P, Bras-Gonçalves R, Lemesre J. 2006. Phenotypical characteristics, biochemical pathways, molecular targets and putative role of nitric oxide-mediated programmed cell death in *Leishmania*. Parasitology. 132 Suppl:S19-32.

Holzmuller P, Hide M, Sereno D, Lemesre J. 2006. *Leishmania infantum* amastigotes resistant to nitric oxide cytotoxicity: Impact on *in vitro* parasite developmental cycle and metabolic enzyme activities. Infect Genet Evol. 6(3):187-97.

Hoover D, Berger M, Hammer C, Meltzer M. 1985. Complement-mediated serum cytotoxicity for *Leishmania major* amastigotes: killing by serum deficient in early components of the membrane attack complex. J Immunol. 135(1):570-4.

Hosein S, Rodríguez-Cortés A, Blake D, Allenspach K, Alberola J, Solano-Gallego L. 2015. Transcription of Toll-Like Receptors 2, 3, 4 and 9, FoxP3 and Th17 Cytokines in a Susceptible Experimental Model of Canine *Leishmania infantum* Infection. PLoS One. 10(10):e0140325. doi: 10.1371/journal.pone.0140325.

Ibrahim M, Barker D. 2001. The origin and evolution of the *Leishmania donovani* complex as inferred from a mitochondrial cytochrome oxidase II gene sequence. Infect Genet Evol. 1(1):61-8.

Ichikawa S, Mucida D, Tyznik AJ, Kronenberg M, Cheroutre H. 2011. Hepatic stellate cells function as regulatory bystanders. J Immunol. 186(10):5549-55. doi: 10.4049/jimmunol.1003917.

Iiai T, Watanabe H, Seki S, Sugiura K, Hirokawa K, Utsuyama M, Takahashi-Iwanaga H, Iwanaga T, Ohteki T, Abo T. 1992. Ontogeny and development of extrathymic T cells in mouse liver. Immunology. 77(4):556-63.

Ilg T, Montgomery J, Stierhof Y, Handman E. 1999. Molecular cloning and characterization of a novel repeat-containing *Leishmania major* gene, ppg1, that encodes a membrane-associated form of proteophosphoglycan with a putative glycosylphosphatidylinositol anchor. J Biol Chem. 274(44):31410-31420.

Inohara, Chamaillard, McDonald C, Nuñez G. 2005. NOD-LRR proteins: role in host-microbial interactions and inflammatory disease. Annual review of biochemistry. 74:355-83.

Jenne C, Kubes P. 2013. Immune surveillance by the liver. Nat Immunol. 14(10):996-1006. doi: 10.1038/ni.2691.

Jinushi M, Takehara T, Tatsumi T, Yamaguchi S, Sakamori R, Hiramatsu N, Kanto T, Ohkawa K, Hayashi N. 2007. Natural killer cell and hepatic cell interaction via NKG2A leads to dendritic cell-mediated induction of CD4 CD25 T cells with PD-1-dependent regulatory activities. Immunology. 120(1):73-82.

João A, Pereira M, Cortes S, Santos-Gomes G. 2006. Canine leishmaniasis chemotherapy: dog's clinical condition and risk of *Leishmania* transmission. J Vet Med A Physiol Pathol Clin Med. 53(10):540-5.

Gil J, Nasser J, Cajal S, Juarez M, Acosta N, Cimino R, Diosque P, Krolewiecki A. 2010. Urban Transmission of American Cutaneous Leishmaniasis in Argentina: Spatial Analysis Study. Am J Trop Med Hyg. 82(3):433–440. doi: 10.4269/ajtmh.2010.09-0113.

Joshi P, Kelly B, Kamhawi S, Sacks D, McMaster W. 2002. Targeted gene deletion in *Leishmania major* identifies leishmanolysin (GP63) as a virulence factor. Mol Biochem Parasitol. 120(1):33-40.

Joshi T, Rodriguez S, Perovic V, Cockburn I, Stäge S. 2009. B7-H1 blockade increases survival of dysfunctional CD8(+) T cells and confers protection against *Leishmania donovani* infections. PLoS Pathogens. 5(5):e1000431. doi: 10.1371/journal.ppat.1000431.

Julia V, Rassoulzadegan M, Glaichenhaus N. 1996. Resistance to *Leishmania major* induced by tolerance to a single antigen. Science. 274(5286):421-3.

Kamhawi S. 2000. The biological and immunomodulatory properties of sand fly saliva and its role in the establishment of *Leishmania* infections. Microbes Infect. 2:1765–73.

Karmakar S, Bhaumik S, Paul J, De T. 2012. TLR4 and NKT cell synergy in immunotherapy against visceral leishmaniasis. PLoS Pathog. 8(4):e1002646. doi: 10.1371/journal.ppat.1002646.

Kassi M, Kasi PM, Marri SM, Tareen I, Khawar T. 2008. Vector control in cutaneous leishmaniasis of the old world: a review of literature. Dermatol Online J. 14(6):1.

Kaszak I, Planellas M, Dworecka-Kaszak B. 2015. Canine leishmaniosis - an emerging disease. Ann Parasitol. 61(2):69-76.

Kawai T, Akira S. 2010. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nature immunology. 11(5):373-84. doi: 10.1038/ni.1863.

Kerr S. 2000. Palaearctic origin of *Leishmania*. Mem Inst Oswaldo Cruz. 95(1):75-80.

Killick-Kendrick R, Killick-Kendrick M, Focheux C, Dereure J, Puech M-P, Cadiergues M. 1997. Protection of dogsfrom bites of phlebotomine sandflies by deltamethrin collars for control of canine leishmaniasis. Medical and Veterinary Entomology. 11(2):105-11.

Killick-Kendrick R, Molyneux D, Ashford R. 1974. *Leishmania* in phlebotomid sandflies. I. Modifications of the flagellum associated with attachment to the mid-gut and oesophageal valve of the sandfly. Proc R Soc Lond B Biol Sci. 187(1089):409-19.

Kita H, Mackay I, Van De Water J, Gershwin M. 2001. The lymphoid liver: considerations on pathways to autoimmune injury. Gastroenterology. 120:1485–1501.

Kopf M, Brombacher F, Köhler G, Kienzle G, Widmann K, Lefrang K, Humborg C, Ledermann B, Solbach W. 1996. IL-4-deficient Balb/c mice resist infection with *Leishmania major*. J Exp Med. 184(3):1127-36.

Korn T, Bettelli E, Oukka M, Kuchroo V. 2009. IL-17 and Th17 Cells. Annu Rev Immunol. 27:485-517. doi: 10.1146/annurev.immunol.021908.132710.

Kropf P, Freudenberg M, Modolell M, Price H, Herath S, Antoniazi S, Galanos C, Smith D, Müller I. 2004a. Toll-like receptor 4 contributes to efficient control of infection with the protozoan parasite *Leishmania major*. Infect Immun. 72(4):1920-8.

Kropf P, Freudenberg N, Kalis C, Modolell M, Herath S, Galanos C, Freudenberg M, Müller I. 2004b. Infection of C57BL/10ScCr and C57BL/10ScNCr mice with *Leishmania major* reveals a role for Toll-like receptor 4 in the control of parasite replication. J Leukoc Biol. 76(1):48-57.

Kuhls K, Mauricio I, Pratlong F, Presber W, Schönian G. 2005. Analysis of ribosomal DNA internal transcribed spacer sequences of the *Leishmania donovani* complex. Microbes Infect. 7(11-12):1224-34.

Kumar R, Engwerda C. 2014. Vaccines to prevent leishmaniasis. Clin Transl Immunology. 3(3):e13. doi: 10.1038/cti.2014.4

Kumar R, Goto Y, Gidwani K, Cowgill K, Sundar S, Reed S. 2010. Evaluation of *ex vivo* human immune response against candidate antigens for a visceral leishmaniasis vaccine. Am J Trop Med Hyg. 82(5):808-13. doi: 10.4269/ajtmh.2010.09-0341.

Lainson R, Shaw J. 1977. Leishmaniasis in Brazil: XII. Observations on cross-immunity in monkeys and man infected with *Leishmania mexicana mexicana, L. m. amazonensis, L. braziliensis braziliensis, L. b. guyanensis and L. b. panamensis*. J Trop Med Hyg. 80(2):29-35.

Laison R. 2010. The Neotropical *Leishmania* species: a brief historical review of their discovery, ecology and taxonomy. Rev Pan-Amaz Saude. 1(2):13-32

Lane R. 1991. The contributions of sand fly control to leishmaniasis control. Ann Soc Belg Med Trop. 71:65–74.

Laufs H, Müller K, Fleischer J, Reiling N, Jahnke N, Jensenius JC, Solbach W, Laskay T. 2002. Intracellular survival of *Leishmania major* in neutrophil granulocytes after uptake in the absence of heat-labile serum factors. Infect Immun. 70(2):826-35.

Lee M, Kim Y. 2007. Signaling pathways downstream of pattern-recognition receptors and their cross talk. Annual review of biochemistry. 76:447-80.

Li F, Tian Z. 2013. The liver works as a school to educate regulatory immune cells. Cell Mol Immunol. 10(4):292–302. doi: 10.1038/cmi.2013.7.

Liese J, Schleicher U, Bogdan C. 2007. TLR9 signaling is essential for the innate NK cell response in murine cutaneous leishmaniasis. Eur J Immunol. 37(12):3424-34.

Lima-Junior D, Costa D, Carregaro V, Cunha L, Silva A, Mineo T, Gutierrez F, Bellio M, Bortoluci K, Flavell R, Bozza M, Silva JS, Zamboni D. 2013. Inflammasome-derived IL-1β production induces nitric oxide-mediated resistance to *Leishmania*. Nat Med. 19(7):909-15. doi: 10.1038/nm.3221.

Liu D, Kebaier C, Pakpour N, Capul A, Beverley S, Scott P, Uzonna J. 2009. *Leishmania major* phosphoglycans influence the host early immune response by modulating dendritic cell functions. Infect Immun. 77(8):3272-83.

Llanos-Cuentas A, Calderón W, Cruz M, Ashman J, Alves F, Coler R, Bogatzki L, Bertholet S, Laughlin E, Kahn S, Beckmann A, Cowgill K, Reed S, Piazza F. 2010. A clinical trial to evaluate the safety and immunogenicity of the LEISH-F1+MPL-SE vaccine when used in combination with sodium stibogluconate for the treatment of mucosal leishmaniasis. Vaccine. 28(46):7427-35. doi: 10.1016/j.vaccine.2010.08.092.

Lukes J, Mauricio I, Schönian G, Dujardin J, Soteriadou K, Dedet J, Kuhls K, Tintaya K, Jirků M, Chocholová E, Haralambous C, Pratlong F, Oborník M, Horák A, Ayala F, Miles M. 2007. Evolutionary and geographical history of the *Leishmania donovani* complex with a revision of current taxonomy. Proc Natl Acad Sci U S A. 104(22):9375-80.

Lüth S, Huber S, Schramm C, Buch T, Zander S, Stadelmann C, Brück W, Wraith D, Herkel J, Lohse A. 2008. Ectopic expression of neural autoantigen in mouse liver suppresses experimental autoimmune neuroinflammation by inducing antigen-specific Tregs. J Clin Invest. 118(10):3403-10. doi: 10.1172/JCI32132.

Luyo-Acero G, Uezato H, Oshiro M, Takei K, Kariya K, Katakura K, Gomez-Landires E, Hashiguchi Y, Nonaka S. 2004. Sequence variation of the cytochrome b gene of various human infecting members of the genus *Leishmania* and their phylogeny. Parasitology. 128(Pt 5):483-91.

Luz KG, da Silva VO, Gomes EM, Machado FC, Araujo MA, Fonseca HE, Freire TC, d'Almeida JB, Palatnik M, Palatnik-de-Sousa CB. 1997. Prevalence of anti-*Leishmania donovani* antibody among Brazilian blood donors and multiply transfused hemodialysis patients. Am J Trop Med Hyg. 57(2):168-71.

Mackay C, Marston W, Dudler L, Spertini O, Tedder T, Hein W. 1992. Tissue-specific migration pathways by phenotypically distinct subpopulations of memory T cells. Eur J Immunol. 22:887–895.

Mackay C, Marston W, Dudler L. 1990. Naive and memory T cells show distinct pathways of lymphocyte recirculation. J Exp Med. 171:801–817.

Maroli M, Pennisi MG, Di Muccio T, Khoury C, Gradoni L, Gramiccia M. 2007. Infection of sandflies by a cat naturally infected with *Leishmania infantum*. Vet Parasitol. 145(3-4):357-60.

Mary C, Faraut F, Lascombe L, Dumon H . 2004. Quantification of *Leishmania infantum* DNA by a real-time PCR assay with high sensitivity. J Clin Microbiol. 42: 5249-5255.

Matsuda J, Mallevaey T, Scott-Browne J, Gapin L. 2008. CD1d-restricted iNKT cells, the "Swiss-Army knife" of the immune system. Curr Opin Immunol. 20(3):358-68. doi: 10.1016/j.coi.2008.03.018.

Mauricio I, Gaunt M, Stothard J, Miles M. 2007. Glycoprotein 63 (gp63) genes show gene conversion and reveal the evolution of Old World *Leishmania.* Int J Parasitol. 37(5):565-76.

McKerrow J, Caffrey C, Kelly B, Loke P, Sajid M. 2006. Proteases in parasitic diseases. Ann Rev Pathol. 1:497 – 536.

Melby P, Tabares A, Restrepo B, Cardona A, McGuff H, Teale J. 2001. *Leishmania donovani:* evolution and architecture of the splenic cellular immune response related to control of infection. ExpParasitol. 99(1):17–25.

Meylan E, Tschopp J, Karin M. 2006. Intracellular pattern recognition receptors in the host response. Nature. 442(7098):39-44.

Miró G, Cardoso L, Pennisi M, Oliva G, Baneth G. 2008. Canine leishmaniosis - new concepts and insights on an expanding zoonosis: part two. Trends Parasitol. 24(8):371-7. doi: 10.1016/j.pt.2008.05.003.

Mohapatra T, Singh D, Sen M, Bharti K, Sundar S. 2010. Comparative evaluation of rK9 rK26 and rK39 antigens in the serodiagnosis of Indian visceral leishmaniasis. J Infect Dev Ctries. 4: 114-117. doi: 10.3855/jidc.544.

Mohapatra S. 2014. Drug resistance in leishmaniasis: Newer developments. Trop Parasitol. 4(1):4-9. doi: 10.4103/2229-5070.129142.

Momen H, Cupolillo E. 2000. Speculations on the origin and evolution of the genus *Leishmania.* Mem Inst Oswaldo Cruz. 95(4):583-8.

Momeni A, Jalayer T, Emamjomeh M, Khamesipour A, Zicker F, Ghassemi R, Dowlati Y, Sharifi I, Aminjavaheri M, Shafiei A, Alimohammadian M, Hashemi-Fesharki R, Nasseri K, Godal T, Smith P, Modabber F. 1999. A randomised, double-blind, controlled trial of a killed *L. major* vaccine plus BCG against zoonotic cutaneous leishmaniasis in Iran. Vaccine. 17(5):466-72.

Moreno J, Vouldoukis I, Schreiber P, Martin V, McGahie D, Gueguen S, Cuisinier A. 2014. Primary vaccination with the LiESP/QA-21 vaccine (CaniLeish) produces a cell-mediated immune response which is still present 1 year later. Vet Immunol Immunopathol. 158(3-4):199-207. doi: 10.1016/j.vetimm.2014.01.011.

Mosser D, Edelson P. 1985. The mouse macrophage receptor for C3bi (CR3) is a major mechanism in the phagocytosis of *Leishmania* promastigotes. J Immunol. 135(4):2785-9.

Mott K, Desjeux P, Moncayo A, Ranque P, de Raadt P. 1990. Parasitic diseases and urban development. Bull World Health Organ. 68(6):691-8.

Muhanna N, Horani A, Doron S, Safadi R. 2007. Lymphocyte-hepatic stellate cell proximity suggests a direct interaction. Clin Exp Immunol. 148(2):338-47.

Muller G, Junnila A, Kaavchenko V, Revay E, Butler J, Schlein Y. 2008. Indoor protection against mosquito and sand fly bites: A comparison between citronella, linalool and geraniol candles. J Am Mosq Control Assoc. 24:150–3.

Müller I. 1992. Role of T cell subsets during the recall of immunologic memory to *Leishmania major*. Eur J Immunol. 22(12):3063-9.

Muraille E, De Trez C, Brait M, De Baetselier P, Leo O, Carlier Y. 2003. Genetically resistant mice lacking MyD88-adapter protein display a high susceptibility to *Leishmania major* infection associated with a polarized Th2 response. J Immunol. 170(8):4237-41.

Murray H, Nathan F. 1999. Macrophage microbicidal mechanisms *in vivo*: reactive nitrogen versus oxygen intermediates in the killing of intracellular visceral *Leishmania donovani*. Journal of Experimental Medicine. 189(4):741–746.

Murray H, Hariprashad J. 1995. Interleukin 12 is effective treatment for an established systemic intracellular infection: experimental visceral leishmaniasis. Journal of Experimental Medicine. 181(1):387–391.

Murray H, Berman J, Davies C, Saravia N. 2005. Advances in leishmaniasis. Lancet. 366(9496):1561-77.

Murray H, Lu C, Mauze S, Freeman S, Moreira A, Kaplan G, Coffman R. 2002. Interleukin-10 (IL-10) in experimental visceral leishmaniasis and IL-10 receptor blockade as immunotherapy. Infect Immun. 70(11):6284-93.

Murray H, Zhang Y, Zhang Y, Raman V, Reed S, Ma X. 2013. Regulatory actions of Toll-like receptor 2 (TLR2) and TLR4 in *Leishmania donovani* infection in the liver. Infect Immun. 81(7):2318-26. doi: 10.1128/IAI.01468-12.

Murray H. 1997. Endogenous interleukin-12 regulates acquired resistance in experimental visceral leishmaniasis. Journal of Infectious Diseases. 175:6,1477–79.

Musa A, Khalil E, Hailu A, Olobo J, Balasegaram M, Omollo R, Edwards T, Rashid J, Mbui J, Musa B, Abuzaid AA, Ahmed O, Fadlalla A, El-Hassan A, Mueller M, Mucee G, Njoroge S, Manduku V, Mutuma G, Apadet L, Lodenyo H, Mutea D, Kirigi G, Yifru S, Mengistu G, Hurissa Z, Hailu W, Weldegebreal T, Tafes H, Mekonnen Y, Makonnen E, Ndegwa S, Sagaki P, Kimutai R, Kesusu J, Owiti R, Ellis S, Wasunna M. 2012. Sodium stibogluconate (SSG) & paromomycin combination compared to SSG for visceral leishmaniasis in East Africa: a randomised controlled trial. PLoS Negl Trop Dis. 6(6):e1674. doi: 10.1371/journal.pntd.0001674.

Musa A, Khalil E, Younis B, Elfaki M, Elamin M, Adam A, Mohamed H, Dafalla M, Abuzaid A, El-Hassan A. 2013. Treatment-Based Strategy for the Management of Post-Kala-Azar Dermal Leishmaniasis Patients in the Sudan. J Trop Med. 2013:708391. doi: 10.1155/2013/708391.

Muyombwe A, Olivier M, Ouellette M, Papadopoulou B. 1997. Selective killing of *Leishmania* amastigotes expressing a thymidine kinase suicide gene. Exp Parasitol. 85(1):35-42.

Myskova J, Svobodova M, Beverley S, Volf P. 2007. A lipophosphoglycan-independent development of *Leishmania* in permissive sand flies. Microbes Infect. 9(3):317-24.

Nagill R, Kaur S. 2011. Vaccine candidates for leishmaniasis: a review. Int Immunopharmacol. 11(10):1464-88.

Nascimento E, Fernandes D, Vieira E, Campos-Neto A, Ashman J, Alves F, Coler R, Bogatzki L, Kahn S, Beckmann A, Pine S, Cowgill K, Reed S, Piazza F. 2010. A clinical trial to evaluate the safety and immunogenicity of the LEISH-F1+MPL-SE vaccine when used in combination with meglumine antimoniate for the treatment of cutaneous leishmaniasis. Vaccine. 28(40):6581-7. doi: 10.1016/j.vaccine.2010.07.063.

Nascimento M, Carregaro V, Lima-Júnior D, Costa D, Ryffel B, Duthie M, de Jesus A, de Almeida R, da Silva J. 2015. Interleukin 17A acts synergistically with interferon γ to promote protection against *Leishmania infantum* infection. J Infect Dis. 211(6):1015-26. doi: 10.1093/infdis/jiu531.

Navin T, Arana B, Arana F, Berman J, Chajón J. 1992. Placebo-controlled clinical trial of sodium stibogluconate (Pentostam) versus ketoconazole for treating cutaneous leishmaniasis in Guatemala. J Infect Dis. 165(3):528-34.

Navin T, Krug E, Pearson R. 1989. Effect of immunoglobulin M from normal human serum on *Leishmania donovani* promastigote agglutination, complement-mediated killing, and phagocytosis by human monocytes. Infect Immun. 57(4):1343-6.

Nieto C, García-Alonso M, Requena J, Mirón C, Soto M, Alonso C, Navarrete I. 1999. Analysis of the humoral immune response against total and recombinant antigens of *Leishmania infantum*: correlation with disease progression in canine experimental leishmaniasis. Vet Immunol Immunopathol. 67(2):117-30.

Nieves E, Pimenta P. 2000. Development of *Leishmania* (*Viannia*) *braziliensis* and *Leishmania* (*Leishmania*) *amazonensis* in the sand fly *Lutzomyia migonei* (*Diptera*: *Psychodidae*). J Med Entomol. 37(1):134-40.

Notas G, Kisseleva T, Brenner D. 2009. NK and NKT cells in liver injury and fibrosis. Clin Immunol. 130(1):16-26. doi: 10.1016/j.clim.2008.08.008.

Noyes H, Morrison D, Chance M, Ellis J. 2000. Evidence for a neotropical origin of *Leishmania*. Mem Inst Oswaldo Cruz. 95(4):575-8.

Noyes H, Pratlong F, Chance M, Ellis J, Lanotte G, Dedet J. 2002. A previously unclassified trypanosomatid responsible for human cutaneous lesions in Martinique (French West Indies) is the most divergent member of the genus *Leishmania* ss. Parasitology. 124(Pt 1):17-24.

Noyes H. 1998. Implications of a Neotropical origin of the genus *Leishmania*. Mem Inst Oswaldo Cruz. 93(5):657-61.

O'Farrelly C, Crispe I. 1999. Prometheus through the looking glass: reflections on the hepatic immune system. Immunol Today. 20:394–398.

Olliaro P, Bryceson A. 1993. Practical progress and new drugs for changing patterns of leishmaniasis. Parasitol Today. 9(9):323-8.

Oskam L, Nieuwenhuijs J, Hailu A. 1999. Evaluation of the direct agglutination test (DAT) using freeze-dried antigen for the detection of anti-*Leishmania* antibodies in stored sera from various patient groups in Ethiopia. Trans R Soc Trop Med Hyg: 93: 275-277.

Palatnik-de-Sousa C, Silva-Antunes I, Morgado Ade A, Menz I, Palatnik M, Lavor C. 2009. Decrease of the incidence of human and canine visceral leishmaniasis after dog vaccination with Leishmune in Brazilian endemic areas. Vaccine. 27(27):3505-12. doi: 10.1016/j.vaccine.2009.03.045.

Palm N, Medzhitov R. 2009. Pattern recognition receptors and control of adaptive immunity. Immunological reviews. 227(1):221-33. doi: 10.1111/j.1600-065X.2008.00731.x.

Pandey K, Pun S, Pandey B. 2012. Relapse of kala-azar after use of multiple drugs: a case report and brief review of literature. Indian J Med Microbiol. 30(2):227-9. doi: 10.4103/0255-0857.96703.

Perrotey S, Mahamdallie S, Pesson B, Richardson K, Gállego M, Ready P. 2005. Postglacial dispersal of *Phlebotomus perniciosus* into France. Parasite. 12(4):283-91.

Peters C, Stierhof YD, Ilg T. 1997. Proteophosphoglycan secreted by *Leishmania mexicana* amastigotes causes vacuole formation in macrophages. Infect Immun. 65(2):783-6.

Peters NC, Kimblin N, Secundino N, Kamhawi S, Lawyer P, Sacks DL. 2009. Vector transmission of *Leishmania* abrogates vaccine-induced protective immunity. PLoS Pathog. 5(6):e1000484. doi: 10.1371/journal.ppat.1000484.

Piarroux R, Fontes M, Perasso R, Gambarelli F, Joblet C, Dumon H, Quilici M. 1995. Phylogenetic relationships between Old World *Leishmania* strains revealed by analysis of a repetitive DNA sequence. Mol Biochem Parasitol. 73(1-2):249-52.

Pillarisetty V, Katz S, Bleier J, Shah A, Dematteo R. 2005. Natural killer dendritic cells have both antigen presenting and lytic function and in response to CpG produce IFN-gamma via autocrine IL-12. J Immunol. 174(5):2612-8.

Pillarisetty V, Shah A, Miller G, Bleier J, DeMatteo R. 2004. Liver dendritic cells are less immunogenic than spleen dendritic cells because of differences in subtype composition. J Immunol. 172(2):1009-17.

Pimenta P, Turco S, McConville M, Lawyer P, Perkins P, Sacks D. 1992. Stage-specific adhesion of *Leishmania* promastigotes to the sandfly midgut. Science. 256(5065):1812-5.

Pinelli E, Gonzalo R, Boog C, Rutten V, Gebhard D, del Real G, Ruitenberg E. 1995. *Leishmania infantum*-specific T cell lines derived from asymptomatic dogs that lyse infected macrophages in a major histocompatibility complex-restricted manner. Eur J Immunol. 25(6):1594-600.

Poinar G, Poinar R. 2004. *Paleoleishmania proterus* n. gen., n. sp., (*Trypanosomatidae*: *Kinetoplastida*) from *Cretaceous Burmese* amber. Protist. 155(3):305-10.

Poot J, Janssen L, van Kasteren-Westerneng T, van der Heijden-Liefkens K, Schijns V, Heckeroth A. 2009. Vaccination of dogs with six different candidate leishmaniasis vaccines composed of a chimerical recombinant protein containing ribosomal and histone protein epitopes in combination with different adjuvants. Vaccine. 27(33):4439-46. doi: 10.1016/j.vaccine.2009.05.043.

Puentes S, Da Silva R, Sacks D, Hammer C, Joiner K. 1990. Serum resistance of metacyclic stage *Leishmania major* promastigotes is due to release of C5b-9. J Immunol. 145(12):4311-6.

Purkait B, Kumar A, Nandi N, Sardar A, Das S, Kumar S, Pandey K, Ravidas V, Kumar M, De T, Singh D, Das P. 2012. Mechanism of amphotericin B resistance in clinical isolates of *Leishmania donovani*. Antimicrob Agents Chemother. 56(2):1031-41. doi: 10.1128/AAC.00030-11.

Quinnell R, Kennedy L, Barnes A, Courtenay O, Dye C, Garcez L, Shaw M, Carter SD, Thomson W, Ollier W. 2003. Susceptibility to visceral leishmaniasis in the domestic dog is associated with MHC class II polymorphism. Immunogenetics. 55(1):23-8.

Racanelli V, Rehermann B. 2006. The liver as an immunological organ. Hepatology. 43, S54–S62.

Rahman A, Aloman C. 2013. Dendritic cells and liver fibrosis. Biochimica et Biophysica Acta. 1832(7):998-1004. doi: 10.1016/j.bbadis.2013.01.005.

Rai A, Thakur C, Singh A, Seth T, Srivastava S, Singh P, Mitra D. 2012. Regulatory T cells suppress T cell activation at the pathologic site of human visceral leishmaniasis. PLoS One. 7(2):e31551. doi: 10.1371/journal.pone.0031551.

Ramiro M, Zárate J, Hanke T, Rodriguez D, Rodriguez J, Esteban M, Lucientes J, Castillo J, Larraga V. 2003. Protection in dogs against visceral leishmaniasis caused by *Leishmania infantum* is achieved by immunization with a heterologous prime-boost regime using DNA and vaccinia recombinant vectors expressing LACK. Vaccine. 21(19-20):2474-84.

Ready P. 2010. Leishmaniasis emergence in Europe. Eurosurveillance. 15(10):19505.

Ready P. 2014. Epidemiology of visceral leishmaniasis. Clin Epidemiol. 6:147-54. doi: 10.2147/CLEP.S44267.

Reis A, Teixeira-Carvalho A, Vale A, Marques M, Giunchetti R, Mayrink W, Guerra L, Andrade R, Corrêa-Oliveira R, Martins-Filho O. 2006. Isotype patterns of immunoglobulins: hallmarks for clinical status and tissue parasite density in Brazilian dogs naturally infected by *Leishmania (Leishmania) chagasi*. Vet Immunol Immunopathol. 112(3-4):102-16.

Reithinger R, Mohsen M,Aadil K,Sidiqi M, Erasmus P, Coleman P. 2003. Anthroponotic Cutaneous Leishmaniasis, Kabul, Afghanistan. Emerg Infect Dis. 9(6):727–729. doi: 10.3201/eid0906.030026.

Rioux J, Lanotte G, Serres E, Pratlong F, Bastien P, Perieres J. 1990. Taxonomy of *Leishmania*. Use of isoenzymes. Suggestions for a new classification. Ann Parasitol Hum Comp. 65(3):111-25.

Ritmeijer K, Davies C, van Zorge R, Wang S, Schorscher J, Dongu'du S, Davidson R. 2007. Evaluation of a mass distribution programme for fine-mesh impregnated bednets against visceral leishmaniasis in eastern Sudan. Trop Med Int Health. 12(3):404-14.

Rivier D, Bovay P, Shah R, Didisheim S, Mauël J. 1999. Vaccination against *Leishmania major* in a CBA mouse model of infection: role of adjuvants and mechanism of protection. Parasite Immunol. 21(9):461-73.

Robert L, Perich M, Schlein Y, Jacobson RL, Wirtz R, Lawyer PG, Githure J. 1997. Phlebotomine sand fly control using bait-fed adults to carry the larvicide *Bacillus sphaericus* to the larval habitat. J Am Mosq Control Assoc. 13(2):140-4.

Roberts M. 2006. Current understandings on the immunology of leishmaniasis and recent developments in prevention and treatment. Br Med Bull. 75-76:115-30.

Rodrigues O, Marques C, Soares-Clemente M, Ferronha M, Santos-Gomes G. 2009. Identification of regulatory T cells during experimental *Leishmania infantum* infection. Immunobiology. 214(2):101-11. doi: 10.1016/j.imbio.2008.07.001.

Rodríguez-Cortés A, Ojeda A, López-Fuertes L, Timón M, Altet L, Solano-Gallego L, Sánchez-Robert E, Francino O, Alberola J. 2007. A long term experimental study of canine visceral leishmaniasis. Int J Parasitol. 37(6):683-93.

Roger A, Hug L. 2006. The origin and diversification of eukaryotes: problems with molecular phylogenetics and molecular clock estimation. Philos Trans R Soc Lond B Biol Sci. 361(1470):1039-54.

Rogers M, Bates P. 2007. *Leishmania* manipulation of sand fly feeding behavior results in enhanced transmission. PLoS Pathog. 3(6):e91.

Rogers M, Chance M, Bates P. 2002. The role of promastigote secretory gel in the origin and transmission of the infective stage of *Leishmania mexicana* by the sandfly *Lutzomyia longipalpis*. Parasitology. 124(Pt 5):495-507.

Rohoušová I, Hostomská J, Vlková M, Kobets T, Lipoldová M, Volf P. 2011. The protective effect against *Leishmania* infection conferred by sand fly bites is limited to short-term exposure. Int J Parasitol. 41(5):481-5. doi: 10.1016/j.ijpara.2011.01.003.

Romero G, de la Gloria Orge Orge M, de Farias M, Paes M, de Olivera Macedo V, de Carvalho E. 2005. Antibody response in patients with cutaneous leishmaniasis infected by *Leishmania* (*Viannia*) *brazilensis* or *Leishmania* (*Viannia*) *guayanensis* in Brazil. Acta Trop. 93:49-56.

Ronet C, Voigt H, Himmelrich H, Doucey M, Hauyon-La Torre Y, Revaz-Breton M, Tacchini-Cottier F, Bron C, Louis J, Launois P. 2008. *Leishmania major*-specific B cells are necessary for Th2 cell development and susceptibility to *L. major* LV39 in BALB/c mice. J Immunol. 180(7):4825-35.

Ryan J, Smithyman A, Rajasekariah G, Hochberg L, Stiteler J, Martin S. 2002. Enzyme-linked immunosorbent assay based on soluble promastigote antigen detects immunoglobulin M (IgM) & IgG antibodies in sera from cases of visceral and cutaneous leishmaniasis. J Clin Microbiol. 40: 1037-1043.

Saad A, Ahmed N, Osman O, Al-Basheer A, Hamad A, Deborggraeve A, Büscher P, Schoone G, Schallig H, Laurent T, Haleem A, Osman O, Eltom A, Elbashir M, El-Safi S. 2010. Diagnostic Accuracy of the *Leishmania* OligoC-TesT and NASBA-Oligochromatography for Diagnosis of Leishmaniasis in Sudan. PLoS Neglec Trop Dis. 4(8):e776. doi: 10.1371/journal.pntd.0000776.

Sacks D, Kamhawi S. 2001. Molecular aspects of parasite-vector and vector-host interactions in leishmaniasis. Annu Rev Microbiol. 55:453-83.

Sacks D, Modi G, Rowton E, Späth G, Epstein L, Turco S, Beverley S. 2000. The role of phosphoglycans in *Leishmania*-sand fly interactions. Proc Natl Acad Sci U S A. 97(1):406-11.

Sanchez M, Diaz N, Zerpa O, Negron E, Convit J, Tapia F. 2004. Organ-specific immunity in canine visceral leishmaniasis: analysis of symptomatic and asymptomatic dogs naturally infected with *Leishmania chagasi*. Am J Trop Med Hyg. 70(6):618-24.

Sanchez-Robert E, Altet L, Sanchez A, Francino O. 2005. Polymorphism of Slc11a1 (Nramp1) gene and canine leishmaniasis in a case-control study. J Hered. 96(7):755-8.

Santos-Gomes G, Gomes-Pereira S, Campino L, Araujo M, Abranches P. 2000. Performance of immunoblotting in diagnosis of visceral Leishmaniasis in human immunodeficiency virus-*Leishmania* sp.-coinfected patients. J Clin Microbiol. 38: 175-178.

Santos-Gomes G, Rosa R, Leandro C, Cortes S, Romão P, Silveira H. 2002. Cytokine expression during the outcome of canine experimental infection by *Leishmania infantum*. Vet Immunol Immunopathol. 88(1-2):21-30.

Saraiva E, de Figueiredo Barbosa A, Santos F, Borja-Cabrera G, Nico D, Souza L, de Oliveira Mendes-Aguiar C, de Souza E, Fampa P, Parra L, Menz I, Dias J, de Oliveira S, Palatnik-de-Sousa C. 2006. The FML-vaccine (Leishmune) against canine visceral leishmaniasis: a transmission blocking vaccine. Vaccine. 24(13):2423-31.

Sarma J, Ward P. 2011. The complement system. Cell Tissue Res. 343(1):227-35. doi: 10.1007/s00441-010-1034-0.

Schamber-Reis B, Petritus P, Caetano B, Martinez E, Okuda K, Golenbock D, Scott P, Gazzinelli R. 2013. UNC93B1 and nucleic acid-sensing Toll-like receptors mediate host resistance to infection with *Leishmania major*. J Biol Chem. 288(10):7127-36. doi: 10.1074/jbc.M112.407684.

Scharton T, Scott P. 1993. Natural killer cells are a source of interferon gamma that drives differentiation of $CD4^+$ T cell subsets and induces early resistance to *Leishmania major* in mice. J Exp Med. 178(2):567-77.

Schleicher U, Liese J, Knippertz I, Kurzmann C, Hesse A, Heit A, Fischer J, Weiss S, Kalinke U, Kunz S, Bogdan C. 2007. NK cell activation in visceral leishmaniasis requires TLR9, myeloid DCs, and IL-12, but is independent of plasmacytoid DCs. J Exp Med. 204(4):893-906.

Schönian G, Kuhls K, Mauricio I. 2011. Molecular approaches for a better understanding of the epidemiology and population genetics of *Leishmania*. Parasitology. 138(4):405-25. doi: 10.1017/S0031182010001538.

Schönian G, Mauricio I, Cupolillo E. 2010. Is it time to revise the nomenclature of *Leishmania*? Trends Parasitol. 26(10):466-9. doi: 10.1016/j.pt.2010.06.013.

Schoone G, Hailu A, Kroon C, Nieuwenhuys J, Schallig H, Oskam L. 2001. A fast agglutination-screening test (FAST) for the detection of anti-*Leishmania* antibodies. Trans R Soc Trop Med Hyg 4: 400-401.

Scott P, Natovitz P, Coffman R, Pearce E, Sher A. 1988. Immunoregulation of cutaneous leishmaniasis. T cell lines that transfer protective immunity or exacerbation belong to different T helper subsets and respond to distinct parasite antigens. J Exp Med. 168(5):1675-84.

Secundino N, Eger-Mangrich I, Braga EM, Santoro MM, Pimenta P. 2005. *Lutzomyia longipalpis* peritrophic matrix: formation, structure, and chemical composition. J Med Entomol. 42(6):928-38.

Shakarian A, Dwyer D. 2000. Structurally conserved soluble acid phosphatases are synthesized and released by *Leishmania major* promastigotes. Exp Parasitol. 95(2):79-84.

Sheth K, Bankey P. 2001. The liver as an immune organ. Curr Opin Crit Care. 7(2):99-104.

Silva K, de Andrade M, Melo L, Perosso J, Vasconcelos R, Munari D, Lima V. 2014. $CD4^{+}FOXP3^{+}$ cells produce IL-10 in the spleens of dogs with visceral leishmaniasis. Vet Parasitol. 202(3-4):313-8. doi: 10.1016/j.vetpar.2014.03.010.

Silva-Almeida M, Pereira B, Ribeiro-Guimarães M, Alves C. 2012. Proteinases as virulence factors in *Leishmania* ssp. infection in mammals. Parasit Vectors. 5:160. doi: 10.1186/1756-3305-5-160.

Singh N, Curran M, Rastogil A, Middleton D, Sundar S. 1999. Diagnostic PCR with *Leishmania donovani* specificity using sequences from the variable region of kinetoplast minicircle DNA. Trop Med Int Health. 4:448-453.

Singh N, Kumar M, Singh R. 2012a. Leishmaniasis: current status of available drugs and new potential drug targets. Asian Pac J Trop Med. 5(6):485-97. doi: 10.1016/S1995-7645(12)60084-4.

Singh O, Stober C, Singh A, Blackwell J, Sundar S. 2012b. Cytokine responses to novel antigens in an Indian population living in an area endemic for visceral leishmaniasis. PLoS Negl Trop Dis. 6(10):e1874. doi: 10.1371/journal.pntd.0001874.

Singh S, Sivakumar R. 2003. Recent advances in the diagnosis of leishmaniasis. J Postgrad Med. 49:55-60.

Šiško-Kraljević K, Jerončić A, Mohar B, Punda-Polić V. 2013. Asymptomatic *Leishmania infantum* infections in humans living in endemic and non-endemic areas of Croatia, 2007 to 2009. Euro Surveill. 18(29):20533.

Smelt S, Engwerda C, McCrossen M, Kaye P. 1997. Destruction of follicular dendritic cells during chronic visceral leishmaniasis. J Immunol. 158(8):3813-21.

Smrkovski L, Larson C. 1977. Antigenic cross reactivity between Mycobacterium bovis (BCG) and *Leishmania donovani*. Infect Immun. 18:561-562.

Solano-Gallego L, Miró G, Koutinas A, Cardoso L, Pennisi M, Ferrer L, Bourdeau P, Oliva G, Baneth G. 2011. LeishVet guidelines for the practical management of canine leishmaniosis. Parasit Vectors. 4:86. doi: 10.1186/1756-3305-4-86.

Späth G, Epstein L, Leader B, Singer S, Avila H, Turco S, Beverley S. 2000. Lipophosphoglycan is a virulence factor distinct from related glycoconjugates in the protozoan parasite *Leishmania major*. Proc Natl Acad Sci U S A. 97(16):9258-63.

Späth G, Garraway L, Turco S, Beverley S. 2003a. The role(s) of lipophosphoglycan (LPG) in the establishment of *Leishmania major* infections in mammalian hosts. Proc Natl Acad Sci U S A. 100(16):9536-41.

Späth G, Lye L, Segawa H, Sacks D, Turco S, Beverley S. 2003b. Persistence without pathology in phosphoglycan-deficient *Leishmania major*. Science. 301(5637):1241-3.

Srivastav S, Kar S, Chande A, Mukhopadhyaya R, Das P. 2012. *Leishmania donovani* exploits host deubiquitinating enzyme A20, a negative regulator of TLR signaling, to subvert host immune response. Immunol. 189(2):924-34. doi: 10.4049/jimmunol.1102845.

Srivastava P, Dayama A, Mehrotra S, Sundar S. 2011. Diagnosis of visceral leishmaniasis. Trans R Soc Trop Med Hyg. 105:1-6.

Srivastava S, Pandey S, Jha M, Chandel H, Saha B. 2013. *Leishmania* expressed lipophosphoglycan interacts with Toll-like receptor (TLR)-2 to decrease TLR-9 expression and reduce anti-leishmanial responses. Clin Exp Immunol. 172(3):403-9. doi: 10.1111/cei.12074.

Stäger S, Maroof A, Zubairi S, Sanos SL, Kopf M, Kaye P. 2006. Distinct roles for IL-6 and IL-12p40 in mediating protection against *Leishmania donovani* and the expansion of IL-10^{+} $CD4^{+}$ T cells. Eur J Immunol. 36(7):1764-71.

Stanley A, Engwerda C. 2007. Balancing immunity and pathology in visceral leishmaniasis. Immunology and Cell Biology. 85(2):138–147.

Stern M, Oca J, Rubin B, Anderson S, Murray H. 1988. Role of $L3T4^{+}$ and $Lyt\text{-}2^{+}$ cells in experimental visceral leishmaniasis. Journal of Immunology. 140(11):3971–3977.

Strober W, Murray P, Kitani A, Watanabe T. 2006. Signalling pathways and molecular interactions of NOD1 and NOD2. Nature Reviews Immunology. 6(1):9-20. doi: 10.1038/nri1747.

Sukmee T, Siripattanapipong S, Mungthin M, Worapong J, Rangsin R, Samung Y, Kongkaew W, Bumrungsana K, Chanachai K, Apiwathanasorn C, Rujirojindakul P, Wattanasri

S, Ungchusak K, Leelayoova S. 2008. A suspected new species of *Leishmania*, the causative agent of visceral leishmaniasis in a Thai patient. Int J Parasitol. 38(6):617-22. doi: 10.1016/j.ijpara.2007.12.003.

Sundar S, Rai M. 2002. Advances in the treatment of leishmaniasis. Curr Opin Infect Dis. 15(6):593-8.

Sundar S, Jha T, Thakur C, Engel J, Sindermann H, Fischer C, Junge K, Bryceson A, Berman J. 2002. Oral miltefosine for Indian visceral leishmaniasis. N Engl J Med. 347(22):1739-46.

Svárovská A, Ant T, Seblová V, Jecná L, Beverley S, Volf P. 2010. *Leishmania major* glycosylation mutants require phosphoglycans (lpg2-) but not lipophosphoglycan (lpg1-) for survival in permissive sand fly vectors. PLoS Negl Trop Dis. 4(1):e580.

Teixeira D, Benchimol M, Rodrigues J, Crepaldi P, Pimenta P, de Sousa W. 2013. Atlas didático: ciclo de vida da *Leishmania*. Rio de Janeiro: Fundação CECIERJ, Consórcio CEDERJ.

Tetreault G, Zayed A, Hanafi H, Beavers G, Zeichner B. 2001. Susceptibility of sand flies to selected insecticides in North Africa and the Middle East. J Am Mosq Control Assoc. 17(1):23-7.

Tewary P, Jain M, Sahani M, Saxena S, Madhubala R. 2005. A heterologous prime-boost vaccination regimen using ORFF DNA and recombinant ORFF protein confers protective immunity against experimental visceral leishmaniasis. J Infect Dis. 191(12):2130-7.

Thomson A, Knolle P. 2010. Antigen-presenting cell function in the tolerogenic liver environment. Nat Rev Immunol. 10:753–766.

Titus R, Ribeiro J. 1988. Salivary gland lysates from the sand fly *Lutzomyia longipalpis* enhance *Leishmania* infectivity. Science. 239:1306–8.

Trigo J, Abbehusen M, Netto E, Nakatani M, Pedral-Sampaio G, de Jesus R, Goto Y, Guderian J, Howard R, Reed S. 2010. Treatment of canine visceral leishmaniasis by the vaccine Leish-111f+MPL-SE. Vaccine. 28(19):3333-40. doi: 10.1016/j.vaccine.2010.02.089.

Tuon F, Neto V, Amato V. 2008. *Leishmania*: origin, evolution and future since the Precambrian. FEMS Immunol Med Microbiol. 54(2):158-66. doi: 10.1111/j.1574-695X.2008.00455.x.

van Zandbergen G, Klinger M, Mueller A, Dannenberg S, Gebert A, Solbach W, Laskay T. 2004. Cutting edge: neutrophil granulocyte serves as a vector for *Leishmania* entry into macrophages. J Immunol. 173(11):6521-5.

Vantourout P, Hayday A. 2013. Six-of-the-best: unique contributions of γδ T cells to immunology. Nat Rev Immunol. 13(2):88-100. doi: 10.1038/nri3384.

Vélez I, del Pilar Agudelo S, Arbelaez M, Gilchrist K, Robledo S, Puerta J, Zicker F, Berman J, Modabber F. 2000. Safety and immunogenicity of a killed *Leishmania (L.) amazonensis* vaccine against cutaneous leishmaniasis in Colombia: a randomized controlled trial. Trans R Soc Trop Med Hyg. 94(6):698-703.

Veras P, Brodskyn C, Balestieri F, Freitas Ld, Ramos A, Queiroz A, Barral A, Beverley S, Barral-Netto M. 1999. A dhfr-ts-*Leishmania major* knockout mutant cross-protects against *Leishmania amazonensis*. Mem Inst Oswaldo Cruz. 94(4):491-6.

Villinski J, Klena J, Abbassy M, Hoel D, Puplampu N, Mechta S, Boakye D, Raczniak G. 2008. Evidence for a new species of *Leishmania* associated with a focal disease outbreak in Ghana. Diagn Microbiol Infect Dis. 60(3):323-7.

Wakid M, Bates P. 2004. Flagellar attachment of *Leishmania* promastigotes to plastic film *in vitro*. Exp Parasitol. 106(3-4):173-8.

Walker P, Scharton-Kersten T, Rowton E, Hengge U, Bouloc A, Udey MC, Vogel J. 1998. Genetic immunization with glycoprotein 63 cDNA results in a helper T cell type 1 immune response and protection in a murine model of leishmaniasis. Hum Gene Ther. 9(13):1899-907.

Warren A, Le Couteur D, Fraser R, Bowen D, McCaughan G, Bertolino P. 2006. T lymphocytes interact with hepatocytes through fenestrations in murine liver sinusoidal endothelial cells. Hepatology. 44(5):1182-90.

Watanabe H, Miyaji C, Kawachi Y, Iiai T, Ohtsuka K, Iwanage T, Takahashi-Iwanaga H, Abo T. 1995. Relationships between intermediate TCR cells and $NK1.1^+$ T cells in various immune organs. $NK1.1^+$ T cells are present within a population of intermediate TCR cells. J Immunol. 155(6):2972-83.

Werts C, Girardin S, Philpott D. 2006. TIR, CARD and PYRIN: three domains for an antimicrobial triad. Cell death and differentiation. 13(5):798-815.

Wiegard C, Wolint P, Frenzel C, Cheruti U, Schmitt E, Oxenius A, Lohse A, Herkel J. 2007. Defective T helper response of hepatocyte-stimulated CD4 T cells impairs antiviral CD8 response and viral clearance. Gastroenterology. 133(6):2010-8.

Wilson M, Jeronimo S, Pearson R. 2005. Immunopathogenesis of infection with the visceralizing *Leishmania* species. Microb Pathog. 38(4):147-60.

Wilson M, Recker T, Rodriguez N, Young B, Burnell K, Streit J, Kline J. 2002. The TGF-beta response to *Leishmania chagasi* in the absence of IL-12. Eur J Immunol. 32(12):3556-65.

Wilson M, Sandor M, Blum A, Young B, Metwali A, Elliott D, Lynch R, Weinstock J. 1996. Local suppression of IFN-gamma in hepatic granulomas correlates with tissue-specific replication of *Leishmania chagasi*. J Immunol. 156(6):2231-9.

Winau F, Quack C, Darmoise A, Kaufmann SH. 2008. Starring stellate cells in liver immunology. Curr Opin Immunol. 20(1):68-74.

Wisse E. 1970. An electron microscopic study of the fenestrated endothelial lining of rat liver sinusoids. J Ultrastruct Res. 31:125-150

Wiwanitkit V. 2012. Interest in paromomycin for the treatment of visceral leishmaniasis (kala-azar). Ther Clin Risk Manag. 8:323-8. doi: 10.2147/TCRM.S30139.

Woelbing F, Kostka SL, Moelle K, Belkaid Y, Sunderkoetter C, Verbeek S, Waisman A, Nigg AP, Knop J, Udey MC, von Stebut E. 2006. Uptake of *Leishmania major* by dendritic cells is mediated by Fcgamma receptors and facilitates acquisition of protective immunity. J Exp Med. 203(1):177-88.

Wong C, Jenne C, Lee W, Léger C, Kubes P. 2011. Functional innervation of hepatic iNKT cells is immunosuppressive following stroke. Science, 334(6052):101-5. doi: 10.1126/science.1210301.

World Health Organization. 2002. Weekly epidemiological record. No. 44, 77, 365–372. http://www.who.int/wer

World Health Organization. 2013. Global Health Observatory Map Gallery. http://gamapserver.who.int/mapLibrary/Files/Maps/Leishmaniasis_2013_VL.png and http://gamapserver.who.int/mapLibrary/Files/Maps/Leishmaniasis_2013_CL.png.

World Health Organization. 2014. Leishmaniasis: the disease and its epidemiology. http:/who.int/leishmaniasis/disease_epidemiology/en/index.html.

World Health Organization. 2015. Fact sheet N°375. Updated February 2015. http://www.who.int/mediacentre/factsheets/fs375/en/.

Wortmann G, Sweeney C, Houng H, Aronson N, Stiteler J, Jackson J, Ockenhouse C. 2001. Rapid diagnosis of leishmaniasis by fluorogenic polymerase chain reaction. Am J Trop Med Hyg. 65: 583-587.

Xin L, Li K, Soong L. 2008. Down-regulation of dendritic cell signaling pathways by *Leishmania amazonensis* amastigotes. Mol Immunol. 45(12):3371-82. doi: 10.1016/j.molimm.2008.04.018.

Yao C, Donelson J, Wilson M. 2003. The major surface protease (MSP or GP63) of *Leishmania* sp. Biosynthesis, regulation of expression, and function. Mol Biochem Parasitol. 132(1):1 – 16.

Yao C, Gaur Dixit U, Barker J, Teesch L, Love-Homan L, Donelson J, Wilson M. 2013. Attenuation of *Leishmania infantum chagasi* metacyclic promastigotes by sterol depletion. Infect Immun. 81(7):2507-17. doi: 10.1128/IAI.00214-13.

Zelazny A, Fedorko D, Li L, Neva F, Fischer S. 2005. Evaluation of 7SL RNA gene sequences for the identification of *Leishmania* ssp. Am J Trop Med Hyg. 72(4):415-20.

Zhang X, Meng Z, Qiu S, Xu Y, Yang D, Schlaak J, Roggendorf M, Lu M. 2009. Lipopolysaccharide-induced innate immune responses in primary hepatocytes downregulates woodchuck hepatitis virus replication via interferon-independent pathways. Cell Microbiol. 11(11):1624-37. doi: 10.1111/j.1462-5822.2009.01353.x.

Zink AR, Spigelman M, Schraut B, Greenblatt CL, Nerlich AG, Donoghue HD. 2006. Leishmaniasis in ancient Egypt and Upper nubia. Emerg Infect Dis. 12(10):1616-7. doi: 10.3201/eid1210.060169

Zufferey R, Allen S, Barron T, Sullivan D, Denny P, Almeida I, Smith D, Turco S, Ferguson M, Beverley S. 2003. Ether phospholipids and glycosylinositolphospholipids are not required for amastigote virulence or for inhibition of macrophage activation by *Leishmania major*. J Biol Chem. 278(45):44708 – 44718.

2 General objectives

This study aims to clarify the natural mechanisms elicited by the liver of the dog, the main reservoir for *Leishmania*, to control the infection by *L. infantum*. Studies of liver immune activation and deactivating processes as well as immune pathogenic mechanisms may contribute to the better management of the disease and to the development of treatment alternatives. It is in this context that an *in vitro* bidimensional (2D) and tridimensional (3D) models of liver were established, allowing the assessment of the effect of *L. infantum* presence in the activity of hepatocytes and KC, by evaluating immunologic and functional cell markers. This system has also been used to evaluate the effect of antileishmanial drugs on liver current activity and on the immune response. The present study also proposes to characterize the resident memory lymphocyte population in the liver, eliciting its importance as an immunological memory organ. To do this task, it was used a murine model of visceral leishmaniasis, the BALB/c mice (*Mus musculus*). It was accessed the liver resident effector cells, effector memory cells and central memory cells that responded to *L. infantum* total soluble antigen or recombinant parasite proteins in healthy (non-infected), infected and in animals treated with a leishmanicidal drug.

Thus, the present work proposes the characterization of the following processes:

- Interaction of *L. infantum* with dog's KC;
- Interaction of *L. infantum* infected-KC with dog's hepatocyte;
- Interaction of *L. infantum* with canine hepatocytes in 2D and 3D culture systems;
- Comparison of immune activation profiles of canine blood macrophages *vs* liver macrophages (KC);
- Assess the impact of commercial anti-leishmanial compound in the culture systems previously described;
- Assess the impact of new potential anti-leishmanial compound in the culture systems;
- Characterize the resident memory lymphocyte population in the liver, eliciting its importance as an immunological memory organ.

3 General Methods

3.1. Experimental design

The present study aimed to a better understanding of the immune response generated by the dog liver when facing an infection by *Leishmania infantum* parasites. Therefore, this section includes the descriptions of the methods used to isolate, culture and analyze liver cells. The liver samples were from dogs (*Canis lupus familiaris*) belonging to the Official Kennel (Lisbon, Portugal).

The experimental design used is represented in Figure . Briefly, livers from healthy stray dogs were collected and hepatocytes and the Kupffer cells were isolated. Bidimensional (2D) and tridimensional (3D) cultures of canine hepatocytes were established and cells were adapting to the new environment (culture medium) for a 72 h period. The cells were exposed to *L. infantum* amastigotes or promastigotes for another 72 h and several parameters were evaluated at successive time points. Cells were collected to assess the innate immunity (i) by evaluating the gene expression of membrane receptors, such as NOD1, NOD2, TLR2, TLR4 and TLR9 and (ii) the mRNA accumulation of pro-inflammatory (TNF-α and IL-6) cytokines and of the immune suppressor IL-10 and, to analyze metabolic parameters through (iii) the quantification of urea and nitric oxide production and by evaluating the activities of cytochrome P450 enzymes (e.g. CYP1A1, CYP1A2, CYP1B, CYP2B11, CYP3A12 and CYP 3A26, alkoxyresorufin O-dealkylation family). Also confocal and electron scanning microscopy techniques were applied to characterize hepatic cell structure and parasite interaction with cell membrane. The impact of an antileishmanial drug in a *L. infantum* liver infection was also analyzed. Meglumine antimoniate, the conventional drug used to treat canine leishmaniasis, was added to the 3D and 2D cultures of parasite-exposed hepatocytes for 72 h. Experimental compounds with antileishmanial activity, such as chalcone, ursolic acid and quercetina were also used. Innate immunity and metabolic parameters, as previously described were also evaluated.

Kupffer cells were cultured for 7 days to ensure macrophage complete maturation and were infected by *L. infantum* amastigotes or promastigotes. Infection dynamics was followed by flow cytometry and the gene expression of innate immune

receptors (NOD1, NOD2, TLR2, TLR4 and TLR9) and of cytokines (TNF-α, IL-12, IL-4, IL-10 and TGF-β) were assessed at different time points. Meglumine antimoniate was added to parallel cultures for 24 h. Cells were analyzed as previously described.

To compare the immune response on Kupffer cells, as liver resident macrophages and peripheral blood macrophages, monocytes were isolated blood from dog blood and differentiated in macrophages after 3-days in culture. Macrophages were then infected with *L. infantum* promastigotes and the gene expression of the innate immune receptors and of cytokines were followed for 24 h.

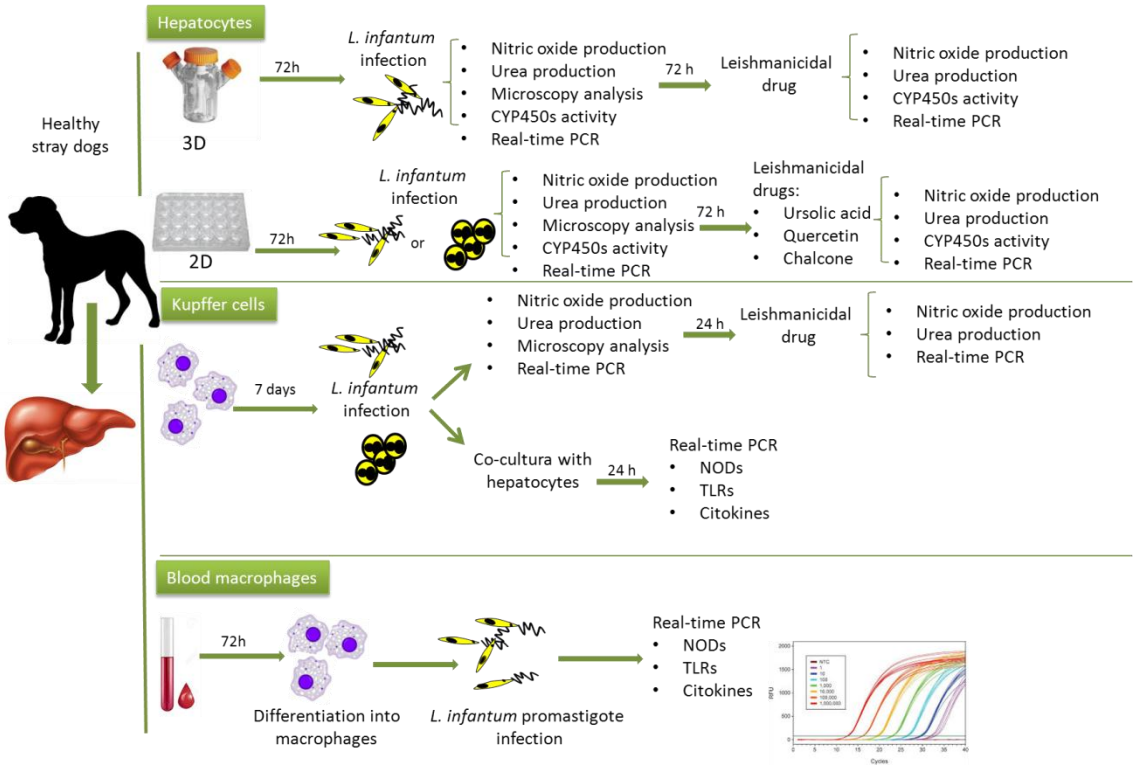


Figure 12: Experimental design.
Hepatocytes and Kupffer cells were isolated from livers of healthy stray dogs. 2D and 3D cultures of canine hepatocytes were established and exposed to *L. infantum* virulent parasites. In 2D culture system, hepatocytes were exposed to both *L. infantum* promastigotes and amastigotes. Antileishmanial conventional and experimental drugs were tested in parasite-exposed hepatocytes, in 2D culture system. After complete differentiation, Kupffer cells were infected with *L. infantum* promastigotes and amastigotes. The effect of a commercial antileishmanial drug (meglumine antimoniate) was also evaluated. Several metabolic and immunological parameters were analyzed at different time points. Peripheral blood was also collected to monocytes isolation and further differentiation into macrophages. Cells were infected with *L. infantum* promastigotes and immune response obtained was compared to liver resident macrophages- the Kupffer cells.

3.2. Animals

This study was performed with 12 healthy dogs weighing between 8 and 28 kg (Table 3). These dogs belonged to the Official Kennel (an organization recognized by the European Convention for the Protection of Small Animals). Unless reclaimed or adopted after a quarantine period, these animals undergo euthanasia. Animals were firstly injected with heparin intravenous (5000 U/ml) 30 minutes prior surgery, to avoid blood coagulation in the liver during extraction. Animals were then anesthetized with ketamine intravenous [5 mg/Kg] (Vetnil), followed by the administration of sodium pentobarbital in accordance with animal weight [<10kg200 mg/kg; >10kg 300mg/Kg] (Eutasil). Livers were them collected. Experiments were monitored by competent veterinary surgeon and approved by the ethical committee of the Faculty of Veterinary Medicine (Lisbon, Portugal).

Table 3: Breed, weight, age and gender of the dogs used in the present study.

Identification number	Breed	Weight (kg)	Apparent age (years)	Gender
1	Boxer	27	6	Male
2	Mongrel	15	7	Male
3	Mongrel	12	6	Female
4	German Shepherd	28	9	Male
5	Portuguese hound	8	3	Male
6	Cross-breed German Shepherd	16	6	Female
7	Crossed Pointer	12	7	Male
8	Mongrel	22	8	Male
9	Mongrel	12	7	Male
10	Mongrel	22	8	Male
11	Pointer	12	6	Male
12	Portuguese hound	10	9	Male

3.2.1. Animal health tests

All animals used were previously tested for parasites and biochemical and hematological parameters were determined to ensure that animals did not had vector borne infections (CVBD – canine vector borne diseases) and that blood parameters were normal. The parasitological and bacterial tests performed to each dog are summarized in Table 4. As biochemical tests quantification of urea and creatinine in circulating blood was performed to assess kidney function. The liver function was evaluated in circulating blood by total bilirubin quantification, aspartate aminotransferase and alanine aminotransferase presence as well as alkaline phosphatase and bile acid. Hematological parameters assessed were hemogram and ionogram (sodium, potassium, clorets) as well as inorganic phosphorus. Albumin and calcium blood concentrations were also measured. Animals were CVBD negative and evidenced normal values for hematological and biochemical parameters.

Table 4: Parasitological and bacterial tests performed in the dogs included in the present study.

Parasitological and bacterial tests
Canine Leishmaniasis Indirect immunofluorescence (IFI), cut off 1:80 DNA amplification by real-time PCR for *L. infantum* (Helhazar *et al.*, 2013)
Dirofilaria immitis in blood Knott test
Babesia/Theleria spp. *Ehrlichia/Anaplasma* spp. DNA amplification by real-time PCR*
Mycoplasma haemocanis Blood smear for microscopy observation
Ricketsia spp. Blood smear for microscopy observation

*According to the protocol implemented in Virology laboratory of FMV, UTL. Primers and probes were designed with Primer 3 and BLAST software.

3.3. Parasites

3.3.1. *Leishmania infantum* promastigotes

L. infantum zymodeme MON-1 (MHOM/PT/89/IMT151) was maintained by successive passages in BALB/c mice. The spleens of infected mice were extracted and homogenized with a tissue disaggregator with 50 μm separator screen (Medicons, Syntec International) to isolate a suspension of single cells. This cell suspension was added to Schneider medium with L-glutamine (SCHN, Sigma-Aldrich) supplemented with 10% (v/v) of heat-inactivated fetal bovine serum (FBS, Sigma-Aldrich) and penicillin-streptomycin (Biochrom) at 100 U/ml and 100 $\mu g.ml^{-1}$ respectively (complete SCHN medium). Spleen cells were incubated at 24 ºC until complete differentiation of promastigotes. Only virulent parasites with less than five passages were used (Santos-Gomes and Abranches, 1996) in the present study. The concentration (C) of promastigote suspension was determinate in a Neubauer-improved chamber using the following equation.

Equation 1:

$$[C] = number\ of\ parasites \times 50 \times dilution\ factor \times 10^3$$

50 and 10^3 are consideration factors for the number of squares counted and the volume of the chamber. Together they stand for counting 5 squares in 0.0001 ml – volume of the Neubauer chamber.

3.3.2. *Leishmania infantum* axenic amastigotes

L. infantum zymodeme MON-1 (MHOM/PT/89/IMT151) was maintained by successive passages in complete SCHN medium (see 3.3.1) until reaching the stationary phase (around three passages). Promastigotes concentration was determinate in a Neubauer chamber (see Equation 1) and the suspension adjusted to 2×10^6 $parasites.ml^{-1}$. Parasites were inoculated into complete SCHN medium (as described in 3.3.1) with acid pH at 5.5 and supplemented with 2% of filtered human urine (from a healthy male donor) for amastigote differentiation. Cultures were incubated at 37 ºC, in a humidified atmosphere with 5% CO_2 during two to three weeks to allow complete amastigote

transformation. The amastigote differentiation was followed by inverted microscopy and confirmed with scanning electronic microscope (JEOL5200-LV).

3.3.3. *Leishmania infantum*-GFP

Transgenic Green Fluorescent Protein (GFP)-expressing *L. infantum* promastigotes were kindly given by Professor Ana Tomás from Instituto de Biologia Molecular e Celular (IBMC), Universidade do Porto (Oporto, Portugal). Briefly the GFP open reading frame was inserted in the trypanosomatid expression plasmid pTEX (Kelly *et al.*, 1992) and the resulting construct electroporated in *L. infantum* promastigotes following standard procedures. *L. infantum* mutants were selected on agar plates using Geneticin (G418 disulfate salt solution, Sigma-Aldrich) at 25μg.ml^{-1}. Individual colonies were grown in culture medium using the same selection conditions, analyzed by southern blotting to confirm that the construct was structurally intact, and by fluorescence to ensure GFP expression. *L. infantum* GFP promastigotes were maintained in culture using SCHN medium supplemented with 25 μg.ml^{-1} of Geneticin. GFP amastigotes were also obtained using the method described in 3.2, but maintaining the 25 μg.ml^{-1}of Geneticinin culture medium to ensure the conservation of the plasmid. Amastigotes differentiation was confirmed under a fluorescent microscope with GFP filter (Nikon eclipse 80i) and camera Nikon DS-Ri1.

3.4. Real-time PCR technique

3.4.1. Basic principles

Monitoring the immune response is critical for the follow-up and treatment evaluation of numerous diseases. Studying the profile of cytokine production can help to clarify functional properties of immune cells, both in research and in clinical diagnosis (Stordeur, 2009). Since the first documentation of real-time PCR (Higuchi *et al.*, 1993) it has been used for increasing the number of applications, including mRNA studies, DNA copy number measurements in genomic or viral DNA, allelic discrimination assays or expression analysis of specific splice variant of genes (Arya *et al.*, 2005). Real-time quantitative PCR offers a powerful tool for the quantification of target nucleic acids, being a very high throughput technique that is faster than

conventional PCR and both accurate and sensitive. Real-time-PCR can virtually detect one copy of a specific sequence, using very small samples with low gene expression. In a conventional PCR, the reaction must be finish before proceeding with the post-PCR analysis, by gel electrophoresis. Real-time PCR overcome this problem by measuring the PCR products at the early stages of reaction, as they accumulate in a real time detection mode, thus measuring the amount of PCR product when the reaction is still in the exponential phase. Real-time PCR allows detection and quantitative measurement of products generated during each cycle of the PCR process that are directly proportional to the amount of the template DNA before the start of the PCR process. This requires the use of an appropriate methodology able to detect the product formed on each cycle and the software must record the results obtained on each amplification cycle in a real time manner (Pestana *et al.*, 2010).

A representative amplification plot of a real-time PCR reaction is shown in Figure 13. In the initial cycles of amplification, there is no significant change in the fluorescent signal and it stays beneath the limits of detection, constituting the baseline. A baseline subtracted amplification plot (ΔRn) is generated by the associated software using the equation ΔRn = Rnf – Rnb, where Rnb is the fluorescent emission of the product at each time point and Rnb is the baseline fluorescence level. The ΔRn values are plotted versus the cycle number. Then a computational algorithm searches for the point on the amplification plot at which the ΔRn value crosses the threshold level. This point defines the threshold cycle (C_T) of a sample. The exponential growth phase of amplification starts from this point on. The C_T value consists in the value (number of cycles) where the reaction curve intersects the threshold line, showing the numbers of cycles need to detect a real signal from the samples. C_T values are inverse to the amount of nucleic acid in the sample and correlate to the number of copies the sample. Lower C_T values indicate high amounts of targeted nucleic acid, while higher C_T values mean lower amounts of target nucleic acid. The standard curve method is used when absolute quantification is critical for the investigator (Arya *et al.*, 2005; Pestana *et al.*, 2010).

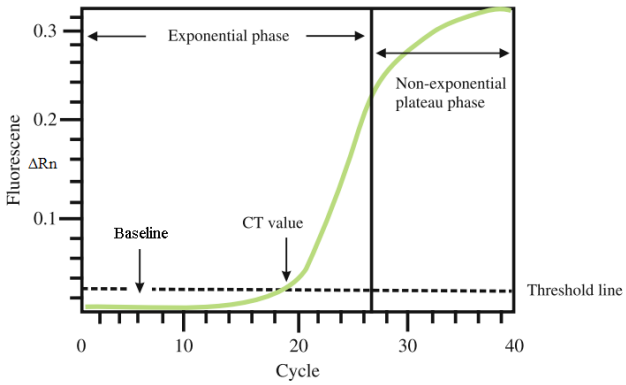


Figure 13: Real-time PCR amplification plot.
Fluorescent emission is measured continuously during the reaction if amplification and a baseline subtracted amplification plot (ΔRn) is generated. The CT value constitutes the point where the fluorescence of the sample is above the threshold (adapted from Pestana *et al.*, 2010).

There are a wide variety of commercial available fluorescent DNA dyes. The most commonly used is SYBR®Green I, an asymmetrical cyanine dye with two positive charges that under PCR conditions contributes to its high double strand DNA (dsDNA) binding affinity. When SYBR®Green dye binds to the minor groove of dsDNA its fluorescence is increased and can be measured in the extension phase of each real-time PCR cycle. Given that nonspecific products and primer dimers can be formed during the PCR process, a melting curve analysis is recommended to check for unspecific amplifications (Navarro *et al.*, 2015). An example of IL-6 melting curve obtained in the current study is shown in Figure 14B. The advantages of the SYBR®Green dye are mainly related to its capacity to monitor the amplification of any dsDNA, as no probe is required, and specificity is ensured by the primer sequence. This reduces the assay running costs and due to the dye characteristics, the generated fluorescent signals are much stronger, increasing sensitivity. SYBR®Green provides a convenient and cost effective technique to screen a large number of samples and targets.

In the present study, absolute quantification was performed to determine the levels of expression of several genes with immunological importance. As said before, absolute quantification determines the copy number of the target in a sample by associating the PCR signal to a standard curve. In order to measure a specific cytokine or innate immune receptor copy number in a sample, a standard curve constructed with known quantities of each gene target was performed. The standard curve is generated by

serial dilutions of known quantities of plasmid with target insert, produced as described in 3.4.3.

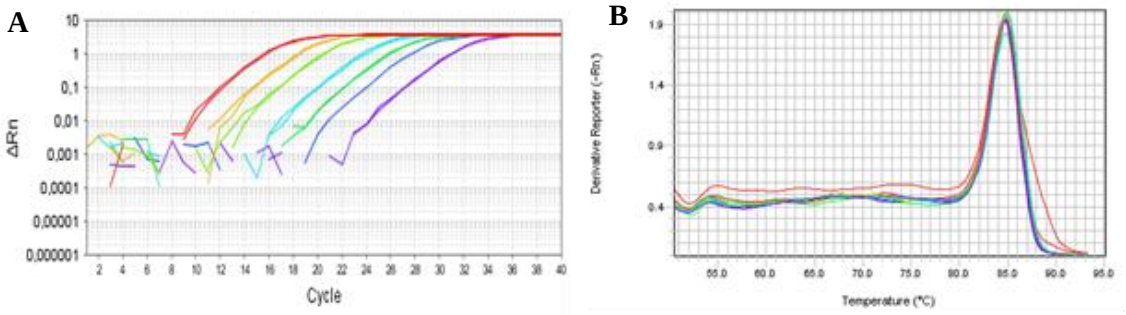


Figure 14: Quantitative analysis of gene expression by real-time PCR.
Quantitative analysis of gene expression by real-time PCR. Exemplificative real-time PCR amplification plot for a standard curve performed with seven duplicate dilutions of a plasmid with the IL-6 insert (A) and the melting curves of IL-6 amplification in different samples (B).

The efficiency of real-time PCR amplification is defined as the rate at which a PCR product is generated and is expressed as a percentage value. The slope of the standard curve (Figure 14A) is used to estimate the PCR amplification efficiency of a real-time PCR reaction. A real-time PCR standard curve is graphically represented by a semi-log regression line plot of C_T value *vs* log of the input nucleic acid. The calculation for the real-time PCR assay efficiency (E) is given by the following equation.

Equation 2:

$$E = 10^{-1/slope}$$

Slope value of the standard curve.

Ideally, the amount of PCR product will perfectly double during each cycle of exponential amplification, meaning that, there will be a 2-fold increase in the number of copies with each cycle. This translates to a reaction efficiency of 2, which correspond to 100% of efficiency in a PCR reaction. In the present study all real-time PCR reactions were performed with 90% to 99% of efficiency, depending on the gene evaluated.

3.4.2. Primer selection and optimization

Primers were selected using data from published works in an extended literature review or were designed using Primer-BLAST software (Ye *et al.*, 2012) (Table 5). For each pair of primers (Stabvida, Portugal) PCR conditions were optimized by changing

the annealing temperature (T_{AN}) and duration of cycles until the desired fragment was the only DNA amplified in each sample. T_{AN} of each gene fragment was calculated subtracting 5 ºC to the primer melting temperature specified by the manufacturer of the primers. To establish the PCR conditions, T_{AN} was gradually increased by 0.5 ºC until the required amplification specifications were set (Figure 15). For all the pair of primers used in this study it was confirmed the absence of cDNA amplification in dog, mouse and *L. infantum* samples, evidencing the high specificity of the primers.

Table 5: List of forward (FW) and reverse (RV) primers, base pair number (bp) of amplified fragment and primer annealing temperature (T_{AN}) for each gene studied.

Gene	Primer	Reference	Fragment size (bp)	T_{AN} (ºC)
β-Actin	FW-5'ACGGAGCGTGGCTACAGC 3' RW-5' TCCTTGATGTCACGCACGA 3'	Sauter *et al.*, 2005	61	60.5
TLR2	FW-5' AATCCCCCGTTCAAGTTGTG 3' RW-5' ATGGTTTTGCGGCTCTTCTC 3'	Ishii *et al.*, 2006	101	61
TLR4	FW-5' CCCCACATCAACCGAGAAACTGGA 3' RW-5' GCAGTTTTGGGAAGTTGGAGAAGCA 3'	*	86	64
TLR9	FW-5' ACCACATCATCACCCTGGCACCT 3' RW-5' CGGCGACAGTTCCCACCCAC 3'	*	82	67
NOD1	FW-5' CCTTGGCTGTCGGAGATTGGCT 3' RW-5' ACCTGCTTACTGGGTCGGTGT 3'	*	82	63
NOD2	FW-5' TGGCGTGGGAGCAGGGTTTC 3' RW-5' CGCTGGGAGGATGTGAAGATGG 3'	*	76	68.5
TNF-α	FW-5' AATCATCTTCTCGAACCCCAAGT 3' RW-5' GGAGCTGCCCCTCAGCTT 3'	Sauter *et al.*, 2005	75	57
IL-12p40	FW-5' CAGCAGAGAGGGTCAGAGTGG 3' RW-5' ACGACCTCGATGGGTAGGC 3'	Peters *et al.*, 2005	109	58
IL-4	FW-5' CATCCTCACAGCGAGAAACG 3' RW-5' CCTTATCGCTTGTGTTCTTTGGA 3'	Huang et al., 2008	83	50
IL-10	FW-5' CAAGCCCTGTCGGAGATGAT 3' RW-5' CTTGATGTCTGGGTCGTGGTT 3'	Yu *et al.*, 2010	78	54
IL-6	FW-5' CTCTCCACAAGCGCCTTCTC 3' RW-5' TGAAGTGGCATCATCCTTGG 3'	Peters *et al.*, 2005	102	61.5
TGF-β	FW-5' CAGAATGGCTGTCCTTTGATGTC 3' RW-5' AGGCGAAAGCCCTCGACTT 3'	Huang *et al.*, 2008	79	60

* indicates that the primers were designed with Primer-BLAST software.

PCR amplifications were done using NZTech master mix PCR (Nzytech genes & Enzymes), according to manufacturer's guidance. A 20 µl of a mix containing 2 µl of a sample and 0.2 µl of 20 pmol.µl^{-1} forward primer solution and of the reverse primer solution were used. Optimized PCR conditions were 5 min at 94 ºC for complete DNA denaturation, 30 cycles of 30 sec. at 94 ºC, 15 sec. at primer/gene specific T_{AN} and 30 sec. at 72 ºC, and a final step of 3 min at 72 ºC to guarantee complete elongation of most PCR products initiated during the last cycle. Samples were analyzed by DNA electrophoresis using a gel with 3% (m/v) agarose in Tris-acetate-EDTA (TAE) buffer (Merck) containing 0.1µl.ml^{-1} of 10000× GelRed Nucleic Acid Stain (Biotium).

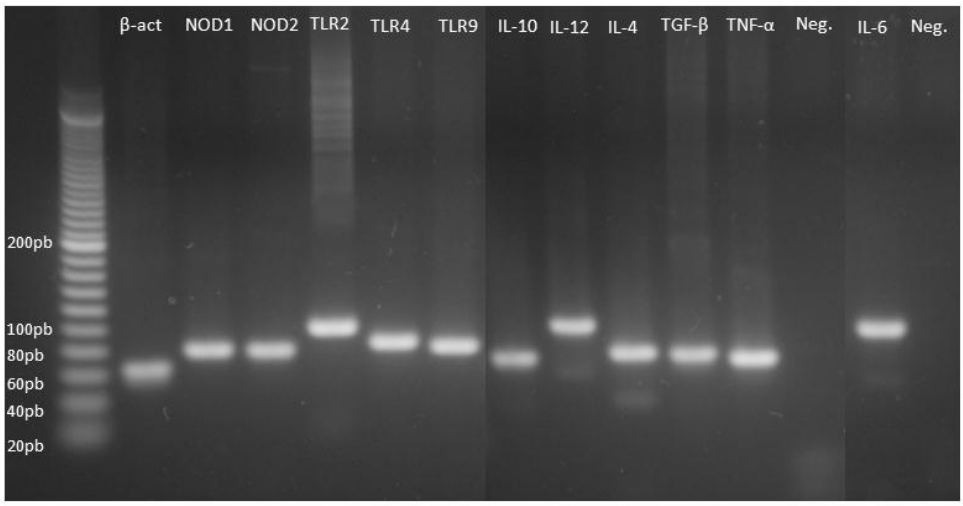


Figure 15: Agarose gel showing the amplified fragments of the dog genes β-actin (β-act), NOD1, NOD2, TLR2, TLR4, TLR9, IL-10, IL-12p40 (IL-12), IL-4, TGF-β, TNF-α and IL-6.
Two negative controls (Neg.) are included.

3.4.3. Plasmid cloning

cDNA isolated from dog macrophages or dog hepatocytes were previously analyzed by PCR and those samples that were positive for the gene of interest were amplified by PCR. The PCR product was analyzed by agarose gel electrophoresis to confirm that the amplification was performed correctly. The remaining PCR product was purified using innuPREP DOUBLE pure Kit (Analytik Jena AG) following the manufacturer's instructions and again visualized by agarose gel electrophoresis to ensure the purity of cDNA fragment. Then the purified fragment was introduced into a circular plasmid using pGEM®-T Easy Vector Systems (Promega) as described in the manufacturer protocol. Briefly, 10 µl of ligation mix, containing the linear form of the

vector, was prepared using 3 µl of PCR product and DNA ligase enzyme and incubated overnight at 4 ºC to ensure the maximum number of transformants. Competent *Escherichia coli* strain JM109 was used for plasmid cloning.

To induce the growth of competent *E. coli* JM109 able to incorporate the plasmid construction, bacteria was grown overnight in solid M9 medium. This medium is prepared with 0.6% Na_2HPO_4 (m/v), 0.3% KH_2PO_4 (m/v), 0.05% NaCl (m/v), 0.1% NH_4Cl (m/v), 0.2% $MgSO_4$ (m/v), 0.01% $CaCl_2$ (m/v), 0.4% glucose (m/v) and 1.5% agar (m/v) in distilled water, and supplemented with 1 mM of thiamine. After incubation a colony was selected and grown in 5 ml of LB broth EZMIX (Sigma-Aldrich) overnight, at 37 ºC. About 5ml of overnight culture were inoculated into 500 ml of LB medium and left to grow at 37 ºC in a rotary shaker at 250 rpm until OD 600 nm reached 0.4 density value. The bacterial culture was then pellet by centrifugation at 1000 *x*g for 10 min at 4 ºC and resuspended in 50 ml of cold transformation and storage solution (TSS). This solution was constituted by Luria broth (LB) medium with 10% (m/v) of PEG 8000, 5% (v/v) of DMSO and 50 mM of $MgCl_2$ (Chung *et al.*,1989). Aliquots were frozen within liquid nitrogen until use.

To proceed with the cloning process, frozen aliquots of competent *E. coli* were then thawed on ice and 50 µl of thawed competent cells transferred to 2 ml tubes previously chilled on ice. These two tubes were added 2µl of plasmid with the gene fragment insert (ligation mixture) and incubated on ice for 30 min. Tubes were then subjected to heat shock in a water bath at 42 ºC for 60 sec. and placed on ice again for 2 min. To each tube 500 µl of Super Optimal broth with Catabolite repression (SOC, Invitrogen) was added and bacteria were incubated at 37 ºC with shaking at 250 rpm for 3 h, to allow the cells to recover from the heat shock. Then 250 µl of the cell solution was plated on LB agar medium [LB with 1.5% (m/v) of agar] supplemented with 100 $\mu g.ml^{-1}$ of ampicillin (Sigma-Aldrich), 80 $\mu g.ml^{-1}$ of X-Gal (Sigma-Aldrich) and 0.5 mM of IPTG (Sigma-Aldrich) and incubated at 37 ºC for 18 h.

The use of this specific *E. coli* strain and the pGEM®-T Easy vector allowed the differentiation of colonies containing the plasmid from the colonies with the plasmid and the insert fragment by blue/white color screening and resistance to ampicillin. Only bacteria with plasmid will be able to grow on the LB-agar plates with ampicillin. In this

method of screening, it is used the β-galactosidase, a bacterial enzyme encoded by the *lac*Z gene of the *lac* operon. In its active state, the enzyme is a homotetramer, however, a mutant β-galactosidase, as *E. coli* JM109 strain has a deletion in the N-terminal residues of the ω-peptide and is unable to form an active tetramer. This mutant form of protein however may return to its active tetrameric state in the presence of an N-terminal fragment of the protein, the α-peptide. The rescue of function of the mutant β-galactosidase by the α-peptide is called α-complementation. The blue/white screening method works by disrupting this α-complementation process. The plasmid carries within the *lac*Zα sequence an internal multiple cloning site (MCS). This MCS, within the *lac*Zα sequence, can be cut by restriction enzymes so that the foreign DNA may be inserted within the *lac*Zα gene, thereby disrupting the gene and thus production of α-peptide. Consequently, in cells containing the plasmid with an insert, no functional β-galactosidase may be formed. Blue colonies result from bacteria with an active β-galactosidase enzyme, which is able to cleaved X-gal, a colorless analog of lactose to form 5-bromo-4-chloro-indoxyl, which then spontaneously dimerizes and oxidizes to form a blue insoluble pigment 5,5'-dibromo-4,4'-dichloro-indigo. Blue colonies contain a vector with an uninterrupted lacZα, therefore no insert, while white colonies, where X-gal is not hydrolyzed, indicate the presence of an insert in lacZα which disrupts the production of an active β-galactosidase. The white colonies were picked and incubated overnight in liquid LB medium supplemented with 100 $\mu g.ml^{-1}$ of ampicillin at 37 ºC with shacking at 250 rpm. The isolation of plasmid DNA was then made using the innuPREP Plasmid Mini Kit (Analytik Jena AG) according to the manufacturer protocol In order to confirm the presence of the insert fragment in the plasmid. The plasmid DNA was amplified by PCR with the respective primers and the PCR product visualized in agarose gel.

3.4.4. RNA extraction and cDNA synthesis

Cellular RNA extraction was performed using NZY Total RNA Isolation kit (Nzytech genes & enzymes) according to manufacturer's indications. Liver cells (hepatocytes and Kupffer cells) and blood macrophages were stored in NR buffer (supplied in the kit) supplemented with β-mercaptoethanol (Sigma-Aldrich) to lysate cells and preserve total RNA. Samples were conserved at -80 ºC until further

processing. After thawed, samples were used for RNA extracted according to manufacturer's instructions followed by digestion of contaminant DNA with DNAse in order to maximize the purity of extracted RNA. The purity level of total RNA was confirmed by Nanodrop® 1000 spectrophotometer (Thermo Fisher Scientific) at the absorbance ratio of 260/280nm. Values ≈2 indicates pure RNA.

Extracted RNA samples were additionally processed into cDNA synthesis using NZY First-strand cDNA Synthesis Kit (Nzytech genes & enzymes) according to manufacturer's indications. Complementary DNA (cDNA) consists of a DNA copy synthesized from mRNA. Reverse transcriptases (RTs), initially isolated from virus, use an RNA template and a short primer complementary to the 3' end of the RNA to direct the synthesis of the first strand cDNA, which can be used directly as a template for a posterior PCR. NZY First-strand cDNA Synthesis Kit includes all the necessary components to synthesize first-strand cDNA, resulting in a single-stranded cDNA suitable for use in real-time PCR. Degradation of the cDNA:RNA hybrids formed after the first-stranded cDNA synthesis is ensured by the addition of RNase H. This step increases RT-PCR sensitivity, as primers will bind more easily to the cDNA.

3.4.5. Real-time PCR running

In order to generate the calibration curves, allowing the exact quantification of each gene copy number, serial dilutions of the plasmid with the insert fragment (described in 3.4.3) were also amplified.

Plasmid DNA was quantified with a Nanodrop® 1000 spectrophotometer (Thermo Fisher Scientific) and for each gene 1:5 serial dilutions were prepared starting from 250 $pg.\mu l^{-1}$ to 0.016 $pg.\mu l^{-1}$ in ultra-pure water. cDNA from cells, produced from total extracted mRNA (as described in 3.4.4) were analyzed in duplicate for each sample and in duplicate for each time point for all genes in the study. For every sample and standard, 20 µl of the real-time PCR reaction mix was prepared with 2 µl of cDNA sample or with a standard dilution, 10 µl of SensiFAST SYBR Lo-ROX **(**Bioline), 0.15 µl of 20 $pmol.\mu l^{-1}$ forward primer solution, 0.15 µl of 20 $pmol.\mu l^{-1}$ reverse primer solution and 7.7 µl of ultra-pure water. Amplification was performed in an Applied Biosystems 7500 Fast Real-Time PCR System thermal cycler (Applied Biosystems) and the conditions were 5 min at 95 ºC for complete DNA denaturation, 40 cycles of 30 sec.

at 95 ºC and 30 sec. at primer/gene specific T_{AN} (Table 5) for annealing and extension, followed by 90 cycles of 10 sec. at starting temperature of 50 ºC with an increment of 0.5 ºC for each cycle to complete a melting curve. The fluorescence levels of each sample were analyzed in real time by the thermal cycler and the amount of gene copies calculated in comparison to the calibration curves for each gene. The number of gene copies was calculated as follows:

Equation 3:

$$Gene\ copy\ number = \frac{9.1 \times 10^{11} \times\ quantity\ (\mu g)}{plasmid + insert\ size\ (kb)}$$

where $9.1x10^{11}$ is the amount of molecules presented in 1µg of DNA with 1000 pb. The length of the plasmid used was 3.015 kp.

The number of copies of each gene and sample was normalized to the housekeeping gene β-actin, in order to normalize differences in relative quantities of initial cDNA in the sample. Normalization was performed by dividing the obtained number of gene copies by 1000 copies of β-actin for each sample. The final results for each gene and sample were expressed in a number of copies per 1000 copies of β-actin.

3.5. References

Arya M, Shergill IS, Williamson M, Gommersall L, Arya N, Patel HR. 2005. Basic principles of real-time quantitative PCR. Expert Rev Mol Diagn. 5(2):209-19.

Helhazar M, Leitão J, Duarte A, Tavares L, da Fonseca IP. 2013. Natural infection of synathropic rodent species *Mus musculus* and *Rattus norvegicus* by *Leishmania infantum* in Sesimbra and Sintra - Portugal. Parasit Vectors. 6:88. doi: 10.1186/1756-3305-6-88.

Higuchi R, Fockler C, Dollinger G, Watson R. 1993. Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. Biotechnology (N Y). 11(9):1026-30.

Huang Y, Hung S, Jan T, Liao K, Cheng C, Wang Y, Chu R. 2008. CD5-low expression lymphocytes in canine peripheral blood show characteristics of natural killer cells. J Leukoc Biol. 84(6):1501-10. doi: 10.1189/jlb.0408255

Ishii M, Hashimoto M, Oguma K, Kano R, Moritomo T, Hasegawa A. 2006. Molecular cloning and tissue expression of canine Toll-like receptor 2 (TLR2), Veterinary Immunology and Immunopathology. 110(1-2):87-95.

Kelly JM, Ward HM, Miles MA, Kendall G. 1992. A shuttle vector which facilitates the expression of transfected genes in *Trypanosomacruzi* and *Leishmania*. Nucleic Acids Res. 20:3963-3969.

Navarro E, Serrano-Heras G, Castaño MJ, Solera J. 2015. Real-time PCR detection chemistry. Clin Chim Acta. 439:231-50. doi: 10.1016/j.cca.2014.10.017.

Pestana A, Belak S, Diallo A, Crowther J, Viljoen G. 2010. Early, Rapid and Sensitive Veterinary Molecular Diagnostics - Real Time PCR Applications. Springer Dordrecht Heidelberg London New York, Springer. Pp 27-43.

Peters IR, Helps CR, Calvert EL, Hall EJ, Day MJ. 2005. Cytokine mRNA quantification in histologically normal canine duodenal mucosa by real-time RT-PCR. Vet Immunol pathol. 103(1-2):101-11.

Santos-Gomes GM, Abranches P. 1996. Comparative study of infectivity caused by promastigotes of *Leishmania infantum* MON-1, *L. infantum* MON-24 and *L. donovani* MON-18. Folia Parasitol. 43:7-12.

Sauter S, Allenspach K, Gaschen F, Grone A, Ontsouka E, Blum J. 2005. Cytokine expression in an *ex-vivo* culture system of duodenal samples from dogs with chronic enteropathies: modulation by probiotic bacteria. Domestic Animal Endocrinology. 29:605-22.

Stordeur P. 2009. Monitoring the Immune Response Using Real-Time PCR. In DNA and RNA Profiling in Human Blood: Methods and Protocols. Mannheim, Germany, Peter Bugert (ed.), Humana Press, Pp 323-338.

Strasser A, Kalmar E, Niedermüller H. 1998. A simple method for the simultaneous separation of peripheral blood mononuclear and polymorphonuclear cells in the dog. Veterinary Immunology and Immunopathology. 62:29-35.

Ye J, Coulouris G, Zaretskaya I, Cutcutache I, Rozen S, Madden TL. 2012. Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. BMC Bioinformatics. 13:134. doi: 10.1186/1471-2105-13-134.

Yu D, Noh D, Song R, Park J. 2010. Ethyl pyruvate downregulates tumor necrosis factor alpha and interleukin (IL)-6 and upregulates IL-10 in lipopolysaccharide-stimulated canine peripheral blood mononuclear cells. J Vet Med Sci. 72(10):1379-81.

CHAPTER 1

Interactions of canine hepatocytes with *L. infantum*

4 Chapter 1: Interactions of canine hepatocytes with *L. infantum*

4.1. Introduction

4.1.1. Hepatocytes: metabolic characteristics

The liver is a key metabolic organ which governs body energy metabolism. It acts as a center to metabolically connect various tissues, including skeletal muscle and adipose tissue. As the main detoxifying organ of the body, the liver also plays a central role in metabolic homeostasis and is a major site for synthesis, metabolism, storage and redistribution of carbohydrates proteins and lipids. Hepatocytes are highly active metabolic cells playing critical roles in synthesizing molecules that are utilized in supporting body homeostasis, regulating energy balances, detoxifying the body from endo or exogenous molecules and also may have immune activity. Hepatocytes constitute about 80% of all hepatic cells, are key cells in maintaining the glucose levels in the blood, which is critical for animal survival. Hepatocytes perform many different metabolic pathways and express dozens of enzymes that are alternately turned on or off depending on whether blood levels of glucose are rising or falling out of the normal range. Glucose resulting from digestion is taken up by the hepatocyte's glucose receptors GLUT1/2 and is phosphorylated by glucokinase to generate glucose 6-phosphate (G6P), leading to a reduction in the intracellular glucose concentration, which further increases glucose uptake (Agius, 2008). This excess of glucose is sequestered as a large polymer, glycogen, by the activity of glycogen synthase in a process called glycogenesis. The major hormone regulating the glucose uptake by hepatocytes is insulin. In the fed state, pancreatic-β cells secrete insulin in response to an increase in blood glucose, amino acids, and fatty acids, stimulating glycogen synthesis and glucose uptake. Later, when blood concentrations of glucose begin to decline, the liver activates other pathways which lead to deploymerization of glycogen (glycogenolysis) and export of glucose back into the blood for transport to all other tissues. During prolonged fasting, glycogen is depleted and hepatocytes synthesize glucose, trough gluconeogenesis, from lactate, pyruvate, glycerol, and amino acids (Rui,

2014). These gluconeogenic substrates are either generated in the liver or delivered to the liver through the circulation from extrahepatic tissues. The ability of the liver to perform gluconeogenesis is of immense importance to carnivores, which, at least in the wild, have diets virtually devoid of starch. Also the immune environment of the liver may influence hepatocyte metabolism. The immune cells present in the liver, as well as hepatocytes, secrete numerous cytokines, which regulate hepatocyte metabolism in an autocrine/paracrine fashion. Insulin-signaling hypothalamus stimulates IL-6 production in the liver, and in turn IL-6 suppresses gluconeogenesis by activating STAT3 (Inoue *et al.*, 2006). IL-13 also stimulates tyrosine phosphorylation of STAT3 in hepatocytes, and genetic deletion of IL-13 increases hepatic gluconeogenesis. IL-13 null mice develop hyperglycemia and intolerance to glucose (Stanya *et al.*, 2013). However, the metabolic activity of hepatocytes is not reduced to the glucose balance. The liver, in the form of hepatocytes, also has its role in the fatty acid metabolism. Hepatocytes are extremely active in oxidizing triglycerides producing energy, breaking down many more fatty acids that needed, and exporting large quantities of acetoacetate into the blood where it can be picked up and readily metabolized by other tissues. The liver is the major site for converting excess carbohydrates and proteins into fatty acids and triglyceride, which are then exported and stored in adipose tissue. This organ also synthesizes large quantities of cholesterol and phospholipids. Some of this is packaged with lipoproteins and made available to the rest of the body. The remains are excreted in bile as cholesterol or after conversion to bile acids. Hepatocytes are also critical cells of protein metabolic process that occur in the liver. At the protein metabolism, the liver has four main functions: generation of plasma proteins, amino acid interconversion, deamination of amino acids and urea synthesis (for ammonia excretion) (Charlton, 1996). Deamination and transamination of amino acids, followed by conversion of the non-nitrogenous part of those molecules to glucose or lipids are also processes performed by hepatocytes. Several of the enzymes used in these pathways (for example, alanine and aspartate aminotransferases) are commonly assayed in serum to assess liver damage. Liver energy metabolism is tightly controlled. Multiple nutrient, hormonal, and neuronal signals have been identified to regulate glucose, lipid, and amino acid metabolism in the liver. Dysfunction of the liver signaling and of the metabolism causes or predisposes to nonalcoholic fatty liver disease (NAFLD) and/or type 2 diabetes (Rui, 2014).

Detoxification of the body by removal of ammonia by synthesis of urea is another critical role played by liver hepatocytes. Ammonia is very toxic and if not rapidly and efficiently removed from the circulation will result in central nervous system disease.

Hepatocytes are also responsible for synthesis of most of the plasma proteins. The liver manufactures a broad spectrum of proteins, including many clotting factors, albumin, accounting for fully 40% of hepatic protein synthesis and synthesized almost exclusively by hepatocytes, thyroid binding globulin, very-low density proteins (VLDL), apolipoprotein B100 and complement. Over 80% of the proteins synthesized by the liver are exported into the systemic circulation. These proteins are central to the maintenance of homeostasis, osmotic pressure, transportation of hormones and lipid and acute phase reactions. Among the many other functions of the liver, it is also responsible for the metabolism of many hormones that have discordant effects on protein metabolism, such as insulin, sex hormones and glucagon. It is thus not surprising that chronic and acute liver diseases are associated with dysfunction of many physiological processes, and can profoundly alter amino acid and protein metabolism. When the liver fails acutely, it is the loss of hepatic regulation of protein metabolism that results rapidly in death. It is widely believed that the changes in amino acid metabolism play a role in the pathogenesis of many of cirrhosis complications (Charlton, 1996). Liver is also the major organ responsible for the detoxification of foreign chemicals and the metabolism of drugs.

4.1.1.1. Cytochrome P450 enzymes

The elimination of external chemicals (xenobiotics), such as drugs and toxins, from the body are an essential process designed to protect against the potential toxicity of feeding. The hypothesis that xenobiotics consumed by animals are transformed into water-soluble substances and excreted through the urine was first put forth in the late 18th century. Only in 1947, R.T. Williams proposed for the first time that these non-reactive compounds could be biotransformed in two phases: functionalization, which uses oxygen to form a reactive site, and conjugation, which results in the addition of a water-soluble group to the reactive site. These would be designated phase I and phase II metabolization (Liska, 1998). Cytochromes P450 (CYP450) constitutes a superfamily of heme enzymes found from bacteria to humans. It is reasonable to suppose that a P450

enzyme is present in every living species on Earth, as this enzyme has been found in archaebacteria, in plants, and in various animal species (Anzenbacher and Anzenbacherová, 2001). The liver is the main metabolic organ where these reactions occur. In the liver, there are two main types of metabolism that deal with xenobiotics, and a third that deals with their transport:

- **Phase I:** The phase I detoxification system, composed mainly by the cytochrome P450 supergene family of enzyme, is usually the first enzymatic defense against foreign compounds. Most pharmaceuticals are metabolized through the phase I biotransformation. In a typical phase I reaction, a cytochrome P450 enzyme (CYP450) uses oxygen and, as a cofactor, NADH, to add a reactive group, such as a hydroxyl radical. These reactions result in small chemical changes that make a compound more hydrophilic, so it can be effectively eliminated by the kidneys. However, as a consequence of this detoxification step, reactive molecules, which may be more toxic than the parent molecule, can be produced. Most information available on the phase I activities has been derived from studies with drug metabolism, but phase I enzymes are also involved in detoxifying endogenous molecules, such as steroids (Anzenbacher and Anzenbacherová, 2001).

- **Phase II:** Phase II enzymes also perform an important role in the biotransformation of endogenous compounds and xenobiotics into more easily excreted forms as well as in the metabolic inactivation of pharmacologically active substances. Phase II metabolism takes place if phase I is insufficient to clear a compound from circulation, or if phase I generates a reactive metabolite. The purpose of phase II biotransformation is to perform conjugating reactions, which usually involve the addition of a large polar group (conjugation reaction) to further increase the compound's solubility. These include glucuronidation, sulfation, methylation, acetylation, glutathione, and amino acid conjugation. In general, the respective conjugates are more hydrophilic than the parent compounds. Phase II drug metabolizing enzymes are mostly transferases and include: UDP-glucuronosyltransferases (UGTs), sulfotransferases (SULTs), N-acetyltransferases (NATs), glutathione S-transferases (GSTs) and various methyltransferases [mainly thiopurine S-methyl transferase (TPMT) and catechol O-methyl transferase (COMT)] (Jancova *et al.*, 2010).

There is an intrinsic relation between phase I and phase II enzyme metabolism. Reactive molecules originated in phase I metabolism not further metabolized by phase II conjugation may cause damage to cell proteins, RNA, and DNA. Several studies have shown evidence of associations between induced phase I and/or decreased phase II enzyme activities and an increased risk of disease, such as cancer, systemic lupus erythematosus, and Parkinson's disease (Bandmann *et al.*, 1997). Compromised phase I and/or phase II activity have also been implicated in adverse drug responses (Lee, 1995).

- **Phase III:** Phase III transporters, for example, P-glycoprotein (P-gp), multidrug resistance-associated proteins (MRPs), and organic anion transporting polypeptide 2 (OATP2) are expressed in many tissues such as the liver, intestine, kidney, and brain, playing crucial roles in drug absorption, distribution, and excretion. Drug transporters move drugs across cellular barriers, and as such can target sites of accumulation. They are located in epithelial and endothelial cells of the liver, gastrointestinal tract, kidney, blood-brain barrier and also in other organs (Xu *et al.*, 2005).

Drug metabolism via the cytochrome P450 system has emerged as an important determinant in the occurrence of several drug interactions that can result in drug toxicities or reduced pharmacological effect, and adverse drug reactions. Recognizing whether the drugs involved act as enzyme substrates, inducers, or inhibitors can prevent clinically significant interactions from occurring. Avoiding co-administration or anticipating potential problems and adjusting a patient's drug regimen early in the course of therapy can provide optimal response with minimal adverse effects. A greater degree of interaction predictability has been achieved through the identification of CYP450 isozymes and some of the drugs that share them (Ogu and Maxa, 2000). CYP450, the main phase I enzymes, is a superfamily of mono-oxygenases that are found in all kingdoms of life, and which show the extraordinary diversity. Mammalian CYP450 enzymes can oxidize both xenobiotics and endogenous compounds, and are important for the detoxification of foreign substances, as well as for controlling the level of endogenous compounds, such as hormone synthesis and breakdown, cholesterol synthesis and vitamin D metabolism. CYP450 enzymes are also involved in vascular auto-regulation, especially in the brain, and are vital to the formation of cholesterol,

steroids and arachidonic acid metabolites. They can also clear the organism of metabolic products such as bilirubin, which arises from the breakdown of hemoglobin. CYP450 enzymes are named because they are bound to membranes within a cell (cyto) and contain a heme pigment (chrome and P) that absorbs light at a wavelength of 450 nm when exposed to carbon monoxide. In mammals, these enzymes are found primarily in the membranes of the endoplasmic reticulum (microsomes) of the cells. CYP450 enzymes are predominantly expressed in the liver, in hepatocytes, but they also occur in the small intestine, reducing drug bioavailability, lungs, placenta, and kidneys (Slaughter and Edwards, 1995). In humans there are more than 50 CYP450 enzymes, but CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP3A4, and CYP3A5 enzymes are responsible for the metabolization of 90% of commercial available drugs (Wilkinson, 2005). Given the importance of prediction of new drug metabolization and of the CYP450 involvement, hepatocytes from several animal models have been used by the pharmaceutical industry for drug screening. One major challenge in drug development is defining the optimal animal species to serve as a model of human metabolism. The most frequently used animal species in metabolism studies have been mouse, rat, rabbit, dog, and monkey. The sequence homology of CYP enzymes among these species is known to be rather high. However, minor differences in the amino acid sequences at the active sites of CYPs can result in a wide variation in substrate specificity and response to chemical inhibitors (Turpeinen *et al.*, 2007). Phase II enzymes have attracted much less attention in clinical pharmacology than cytochromes P450 because drug interactions involving these enzymes are relatively rare. Nevertheless, the reduced metabolizing capacity of the phase II enzymes can lead to the manifestation of the toxic effects of clinical drugs. Although phase II reactions are generally detoxifying, the conjugates formed may also mediate adverse effects (for example conjugates acting as carriers for potentially carcinogenic compounds in the activation of benzylic alcohols, polycyclic aromatic hydrocarbons, aromatic hydroxylamines, hydroxamic acid and nitroal-kanes by sulphotransferases) (Glatt, 2000). Phase III constitute the less studied group of the liver xenobiotics metabolism. Along with phase I and phase II enzymes induction, pre-treatment with several kinds of inducers has been shown to alter the expression of phase III transporters, and alter the excretion of xenobiotics, which implies that phase III transporters may also be similarly regulated in a coordinated

fashion, and provides an important mean to protect the body from xenobiotics accumulation (Xu *et al.*, 2005).

Since the detoxification system function help in the management of exposure to exogenous compounds, the body has developed several mechanisms to regulate detoxification activity. Phase I and/or phase II enzymes can be induced, leading to more enzyme being present, increasing the rate of xenobiotic metabolization. Specific pathways may be induced or inhibited depending on the presence of various compounds, age and sex of the individual, genetic background and lifestyle habits (Meyer *et al.*, 1990; Park *et al.*, 1996). Furthermore, the disease can also influence the enzyme activity. In some disease states detoxification activities appear to be induced or up-regulated whereas in other conditions these activities may be inhibited or, the enzymes were not produced at higher levels (Lee, 1995), which may compromise the response to treatment and the disease outcome. It has also been described that immune environment can influence CYP450 activities (Sunman *et al.*, 2004). Inducers are classified as mono-functional, if their action is on one particular CYP450 or multi-functional, if their influence extends to several CYP450 or even to phase II enzymes. Inducers and inhibitors are well characterized in humans. Mono-functional inducers, such as polycyclic hydrocarbons from cigarette smoke and aryl amines from charbroiled meats, result in dramatic induction of the CYP1A1 and CYP1A2 enzymes, leading to a substantial increase in phase I activity, with little or no induction of phase II enzymes (Guengerich, 1984). Pharmacological drugs may also lead to the same result: induction of phase I enzyme as seen in glucocorticoids and anti-convulsants inducing CYP3A4 activity, and ethanol, acetone and isoniazid induce CYP2E1 (Denison *et al.*, 1995). Induction of these enzyme activities without co-induction of phase II metabolism may lead to an uncoupling of the phase I and phase II balance of activity and, therefore, a higher level of reactive intermediates, which can cause damage to DNA, RNA, and proteins (Park *et al.*, 1996). The multi-functional inducers include many of the flavonoid molecules found in fruits and vegetables. For example, ellagic acid found in red grape skin has been shown to induce several phase II enzymes while decreasing phase I activity (Barch *et al.*, 1995; Manson *et al.*, 1997). Many other compounds regularly found in food, as garlic oil, rosemary, soy, cabbage, and brussel sprouts all contain compounds that can induce several phase II enzyme activities (Liska, 1998).

Commonly, the glutathione S-transferase and glucuronyltransferases are induced by multi-functional inducers. In general, phase II increase supports better detoxification and helps to promote and maintain a healthy balance between phase I and phase II activities. The enhancement of phase II activity has been proposed to explain, at least in part, the ability of fruits and vegetables to protect against many cancers (Elangovan *et al.*, 1994; Manson *et al.*, 1997). The functionality of phase I and phase II enzymes can also be inhibited. Inhibition can occur by competition between two or more compounds for the same detoxifying enzyme. Increased toxic load may lead to inhibition of detoxification of a number of compounds by simply overwhelming the system and competing for detoxification enzyme activities. Moreover, some compounds selectively inhibit only one detoxifying activity. Quinidine, which inhibits CYP2D6 activity and cimetidine are examples of compounds that can inhibit phase I enzymes. The first by competition and the second by binding directly to the iron of cytochrome P450 reactive site, abolishing phase I enzyme activity (Liska, 1998). For phase II enzymes, a common mechanism of inhibition is the depletion of cofactors necessary for enzymatic activity. In humans, sulfation is a classical example, particularly susceptible to inhibition due to compromised cofactor status.

Genetic differences in the ability of an individual to metabolize xenobiotics are related to the presence of genetic polymorphisms: different versions of the gene encoding CYP450 or phase II enzymes. Polymorphisms in CYP450 are described in several species, as humans, mouse and dog. In humans, CYP2D6 is the classic example of the influence of genetic polymorphism on phenotype. The polymorphism of the enzyme results in poor, intermediate, efficient or ultrarapid metabolizers of CYP2D6 drugs. The enzyme is largely non-inducible and metabolizes approximately 25% of current drugs. Typical substrates of CYP2D6 are largely lipophilic bases and include some antidepressants, antipsychotics, antiarrhythmics, antiemetics, beta-adrenoceptor antagonists (beta-blockers) and opioids. Adverse side-effects occurring from these drugs may be reduced by decreasing dosages in those individuals who are CYP2D6 slow metabolizers (Ingelman-Sundberg, 2005; Zhou, 2009). Polymorphisms in CYP2D6 have also been associated with a high risk of early onset Parkinson's disease (Lu *et al.*, 2013). Polymorphisms in the phase II activity of N-acetyltransferase can also lead to slow metabolizers. Associations have been found between slow metabolism

through the N-acetyltransferase pathway and high risk of some types of cancer and Parkinson's disease (Daly *et al.*, 1993). As dogs are widely used by the pharmaceutical companies to test new drugs, some studies were performed to evaluate canine drug metabolism and CYP450 activities. The most comprehensively studied on canine CYP genetic polymorphism is the premature stop codon mutation located in the coding region of the CYP1A2 gene that results in complete loss of hepatic CYP1A2 protein associated with enzyme activity failure. It was also found that some dogs had a partial or complete gene deletion of CYP2C41 and amino acid variants in the coding sequence of CYP2D15, CYP2E1 and CYP3A12 are also described, evidencing great variability in drug metabolism by CYP (Court, 2013).

4.1.2. Hepatocyte innate immune role

The structural organization of the liver has profound implications for its immune function. Blood enters the hepatic parenchyma mainly via terminal portal vessels, and then passes through a network of liver sinusoids, leaving the parenchyma via the central hepatic veins. Due to the small diameter of the sinusoids, minimal increases in systemic venous pressure and perturbations of sinusoidal flow result in stasis, which lengthens the contact between lymphocytes and antigen-presenting cells and promotes lymphocyte extravasation. Extravasation is further facilitated by fenestrations in the monolayer of sinusoidal endothelial cells (LSEC) that allow lymphocytes to access the space of Disse via cytoplasmic extensions and to close contact the underlying extracellular matrix, stellate cells and hepatocytes. Hepatocytes play a key role in controlling systemic innate immunity via production of secreted pattern recognition receptors (PRRs) and complement components found in plasma. Expression of genes encoding these proteins is controlled by liver-specific transcription factors, such as hepatocyte nuclear factors, nuclear factor-1, and CCAAT-enhancer-binding protein, which account for their liver-specific expression. During an acute phase or systemic inflammatory response, a variety of pro-inflammatory cytokines, such as interleukin-6 (IL-6), IL-1, tumor necrosis factor α (TNF-α), and interferon (IFN)-γ, can stimulate hepatocytes to produce high levels of complement and secreted PRRs (Gao *et al.*, 2008). The complement system consists of more than 35 plasma or membrane proteins that interact with one another in a cascading fashion to protect against infections. Three different pathways have been identified that

activate the complement system. These include the classical pathway (target-bound antibody), the lectin pathway (microbial repetitive polysaccharide structures), and the alternative pathway (recognition of other foreign surface structures). After activation, the complement system generates a wide range of biologic activities such as opsonic, inflammatory, and cytotoxic functions. Hepatocytes are a major site that biosynthesizes complement components found in plasma. These include C1r/s, C2, C4, and Cbp of the classical pathway, C3 and factor B of the alternative pathway, mannan-binding lectin, mannan-binding lectin-associated serine proteases 1-3, and mannan-binding lectin-associated protein 19 of the lectin pathway, and terminal components C5, C6, C8, and C9 of the complement system (Qin and Gao, 2006). Although immune cells and endothelial cells also produce these components their contributions are minor compared to those of hepatocytes. Additionally, hepatocytes are also primarily responsible for the biosynthesis of several complement regulator proteins found in plasma, such as factor I, factor H, and the C1 inhibitor (Morgan and Gasque, 1997). In addition to being an important part of innate defenses against infection, the complement system also contributes to the pathogenesis of a variety of liver disorders, including liver fibrosis, alcoholic liver disease and liver ischemia (Pritchard *et al.*, 2007). However, the molecular mechanisms underlying the involvement of complements in liver injury and repair remain obscure. Hepatocytes are also the major source for production of secreted PRRs, which have two main functions: complement activation and microbial cell opsonization for phagocytosis. Four major classes of soluble PRRs have been identified according to their domain composition: collectins, pentraxins, lipid transferases, and peptidoglycan-recognition proteins. Many of these proteins are synthesized by hepatocytes and released into the bloodstream, thereby playing an important role in innate immunity against local and systemic microbial infection. In addition, the liver is also a major source of many other acute phase proteins, which play key roles in innate defenses against infection and in reducing tissue damage through inactivation of proteinases released by pathogens and death or dying cells (Gao *et al.*, 2008). The liver not only is the major source of secreted PRRs but also expresses membrane-bound PRRs, such as Toll-like receptors (TLRs). TLRs are a family of proteins that recognize pathogen associated molecular patterns (PAMPs). TLRs are expressed on all major subsets of liver cells. Both exogenous ligands derived from pathogens, and endogenous

ligands that are products of cellular injury, can engage these receptors and activate innate immunity. TLRs play a vital role in viral and parasitic infections of the liver, in ischemia-reperfusion injury, and in toxic liver damage, promoting antipathogen immunity but also hepatocellular injury and fibrogenesis. There are 13 different TLRs identified so far. Each of them recognizes specific PAMPS and activates specific signaling pathways and antimicrobial responses (Akira and Takeda, 2004). However, hepatocytes express messenger RNAs for all known TLRs their role on hepatocyte defenses against invading pathogens is less clear. Hepatocytes TLR2 and TLR4 are responsive to LPS. TLR4 has been detected on all types of liver cells and is likely involved in the uptake and clearance of endotoxins, production of pro- and anti-inflammatory cytokines, and generation of reactive oxidative stress (Szabo *et al.*, 2006; Schwabe *et al.*, 2006). Recently, several other cytoplasmic PRRs have been identified in hepatocytes, including NOD-like receptors and the RIG-like helicases. Among them, RIG-1 serves as a pathogen receptor to regulate cellular permissiveness to hepatitis C virus (HCV) replication (Sumpter *et al.*, 2005). In addition, activation of several TLRs has been shown to inhibit hepatitis B virus (HBV) and HCV infection, providing novel strategies to treat hepatitis viral infection. NOD-like receptor's role is not yet well defined in liver immunology (Isogawa *et al.*, 2005; Thomas *et al.*, 2007).

4.2. Chapter objectives

In the present chapter it is proposed to investigate how liver hepatocytes sense and react to *L. infantum* presence. Hepatocytes are major metabolic cells of the liver, a target organ for *L. infantum* infection. To understand the roles played by these cells during infection outcome in the dog's liver, several specific goals were established:

- Characterize *L. infantum* interaction with canine hepatocytes using two different culture systems: bi-dimensional (2D) and three-dimensional (3D).
- Analyze the immunological status of hepatocytes exposed to *L. infantum* by assessing innate immune receptors and cytokine gene expression, as well as metabolic parameters.
- Investigate the relation between the immune status of hepatocytes and the activity of cytochrome P450 enzymes.
- Evaluate the impact of a leishmanicidal drug in hepatocytes exposed to the parasite by assessing innate immune receptors and cytokine gene expression, and metabolic parameters.
- Characterization of the potential leishmanicidal activity of new compounds: ursolic acid, chalcone and quercetin. Impact of these compounds in the canine hepatocytes.

Some of the techniques used in the present work, mainly confocal microscopy and scan electron microscopy (SEM) were the fruit of a generous collaboration with Professor Graça Alexandre-Pires of the Faculdade de Medicina Veterinária - Universidade de Lisboa and, additional processing of the images for artificial coloration was performed by Tiago Pereira (MSc) under my tutorial guidance.

4.3. Methods

4.3.1. Hepatocyte isolation

Isolation of dog hepatocytes was achieved by two step perfusion protocol followed by collagenase digestion and a Percoll® gradient centrifugation, as described in Miranda *et al.,* (2009 and 2010) with some adaptations. A hepatic lobule was cleaned externally with a NaCl solution supplemented with 0.9% of penicillin/streptomycin. To get rid of the internal blood a first perfusion was performed with 500 ml of perfusion I solution [2.4 M ethylene glycol tetra acetic acid (EGTA), 142 mM NaCl, 6.7 mM KCl and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.4] heated at 38 ºC (Figure 16A). The second perfusion was implemented in order to digest the collagen of the tissue to allow the isolation of single hepatocytes. The Perfusion II solution (67 mM NaCl, 6.7 mM KCl, 100 mM HEPES, 4.8 mM $CaCl_2$, pH 7.6) was supplemented with 250 mg of collagenase H (Roche) and 0.5% albumin (Prolabo®) and, heated at 38 ºC. Following digestion, a cell suspension was obtained by gently scratch the lobule surface with a scalpel. Cellular suspension was filtered through a funnel with sterile gauze and centrifuged at 50 *x*g for 10 min at 4 ºC. Pellets were resuspended in Williams'E medium (Sigma-Aldrich) and concentration of viable cells was estimated in trypan blue under optical microscope through Equation 4.

Equation 4:

$$[\mathrm{C}](\mathrm{cells/ml}) = \frac{\text{number of cells}}{4} \times 10^4 \times \mathrm{DF}$$

[C]= Concentration of cell suspension
DF= dilution factor
4= number of counting squares in a Neubauer chamber
10^4=Volume of Neubauer chamber correspond to 0.1 mm^3=0.0001 ml. For determination of the cell number in 1 ml, it is applied a 10^4 multiplication factor.

Cells (3.5×10^6 $cells.ml^{-1}$, in a maximum of 6 ml) were overlaid into 20 ml of a solution of 25% Percoll® (GE Healthcare) in PBS 1*x* and centrifuged at 1300 *x*g at 4 ºC for 20 min (Figure 16B). After centrifuged, viable hepatocytes obtained in the pellet were then resuspended in cold PBS and washed two times. Final pellet was resuspended in supplement Williams'E medium [Penicillin (100 U/ml)/streptomycin (100 $\mu g.ml^{-1}$) (Sigma-Aldrich), 1.4 µM hydrocortisone (Sigma-Aldrich), 15 mM HEPES (Sigma-Aldrich), 1 mM sodium pyruvate (Sigma-Aldrich), 1 mM non-essential amino acids

(NEAA) (Biochrom), 40 µg.ml^{-1} gentamycin (Sigma-Aldrich) and 10% (v/v) FBS9] and concentration of viable cells estimated under optical microscope using the exclusion dye trypan blue. This procedure allowed the isolation of a solution of pure hepatocytes with ≈80% of viability.

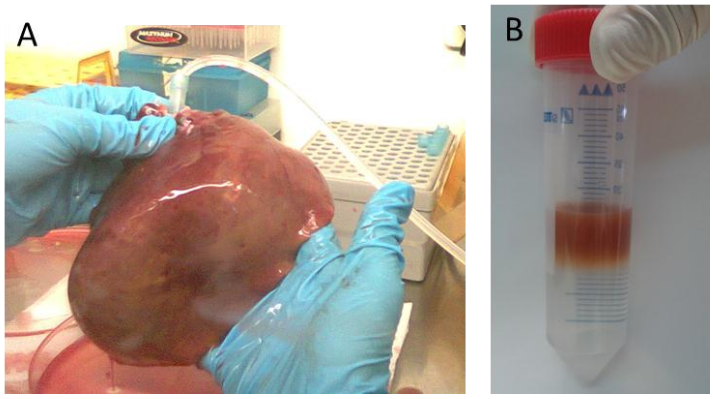


Figure 16: Isolation of canine hepatocytes by a two-step methodology.
Perfusion of an excised liver lobule to clean the peripheral blood (A) and cellular suspension overlaid into a Percoll® gradient in order to purify hepatocytes (B).

4.3.1.1. 3D-hepatocyte culture

3D-hepatocyte culture was established in a 250 ml Spinner vessel (Wheaton) (Figure 17). Prior to cell culture, the spinner vessel was washed with 2 ml of tuloene (Sigma-Aldrich) and siliconized with 2 ml of dichlorodimethylsilane (Sigma-Aldrich). Dichlorodimethylsilane is a tetrahedral, organosilicon compound [$Si(CH_3)_2Cl_2$] that reacts to form both linear and cyclic Si-O chains on the glass inner superficies, which become hydrophobic, avoiding cellular deposition on the vessel walls. The spinner vessel was then sterilized by autoclave. Cells (1.2×10^5 cells.ml^{-1}) were inoculated in supplemented Williams'E medium, extra supplemented with 15% of FBS (v/v), to promote cell aggregation. At 24 h post-inoculation, half of the medium was replaced with fresh medium and, at 72 h the total medium of the vessel was replaced with fresh medium. The cell suspension was collected to 50 ml tubes and centrifuged at 50 *x*g for 10 min at room temperature. Pelleted cells were resuspended in fresh culture medium and inoculated again in the spinner vessel to ensure no loss of cells. The spinner was maintained at 50 rpm rotation in a cell incubator (Sanyo MCO-18AIC) at 37 ºC in a humidified atmosphere with 5% CO_2. Cells were followed by optical microscopy

observation under an inverted microscope (Olympus, CKX41) and digital images were acquired with an Olympus CS30 camera.

Figure 17: Dog 3D hepatocyte culture system.
Hepatocyte aggregates were established in a spinner vessel with controlled atmosphere and in a constant low rotation.

4.3.1.2. 2D-hepatocyte culture

2D-hepatocyte cultures were established in 24 well plates (VWR). Cells (5×10^5 cells.ml^{-1}) were plated in the supplemented Williams'E medium. Medium was replaced at 24 h, 48 h and 72 h. At 72 h, *L. infantum* parasites were added and the plates were kept for about 10 days in an incubator (Sanyo MCO-18AIC) at 37 ºC, in a humidified atmosphere with 5% CO_2. Cells were followed by optical microscopy observation under an inverted microscope (Olympus, CKX41) and digital images were acquired using an Olympus CS30 camera.

4.3.2. Hepatocyte exposure to *L. infantum* virulent promastigotes or axenic amastigotes

After 72 h in 2D- or 3D-culture systems, hepatocytes were exposed to *L. infantum* parasites. *L. infantum* virulent promastigotes and axenic amastigotes were obtained as described in the General Methods chapter, under sub-headings 3.3.1 and 3.3.2, respectively. Parasites were added to hepatocyte culture in a proportion of three promastigotes or amastigotes per one hepatocyte. The 3D-system was maintained at 50 rpm, 37 ºC in a humidified atmosphere with 5% CO_2 and the parasite as let to interact with hepatocyte aggregates for 72 h. In the 2D-system the parasites were left to interact with hepatocytes for 72 h at 37 ºC, in a humidified atmosphere with 5% CO_2. Samples of medium and cells were collected at 1.5 h, 3 h, 5 h, 24 h, 48 h and 72 h incubation

from both culture systems and used for determination of several parameters. Cells were followed by optical microscopy observation under an inverted microscope (Olympus, CKX41) and digital images were acquired using an Olympus CS30 camera.

4.3.2.1. Positive controls

Escherichia coli lipopolysaccharide (LPS, Sigma-Aldrich) was used as a positive control of inflammation. LPS was added to hepatocyte cultures at 1 $\mu g.ml^{-1}$.

After 72 h of 2D-heptocyte culture, the medium was carefully replaced by supplement William's E fresh medium with LPS and cells were left to incubate for more 72 h at 37 ºC in a humidified atmosphere with 5% CO_2. Samples of medium and cells were collected at 1.5 h, 3 h, 5 h, 24 h, 48 h and 72 h of incubation and used as positive controls for further immune tests.

4.3.3. Treatment

Since the liver is a central organ in drug metabolism, the effect of leishmanicidal compounds was tested in hepatocytes exposed to *L. infantum*. The goal of this specific study was to evaluate the effect of drugs in the metabolism and in the immune response of hepatocytes. The following drugs were assessed: meglumine antimoniate, a commercially available and well known antileishmanial drug used for leishmaniasis treatment (see 4.3.3.1) and, three new compounds with leishmanial activity (see 4.3.3.2). The drugs were added to 3D- and 2D-culture systems. After 72 h of hepatocyte incubation with *L. infantum*, drugs were added and cells and parasites were left to incubate for more 72 h. Samples of medium and cells were collected at 1.5 h, 3 h, 5 h, 24 h, 48 h and 72 h of incubation and used for the evaluation of several parameters.

4.3.3.1. Meglumine antimoniate

Meglumine antimoniate is a drug commercially available as Glucantime® (Merial), presented in a formulation constituted by 81 mg of the $compound.ml^{-1}$. This drug belongs to a class of compounds known collectively as pentavalent antimonials used in both human and veterinarian medicine. After 72 h in contact with *L. infantum* virulent promastigotes or axenic amastigotes, the medium of 2D-hepatocyte culture was carefully removed, avoiding disturb the bottom of the well, and supplement William's E

fresh medium with 100 µM of meglumine antimoniate was gently added. Medium of 3D-hepatocyte culture was also removed and the cell aggregates were resuspended in supplemented William's E fresh medium with 100 µM of meglumine antimoniate. Cells of both culture systems were left to incubate for more 72 h at 37 ºC in a humidified atmosphere with 5% CO_2. Samples of medium and cells were collected at 1.5 h, 3 h, 5 h, 24 h, 48 h and 72 h incubation for the evaluation of several parameter analyses.

4.3.3.2. New leishmanicidal compounds

In the present work were used three new compounds with previous proven leishmanicidal activity. Ursolic acid (URS) gently offered by Prof. Doutor Luiz Felipe Passero (LPMI-FMUSP, S. Paulo, Brazil) and Prof. Doutor João Lago (ICAQF-UFSP, S. Paulo, Brazil), and quercetin (QC) and chalcone (CH8) cordially given by Prof. Doutora Bartira Rossi Bergmann (IBCCF-UFRJ, Rio de Janeiro, Brazil). These compounds were dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich) assuring that the DMSO concentration in the final suspension was inferior to 1% (v/v). The concentration of drugs used in hepatocyte cultures follows the amounts previously used in a MØ-like murine cell line (Rafael-Fernandes, 2015). In the present study, hepatocytes were exposed to the IC90 determinate for murine MØ cell line, ensuring the survival of 90% of cells. Therefore the concentrations used in hepatocyte cultures were 0.091 µg.ml^{-1}of URS, 1.52 µg.ml^{-1} QC and 65.8 µg.ml^{-1} of CH8.

4.3.4. Hepatocytes viability

To ensure that hepatocytes were viable during the treatment, the mitochondria's reducing ability was evaluated, using resazurin. The fluorescent compound resazurin (7-hydroxy-3H-phenoxazin-3-one-10-oxide) (Sigma-Aldrich) is a redox indicator of viability that is converted by metabolically active cells into resorufin. Cells (5×10^4 cells.ml^{-1}) were plated for 72 h in a sterile 96 well back plate (Nunc), using a final volume of 200 µl of supplemented William'E medium that was replaced daily with fresh medium. Then William'E medium plus the drug (described in 4.3.3.1 and 4.3.3.2) was added to hepatocyte and cells were left to incubate for 1.5 h, 3 h, 5 h, 24 h, 48 h and 72 h at 37 ºC in a humidified atmosphere with 5% CO_2. At each time point, 25 µl of a 1.25 mM resazurin solution in PBS 1*x* was added to each well and incubated for 24 h.

Plates were read in a fluorometer (TRIADTM 1065, DYNEX Technologies) with excitation and emission wavelengths of 535 NM and 595 NM, respectively. Hepatocytes in the absence of drugs (viable cells) and non-viable hepatocytes were used as controls. Non-viable hepatocytes were obtained by the addition of 2% (m/v) paraformaldehyde (Sigma-Aldrich) in PBS.

4.3.5. Parasite viability

To estimate the viability of parasites that were in contact with hepatocytes in the presence of drugs, an approximation to the limit dilution assay (LDA) was performed with some adaptations. For each of the treatment time points (described in 4.3.3.), 1 ml of cell suspension of the 3D-culture system or one well in the 2D-culture system were collected and centrifuged. For each time point, samples were collected in quadruplicate. Sediments were resuspended in 1 ml of complete SCH medium and 200µl of cell suspension was added to the first column wells of a sterile 96 transparent well plate and used for 1:4 serial dilutions. Plates were sealed and incubated at 24 ºC for 15 days. After this period, each well was observed under optical microscope and the presence of motile promastigotes was registered as positive titers. Parasite viability was estimated by the rate between the reciprocal of the obtained titers and the reciprocal of the highest positive titers obtained for amastigote differentiation without previous contact with hepatocytes or drugs and plated in the same concentration as used in 4.3.2.

4.3.6. Microscopy techniques

4.3.6.1. Confocal microscopy

Hepatocytes in William´s E medium (2.5×10^5 cells.ml^{-1}) were also cultured in a 8-well chamber slide with cover (Lab-Tek II, Nunc) and incubated at 37 ºC in a humidified atmosphere with 5% CO_2 for 72 h. *L. infantum* GFP-amastigotes or GFP-promastigotes were added to cultured cells in a proportion of 3 parasites per cell. Cells were incubated with the parasite for 1.5 h, 3 h, 5 h, 24 h, 48 h and 72 h. Adherent cells were washed with PBS 1x and fixed with 2% (m/v) paraformaldehyde in PBS for 20 min on ice. Slides were maintained in sterile PBS 1*x* at 4 ºC until further processing. For immunostaining, cells were permeabilized overnight at 4 ºC with a solution of PBS 1% Tween-20 (v/v) (Sigma-Aldrich) and 0.2% fish gelatin (m/v) (Sigma-Aldrich). For

antibodies incubation buffer, it was used a solution of PBS 0.125% fish gelatin (m/v) and 1% Tween-20 (v/v). The incubation with primary antibodies was performed during 90 min at room temperature, kept in the dark. For observation of cell morphology, actin, the cytoskeleton protein of the cell, was stained with an actin goat polyclonal antibody FITC conjugated (sc-1616, Santa Cruz), raised against a peptide at the C-terminus of actin of human origin and diluted 1:500 according to manufacturer's recommendation. Therefore, no secondary antibody was necessary and cells were washed three times with PBS 0.08% tween-20 (v/v) and finishing with three more washes with PBS 1x. Ferritin is a protein of iron storage and transport present in functional hepatocytes. Goat polyclonal antibody (sc-14416, Santa Cruz) raised against a peptide near the N-terminus of the ferritin heavy chain of human origin was used in a 1:100 dilution accordingly to manufacturer's instructions. Polyclonal antibodies (anti-actin and anti-ferritin) were described as having cross reactivity with several species, including dogs. For the anti-ferritin staining, a secondary rabbit polyclonal anti-goat IgG Alexa Fluor® 647 (ab150143, Abcam) antibody was used at a dilution of 1:500. Between primary and secondary antibodies, cells were washed three times with buffer PBS 0.08% tween-20 (v/v) and finishing with three more washes in PBS 1*x*. Control slides were prepared with only one antibody staining. Slides were prepared for fluorescence microscopy using TO-PRO®-3 or Vectashield® antifade mounting medium with a red-fluorescent compound and DAPI to localize nucleus and chromosomes, respectively. Slides were observed under a Leica TCS SP2 Laser Scanning Confocal Microscope and digital images were acquired.

4.3.6.2. Electron scanning microscopy

Cultured hepatocytes were washed with cold PBS 1x and fixed with 2% paraformaldehyde (m/v) in PBS for 20 min on ice. Cells were then washed again with cold PBS 1*x* by centrifugation at 350 *x*g for 10 min. Cells were filtrated through a Millipore mesh and subsequently dehydrated in a graded ethanol series [30, 50, 70, 80, 90 and 100% (v/v)]. Samples were dried using the critical point drying method, coated with gold palladium and mounted on stubs. Cells were then observed in a scanning electronic microscope (JEOL5200-LV) and digital images were acquired.

4.3.7. Liver enzymes

The elimination of foreign compounds from the body (xenobiotics) such as drugs and toxins is an essential process designed to protect against potential toxicity from the food and the environment. In the liver, there are two main types of metabolism that deal with xenobiotics – phase I and phase II – and a third that are associated with the transport of xenobiotics metabolites.

4.3.7.1. Phase I enzymes

The activity of Phase I enzymes causes small chemical changes, generating more hydrophilic compounds able to be effectively eliminated by the kidneys. These chemical reactions usually involve either adding or removing a hydroxyl group or other hydrophilic groups such as amine or sulphydryl groups, directing hydrolysis and oxidation or reduction. Cytochrome P450 enzymes are responsible for most reactions of phase I. In the present work the activity of alkoxyresorufin O-dealkylation (AROD) of CYP450 family was analyzed with the appropriate substrates, 7-ethoxyresorufin (EROD), 7-methoxyresorufin (MROD), 7-pentoxyresorufin (PROD), or 7-benzyloxyresorufin (BROD) (see Table 6).

EROD, MROD, PROD and BROD reactions were performed throughout all the sampling time points of this study of the diverse conditions (2D- and 3D-culture systems; non-exposed hepatocytes; *L. infantum* exposed hepatocytes; drug treated cells and inflammation controls). All substrates were purchased at Sigma-Adrich dissolved in DMSO at a final concentration of 10 µM in William's E culture medium. In the 3D-culture system, 1 ml of cell suspension was taken and 10 µl of the correct substrate was added. The cell suspension was then incubated during 1 h at 37 ºC in a humidified atmosphere with 5% CO_2, allowing substrate metabolization by hepatocytes. After incubation, cells were centrifuged at 500 *x*g for 10 min at 4 ºC and supernatants were collected and stored at -20 ºC until further analysis. In 2D-culture system, after carefully removing the medium of a well, new culture medium containing 10 µl of substrate was added and cells were left to incubate as described above. After incubation, the supernatant was collected, centrifuged to eliminate any cell or debris, and stored at -20 ºC until further processing. Samples were analyzed by fluorescence on a fluorometer with excitation and emission wavelengths of 535 nm and 595 nm,

respectively. After thawed, samples were vigorously vortex and 200 µl of each sample were plated in duplicate in a sterile 96 well black plate (Nunc). A standard curve with resorufin (Sigma-Aldrich), the product of the O-dealkylase reaction performed by the hepatocytes, was established. The starting dilution was 0.16 µM of resorufin and serial 1:2 dilutions were performed in William's E medium. A blank prepared with William's E medium and an assay control prepared with the substrate added to fresh culture medium (free of hepatocytes) and incubated for 1 h (as described above) were also included in the assay.

Table 6: List of phase I enzymes and the respective substrate.
Evaluated enzymes are collectively designated as alkoxyresorufin O-dealkylases (AROD) since all produce the same product – resorufin – from different substrates. Identification of the principal CYP450 responsible for the metabolization of each substrate in dog, mouse and man (Cai *et al.*, 1996; Dayal *et al.*, 1999; Martignoni *et al.*, 2006; Mise *et al.*, 2008; Hagemeyer *et al.*, 2010).

Test	Substrate	Canine enzyme	Mouse enzyme	Human enzyme
MROD (7-methoxyresorufin O-dealkylase)	Resorufin methyl ether	CYP1A2 CYP1B	CYP1A2	CYP1A2
PROD (7-pentoxyresorufin O-dealkylase)	Resorufin penthyl ether	CYP2B11	CYP2B9 CYP2B10	CYP2B6 CTP2B7
EROD (7-ethoxyresorufin O-dealkylase)	Resorufin ethyl ether	CYP1A1 CYP1A2	CYP1A1 CYP1A2	CYP1A1 CYP1A2
BROD (7-benzyloxyresorufin O-dealkylase)	Resorufin benzyl ether	CYP2B11 CYP3A12 CYP3A26	CYP3A11	CYP3A4

4.3.7.2. Phase II enzymes

The metabolites resultant of xenobiotics chemically altered by conjugation with charged species, such as glutathione (GSH), sulfate, glycine, or glucuronic acid, are generated due to the intervention of phase II enzymes. The addition of large anionic groups (such as GSH) detoxifies reactive electrophiles and produces more polar metabolites that cannot diffuse across membranes, and may, therefore, be actively transported (phase III).

4.3.7.2.1. UGT

Uridine 5'-diphospho-glucuronosyltransferase (UDP-glucuronosyltransferase, UGT) is a cytosolic glycosyltransferase that catalyzes the transfer of the glucuronic acid component of UDP-glucuronic acid into a small hydrophobic molecule. It is considered

one of the most important phase II enzymes. In the present work UGT activity was evaluated by adding a final concentration of 10 mM of 4- methylumbelliferone (4-MU) (Sigma-Aldrich) to the culture medium. The base for this assay is the difference in fluorescence of 4- methylumbelliferone (4-MU), which exhibits high levels of fluorescence, to its conjugated 4-methylumbelliferone-β-d-glucuronide (4MUG), with little fluorescence levels. In the 3D-culture system, 1 ml of cell suspension was taken and 4-MU was added. Cell suspensions were incubated during 1 h at 37 ºC in a humidified atmosphere with 5% CO_2. The assay was performed in duplicate. After incubation, cells were centrifuged at 500 *x*g, 10 min at 4 ºC, and supernatants were collected and stored at -20 ºC until further analysis. In the 2D-system, after removing the medium from a well, fresh culture medium containing the substrate was added and cells were left to incubate in the same conditions. The assay was also performed in duplicate. After incubation, the supernatant was collected and centrifuged to clean it from any cell or debris, and stored at -20 ºC until further processing. Samples were analyzed by fluorescence emission on a fluorometer (BIOTEK® FLx800) with excitation at 360/40 nm and emission at 460/40 nm. The blank was considered as William's E culture medium and a control assay of substrate and culture medium incubate without cells, was also included. After thawed, samples were vigorously vortex and, 250 µl of each sample were plated in duplicate in sterile 96 black well plates (Nunc). A standard curve with 4-MU, was performed, starting at 10 mM and followed by 1:2 serial dilutions. 4-MU mobilization was calculated by subtracting the amount detected in samples to the initial amount of 4-MU.

4.3.8. Urea production

Production of urea is a metabolic characteristic of fully differentiated active hepatocytes. In this study, urea was analyzed in the supernatant of cell samples by a commercial kit QuantiChrom™ Urea Assay Kit -DIUR-500 (BioAssay System) for colorimetric determination of urea. In the 3D-hepatic culture system, 1 ml of cell suspension was taken and in the 2D-hepatic culture system, the medium of each well was collected in duplicate. Cell suspensions were centrifuged at 500 *x*g, for 10 min at 4 ºC, and supernatants were collected and stored at -20 ºC until further analysis.

The urea assay kit is designed to measure urea directly in biological samples without any pre-treatment. The improved Jung method uses a chromogenic reagent that forms a colored complex specifically with urea. The intensity of the color measured at 450 nm is directly proportional to the urea concentration in the sample. After thawed, samples were vigorously vortex and 50 µl of each sample were plated in duplicate in a sterile 96 well transparent plate (Nunc). Standard urea concentration [5 $mg.dl^{-1}$] and the blank (water) were included and urea was estimated accordingly manufacturer's instructions (equation 5). For posterior analysis sterile William's E culture medium reading was subtracted to each sample reading.

Equation 5:

$$[\mathrm{C}](\mathrm{mg.ml^{-1}}) = \frac{\text{OD sample} - \text{OD blank}}{\text{OD standard} - \text{OD blank}} \times \mathrm{n} \times \text{standard concentration (mg.ml)}$$

Where OD indicates optical density and n the dilution factor (in the present study n=1)

4.3.9. Nitric oxide production

In biological systems, nitric oxide (NO) is synthesized by the enzyme nitric oxide synthase (NOS). NOS acts on molecular oxygen, arginine and NADPH, producing NO, citrulline and NADP.

In the present study, a commercial kit Nitrate/Nitrite Colorimetric Assay kit (Abnova) was used accordingly to the manufacture's recommendation to quantify the nitrate/nitrite production in cell supernatants. This kit provides an accurate and convenient method to measure total nitrate/nitrite production in a two-step protocol. In the first step there is the conversion of nitrate to nitrite by the enzyme nitrate reductase. In the second step occurs the addition of Griess reagent which converts the nitrite into a deep purple azo compound that can be measured by absorbance at 550 nm (BioRad-680, microplate reader), determining the NO_2^- concentration. Samples of cell suspension (1 ml) were taken from the 3D-culture system and centrifuged at 500 *x*g, for 10 min at 4 ºC. Supernatants were collected and stored at -20 ºC until further analysis. In the 2D-culture system, medium was removed from each well and centrifuged as indicated above. After thawed, samples were vigorously vortex and plated (80 µl) in

sterile 96 well transparent plate (provide by the manufactures). Samples were performed in duplicate. A standard curve was constituted by 1:5 nitrate serial dilutions. The blank performed with sterile William's E culture medium was also included in the plate and the production of NO was estimated through the following equation (equation 6), in accordance with manufacturer's instructions:

Equation 6:

$$[\text{Nitrate} + \text{Nitrite}](\mu\text{M}) = (\text{OD550nm} - \text{slope standard curve}) \times \text{SV} \times \text{DF}$$

Where OD indicates optical density, SV is sample volume (µl) and DF, the dilution factor.

4.3.10. Real-time PCR analysis

Collection of samples for RNA extraction, followed by cDNA synthesis and real-time PCR analysis were described in the chapter General Methods (section 3.4). β-actin was used for normalization of gene expression of innate immune receptors NOD1, NOD2, TLR2, TLR4 and TLR9 and of cytokines IL-6, IL-10 and TNF-α, correcting sample to sample variations in RNA extraction and minimal changes of real time PCR run-to-run. Samples from 3D- and 2D-culture system exposed to *L. infantum* promastigotes and amastigotes (only 2D cultures) and hepatocytes exposed to the parasite and leishmanicidal drugs, were collected and analyzed. In parallel, positive controls of inflammation and cells not exposed to the parasite were also evaluated.

4.3.11. Statistical analysis

The Wilcoxon test for paired samples was used to perform the statistical analysis as is the non-parametric equivalent of the paired samples t-test. It should be used when the sample data are not normally distributed, as is the case of the present study.

To explore the differences between time points and experimental conditions, the non-parametric Wilcoxon test for two related samples was used. All the data analysis was performed using the software IBM SPSS Statistics version 16.0 (IBM, USA). A significance level of 5% ($p<0.05$) was used to evaluate statistical significance of the data analyzed.

4.4. Results

4.4.1. Establishment of 2D and 3D *in vitro* cultures of dog hepatocytes

Dog hepatocytes were successfully isolated and purified from liver lobules. The isolated cells were cultured in two different culture systems: in the traditional bidimensional (2D) 24 well plates and in a tridimensional (3D) form, exploiting the natural tendency of hepatocytes to aggregate and form cellular spheroids.

4.4.1.1. 2D-culture system preserves normal morphology of dog hepatocytes

The plated hepatocytes presented the typical hepatocyte morphology. The cells exhibited a round shape with a nucleus and an abundance of intracellular organelles associated with metabolic and secretory functions easily observed in the refrangibility of the light under an optical microscope (OM, Figure 18A, B and C). Scanning electron microscopy (SEM, Figure 18D, E and F) allowed to observe the hepatocyte cellular membrane with a smooth aspect pointing to the absence of major perturbation after isolation. As expected, hepatocytes also exhibited the characteristic tendency to establish cellular aggregates.

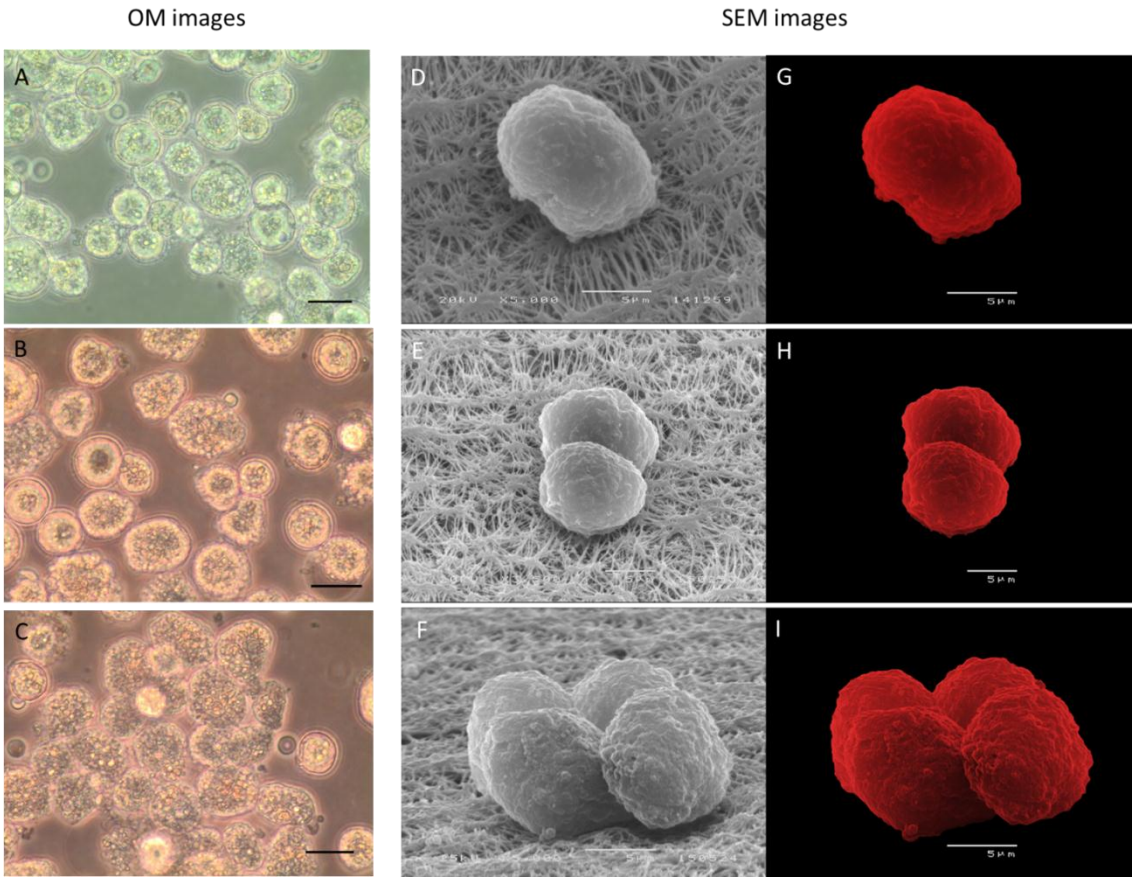


Figure 18: Morphology of dog hepatocytes in 2D-culture system.
Samples of cultured cells with 24 h (A), 48 h (B) and 72 h (C) of incubation were observed under an optical inverted microscope (A-C, scale bar 20 µm, 400*x* magnification) and a scanning electron microscope (D-F), from where images were acquired. Individualized cells with round shape, nucleus and abundance of intracellular organelles (A, B, C) with tendency to aggregate (C and F) were identified. Hepatocytes with a smooth cell membrane were observed 24 h after isolation (D-F). Artificial color (G-I) was applied on cells (red), to evidence the cell morphology.

Isolated cells were stained for actin and ferritin. Actin is a component of the cell cytoskeleton microfilaments and ferritin is an intracellular protein that stores and releases iron in a controlled way. Culture hepatocytes exhibited distinctly rounded nuclei and majority of the cells presented a single nucleus. Cells also exhibited spots positively staining for ferritin, and it is known that hepatocytes have high levels of this protein (Figure 19). Several spots of actin deposition (green spots) were observed, probably indicating secretory vesicles, and some of those were co-localized with ferritin. Hepatocytes are cell rich in organelles, including the endoplasmic reticulum (smooth and rough) and Golgi apparatus that assure secretory functions.

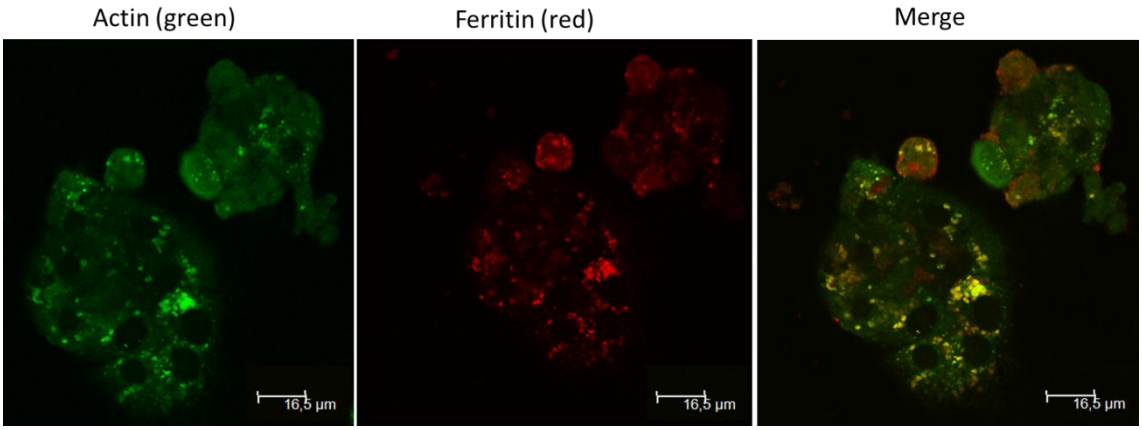


Figure 19: Actin and ferritin in cultured hepatocytes.
Dog hepatocytes incubated for 72 h were stained for actin (green), a component of cell cytoskeleton, and for ferritin (red), usually prevailing in secretory vesicles. Cells were observed under fluorescent confocal microscope and images were acquired. The characteristic rounded shape of individualized hepatocytes, nuclei (black round spots) and cell aggregates is also observed.

4.4.1.2. *In vitro* generation of hepatic spheroids

Culturing hepatocytes as three-dimensional (3D) structures provide cells with an *in vivo*-like environment, enabling the retention of important hepatic functions without the need of extracellular matrix components. The spinner vessel in conjugation with the aggregation properties naturally evidenced by hepatocytes generated cellular spheroids (OM, Figure 20A-C). The cellular spheroids constituted for several cells were cultured in suspension. Spheroid sizes range between 30 µm – 50 µm and their number increased during the first 72 h in culture after hepatocyte isolation, reflecting good cell viability. In SEM images (Figure 20D-I) it was possible to observe that hepatocyte membrane maintained the smooth appearance and cells were united with extracellular matrix produced by the hepatocytes forming cellular aggregates. The production of extracellular matrix enables cells to recreated cell-to-cell communications structures and mimetize the *in vivo* tissue environment.

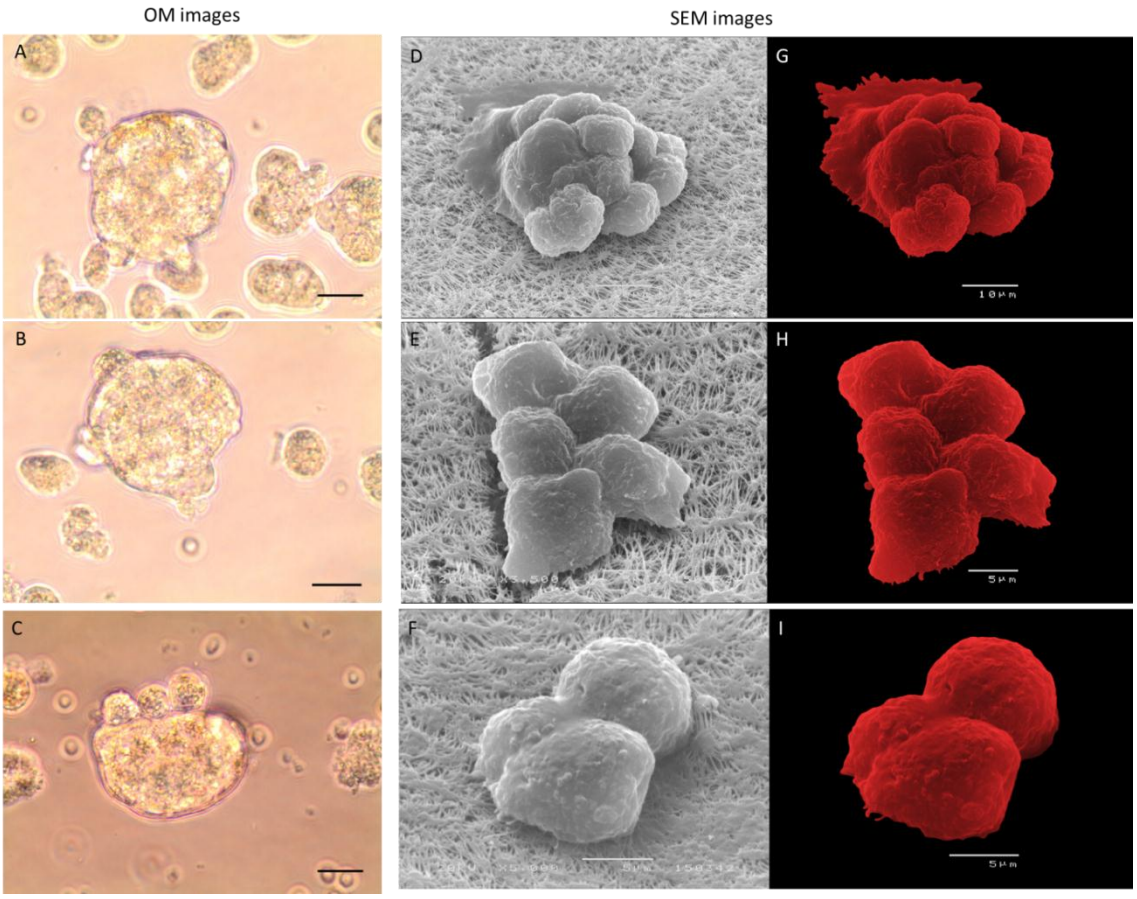


Figure 20: Morphology of hepatic spheroids.
Samples of cells cultured in a 3D-system for 72 h were observed under an inverted microscope (A-C, scale bar 20 µm, 400*x* magnification), a scanning electron microscope (D-F) and images were acquired. Artificial color (G-I) was applied on cells (red), to better evidence cells morphology. Hepatic spheroids with diverse levels of complexity and the nearly complete cell fusion as a consequence of extracellular matrix production were observed.

Hepatic aggregates were also stained for actin and ferritin (Figure 21), revealing a three-dimensional cell arrangement (with cell superposition). Actin presented the expected cytoplasmic network feature with some intense spots, probably due to secretory vesicles. Ferritin spots are also identified in the spheroids, indicating possible storing vesicles.

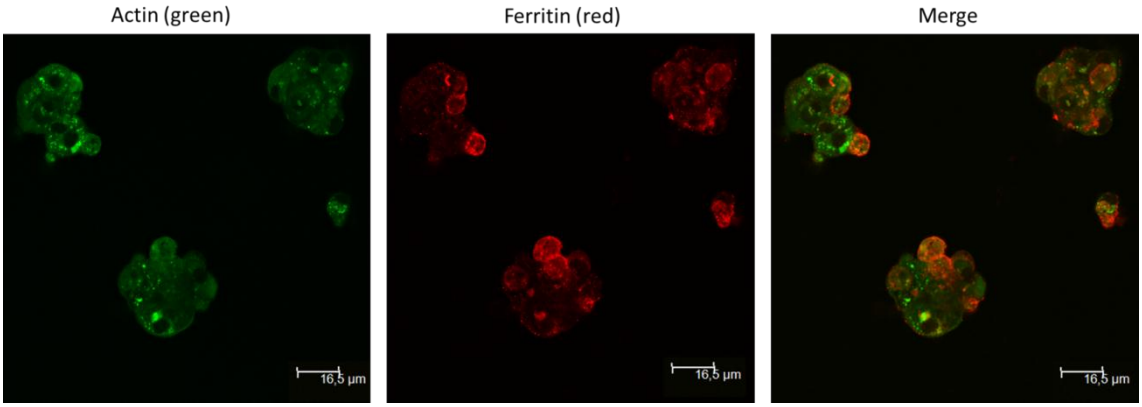


Figure 21: Actin and ferritin in hepatic spheroids.
Hepatic spheroids generated in 3D-culture system were stained for actin (green) and ferritin (red). Spheroids were observed under fluorescent confocal microscope and images were acquired. Tree-dimensional cell superposition, nuclei (black spots), actin network, actin spots of high fluorescence, and spots of ferritin can be observed.

4.4.1.3. 2D- and 3D-culture systems preserves viability and metabolic functions of canine hepatocytes

Characteristics of viable and differentiated hepatocytes were assessed in both culture systems. Urea production and CYP450 enzyme activity were assessed. CYP450 activity was evaluated through EROD, BROD, MROD and PROD assays. (Figure 22) EROD (CYP1A1 and CYP1A2 enzymes) and PROD (CYP2B11) assays revealed the metabolizing activity of hepatocytes in both culture systems, being the higher activity registered in the 3D-system. The 3D-culture system allowed hepatocytes to recover more rapidly from isolation trauma and recover their natural CYP450 enzyme activity, since after 24 h of incubation exhibited EROD and PROD activity. 2D-cultured hepatocytes needed more time to recover (48 h in culture) but achieved similar EROD activity levels compared to the 3D-culture system. Although BROD (CYP2B11, CYP3A12 and CYP3A26) and MROD (CYP1A2 and CYP1B) assays revealed a similar pattern a significant difference (P_{BROD}=0.0469, P_{MROD}=0.0313) in the enzyme activity level was detected when comparing hepatocytes from 2D- and 3D-culture system with 24 h of incubation. Phase II enzymes assessed by the UGT reaction evidenced stable and similar metabolization levels throughout the 72 h of incubation in both culture systems. Altogether, these findings evidenced that *in vitro* conditions established in the present study hepatocytes isolated from dog liver preserve the enzymatic activity of CYP450 and therefore the key metabolic function is assured.

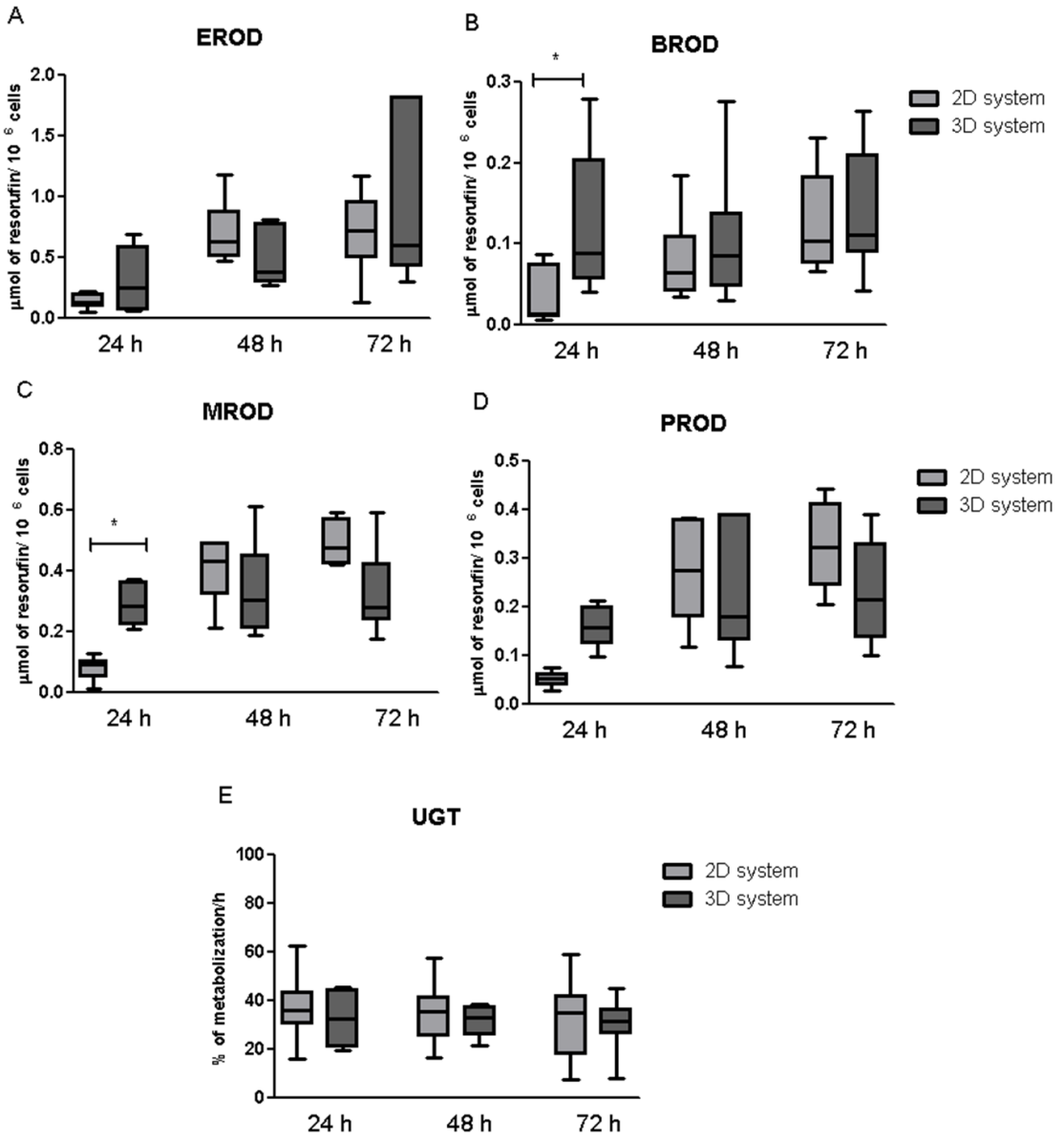


Figure 22: Phase I and phase II enzyme activity of dog hepatocytes.
Samples of 2D- and 3D-culture systems were used to evaluate EROD(A), BROD(B), MROD(C) PROD(D) and UGT(E) enzyme activity at 24 h, 48 h and 72 h of hepatocyte in culture. Samples were performed in triplicate and represented by box plots and whiskers (minimum to maximum). The non-parametric Wilcoxon test was used for statistical comparisons. * represents values of statistical significance ($P<0.05$) between 2D- and 3D-culture systems.

In order to confirm that hepatocytes were active, beyond CYP450 activities urea production was also assessed. Urea constitutes a characteristic metabolite of active and viable hepatocytes. At the initial 24 h, 2D- and 3D-culture systems showed similar urea production rate indicating that in both culture systems hepatocytes are active (Figure 23). In the 2D-culture system the medium was replaced by fresh medium every 24 h,

reason why the *de novo* urea production rate remains similar at 24 h, 48 h and 72 h in culture. However, the medium of 3D-cultures was only replaced after the first 72 h, allowing urea accumulation and relatively decrease of *de novo* production rate. There were significant differences in the *de novo* urea production rate of between the two culture systems at 48 h (P=0.0039) and 72 h (P=0.0039) when compared with the level of urea at 24 h.

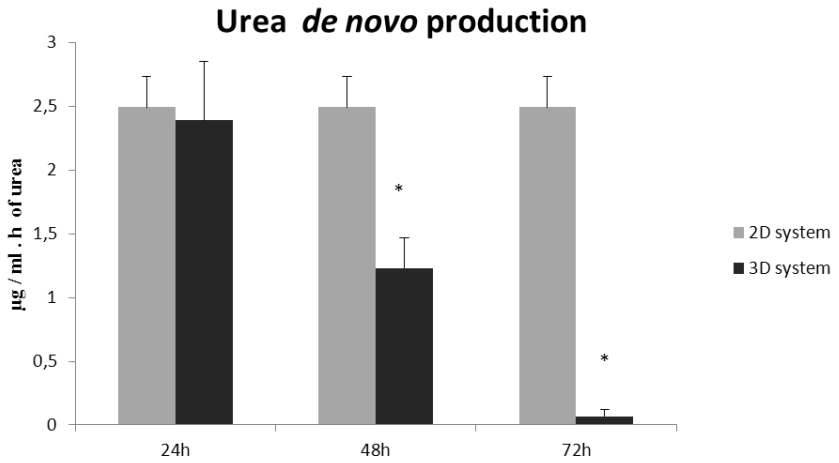


Figure 23: Urea *de novo* production rate in cultured dog hepatocyte, the 2D- and 3D-culture systems.
Samples of 2D- and 3D-culture systems were used to evaluate urea production at 24 h, 48 h and 72 h of hepatocyte incubation. Values are represented as mean plus standard error of samples performed in triplicate. The non-parametric Wilcoxon test was used for statistical comparisons. * represent values of statistical significance (P<0.05) between 2D and 3D culture systems.

Therefore, these findings indicate that dog hepatocytes were viable and metabolic active in both culture systems, allowing the continuation of the study by the evaluation of the immune parameters.

4.4.2. Activation of dog hepatocytes and development of immune response direct to *L. infantum* parasites

4.4.2.1. *L. infantum* parasites interact closely with canine hepatocytes

To investigate how hepatocytes sensed and respond to the presence of *L. infantum* parasite, plated hepatocytes were exposed to axenic amastigotes and to virulent promastigotes. At first, the *L. infantum* morphological forms differentiated in cultured were confirmed by SEM. In fact, parasites cultured at 24 ºC exhibited an

elongated shape and a long free flagellum that emerges from the anterior part of the cell (Figure 24A and C) corresponding to the promastigote morphological form. Parasites cultured at 37 ºC presented around shape with no visible flagellum, characteristics of amastigote morphological form (Figure 24B and D).

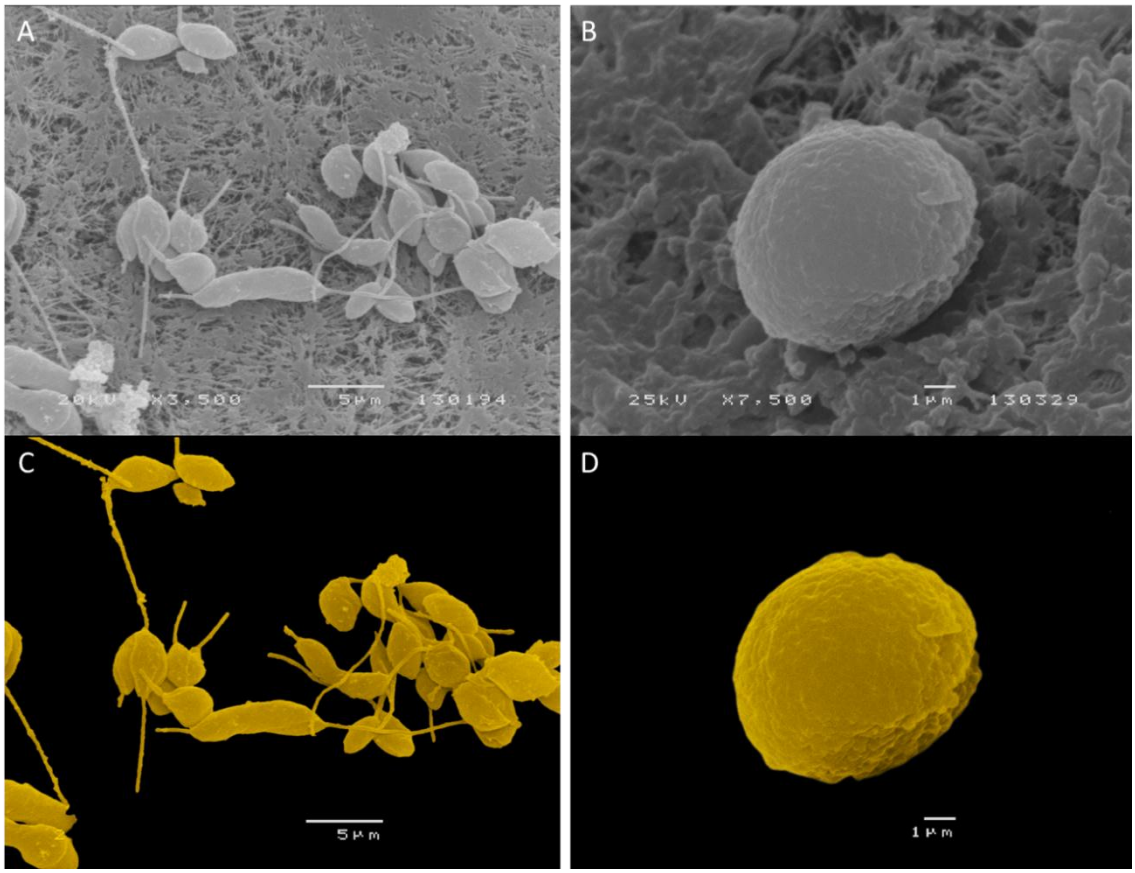


Figure 24: *L. infantum* morphological forms differentiated in different culture conditions.
Parasites were grown at 24 ºC or a 37 ºC and at neutral or acidic pH in axenic culture medium, observed under scanning electron microscope and images were acquired. Promastigote (A) and amastigote (B) morphological forms of *L. infantum* were observed. Artificial color (C and D) was applied on cells (yellow) to better evidence cells morphology.

The addition of virulent *L. infantum* promastigotes to 2D-cultured hepatocytes evidenced a strong promastigote tropism to hepatocytes, as parasites seem to be highly attracted by dog hepatocytes, directly interacting with the cell membrane (Figure 25). After 5 h of promastigote exposure, parasites appeared to be strongly attached to hepatocyte membrane, remaining linked to dog cells over the 72 h of incubation. With time, promastigotes seem to reduce motility and the elongated form, becoming more similar to amastigotes. However, under optical microscopy, no evidences of promastigotes or amastigotes-like parasites entering the canine hepatocytes were found.

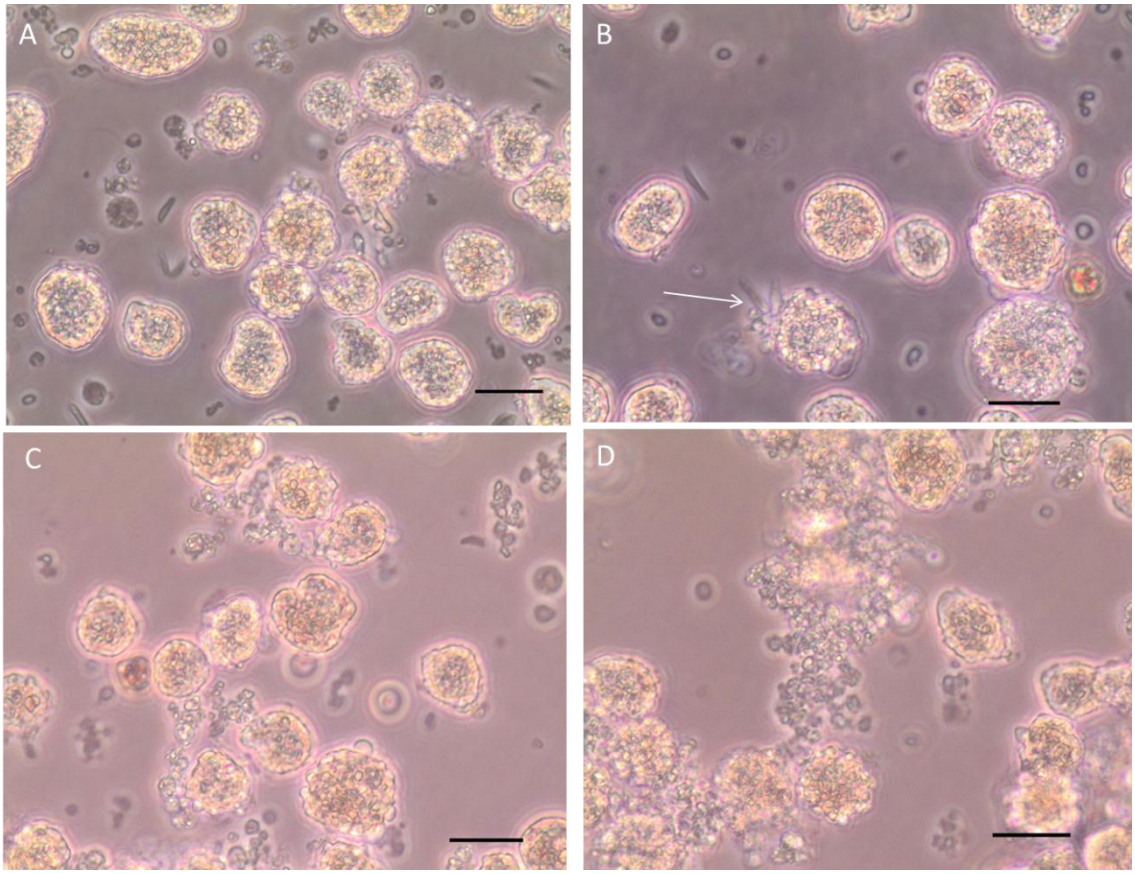


Figure 25: Canine hepatocytes exposed to *L. infantum* virulent promastigotes.
Culture samples obtained at 1.5 h (A), 5 h (B), 24 h (C) and at 72 h (D) of parasite exposure were observed under inverted microscope (scale bar 20 µm, 400*x* magnification) and images were acquired. The interaction of the parasite with cell membrane (B, white arrow) and parasites exhibiting morphological characteristics of amastigote (C and D) are observed.

Interaction of *L. infantum* promastigotes with the canine hepatocyte was also observed with SEM (Figure 26). An intense promastigote tropism to canine hepatocyte was also observed and, in a few cases, parasite flagellum deeply linked to the hepatocyte membrane (Figure 27).

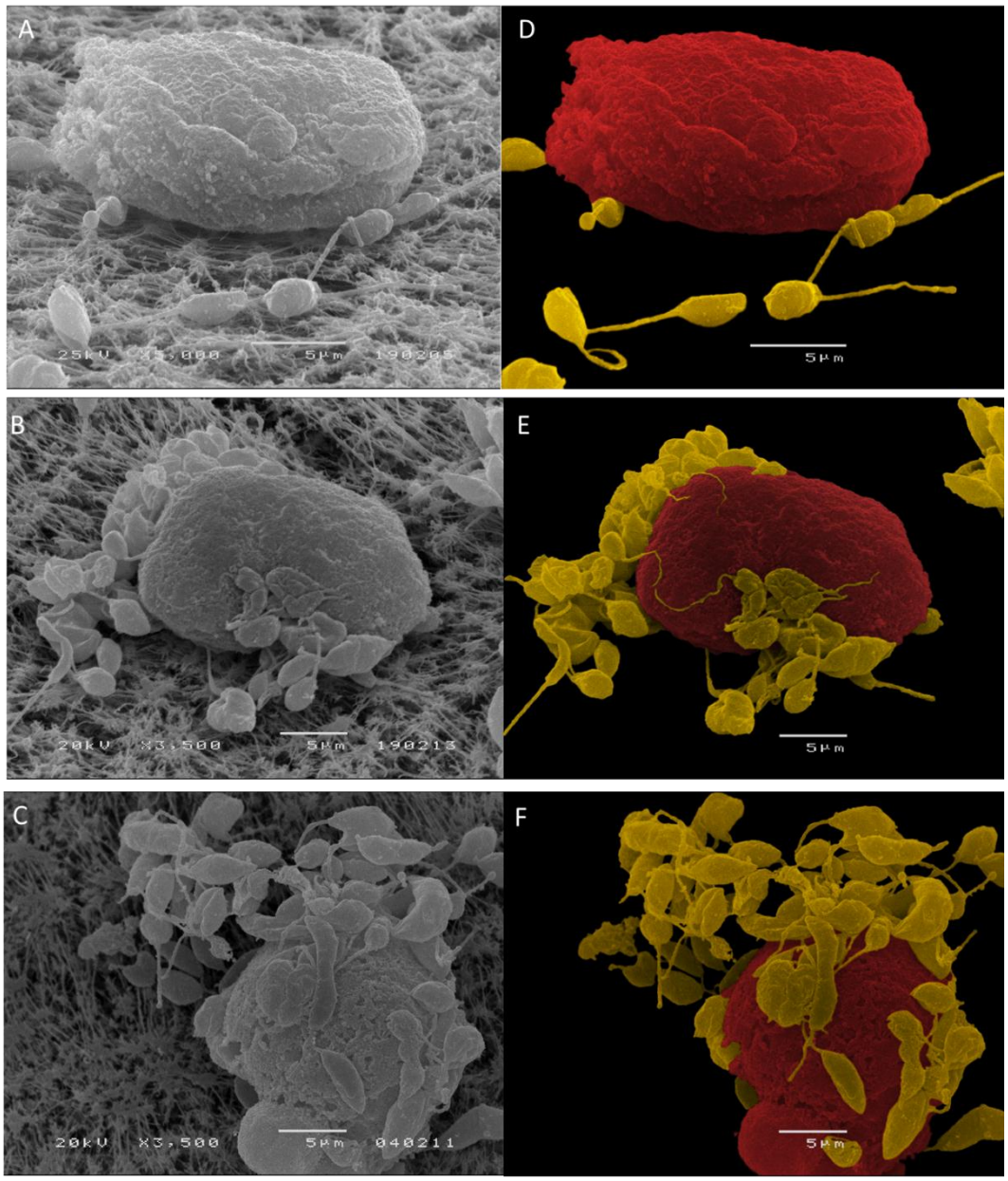


Figure 26: Dog hepatocytes exposed to *L. infantum* virulent promastigotes.
Culture samples obtained at 1.5 h (A), 5 h (B) and 24 h (C) of parasite exposure were observed under SEM and images were acquired. At early parasite exposure, promastigotes are near the hepatocyte (A) but, with time, promastigotes surround the hepatocyte and parasite interaction with the cell membrane of hepatocyte become evident (B, C). Images with artificial color (D-F) evidenced the close interaction of the promastigote form (yellow) and the hepatocyte membrane (red).

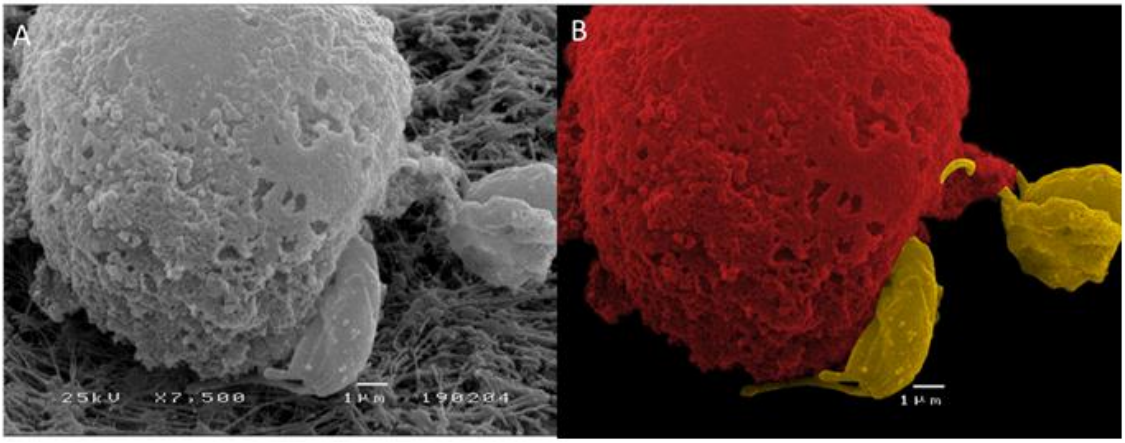
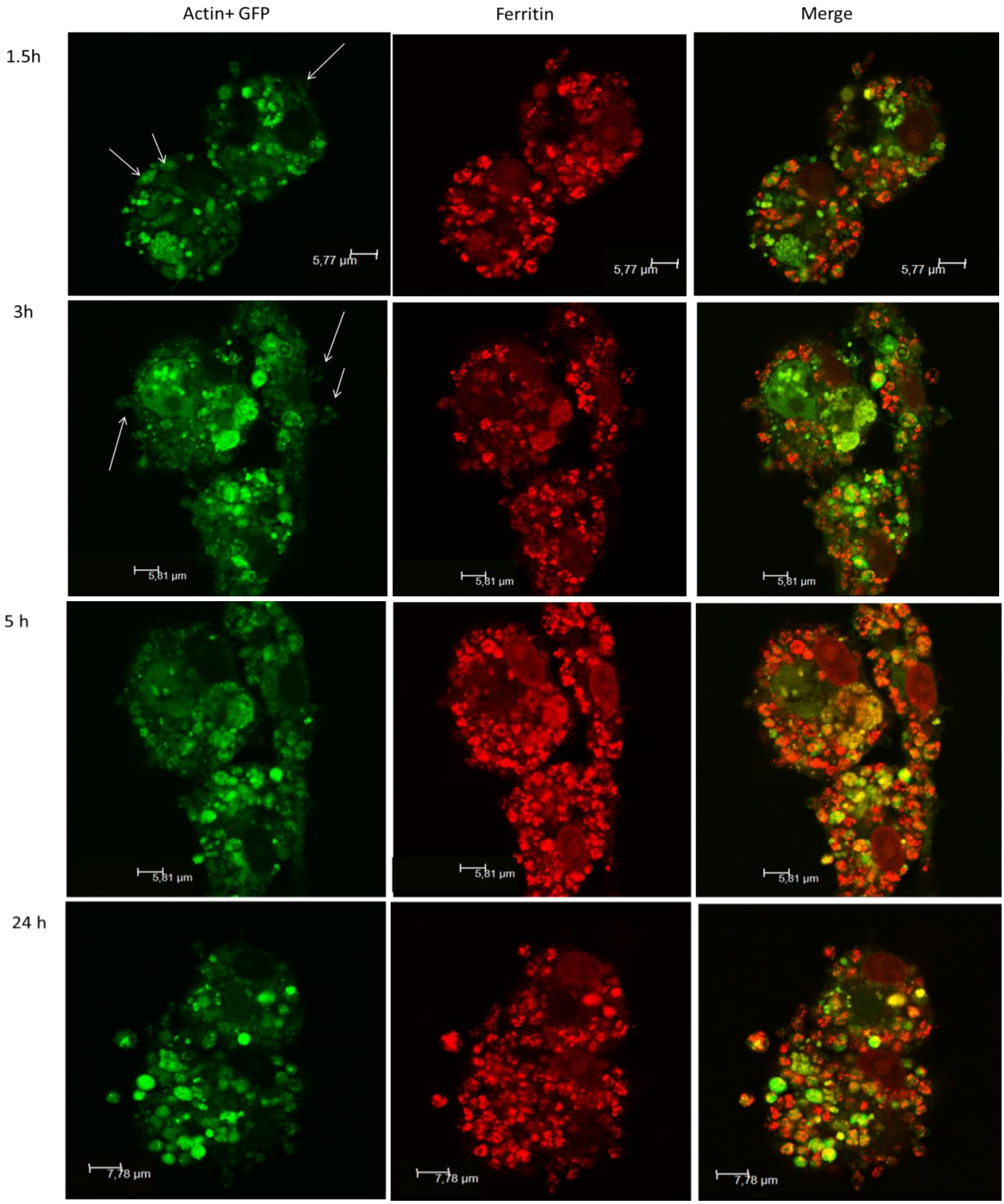


Figure 27: Detail of *L. infantum* virulent promastigotes interaction with canine hepatocytes.
Culture samples obtained after 24 h (A) of parasite exposure were observed under SEM and images were acquired. Promastigotes interact closely with the membrane of the hepatocyte (A) evidencing to be attached to cell. Artificial colored image (B) highlighting the close interaction of the promastigote form (yellow) and the hepatocyte membrane (red).

2D-cultured hepatocytes were also exposed to GFP-*L. infantum* promastigotes and stained for actin and ferritin. Observation under fluorescence confocal microscope (Figure 28) also revealed the high promastigote tropism to dog hepatocytes. Promastigotes were apparently attached to the hepatocyte and in some cases, even seem to be able to enter the hepatocyte, but in a non-conclusive way. In addition, promastigote seems to induce hepatocyte to generate more vesicles. These vesicles presented actin filaments, probably related to increased secretion levels by hepatocytes as a consequence of parasite contact. Curiously, promastigotes appear to progressively lose motility and, later on, parasite elongated body also suffer some changes, acquiring a more round shape (48 h). Ferritin, an iron storage protein, increased its content in hepatocytes at 5 h of exposure and then, ferritin appears to be sequestrated by promastigotes. After 48 h, hepatocyte evidenced a decrease in ferritin and promastigotes presented ferritin localized into a small intracellular vesicle.


Actin+ GFP
Ferritin
Merge
1.5h
3h
5 h
24 h
5,77 µm
5,81 µm
7,78 µm

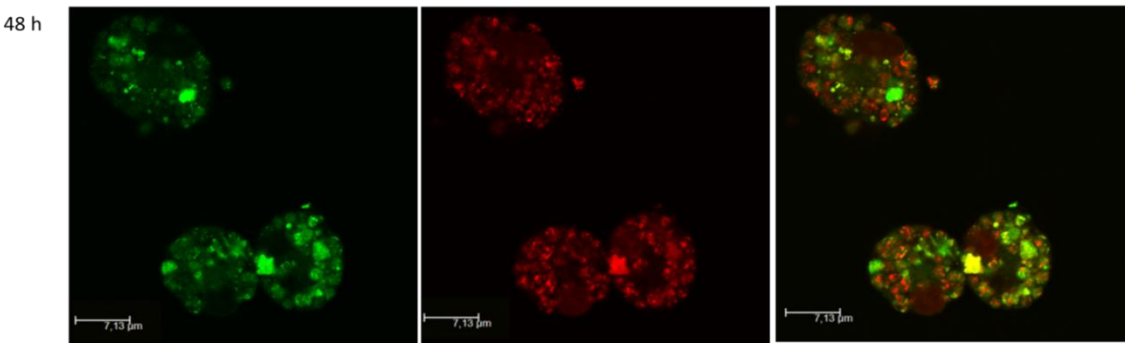


Figure 28: Actin and ferritin in cultured hepatocyte exposed to *L. infantum* virulent promastigotes.
Dog hepatocytes incubated for 48 h in the presence of *L. infantum* promastigotes (with arrows) were stained for actin (green), a component of cell cytoskeleton, and for ferritin (red), frequently present in secretory vesicles. Cells were observed under fluorescent confocal microscope and images were acquired. Promastigotes exhibited a high tropism to hepatocytes, and in some cases appear to enter the cells (24 h – 48 h), but in non-conclusive way. Hepatocytes presented a large round nucleus (stained red by TO-PRO®). The presence of the parasite seemed to induce hepatocyte to generate more vesicles and sequester ferritin inside the cell (5 h).

2D plated canine hepatocytes were also exposed to *L. infantum* axenic amastigotes in order to investigate the possible differences in sensing the parasite, since the amastigote is the parasite morphological form that reaches the dog liver in a natural infection. Hepatocytes were exposed to amastigotes for 72 h (similar to promastigote exposure) and amastigotes also evidenced hepatocyte tropism (Figure 29). After 5 h of exposure, amastigotes were attached to hepatocyte, interacting with the cell membrane throughout.

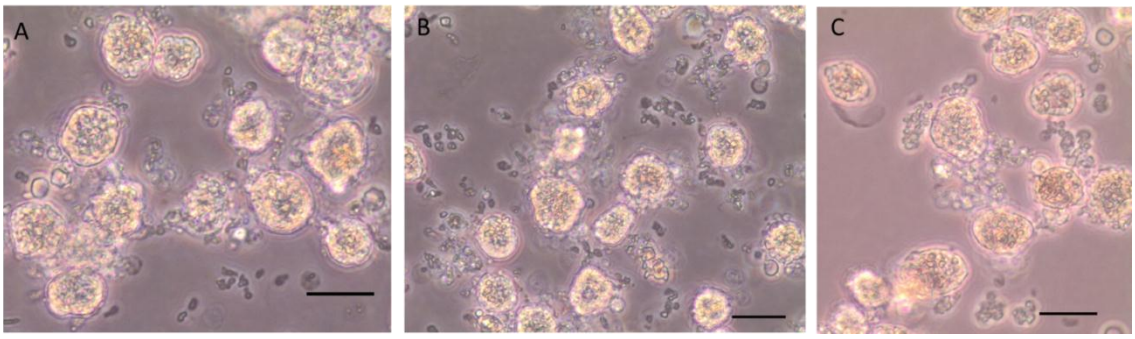


Figure 29: Canine hepatocytes exposed to *L. infantum* axenic amastigotes.
Culture samples obtained at 5 h (A), 24 h (B) and 72 h (C) of parasite exposure were observed under inverted microscope (scale bar 20 µm, 400*x* magnification) and images were acquired. Amastigotes, motionless ovoid body shapes, surround hepatocytes and appear to be attached to the cell membrane.

Observation of 2D-cultured hepatocytes under SEM also demonstrated amastigotes attached to dog hepatocytes, closely interacting with the cell membrane of hepatocyte (Figure 30) throughout the incubation time.

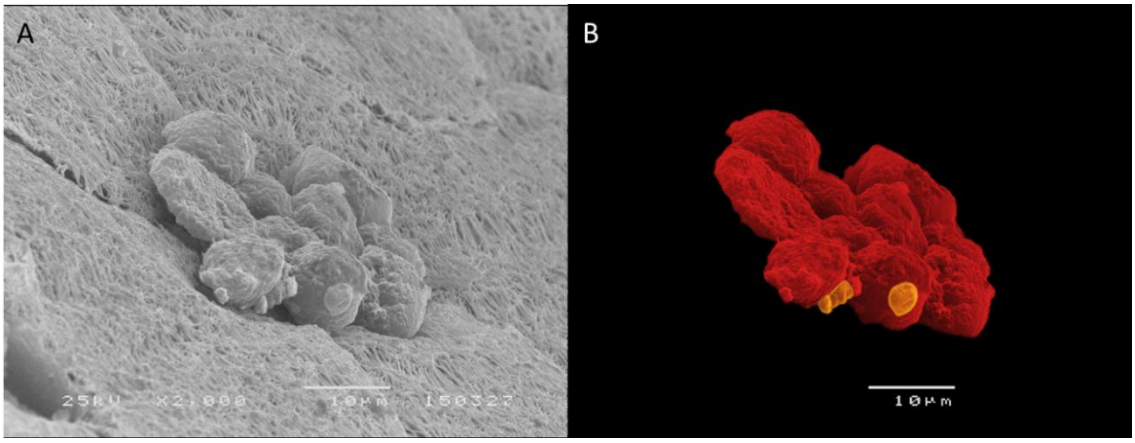


Figure 30: Dog hepatocytes exposed to *L. infantum* axenic amastigotes**.**
Culture samples obtained at 72 h of parasite exposure were observed under SEM. Image directly acquired (A) and after being artificial painted (B) are shown. Amastigote (yellow) interacting with the cell membrane of dog hepatocyte (red) is observed.

4.4.2.2. *L. infantum* parasites disturb dog hepatocytes

In order to investigate how hepatocyte senses and reacts to *L. infantum* presence some metabolic parameters were assessed. Urea is produced by hepatocytes as detoxification product and is highly influenced by the presence of inflammatory stimuli.

Hepatocytes non-exposed to *L. infantum* presented a steady urea production, but when exposed to amastigotes (P=0.0313), promastigotes (P=0.0156) or lipopolysaccharide (LPS) (P=0.0313) which may cause stress, hepatocytes reacted with the production of a burst of urea in first 1.5 h of contact (Figure 31A). After 24 h, hepatocytes significantly reduced the urea production ($P_{amastigotes}$=0.0313, $P_{promastigotes}$=0.0156) and at 72 h urea production was almost completed inhibited ($P_{amastigotes,promastigotes}$=0.0313), even at LPS-stimulated hepatocytes (P=0.0313).

Compared with non-exposed hepatocytes that exhibit a gradual accumulation over incubation time, hepatocytes exposed to amastigotes ($P_{1.5\ h,\ 5\ h,\ 24\ h,\ 72\ h}$=0.0313), promastigotes ($P_{1.5\ h,\ 24\ h}$ =0.0156; $P_{5\ h}$=0.0313) or LPS ($P_{1.5\ h,\ 5\ h,\ 24\ h,\ 72\ h}$=0.0313) showed an intense urea accumulation (Figure 31B) early after contact with the inflammatory stimuli or with the parasite, regardless the morphological form.

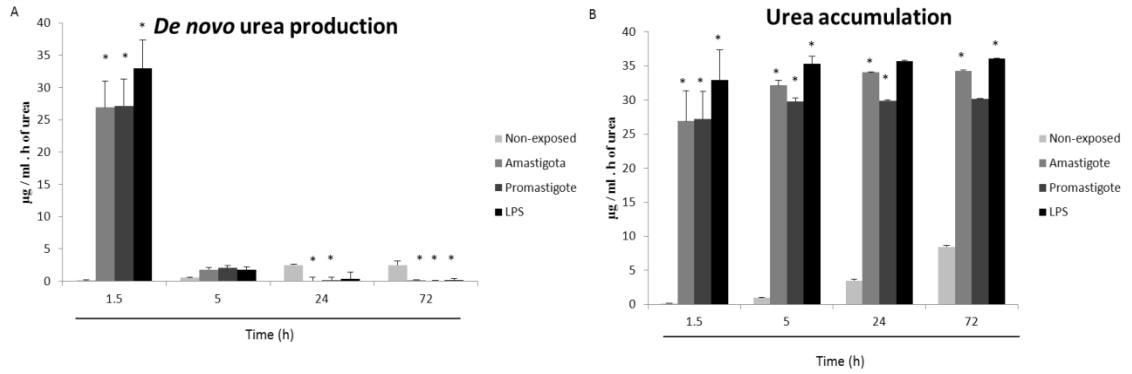


Figure 31: Production and accumulation of urea by hepatocytes exposed to *L. infantum*.
Samples of 2D-cultured hepatocytes were used to evaluate urea *de novo* production (A) and urea accumulation (B) after 72 h of exposure to amastigotes or promastigotes. In parallel, non-exposed hepatocytes and LPS-stimulated hepatocytes were also evaluated. Results of samples performed in triplicate are represented as mean and standard error. The non-parametric Wilcoxon test was used for statistical comparisons. * represents values of statistical significance ($P<0.05$) when comparing non-exposed hepatocytes *vs* the other conditions.

CYP450 enzymes, also designated as phase I enzymes, were assessed in order to investigate how inflammatory stimuli might influence hepatocyte metabolism. EROD, BROD, MROD and PROD assays were performed (Figure 32). The metabolization values of EROD (CYP1A1 and CYP1A2 enzymes) and PROD (CYP2B11) assays were similar between non-exposed hepatocytes, LPS-stimulated hepatocytes and hepatocytes exposed to *L. infantum* amastigotes or promastigotes. BROD assay (CYP2B11, CYP3A12 and CYP3A26) revealed a similar tendency for maintaining metabolization values. However, after 72 h of exposure to *L. infantum* amastigotes, BROD metabolization was significant lower in comparison with non-exposed hepatocytes ($P=0.0469$). MROD (CYP1A2 and CYP1B) activity also showed similar results throughout the evaluation period, except for 72 h of LPS-stimulated hepatocytes that revealed a significant metabolic increase ($P=0.0313$). Phase II enzymes were assessed through the UGT reaction. In this particular case, when compared with the activity evidenced by hepatocytes non-exposed to parasites, phase II enzyme abruptly decreased their metabolization levels in hepatocytes early after exposure to *L. infantum* promastigotes ($P=0.0234$) or amastigotes ($P=0.0156$).

Altogether, these results indicate that *L. infantum*, despite the parasite morphological form used are able to negatively disturb the normal activity of phase II enzymes and also interferes with phase I CYP2B11, CYP3A12 and CYP3A26 normal metabolization.

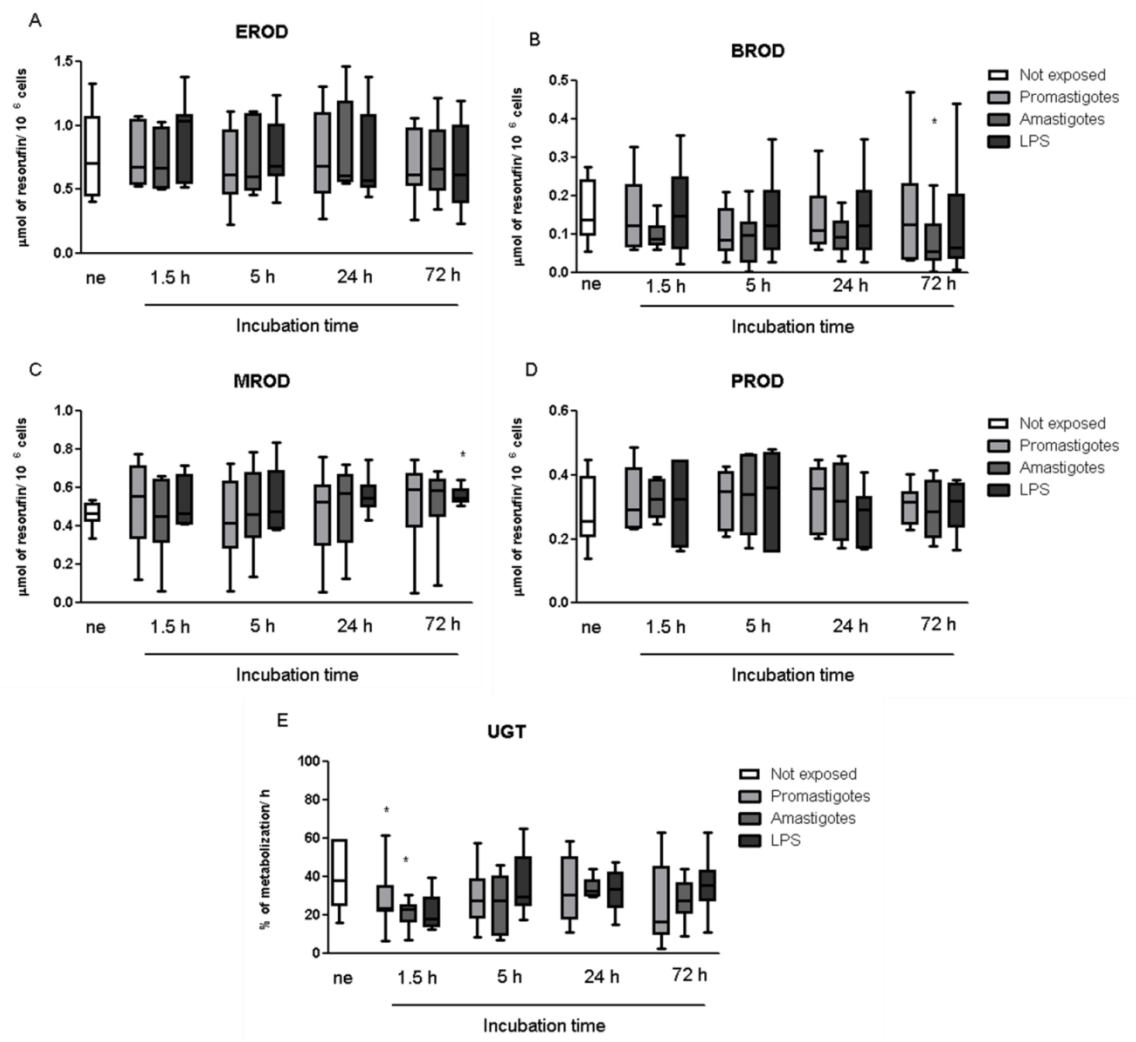


Figure 32: Phase I and phase II enzyme activity of dog hepatocytes exposed to *L. infantum*.
Samples of 2D-cultured hepatocyte were used to evaluate EROD (A), BROD (B), MROD (C) PROD (D) and UGT (E) enzyme activity at 1.5 h, 5 h, 24 h, and 72 h of exposure to amastigotes or promastigotes. Non-exposed hepatocytes and LPS-stimulated hepatocytes were also evaluated. Results of samples performed in triplicate are represented by box plots and whiskers (minimum to maximum). The non-parametric Wilcoxon test was used for statistical comparisons. * represents values of statistical significance ($P<0.05$) between non-exposed hepatocytes *vs* the other conditions.

The synthesis of nitric oxide (NO) by hepatocytes was also assessed. Hepatocytes non-exposed to *L. infantum* evidenced a residual NO production and the exposure to inflammatory stimuli increased the NO production by hepatocytes (Figure 33). When comparing the different stimuli some statistical differences were found

($P_{amastigote\ 24\ h\ vs\ LPS\ 24\ h}$=0.0156, $P_{LPS\ 1.5\ h\ vs\ LPS\ 24\ h}$=0.0313, $P_{1.5\ h\ amastigotes\ vs\ 72\ h\ amastigote}$=0.0234).

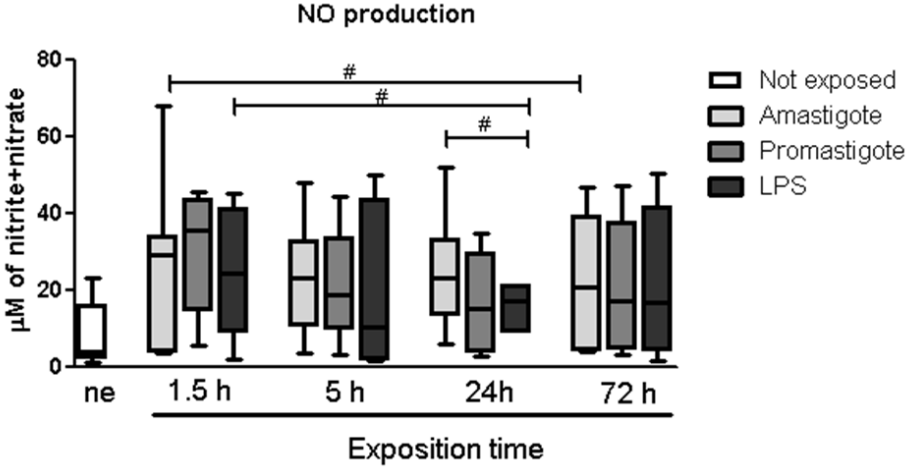


Figure 33: Nitric oxide production by canine hepatocytes exposed to *L. infantum.*
Samples of 2D-cultured hepatocytes were used to evaluate NO production at 1.5 h, 5 h, 24 h, and 72 h of exposure to amastigotes (A) or promastigotes (B). Non-exposed hepatocytes and LPS-stimulated hepatocytes were also evaluated. Results of samples performed in triplicate are represented by box plots and whiskers (minimum to maximum). The non-parametric Wilcoxon test was used for statistical comparisons ($P<0.05$). * represents values of statistical significance when comparing hepatocytes exposed to different stimuli, and # indicates significant differences when comparing hepatocytes exposed to the same stimuli.

Early immune response developed by canine hepatocyte when exposed to *L. infantum* amastigotes or promastigotes was assessed by evaluating the gene expression of the innate immune receptors TLR2, TLR4, TLR9, NOD1 and NOD2 and of the cytokines IL-10, TNF-α and IL-6 by real-time PCR. Gene expression of NOD1 increased with exposure to amastigotes and with the incubation time (Figure 34A). From 1.5 h to 5 h of amastigote exposure, NOD1 mRNA levels increased (P=0.0313) and at 24 h the high accumulation of NOD1 mRNA was different from hepatocytes not exposed to parasites (P=0.0371). NOD2 gene expression in not-exposed hepatocytes was low and exposition to inflammatory stimuli transiently increases NOD2 activation (Figure 34B). Amastigote exposition for 1.5 h leads to a transient increase of NOD2 mRNA (P=0.0195). Hepatocytes exposed to promastigotes exhibited a progressive increase of NOD2 mRNA that reaches significant values at 5 h (P=0.0020) and at 24 h (P=0.0353) when compared with not-exposed cells. After 24 h of LPS stimulation hepatocytes revealed a significant increase in NOD2 gene expression (P= 0.0049) when compared with hepatocytes non-exposed to parasite. The increase in NOD2 expression

on LPS-stimulated cells was statistically significant compared to the presence of amastigotes (P= 0.0020) or promastigotes (P= 0.0342).

Taken together, these findings suggest that although the parasite disturbs the immune status of dog hepatocytes, enhancing the gene expression of NOD1 and NOD2, the parasite also seems to exert a negative regulation of these innate immune receptors, with exposition time, pointing for a balance between hepatocyte activation and tolerance.

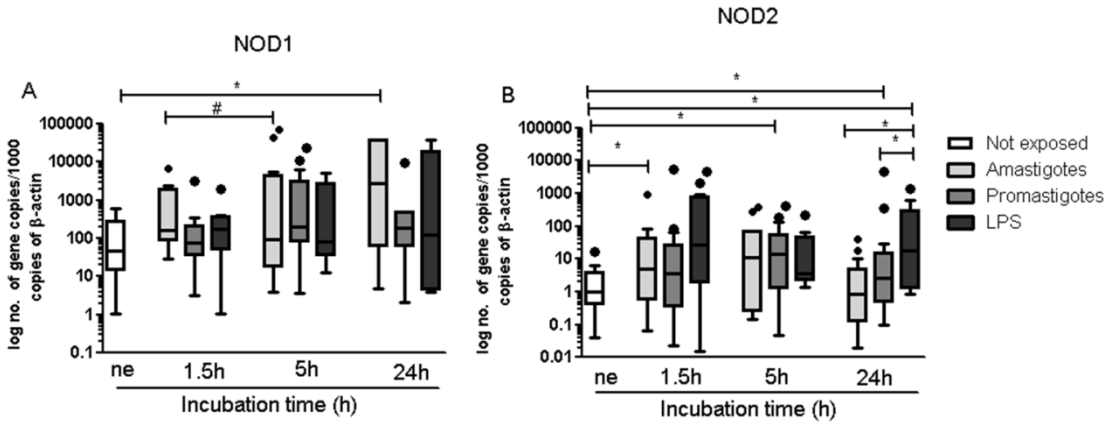


Figure 34: NOD1 and NOD2 gene expression in canine hepatocytes exposed to *L. infantum*.
Samples of 2D-cultured hepatocyte were used to evaluate NOD1 and NOD2 mRNA accumulation at 1.5 h, 5 h and 24 h of exposure to amastigotes or promastigotes. Hhepatocytes not exposed to parasites and LPS-stimulated hepatocytes were also evaluated. Results of samples performed in triplicate are represented by Tukey graphs. Black dots are indicative of outlier values. The non-parametric Wilcoxon test was used for statistical comparisons (P<0.05). * represents values of statistical significance when comparing not-exposed hepatocyte *vs* the other conditions and # indicates significant differences when comparing hepatocytes exposed to the same stimuli.

The contact of *L. infantum* virulent promastigotes or axenic amastigotes with dog hepatocytes did not cause important changes in TLR2 (Figure 35A), TLR4 (Figure 35B) and TLR9 (Figure 35C) gene expression.

TLR2 expression exhibits a tendency to increase with the exposition to inflammatory stimuli. Amastigotes exposition lead to a significant increase in TLR2 expression after 5 h incubation (P= 0.0195) compared to initial expression levels. TLR4 expression in stimulated hepatocytes remains similar to hepatocytes not-exposed to parasites. Amastigotes registered a significant decrease in TLR4 expression after 24 h exposition (P= 0.0469).

TLR9 expression revealed a tendency to decrease by the presence of *L. infantum*. Amastigote exposition lead to a decrease in TLR9 expression after 5 h (P= 0.0273) and in promastigote exposition after 24 h (P= 0.0244). LPS lead to an initial increase in TLR9 expression, achieving the higher expression level.

Although accumulation fluctuations of TLR mRNA were observed these results seems to reflect a probable hepatic tolerance to this parasite as no major expression increase was observed.

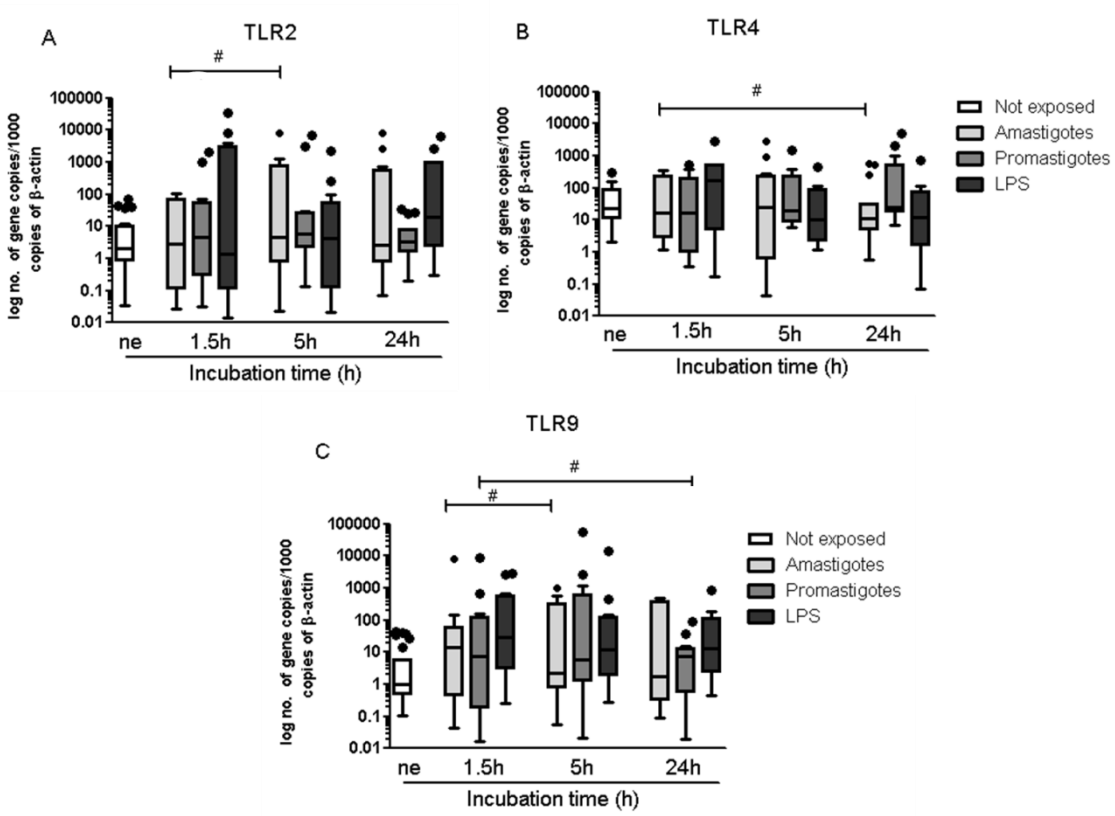


Figure 35: TLR2, TLR4 and TLR9 gene expression in canine hepatocytes exposed to *L. infantum*. Samples of 2D-cultured hepatocytes were used to evaluate TLR2 (A), TLR4 (B) and TLR9 mRNA accumulation at 1.5 h, 5 h and 24 h of exposure to amastigotes or promastigotes. Hepatocytes not exposed to parasites and LPS-stimulated hepatocytes were also evaluated. Results of samples performed in triplicate are represented by Tukey graphs. Black dots are indicative of outlier values.* represents values of statistical significance when comparing not-exposed hepatocyte *vs* the other conditions and # indicates significant differences when comparing hepatocytes exposed to the same stimuli.

Gene expression of pro- and anti-inflammatory cytokines by dog hepatocytes exposed to *L. infantum* parasites were also assessed by real-time PCR. The gene expression of the anti-inflammatory interleukin 10 (IL-10) was significantly increased in LPS-stimulated hepatocytes at 1.5 h (P=0.0024) and at 5 h (P=0.0049) when

compared with hepatocytes not-exposed to parasite (Figure 36). The amastigote exposition lead to an initial and transitory burst of IL-10 mRNA (P=0.0269). Promastigote exposition only generated a notorious augment of IL-10after 24 h (P=0.0186). Gene expression of pro-inflammatory interleukin 6 (IL-6) by hepatocytes was low throughout the study. The only break trough was observed after 5 h of hepatocyte contact with promastigotes (P= 0.0342). When compared with hepatocytes not-exposed to parasites, gene expression of pro-inflammatory tumor necrosis factor α (TNF-α) by LPS-stimulated hepatocytes ($P_{1.5\,h}$= 0.0186, P_{5h}=0.0210) and by hepatocytes exposed to promastigotes ($P_{1.5\,h}$=0.0391, P_{5h}= 0.0389) were significantly increased from 1.5 h to 5 h. Hepatocytes exposed to amastigotes for 24 h exhibited significant accumulation of TNF-α mRNA (P=0.0245).

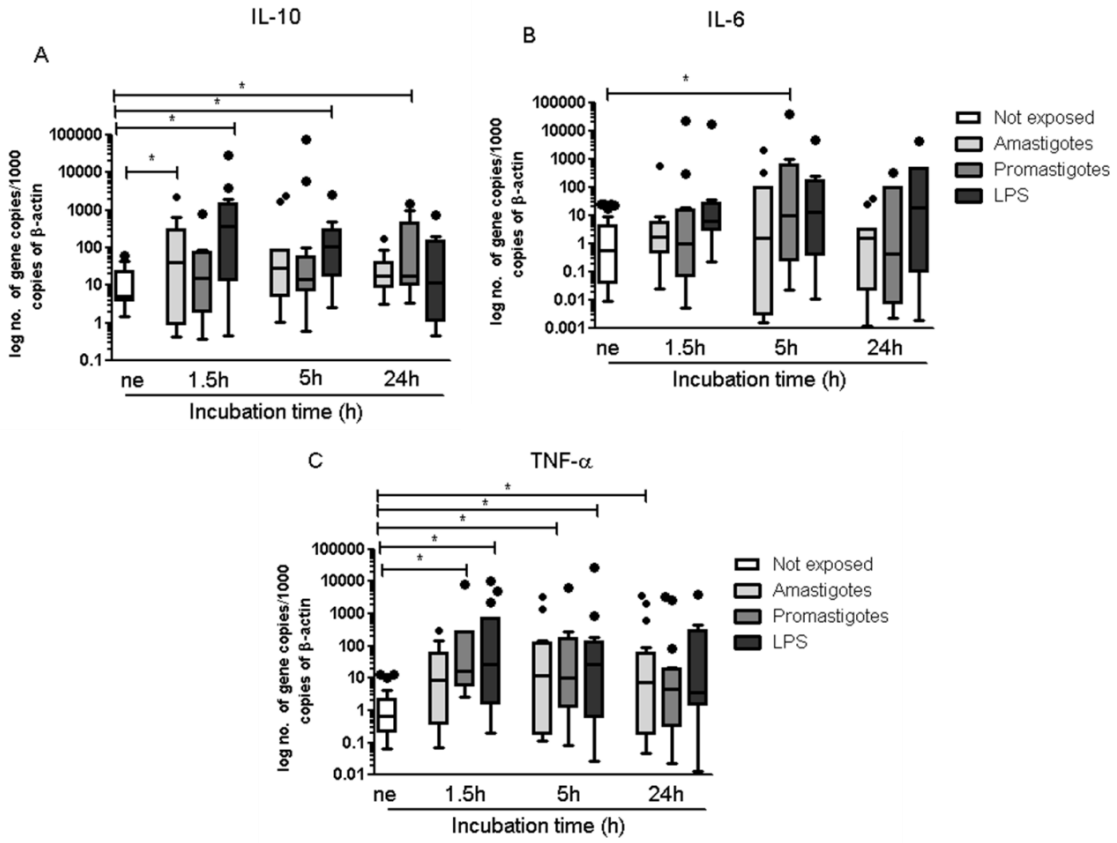


Figure 36: Gene expression of IL-10, IL-6 and TNF-α by canine hepatocytes exposed to *L. infantum*. Samples of 2D-cultured hepatocytes were used to evaluate IL-10 (A), IL-6 (B) and TNF-α (C) mRNA accumulation at 1.5 h, 5 h and 24 h of exposure to amastigotes or promastigotes. Not-exposed hepatocytes and LPS-stimulated hepatocytes were also evaluated. Results of samples performed in triplicate are represented by Tukey graphs. Black dots are indicative of outlier values. * represents values of statistical significance when comparing not-exposed hepatocyte *vs* the other conditions and # indicates significant differences when comparing hepatocytes exposed to the same stimuli.

These findings indicate that the contact with the parasite induce dog's hepatocyte to generate pro- and anti-inflammatory cytokines.

4.4.2.3. *L. infantum* parasites seem to have more difficulty in activating metabolic and immune pathways dog hepatic spheroids

After the establishment of a 3D-culture system constituted by hepatocyte spheroids that better mimic the *in vivo* three-dimensional environment of the cells, the spheroids were exposed to *L. infantum* promastigotes and the direct physical interaction with dog hepatocytes were assessed and the effect on the biochemical and immune parameters were evaluated.

L. infantum promastigotes close interacted with hepatocyte membrane and remain strongly attached to the cell membrane (Figure 37).

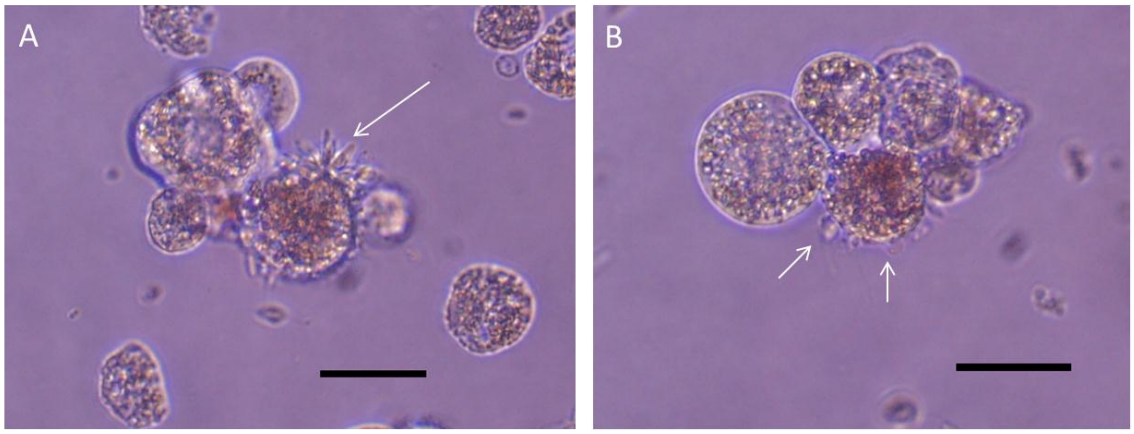


Figure 37: 3D Canine hepatic spheroids exposed to *L. infantum* promastigotes.
3D-culture samples obtained at 5 h (A) and 24 h (B) of parasite exposure were observed under inverted optical microscope (400*x* magnification). Parasites were found in suspension in the medium and in closed contact with the hepatic spheroids (white arrows).

Cellular spheroids exposed to *L. infantum* promastigotes were stained for actin and ferritin (Figure 38). Actin staining revealed the cytoskeleton of the spheroids with cell superposition and fused hepatocytes. In conjugation with the exposition time, it was observed an increase in hepatocyte vesiculation (green dots), particularly visible at 24 h of exposition to promastigotes, indicating that the close interaction of the promastigotes leads spheroids to produce more vesicles, similar to 2D-culture system. Ferritin spots were observed in spheroids exposed to promastigotes during 5 h. After 24 h, a protein reduction within the spheroids was visible. In addition, ferritin appeared to be stored in

vesicles at hepatocyte border (Figure 38, 24 h), probably as a result of promastigote interaction with the cell membrane of hepatocytes, as it was observed in 2D-cultured hepatocytes.

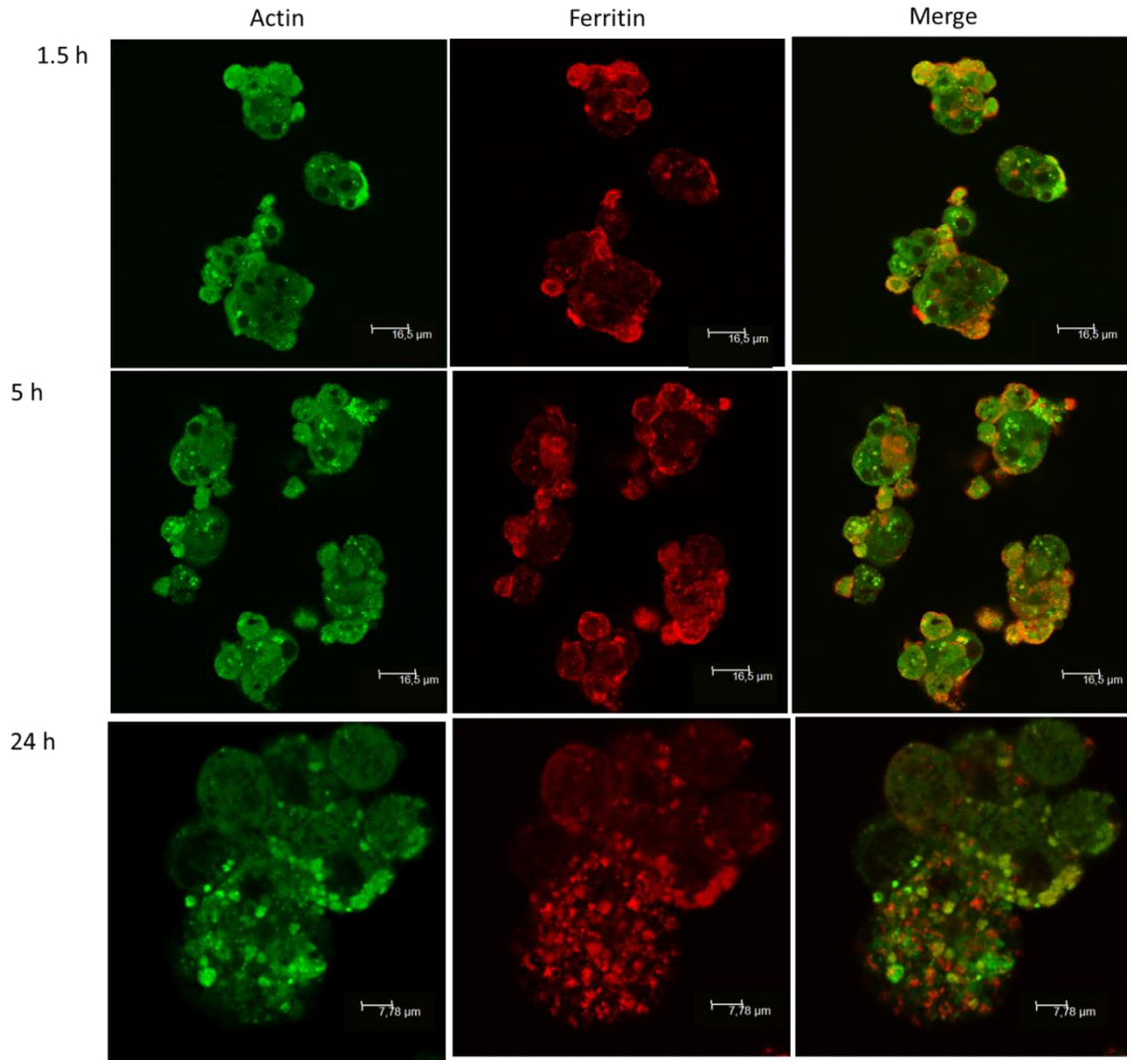


Figure 38: Hepatocyte aggregates exposed to *L. infantum* promastigotes and stained for actin (green) and ferritin (red).
3D-culture samples obtained at 1.5 h, 5 h and 24 h of parasite exposure were observed under confocal fluorescent microscope and images were acquired. Hepatocytes actin (green) increased the number of vesicles after 24 h exposition to the parasite and ferritin (red) appear to increase inside hepatocytes after 5 h parasite exposition and decreases its expression and concentrate in small points after 24 h exposition to *L. infantum* promastigotes. Back spots correspond to the hepatocyte nucleus.

Metabolic characteristics of hepatocytes, such as urea production and CYP450 enzymes were investigated in order to evaluate the impact of the promastigote presence in the 3D hepatocyte aggregate. The interaction of *L. infantum* promastigotes with the hepatic spheroids led to an early transitory burst of urea production (P=0.0156). At 24 h

of parasite exposure, the *de novo* urea production was apparently abolished when compared with non-exposed spheroids (P= 0.0156) that were still able to produce urea (Figure 39). During the entire observation period urea accumulation was highly increased ($P_{1.5\ h}$=0.0156; $P_{5\ h}$=0.0156; $P_{24\ h}$=0.0156; $P_{72\ h}$=0.0156) (Figure 39B) as a probable consequence of the parasite interaction with the hepatic spheroids.

Urea production and accumulation follow a pattern similar in both 2D- and 3D-cultures hepatocytes when faced with *L. infantum* virulent promastigotes, confirming that hepatocytes as an individualized cell or as cell aggregates becomes disturbed when interact with the parasite, reacting in an identical way.

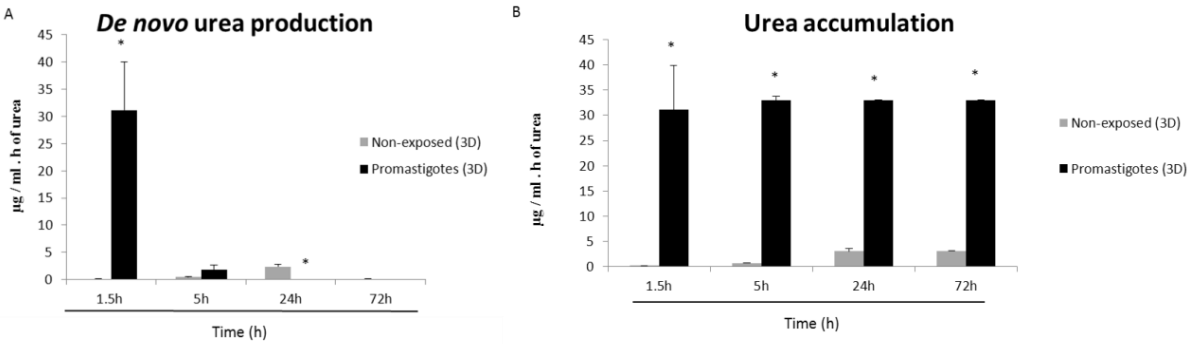


Figure 39: Urea production and accumulation by hepatic spheroids exposed to *L. infantum* promastigotes.
Samples of 3D-cultured hepatocyte were used to evaluate *de novo* production of urea (A) and urea accumulation (B) after 1.5 h, 5 h, 24 h and 72 h of promastigote exposure and compared to non-exposed spheroids. Results of samples performed in triplicate are represented by the mean and standard error. The non-parametric Wilcoxon test was used for statistical comparisons. * represents statistical significance values (P<0.05) when comparing spheroids non-exposed to parasite *vs* spheroids exposed to promastigotes.

Hepatic spheroid showed reduced enzymatic activity when in the presence of *L. infantum* promastigotes, similar to what was previously observed in the 2D-culture system with *L. infantum* parasites or LPS. EROD (CYP1A1 and CYP1A2) revealed a progressive decrease in enzyme activity (P= 0.0319) (Figure 40A). PROD (CYP2B11) assay did not exhibit important changes (Figure 40D). BROD assay (CYP2B11, CYP3A12 and CYP3A26) revealed no induction in the presence of promastigotes. Compared with spheroids non-exposed to *L. infantum*, the early interaction with the parasite caused significant suppression of enzymatic activity (P= 0.0156). Identical significant suppression was also observed at 72 h (P=0.0391) (Figure 40B). MROD (CYP1A2 and CYP1B) activity registered a significant decrease after 5 h of

promastigote exposure (P=0.0469) followed by another significant decrease at 24 h (P=0.0469) (Figure 40C).

Phase II enzyme, UGT, of promastigote-exposed spheroids registered stabilization in metabolization rate compared to non-exposed spheroids (Figure 40E).

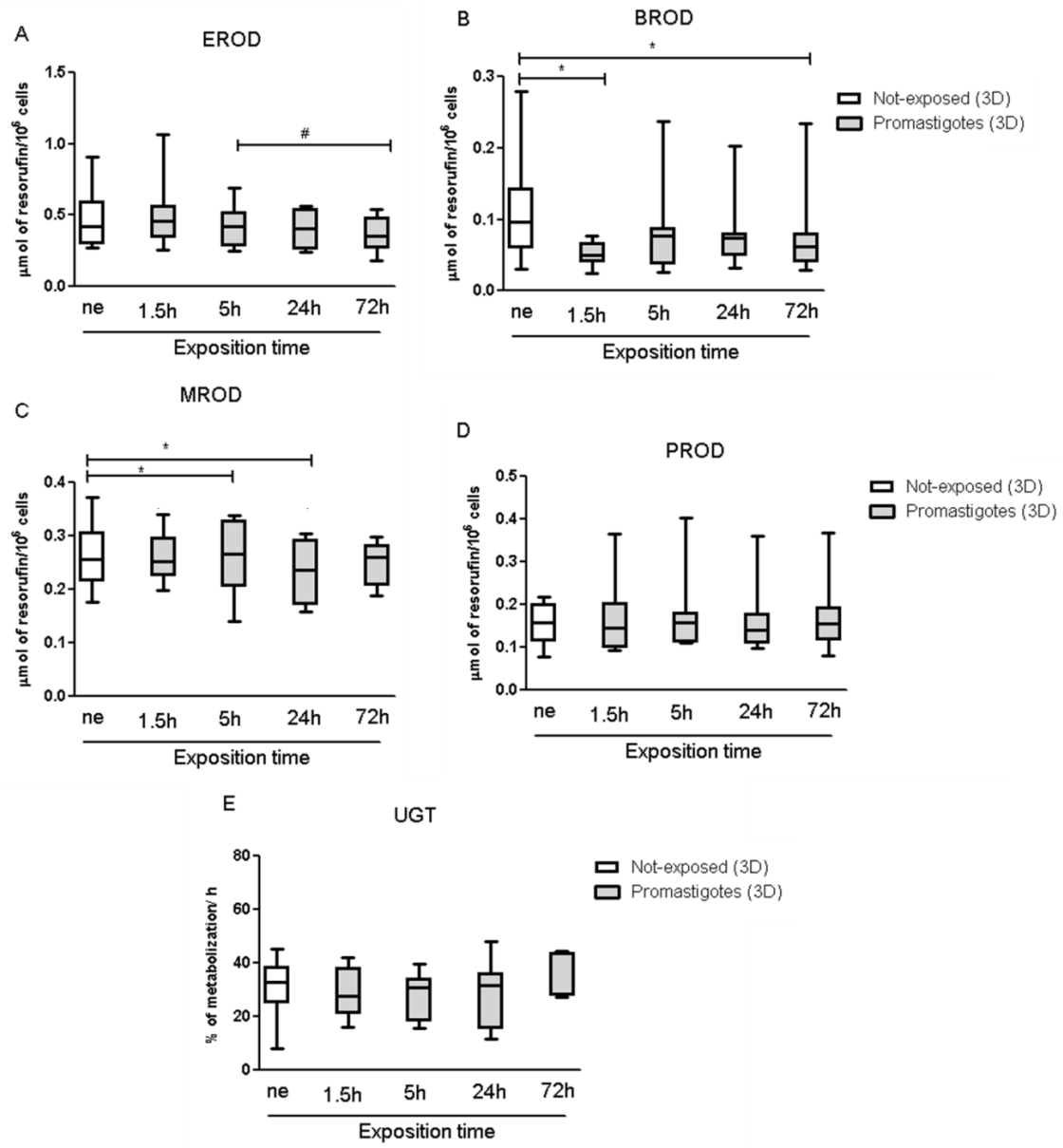


Figure 40: Phase I and phase II enzyme activity of hepatic spheroids exposed to *L. infantum* virulent promastigotes.
Samples of 3D-cultured hepatocytes were used to evaluate EROD (A), BROD (B), MROD (C) PROD (D) and UGT (E) enzyme activity at 1.5 h, 5 h, 24 h and 72 h of exposure to promastigotes and compared to spheroids non-exposed to parasites. Results of samples performed in triplicate are represented by box plots and whiskers (minimum to maximum). The non-parametric Wilcoxon test was used for statistical comparisons. * represents statistical significance values (P<0.05) when comparing non-exposed spheroids *vs* promastigote-exposed spheroids.

Hepatic spheroids non-exposed to inflammatory stimuli produced residual NO. However, when exposed to promastigotes for 5 h (P= 0.0313) and 24 h (P= 0.0313) significant higher levels of NO were detected (Figure 41).

These findings indicate that the close cell-to-cell contact of spheroids may have implications in the oxidative stress.

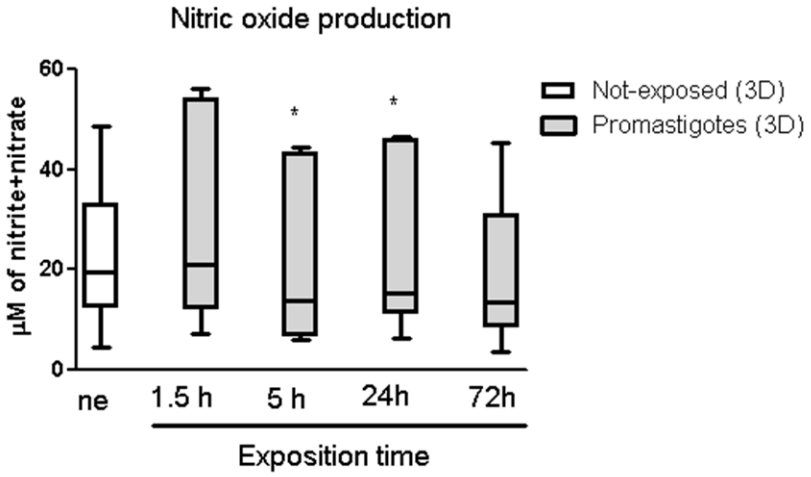


Figure 41: Nitric oxide production by canine hepatic spheroids exposed to *L. infantum* virulent promastigotes.
Samples of 3D-cultured hepatocytes were used to evaluate NO production at 1.5 h, 5 h, 24 h and 72 h of parasite exposure and compared to hepatic spheroids non-exposed to *L. infantum*. Results of samples performed in triplicate are represented by box plots and whiskers (minimum to maximum). The non-parametric Wilcoxon test was used for statistical comparisons (P<0.05). * represents statistical significance values when comparing non-exposed spheroids *vs* promastigote-exposed spheroids.

Immune activation of hepatic spheroids by contact with *L. infantum* promastigotes was assessed by real-time PCR. NOD1 (Figure 42A) and NOD2 (Figure 42B) gene expression did not differ from spheroids not-exposed to promastigotes throughout the incubation time. These findings indicate that *L. infantum* virulent promastigotes do not induce changes in the gene expression of these innate receptors in hepatic spheroids.

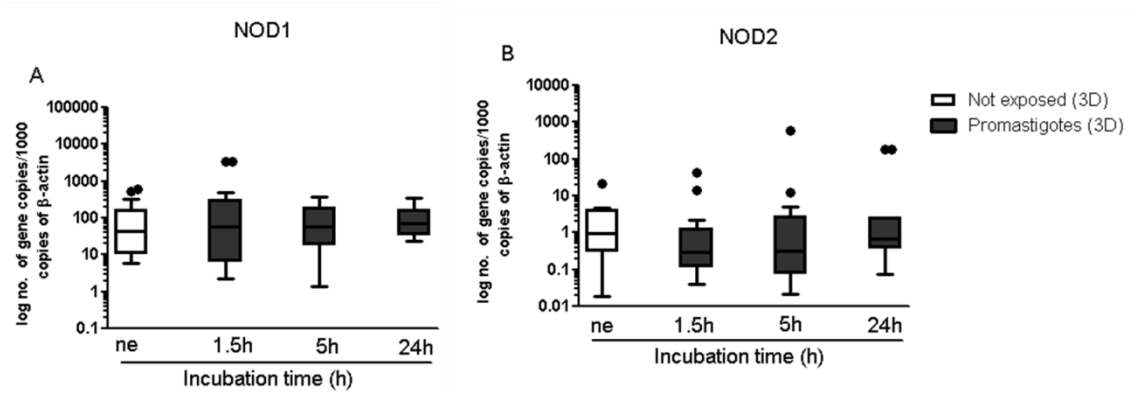


Figure 42: NOD1 and NOD2 gene expression by canine hepatic spheroids exposed to *L. infantum* virulent promastigotes.
Samples of 3D-cultured hepatocytes were used to evaluate NOD1 and NOD2 mRNA accumulation at 1.5 h, 5 h and 24 h of exposure to promastigotes and compared to *L. infantum* non-exposed spheroids. Results of samples performed in triplicate are represented by Tukey graphs. Black dots are indicative of outlier values.

When compared to hepatic spheroids not-exposed to virulent promastigotes, TLR2 gene expression registered a significant increase in promastigote-exposed spheroids for 5 h (P= 0.0105) and 24 h (P= 0.0353), indicating that exposition to the parasite trigger TLR2 receptor (Figure 43A). On the contrary, the amounts of TLR4 and TLR9 mRNA did not reveled major changes in spheroids exposed to parasites, during the entire observation period (Figure 43B and C). Taken together, these results indicate that it is probable that hepatocyte's TLR2 recognize *L. infantum* promastigotes where the others TLRs and NODs evaluated in the present study do not seem to be signalized by parasite PAMPs.

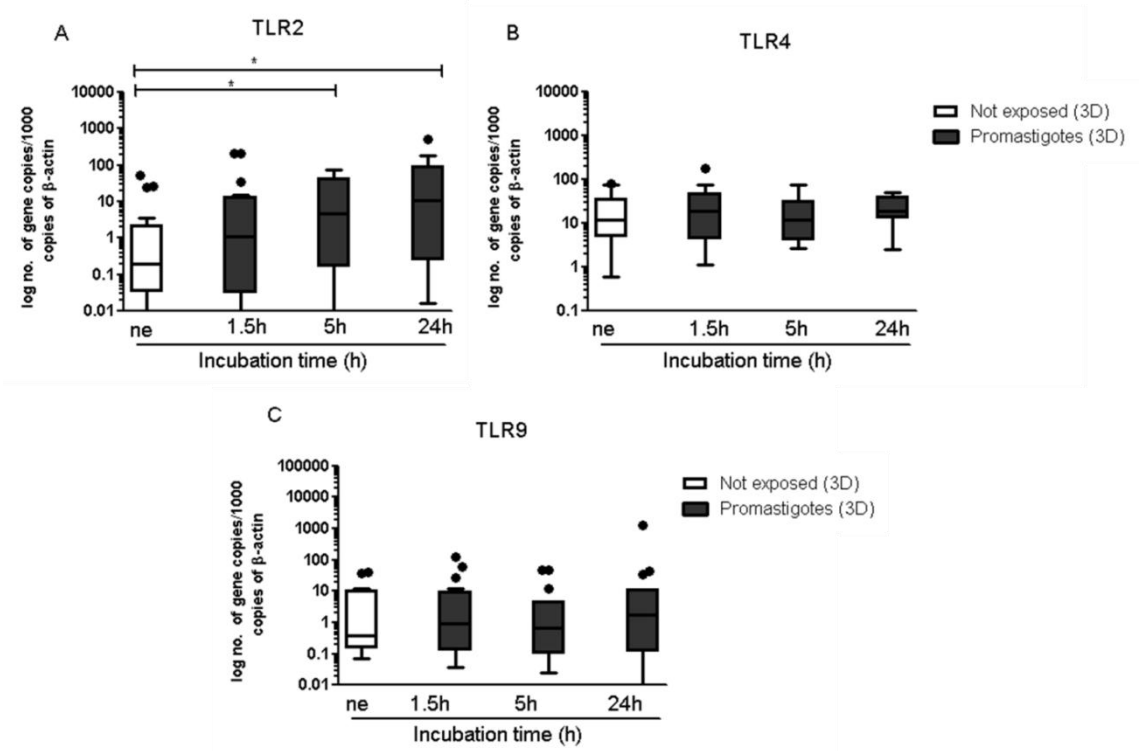


Figure 43: TLR2, TLR4 and TLR9 gene expression in hepatic spheroids exposed to *L. infantum* virulent promastigotes.
Samples of 3D-cultured hepatocytes were used to evaluate TLR2 (A), TLR4 (B) and TLR9 (C) mRNA accumulation at 1.5 h, 5 h and 24 h of exposure to promastigotes and compared with Non-exposed spheroids. Results of samples performed in triplicate are represented by Tukey graphs. Black dots are indicative of outlier values. The non-parametric Wilcoxon test was used for statistical comparisons. * represents statistical significance values ($P<0.05$) when comparing non-exposed spheroids *vs* parasite-exposed spheroids.

In comparison with spheroids not-exposed to promastigotes, gene expression of the anti-inflammatory cytokines IL-10 (Figure 44A) and IL-6 (Figure 44B) and of pro-inflammatory TNF-α (Figure 44C) by promastigotes spheroids incubated with *L. infantum* promastigotes did not reveal important changes during the entire observation period. Only an increase in IL-10 expression ($P= 0.0161$) after 24 h exposition to promastigotes was observed. As a consequence of cells superimposition and aggregation in spheroids, the parasite direct contact of with hepatocytes is limited, therefore promoting a tolerant immune environment.

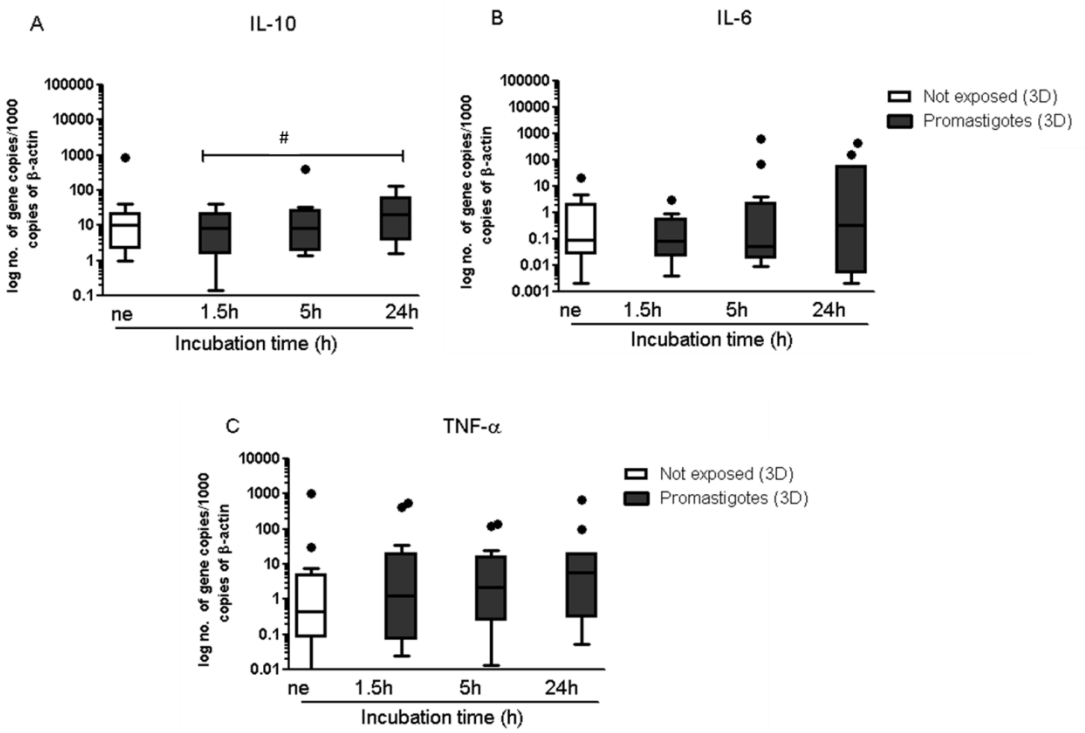


Figure 44: Gene expression of IL-10, IL-6 and TNF-α by canine hepatic spheroids exposed to *L. infantum* virulent promastigotes.
Samples of 3D-cultured hepatocytes were used to evaluate the accumulation of IL-10 (A), IL-6 (B) and TNF-α (C) mRNA at 1.5 h, 5 h and 24 h of exposure to promastigotes and compared to not-exposed cell. Results of samples performed in triplicate are represented by Tukey graphs. Black dots are indicative of outlier values. The non-parametric Wilcoxon test was used for statistical comparisons. # represents statistical significance values ($P<0.05$).

4.4.3. Leishmanicidal drugs stimulate metabolism and immunity of dog hepatocytes when in the presence of *L. infantum* parasites

4.4.3.1. MgA and other drugs increase CYP450 activity in canine hepatocytes

To assess the impact of leishmanicidal drugs on canine hepatocytes exposed to *L. infantum* axenic amastigotes (during 72 h), four different compounds were tested. Meglumine antimoniate (MgA), a commercial antileishmanial drug (Glucantime®) and other three experimental compounds with anti-leishmanial potential: ursolic acid (URA), chalcone-8 (CH-8) and quercetin (QC). In order to verify the survival of hepatocytes in the presence of these compounds, a resazurin assay was performed (Figure 45). Resazurin is a blue dye that is reduced by mitochondria of active cells to a

pink color and highly red fluorescent compound – resorufin. The assay demonstrated that the addition of leishmanicidal compounds to cultured hepatocytes did not disturb the viability of hepatocytes throughout the 72 h of observation.

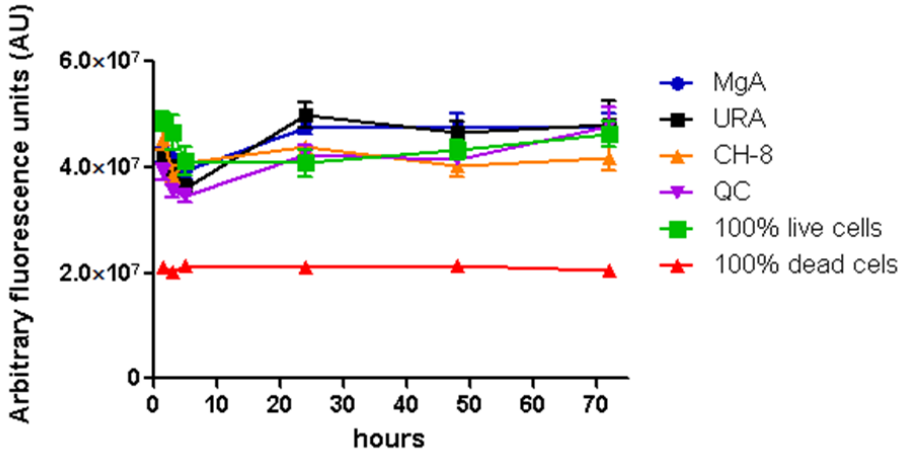


Figure 45: Viability of dog hepatocytes exposed to *L. infantum* treated with antileishmanial compounds.
The viability of 2D-cultured hepatocytes 72 h exposed to axenic hepatocytes and incubated with MgA, URA, CH-8 and QC were periodically evaluated by a resazurin assay. Non-exposed and non-treated hepatocytes (100% live cells) and hepatocytes fixated with paraformaldehyde (100% dead cells) were evaluated in parallel. Results of samples performed in triplicate are represented by XY graph with connected lines. Results are represented be mean values and standard deviation.

Urea production by hepatocyte exposed to amastigotes and tested with antileishmania compounds was influenced by the addition of leishmanicidal compounds producing an initial burst of urea (P_{MgA} =0.0156; P_{URA} =0.0078; P_{CH-8} =0.0078 and P_{QC} =0.0078) when compared with the urea production values of treated hepatocytes. However, no statistical difference was found between the urea production induced by the parasite (amastigote) and the addition of leishmanicidal compounds to parasite-exposed hepatocytes (Table 7). URA was the compound that induced higher production of urea by amastigote-exposed hepatocytes, presenting a significant difference when compared with CH-8 (P=0.0416). After 5 h, urea values produced by amastigote-exposed hepatocytes were minimal. However, only amastigote-exposed hepatocytes treated with URA differ significantly from non-exposed hepatocytes (P=0.0313). After 24 h, amastigote-exposed treated hepatocytes presented a significant decrease in urea production when compared to non-exposed hepatocytes (P_{MgA} =0.0313; P_{URA} =0.0078; P_{CH-8} =0.0078; P_{QC} =0.0078). At 72 h of incubation, the reduction observed in urea production by amastigote-exposed hepatocytes in contact with the

drugs still was statistically important (P_{MgA}=0.0313; P_{URA}=0.0078; P_{CH-8}=0.0078; P_{QC}=0.0078). The difference in urea production between amastigote-exposed hepatocytes treated with CH-8 and QC for 72 h was statistically significant (P=0.0140).

Table 7: *De novo* urea production by hepatocytes exposed to *L. infantum* amastigotes for 72 h and treated with the leishmanicidal compounds MgA, ursolic acid (URA), chalcone-8 (CH-8) and quercetin (QC).
Urea produced by non-exposed hepatocytes and amastigote-exposed hepatocytes were also compared. Mean urea values and standard error are indicated. The non-parametric Wilcoxon test was used for statistical comparisons (P<0.05). * represents statistically significant values when comparing non-exposed hepatocytes *vs* parasite exposed treated hepatocytes and # represents statically significant values when comparing different treatments. Units: $\mu g.ml^{-1}.h^{-1}$ of urea

Incubation time (h)	Non-exposed hepatocytes	Amastigote-exposed hepatocyte	Amastigote-exposed hepatocytes and treated with			
			MgA	URA	CH-8	QC
1.5	0.17±0.0008	26.91±4.45	28.27±2.17*	29.10±1.08*	26.53±0.96*#	26.47±2.97*
5	0.56±0.003	1.78±0.77	1.40±0.67	2.49±0.92*	0.77±0.45	0.38±0.033
24	2.69±0.13	0.11±0.04	0.05±0.03*	0.22±0.09*	0.22±0.17*	0.13±0.05*
72	2.07±0.19	0.07±0.04	0.1±0.04*	0.05±0.05*	0.05±0.05*	0.28±0.03*#

To verify if the addition of leishmanicidal compounds affects the activity of metabolizing enzymes in amastigote-exposed hepatocytes, Phase I enzymes (CYPs450) were assessed by EROD, BROD, MROD and PROD assays and phase II enzyme trough UGT assay. All of the evaluated enzymes showed an increase activity rate in the presence of the drugs (Figure 46)

The addition of MgA to amastigote-exposed hepatocytes induced the significant augment of EROD ($P_{1.5\ h,\ 24\ h,\ 72\ h}$ = 0.0156; $P_{5\ h}$= 0.0313) (Figure 46A), BROD ($P_{1.5\ h,\ 5\ h,\ 24\ h,\ 72\ h}$ = 0.0156) (Figure 46B) and MROD ($P_{1.5\ h,\ 5\ h,\ 24\ h,\ 72\ h}$ = 0.0313) (Figure 46C) activities throughout the times points evaluated when compared with untreated amastigote-exposed hepatocytes.

These results reflect an important increase in CYP450 activities, indicating that the addition of MgA decreases inflammation levels on hepatocytes helping CYPs to restore their metabolization rate and also that MgA probable to be metabolized by CYP1A1, CYP1A2, CYP2B11, CYP3A12, CYP3A26 and CYP1B enzymes.

After MgA treatment, PROD assay also revealed significant high enzymatic activity by MgA treated amastigotes-exposed hepatocytes ($P_{5\ h,\ 24\ h,\ 72\ h}$=0.0313) in comparison with untreated amastigote-exposed hepatocytes (Figure 46D). In this particular case, although the increment of enzymatic activity only was detected after 5 h of incubation with the drug these findings confirm that MgA influence on CYP2B11 metabolization rate.

After 24 h incubation, a transient increase in EROD metabolization by amastigote-exposed hepatocytes treated by URA was statistically different from non-treated amastigote-exposed hepatocytes (P=0.0313). URA addition to amastigote-exposed hepatocytes increased CYPs activity in all the assays performed. CH-8 treatment also induced an increase in all performed assays, with more evident results with EROD. In EROD (CYP1A1 and CYP1A2 enzymes) and PROD (CYP2B11) assay of hepatocytes exposed to amastigotes and treated with CH-8, the enzymes activity were increased with incubation time ($P_{24\ h\ EROD}$ = 0.0345; $P_{24\ h\ PROD}$ = 0.0345). QC treatment similarity to other treatments induced increasing in the assessed CYPs, but to lesser extent.

These results reveal the used drugs were able to increase in CYP450 activities, especially MgA and URA, demonstrating that the addition of leishmanicidal drugs decreases inflammation levels on hepatocytes aiding CYPs to restore their metabolization rate and also that probably the used drugs are metabolized by the assessed phase I enzymes.

Phase II UGT revealed differential tendencies with the drugs used. MgA and URA treated hepatocytes maintained a similar metabolization profile to non-treated and amastigote-exposed hepatocytes, revealing no induction (Figure 46E). CH-8 and QC treated hepatocytes presented a tendency to increase phase II activity, especially in the first 5 h incubation with the drugs ($P_{CH\text{-}8}$ = 0.0345).

The present results reveal some tendency to impairment of phase I and phase II enzymes by the application of drugs, particularly MgA and URA. These induce phase I and not phase II enzymes, which lead to the accumulation of toxic metabolites on cells.

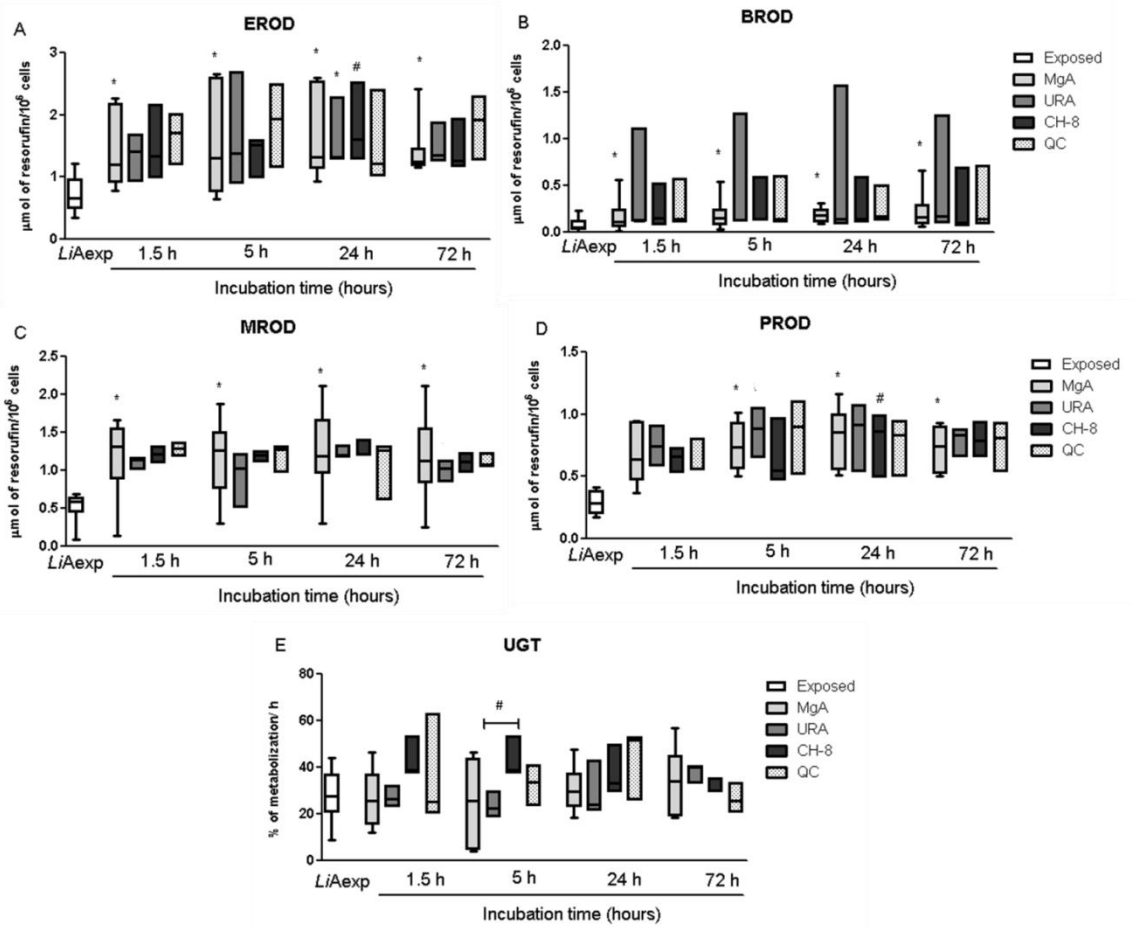


Figure 46: Phase I and phase II enzyme activity after treatment of hepatocytes exposed to *L. infantum* axenic amastigotes.
Samples of 2D-cultured hepatocytes were used to evaluate EROD (A), BROD (B), MROD (C) PROD (D) and UGT (E) enzyme activity at 1.5 h, 5 h, 24 h and 72 h of treatment with MgA, URA, CH-8 or QC and compared to non treated amastigote-exposed hepatocytes (*Li*Aexp). Results of samples performed in triplicate are represented by box plots and whiskers (minimum to maximum). The non-parametric Wilcoxon test was used for statistical comparisons. * represents statistical significance values ($P<0.05$) when comparing non-treated amastigote-exposed hepatocytes vs treated amastigote-exposed hepatocytes. # represents statistical significance values ($P<0.05$) when comparing treated amastigote-exposed hepatocytes with different drugs.

The leishmanicidal potential of the drugs was evaluated trough an adapted limiting dilution assay. The viability of amastigotes that were in culture with dog hepatocytes and then treated for 72 h were evaluated by the parasite's ability to fully revert to promastigotes in complete Schneider medium at 24 ºC. The reciprocal of the last positive dilution was considered a measure of parasite viability (Figure 47). Axenic amastigotes used as control are indicative of maximum viability (100%). As expected, all the compounds exhibited leishmanicidal activity that increased with the time of contact. At 1.5 h incubation with the leishmanicidal drugs (Figure 47A), a significant

inhibition of parasite viability was obtained with URA (P= 0.0355) and CH-8 (P= 0.0355) treatments. After 5 h incubation (Figure 47B) all the drugs presented a significant reduction in parasite viability (P_{MgA}= 0.0271; P_{URA}= 0.0335; P_{CH-8}=0.0272; P_{QC}= 0.0257) and after 24 h treatment the inhibition of parasite growth reached the 100% and no viable parasites were observed.

Altogether, these results confirm the effect of the evaluated drugs in parasite viability. However, in the conditions of this study, the drug classical used to treat leishmaniasis (MgA), including canine leishmaniasis, seems to be the less efficient in comparison with the experimental compounds used in the present study.

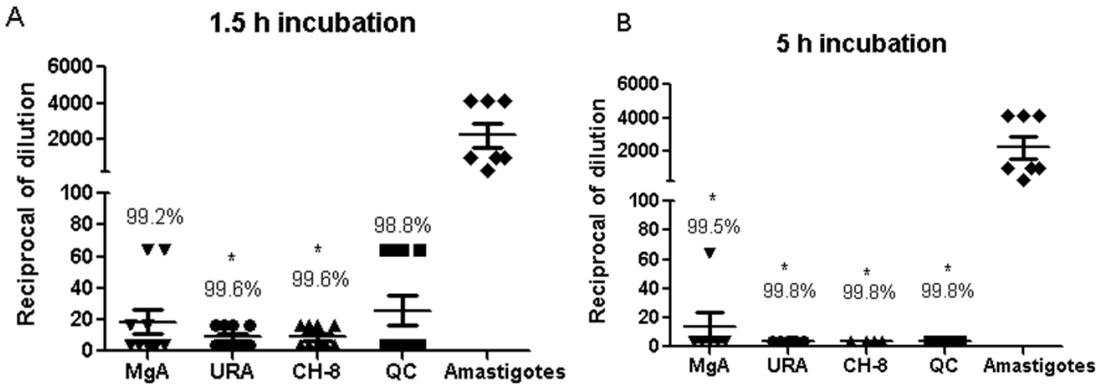


Figure 47: Viability of *L. infantum* axenic amastigotes exposed to dog hepatocytes and leishmanicidal drugs.
Samples of amastigote-exposed hepatocytes treated with MgA, URA, CH-8 and QC for 1.5 h (A) and 5 h (B) were plated in SCH medium and incubated at 24 ºC. In parallel, platted axenic amastigotes were also allowed to differentiate in promastigotes in the same conditions. The reciprocal of last promastigote positive dilution was considered as indicative of viability. The reciprocal of the dilution obtained for each condition is represented by the mean and standard error. The non-parametric Wilcoxon test was used for statistical comparisons. * indicates statistically important differences (P<0.05). Inscribed values indicate the reduction obtained when compared to the amastigote reciprocal dilution (100% viability).

The immune response, eventually potentiated by the addition of leishmanicidal drugs was evaluated by analyzing the gene expression of innate immune receptors and of cytokines.

Curiously, after treatment NOD1 gene expression of amastigotes-exposed hepatocytes stay similar to non-treated amastigotes-exposed hepatocytes or evidenced a significant reduction ($P_{QC1.5\ h}$=0.0273, $P_{URA,\ 5h;\ CH-8,\ 24\ h}$=0.0078, Figure 48A). On the contrary, the addition of MgA to amastigotes-exposed hepatocytes for 5 h led to the increase of NOD2 mRNA (P=0.0162, Figure 48B).

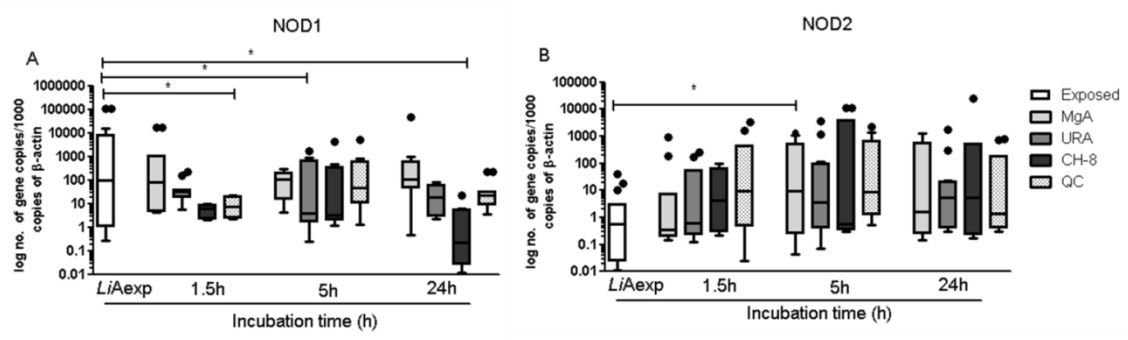


Figure 48: NOD1 and NOD2 gene expression by amastigote-exposed hepatocytes after treatment. Samples of amastigote-exposed hepatocytes treated with MgA, URA, CH-8or QC were used to evaluate NOD1 (A) and NOD2 (B) mRNA accumulation at 1.5 h, 5 h and 24 h of treatment and values compared to amastigote-exposed hepatocytes (*Li*Aexp). Results of samples performed in triplicate are represented by Tukey graphs. Black dots are indicative of outlier values. The non-parametric Wilcoxon test was used for statistical comparisons. * indicates statistically important differences ($P<0.05$) when comparing treated and non-treated hepatocytes.

After treatment with different leishmanicidal drugs, gene expression of TLRs stay similar to non treated amastigote-exposed hepatocytes or significantly decreased (Figure 49). TLR2 expression revealed a accentuated decrease during treatment with URA ($P_{URA,1.5\ h} = 0.0391$, $P_{URA,5\ h} = 0.0137$, $P_{URA,24h}=0.0049$) (Figure 49A). Quercetin treatment also demonstrated a significant decrease during early treatment time ($P_{QC,1.5\ h}= 0.0371$). TLR4 expression exhibited the unique exception, presenting a transient increase of TLR4 mRNA 5 h after MgA treatment ($P= 0.0049$), as a possible consequence of high availability of parasite antigens (Figure 49B). TLR9 expression levels were kept similar between amastigote-exposed hepatocytes and not treated and treated cells, revealing that parasite antigen's apparently do not activate this innate immune receptor.

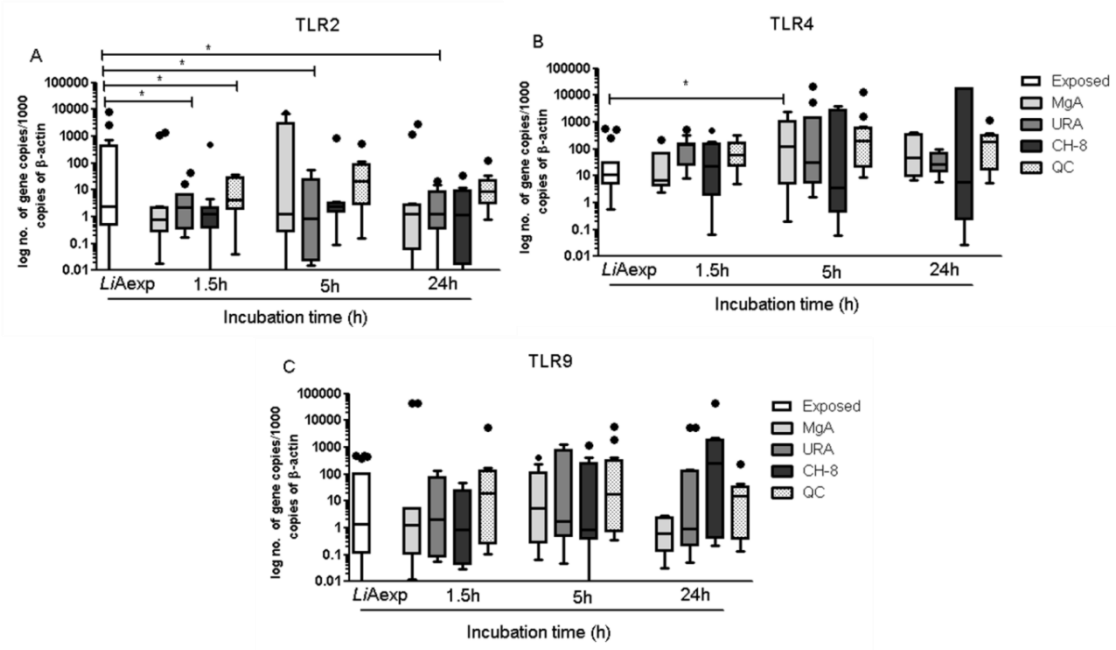


Figure 49: TLR2, TLR4 and TLR9 gene expression after treatment of canine hepatocytes exposed to *L. infantum* axenic amastigotes.
Samples of amastigote-exposed hepatocytes treated with MgA, URA, CH-8or QC were used to evaluate TLR2 (A), TLR4 (B) and TLR9 (C)mRNA accumulation at 1.5 h, 5 h and 24 h after treatment and compared to not treated amastigote-exposed hepatocytes (*Li*Aexp). Results of samples performed in triplicate are represented as Tukey graphs. Black dots are indicative of outlier values. The non-parametric Wilcoxon test was used for statistical comparisons. * represents statistical significance values ($P<0.05$) when comparing not treated *vs* treated hepatocytes.

The addition of leishmanicidal drugs led to transient changes cytokine environment in treated hepatocytes (Figure 50). In the IL-10 profile exhibit by amastigote-exposed hepatocytes after 1.5 h of URA treatment was observed an increase of IL-10 mRNA accumulation ($P=0.0020$) and 24 h incubation with the drug ($P= 0.0098$). The treatment with QC also increase IL-10 gene expression at the beginning ($P_{1.5\ h}=0.0078$) and again after 24 h treatment ($P_{24\ h}=0.0371$). After 24 h of treatment, MgA significantly increased IL-10 gene expression ($P= 0.0098$) when compared with non-treated hepatocytes (Figure 50A). However, after application of the leishmanicidal drugs gene expression of IL-6 and TNF-α did not show important differences when compared with non-treated hepatocytes, although IL-6 expression evidenced an increasing tendency (Figure 50B and C). Altogether, these findings indicate that some of the drugs are able to induce dog hepatocytes to generate transient peaks of the anti-inflammatory cytokine IL-10, probably in an attempt to preserve immune homeostasis.

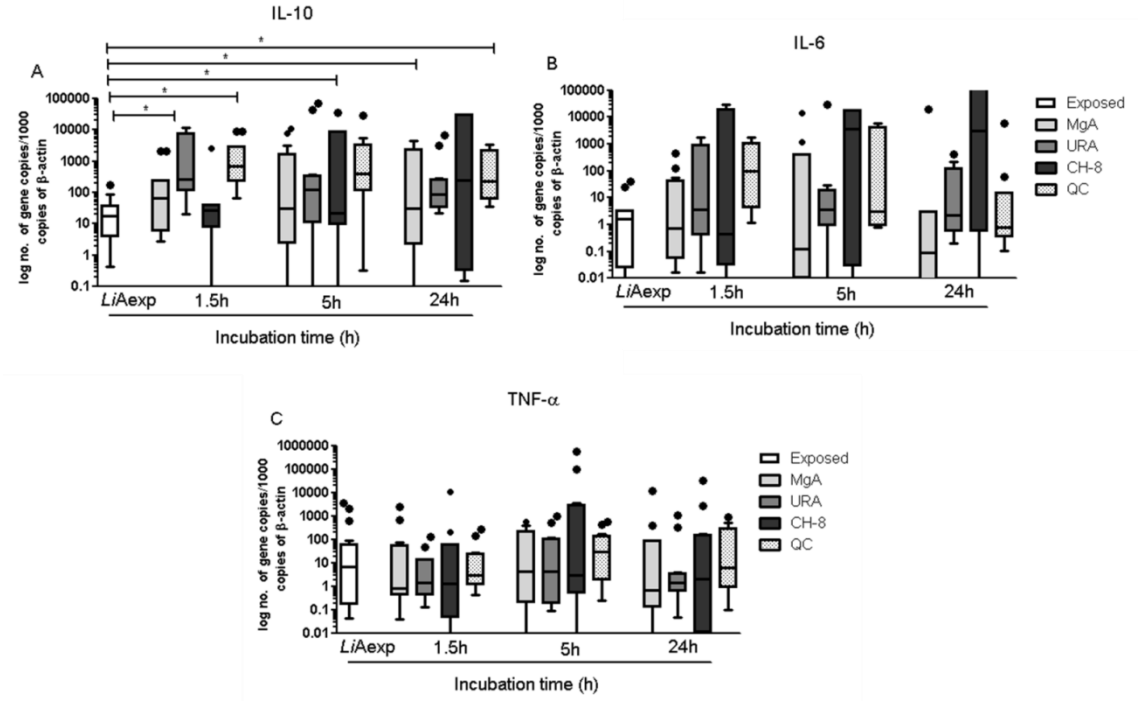


Figure 50: Gene expression of IL-10, IL-6 and TNF-α after treatment of hepatocytes exposed to *L. infantum* axenic amastigotes.
Samples of amastigote-exposed hepatocytes treated with MgA, URA, CH-8 or QC were used to evaluate IL-10, (A), IL-6 (B) and TNF-α (C) mRNA accumulation at 1.5 h, 5 h and 24 h after treatment and compared to not treated amastigote-exposed hepatocytes(*Li*Aexp). Results of samples performed in triplicate are represented by Tukey graphs. Black dots are indicative of outlier values. The non-parametric Wilcoxon test was used for statistical comparisons. * represents statistical significance values ($P<0.05$) when comparing not treated *vs* treated hepatocytes.

4.4.3.2. Dog hepatic spheroid become activated by parasite antigens and nucleic acids released during MgA treatment

Hepatic spheroids cultured in a 3D-system were previously exposed to *L. infantum* promastigotes for 72 h and then MgA was added to the system in order to evaluate the impact of the leishmanicidal drug into hepatocyte aggregates. Although not observed under optical microscopy, SEM revealed that the morphology of the cell membrane was altered (Figure 51C and E). Hepatocytes remain attached to each other by the extracellular matrix but the exposition to promastigotes and MgA seem to increased membrane roughness probably associated with high secretion levels and disrupted some of the cellular initial cohesion. Furthermore, was also apparent that the initially added promastigotes differentiated into amastigotes (ovoid body form without a free flagellum) and remain strongly attached to the spheroid membrane (Figure 51D-F).

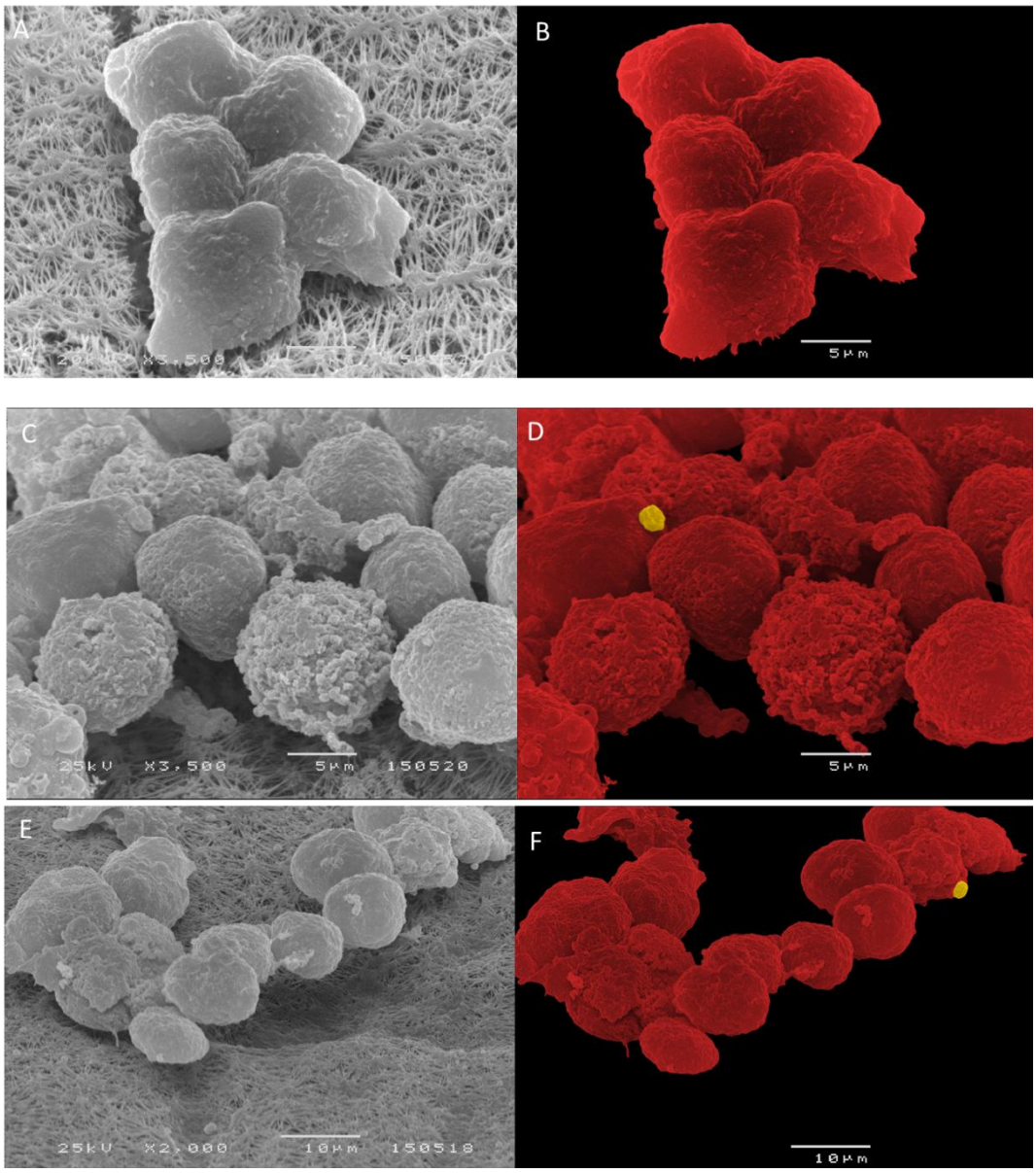


Figure 51: Morphology of promastigote-exposed spheroids after treatment.
Samples of 72 h promastigote-exposed hepatic spheroids and 72 h treated with MgA (C and E) were observed under SEM. Images were acquired and artificial colored to evidence the transformation of promastigotes into amastigotes (yellow) while attached to the hepatic spheroid membrane (red). Compared to the initial spheroids (A and B) the exposition to *L. infantum* promastigotes and MgA increased the membrane roughness of hepatic spheroids (C-F).

Nitric oxide (NO) production in the 3D-culture system was not altered by the addition of MgA, presenting identical high production levels during the 72 h of treatment. In addition, similar NO production levels were observed in no treated promastigote-exposed spheroids (Figure 52A). Urea production by promastigote-exposed spheroids follows the anterior described pattern, producing an initial burst when facing a stress situation. The addition of MgA does not cause

important changes, inducing a peak (1.5 h) of *de novo* synthesis urea followed by a major decrease. Then, high levels of urea accumulation remained constant (Figure 52B).

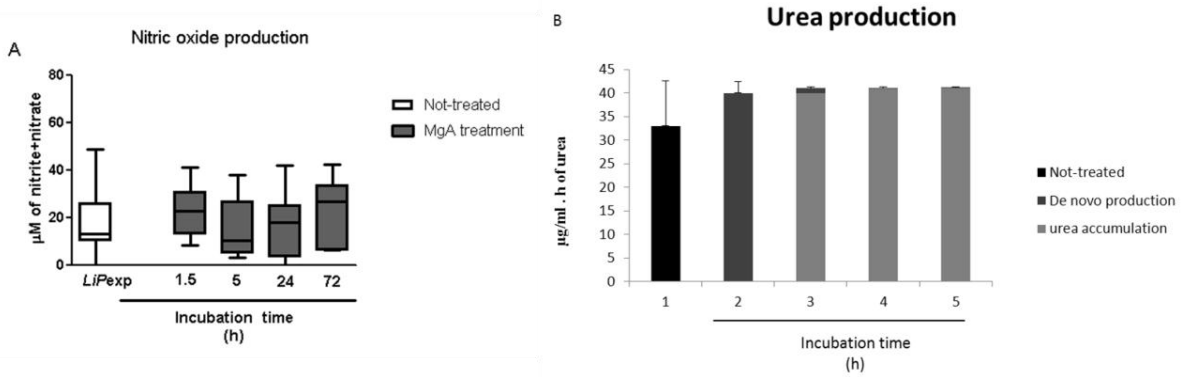


Figure 52: Nitric oxide and urea production by promastigote-exposed spheroids after MgA treatment.
Supernatant samples of hepatic spheroids exposed to L. *infantum* promastigotes for 72 h and treated with MgA for more 72 h were used to evaluate NO and urea production, supernatants of untreated promastigote-exposed spheroids (*Li*Pexp) were used for comparison. NO results are represented by box plots and whiskers (minimum to maximum) (A) and urea production by mean and standard deviation (B).

Phase I enzyme of promastigote-exposed spheroids treated with MgA exhibited activity levels similar to untreated promastigote-exposed spheroids. With the exception of EROD metabolization that exhibited a significant decrease (P=0.0391) at the beginning of treatment (1.5 h) in comparison with untreated hepatocytes (Figure 53). Phase II enzymes exhibited a decreasing tendency with statistical significance after 24 h incubation with MgA (P=0.0234).

Taken together, these results indicated that treatment with MgA has minimal effect on the CYP450 metabolization and of urea and NO production of dog hepatic spheroids that have established contact with *L. infantum* promastigote parasites.

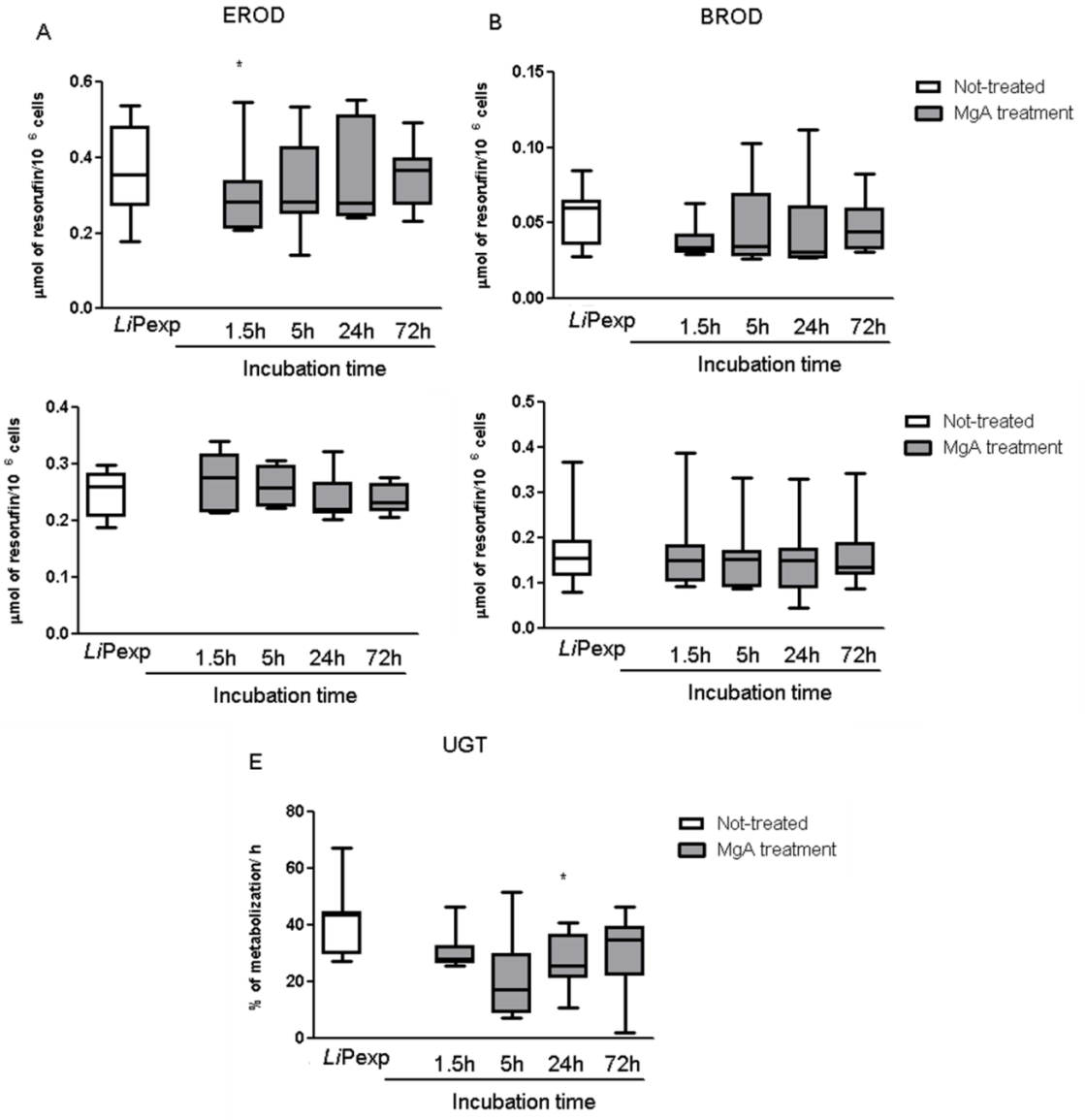


Figure 53: Phase I and phase II enzyme activity after MgA treatment of hepatic spheroids exposed to *L. infantum* axenic promastigotes.
Samples of 3D-cultures supernatants were used to evaluate EROD (A), BROD (B), MROD (C) PROD (D) and UGT (E) enzyme activity at 1.5 h, 5 h, 24 h and 72 h of MgA treatment. Untreated and 72 h amastigote-exposed hepatocytes (*Li*Aexp) were used for comparison. Results of samples performed in triplicate are represented by box plots and whiskers (minimum to maximum). The non-parametric Wilcoxon test was used for statistical comparisons. * represents statistical significance values ($P<0.05$) when comparing untreated *vs* treated amastigote-exposed spheroids.

Gene expression of the innate immune receptors NOD1 and NOD2 (Figure 54) were assessed after addition of MgA to spheroids previously exposed for 72 h to *L. infantum* promastigotes. When compared with exposed and untreated spheroids, the addition of MgA induces a significant increase of NOD1 expression ($P=0.0342$) after 24 h incubation with the drug. NOD2 exhibited a similar tendency with an increased

mRNA accumulation 24 h of MgA treatment (P=0.0166) and compared to spheroids exposed to *L. infantum* and not-treated (P= 0.0049). These results together with results obtained from treated 2D cultures, indicates that NOD2 receptors may play a role in recognize parasite antigens.

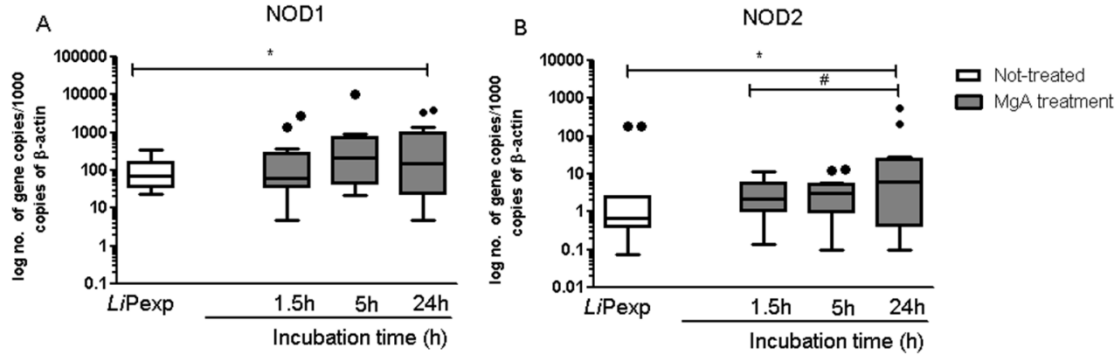


Figure 54: NOD1 and NOD2 gene expression after MgA treatment of promastigote-exposed spheroids.
Samples of 3D-hepatocytes cultures treated with MgA, were collected and NOD1 (A) and NOD2 (B) mRNA accumulation at 1.5 h, 5 h and 24 h of treatment assessed and compared with samples of untreated 72 h promastigote-exposed hepatocytes (*Li*Aexp). Results of samples performed in triplicate are represented by Tukey graphs. Black dots are indicative of outlier values. The non-parametric Wilcoxon test was used for statistical comparisons. * indicates statistically important differences (P<0.05) when comparing treated and non-treated hepatocytes. # * indicates statistically important differences (P<0.05) when comparing treated spheroids throughout incubation time.

The application of a leishmanicidal drug lead to an increase in TLR2 expression in treated promastigote-exposed spheroids after 24 h incubation with MgA (P=0.0353) (Figure 55A). TLR4 expression levels did not revealed differences caused by the addition of MgA and TLR9 gene expression registered an increase after 24 h incubation with MgA, when compared with untreated spheroids (P=0.0353) (Figure 55C).

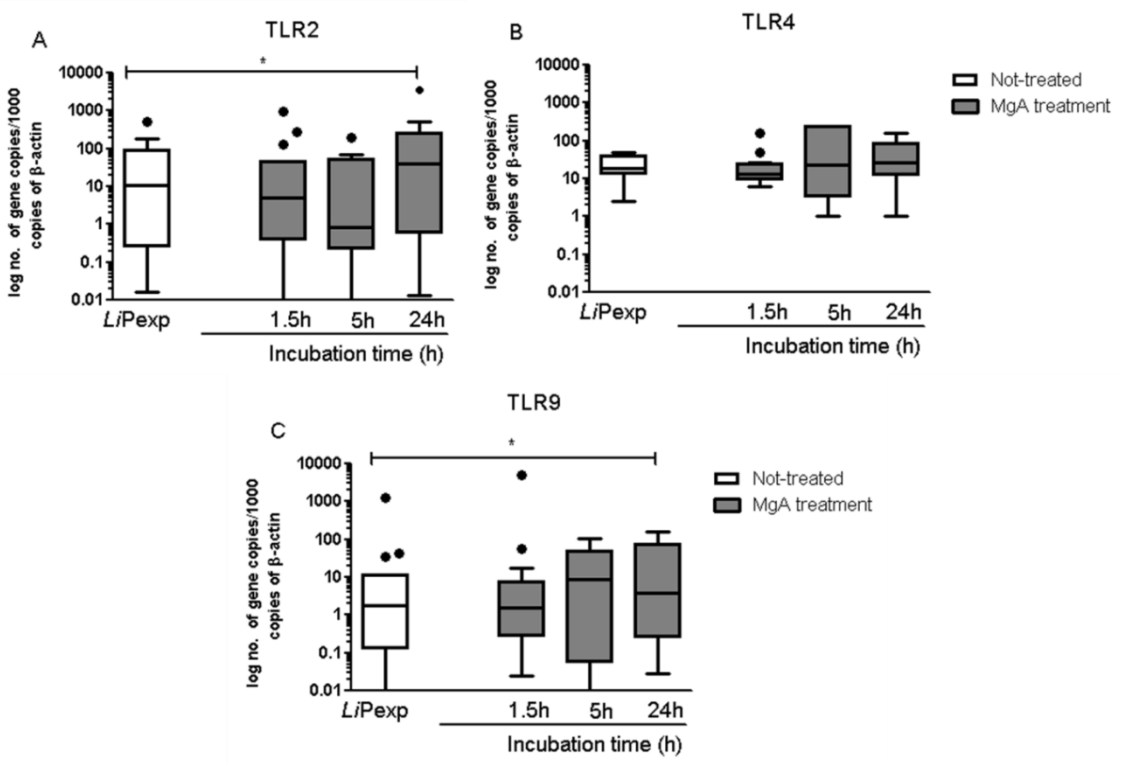


Figure 55: TLR2, TLR4 and TLR9 gene expression after treatment of hepatic spheroids exposed to *L. infantum* promastigotes.
Supernatant of 3D-cultures treated with MgA were used to evaluate TLR2 (A), TLR4 (B) and TLR9 (C)mRNA accumulation at 1.5 h, 5 h and 24 h after treatment and compared to untreated promastigote-exposed spheroids (*Li*Aexp). Results of samples performed in triplicate are represented by Tukey graphs. Black dots are indicative of outlier values. The non-parametric Wilcoxon test was used for statistical comparisons. * represents statistical significance values ($P<0.05$) when comparing not treated *vs* treated spheroids.

After MgA treatment of promastigote-exposed spheroids, gene expression of the anti-inflammatory IL-10 and IL-6 (Figure 56A, B) were kept similar to the untreated promastigote-exposed spheroids, although IL-6 presented a transient increase at 5 h incubation with MgA. However, the pro-inflammatory TNF-α registered a significant increased gene expression ($P=0.0031$) after 24 h of treatment (Figure 56C).

Altogether, these findings demonstrated that only a prolonged treatment (24 h) with MgA induced changes in the gene expression of a few innate receptors (internal and external) and of a pro-inflammatory cytokine of spheroids previously exposed to *L. infantum* parasites suggesting the spheroid activation by recognition of parasite antigens and nucleic acids, released as a consequence of treatment, and the establishment of a pro-inflammatory environment.

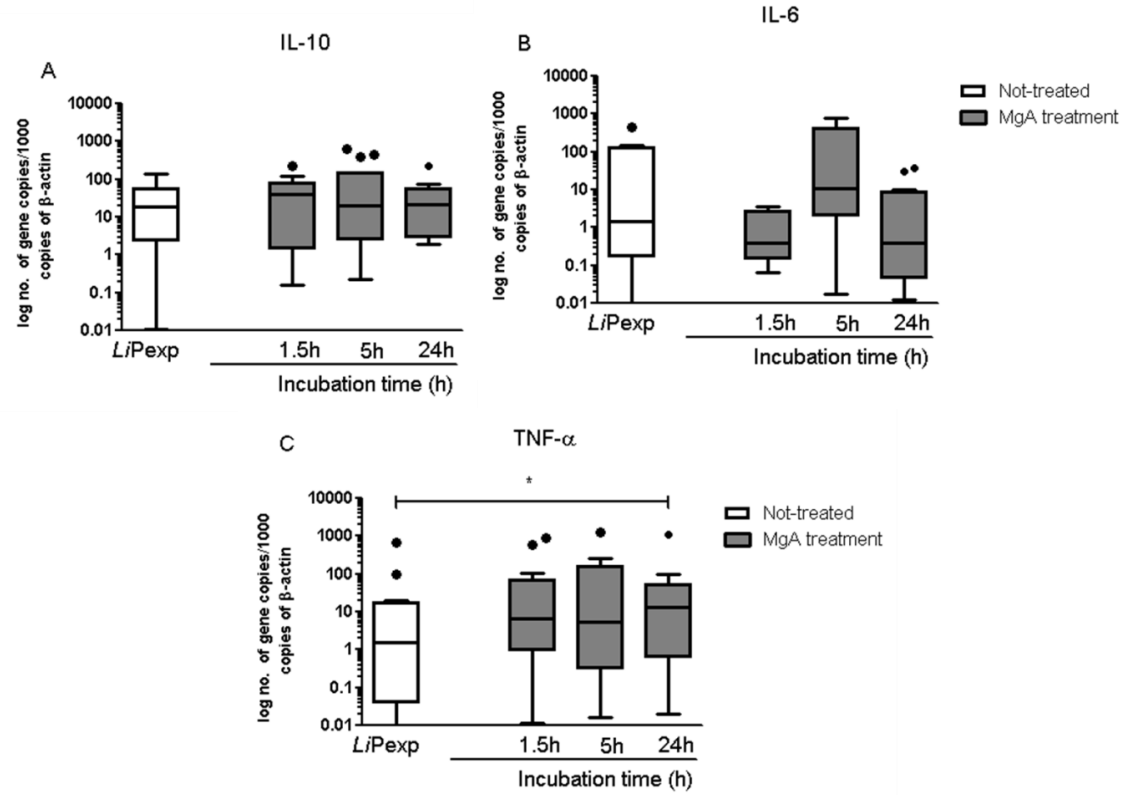


Figure 56: Gene expression of IL-10, IL-6 and TNF-α after treatment of hepatic spheroids exposed to *L. infantum* virulent promastigotes.
Samples of promastigote-exposed spheroids treated with MgA were used to evaluate IL-10, (A), IL-6 (B) and TNF-α (C) mRNA accumulation at 1.5 h, 5 h and 24 h after treatment and compared to untreated promastigote-exposed spheroids (*Li*Aexp). Results of samples performed in triplicate are represented by Tukey graphs. Black dots are indicative of outlier values. The non-parametric Wilcoxon test was used for statistical comparisons. * represents statistical significant values ($P<0.05$) when comparing untreated *vs* treated spheroids.

4.5. Discussion

Hepatocytes are cells of the main parenchymal tissue of the liver. These cells, which constitute 70-85% of the liver's mass, display vital roles in protein synthesis and storage, carbohydrate metabolism, cholesterol synthesis, detoxification and in the modification and excretion of exogenous and endogenous substances. The typical hepatocyte presents cubical shape with lateral sides of 20-30 µm. Exhibits eosinophilic cytoplasm, reflecting the numerous mitochondria present, and basophilic stippling due to the large amounts of rough endoplasmic reticulum and free ribosomes that support the immense protein synthesis. Hepatocyte nuclei are round with a dispersed chromatin and prominent nucleoli. Variation in the size of the nuclei is common and often reflects tetraploidy and other degrees of polyploidy, a normal feature of 30-40% of hepatocytes in the adult human liver. Binucleate cells are also common (Celton-Morizur *et al.*, 2010). Cells isolated from dog liver correspond to this description with a cytoplasm full of organelles positively staining for ferritin, the major intracellular protein. Constituted by 24 protein subunits of two types, the heavy (H) and light (L) subunits, this protein is involved in the storage and release of intracellular iron. Ferritin corresponds to ion Fe^{2+} that when oxidized originates the ion Fe^{3+}, which in turn can be complexed with ferritin in the cytosol of cells, transferrin in serum or with lactoferrin in mucosal secretions (Theil, 2003). Ferritin, a non-toxic form of iron storing is deposited within hepatocyte which has a large capacity for iron storage and transport. Free iron is toxic to cells as it acts as a catalyst in the formation of free radicals from reactive oxygen species. However, iron is essential for diverse cellular functions such as oxygen transport, electron transfer, nitrogen fixation, DNA synthesis, and the production of hemoproteins like hemoglobin and myoglobin. Therefore, vertebrates evolve an elaborate set of protective mechanisms to bind iron in various tissue compartments. When hepatocytes become iron overload, an insoluble hemosiderin is derived from iron-rich ferritin (Koorts and Viljoen, 2007). Ferritin is widely distributed in cells and is found in the cytosol, nucleus, mitochondria and lysosomes and appears in plasma as a result of cellular secretion. This specific distribution enables ferritin to supply, in particular enzymes and other proteins with appropriate amounts of iron, and, equally important, places ferritin in close proximity to sites where large amounts of iron are metabolized (Koorts and Viljoen, 2007). Ferritin synthesis is stimulated during cell differentiation by

pro-inflammatory cytokines as well as by some hormones (Festa *et al.*, 2000). An important role for ferritin during the acute phase response is to restrict the availability of iron by sequestration into the cavity of the ferritin protein shell. Furthermore, ferritin can modulate many immune functions, play a role in pro-apoptotic and anti-apoptotic pathways, and is implicated in the pathology of cancer (Koorts and Viljoen, 2011). During infection, the pathogen usurps host iron for its survival and pathogenicity, thus maintenance of the plasma ferritin level during infection is a crucial host defense mechanism. Endotoxin is an inducer of the gene encoding for ferritin, thus causing the rise of ferritin concentration. The concentration of ferritin has been shown to increase in response to stress, such as anoxia, suggesting that can be considered as an acute phase protein (Tsuji *et al.*, 2000). Vertebrate ferritin has been indirectly linked to innate immune response since its synthesis is regulated by pro-inflammatory cytokines at both the transcriptional and translational levels. In the present work ferritin seem to be augmented in canine hepatocytes early after exposure to *L. infantum* promastigotes, in both 2D- and 3D-culture systems, although more evident in the 2D-culture, as a consequence of an initial inflammatory response. Curiously, this increase in ferritin content of protein, correspond to the generation of IL-6 and TNF-α, both potent inflammatory cytokines. IL-6 is a pleiotropic cytokine with beneficial effects in the liver. This cytokine promotes liver regeneration and protects against a multitude of liver-damaging influences such as alcohol and CCl4 (carbon tetrachloride) intoxication (Mizuhara *et al.*, 1994). IL-6 also prevents apoptosis and exhibits positive effects in both ischemia models and fatty liver (Kovalovich *et al.*, 2000). However, the IL-6–triggered molecular mechanisms leading to liver protection are not completely understood. Panesar *et al.* (1999) related that murine hepatocytes release significant amounts of IL-6 when exposed to endotoxin or pro-inflammatory cytokines. In the present work, canine hepatocytes reacted to the presence of LPS with the generation of TNF-α similar to the response given by hepatocytes exposed to *L. infantum* amastigotes or promastigotes, and in this case together with the expression of IL-6, indicating that hepatocytes sense and react to the presence of *L. infantum* parasites, launching a classical inflammatory response, not specific for the parasite. In addition, IL-6, together with TNF-α and IL-1, is required for induction of the acute phase response which comprises fever, corticosteroid release, and hepatic production of acute phase proteins.

However, IL-6 is the major mediator for the hepatocytic secretion of most of the acute phase proteins. Overall, induction of the acute phase response by IL-6 has been regarded as part of an attempt to maintain homeostasis (Streetz *et al.*, 2000) and also seem to be associated with some of LV characteristic symptoms, such as fever. Inflammation in the liver has other consequences for the host. In laboratory animal species, IL-1, IL-6, and TNF-α have been found to modulate intermediary metabolism of carbohydrate, fat, and protein substrates, regulate hypothalamic-pituitary outflow, and act in the brain to reduce food intake (Johnson *et al.*, 1993, Johnson, 1997). Furthermore, TNF-α causes muscle catabolism that is also mediated by glucocorticoids, as well as glucagon-induced hyperglycemia and amino acid uptake by the liver, influencing the metabolic state of the host. The acute phase response changes blood plasma composition is thought to be beneficial to the organism by preventing microbial growth and helping to restore homeostasis. Some acute phase proteins can promote microorganism opsonization and activate complement, others scavenge cellular remnants and free radicals, or neutralize proteolytic enzymes (Gruys *et al.*, 2005). After stimulation by the pro-inflammatory cytokines, KCs release IL-6 that are recognized by hepatocytes. In this phase, IL-6 depresses mononuclear production of IL-1 and TNF-α (Schindler *et al.*, 1990) thus mitigating the whole cascade reaction and controlling inflammation. Downregulation of the hepatocytic acute phase receptors is achieved by rapid hepatic removal of circulating cytokines (Heinrich *et al.*, 1998) and release of IL-10 by KCs, inducing tolerance and suppressing the local IL-6 production. Acute phase protein production can be regulated by cytokines. Although cytokine production is also influenced by the presence of some acute phase proteins, IL-1 and IL-4 suppress acute protein synthesis (Loyer *et al.* 1993). A recent work by Silvestrini *et al.* (2013) addressed the relation between iron levels and C-reactive protein levels in canine leishmaniasis, concluding that inflammation contributes for the alteration of iron molecular status, but other mechanisms are likely involved. Low total iron-binding capacity and increased C-reactive protein are risk factors for the outcome of canine leishmaniasis. In summary, the exposure of canine hepatocytes to *L. infantum* parasites promote the increase in ferritin protein simultaneously with the generation of pro-inflammatory IL-6 and TNF-α cytokines, corroborating what was described in literature about inflammation increased ferritin levels. This enhancing ferritin levels

may be an attempt to transiently reduce iron availability for invading pathogens at the same time that hepatocytes react to the presence of *L. infantum* parasites by orchestrating an inflammatory response. Also, it has been described that in order to suppress pathogen growth the host responds by sequestering iron as evidenced by increased ferritin deposits in the trypanosomiasis chronic phase (Stijlemans *et al.*, 2008).

Since iron is an essential element for survival, dimorphic protozoan *Leishmania* spp. parasites had to evolve different strategies to acquire iron. *Leishmania* spp. are defective in the heme biosynthetic pathway and require exogenous heme for growth (Chang *et al.*, 1985). Iron is critically required as a cofactor of various important enzymes and can also influence the differentiation of *Leishmania* promastigotes into virulent amastigote forms (Mittra and Andrews, 2013). In *Leishmania* amastigotes which replicates inside phagolysosomes of MØ, iron superoxide dismutases are important for protection against the oxidative damage resulting from activation of the NADPH oxidase (Paramchuk *et al.* 1997). *In vitro*, promastigotes or amastigotes are supplemented with serum, which has high levels of ferritransferrin or with blood (lysed erythrocytes) rich in hemoglobin. Wilson *et al.* (1994) demonstrated that *L. chagasi* promastigotes are able to use iron bound to transferrin, lactoferrin or hemin, showing plasticity in the iron uptake. However, according with the same study, ferritin, the principal intracellular form of eukaryotic iron storage was unable to support promastigote growth. Although *Leishmania* appears able of acquiring Fe^{3+} complexed with lactoferrin or transferrin, the existence of a specific receptor-based uptake mechanism in these parasites has not been confirmed (Huynh *et al.*, 2006). Experiments with *L. chagasi* revealed the presence of NADPH-dependent ferric reductase activity associated with the cell surface of live parasites (Wilson *et al.*, 2002), indicating the existence of a potential pathway for direct membrane translocation of Fe^{2+}. The LFR1 (*L. amazonensis* plasma membrane-associated ferric reductase) was identified by Flannery *et al.*, 2011. LFR1 revealed to be essential to the intracellular growth of parasites and development of cutaneous lesions in mice (Huynh *et al.*, 2006). Together, LFR1 and LIT1 provide *Leishmania* with an inorganic iron acquisition pathway that has several similarities with the system found in plants. However, the ability of *Leishmania* to store iron in the cytosol is still debated (Flannery *et al.*, 2013). In fact, *Leishmania*

may not have a system to store cytosolic iron. This possibility may explain the observation that moderate amounts of Fe-NTA (ferric nitriloacetate) are toxic to *Leishmania* (Mittra *et al.*, 2013). Another possibility is that alternatively to ferritin, *Leishmania* utilize frataxin to store iron in the mitochondria (Adamec *et al.*, 2000). In the present study *L. infantum* promastigote appear to be also positive to ferritin. However, this result might be caused by a cross reaction from the polyclonal antibody anti-ferritin with the bovine ferritin found in bovine serum added to the parasite cultures as medium supplementation. This can cause a positive reaction due to the polyclonal antibody and species cross reactivity. Consequently, positive ferritin in *L. infantum* promastigotes in the present study should be carefully interpreted, even though, canine hepatocytes appear to increase their levels in ferritin early after exposure to the parasite. Therefore, it is possible that hepatocytes initially react to parasite presence with an inflammatory response accompanied by lowering extracellular iron levels in order to difficult pathogen survival, similar to what occurs with MØ. To avoid iron acquisition by invading organisms, MØ sequester iron using different strategies, including expression reduction of principal iron uptake protein transferrin receptor-1 (Mulero and Brock, 1999), increase synthesis of iron storage protein ferritin (Drakesmith *et al.*, 2005) or decrease expression of iron release gene ferroportin (Nairaz *et al.*, 2007). However, hepatocytes decreased ferritin content after 48 h of exposure to *L. infantum*. Direct release of cellular ferritin from damaged cell membranes has also been proposed (Tran *et al.*, 1997). During infection, it is speculated that plasma ferritin scavenges and helps to detoxify Fe^{2+} leaking from damaged cells. Evidence of ferritin secretion has been shown in rat hepatoma cells where it is regulated by inflammatory cytokines and iron (Tue *et al.*, 1997). In the present study it was evident a close contact between the parasite and the hepatocyte membrane, stressing hepatocytes. It is hypothesized that hepatocytes may release ferritin as a consequence of membrane damage caused by a longer interaction with *L. infantum* parasites.

Close parasites interaction with hepatocyte membrane lead to the hypothesis that *L. infantum* can be internalized by dog hepatocytes. It is known that hepatocytes can perform phagocytosis under special circumstances. The phagocytic action of KCs is well known in the liver, but such a phenomenon has not been well documented for hepatocytes. Soji *et al.* (1992) referred that hepatocytes can perform phagocytosis

although not in a uniform manner. There are evidences of the presence of *L. donovani* amastigotes in the liver biopsies from human patients with VL (Duarte *et al.*, 1989). Amastigotes were found within hepatocytes, using electron microscopy, and after patients gone through successful therapy. Gangneux *et al.* (2005) demonstrated *in vitro* that murine, rat and human primary hepatocytes were permissive to *L. donovani* promastigote infection, but parasites do not massively proliferate. This slight infection resulted in limited damage to hepatocytes. However, these results bring into question a possible role for hepatocytes as a parasite reservoir during latent infection. The present work is not conclusive regarding the question of *L. infantum* promastigotes or amastigotes entering the hepatocytes. *L. infantum* exhibited a clear tropism to hepatocytes and the interaction that parasite establish with the cell membrane seem to be very strong, as parasite remain attached to the hepatocytes throughout all the experimental period of time. *Leishmania* species are known to trigger MØ phagocytosis by establishing ligation with the surface receptors. Receptors reported to facilitate *Leishmania* internalization include the third complement receptor (CR3), first complement receptor (CR1) mannose receptor (MR), Fc gamma receptors (FcγRs, in particular FcγRII-B2) and fibronectin receptors (FnRs) (Ueno and Wilson, 2012). It is described the presence of mannose receptor (Ohnishi *et al.*, 2012) and fibronectin receptors (Johansson and Lundgren, 1987) in hepatocytes. A number of pathogenic microorganisms, including *C. albicans* (Maródi *et al.*, 1991), *Pneumocystis carinii* (Ezekowitz *et al.*, 1991) and *L. donovani* (Chakraborty *et al.*, 2001) display glycans on their surfaces with terminal mannose residues that are recognized by the C-type CRDs of the mannose receptor, thereby acting as a non-self marker. In phagocytic cells, upon recognition, the receptor internalizes the bound pathogen and transports it to lysosomes for degradation via the phagocytic pathway. In this way, the mannose receptor acts as a pattern recognition receptor (PRR). However, the ability of the mannose receptor to assist pathogen internalization is also thought to facilitate infection of intracellular pathogens as *M. tuberculosis* or *Leishmania* spp.. *M. tuberculosis* also reside and multiply inside MØ, preventing formation of the phagolysosome to avoid degradation. Hence, by mediating their entrance into the MØ, the mannose receptor helps these pathogens to infect and growth in their target cell (Gazi and Martinez-Pomares, 2009). In MØ, fibronectin receptors act synergistically with complement receptor to phagocyte

pathogens. Fibronectin receptors alone may retain the pathogen, possibly being the major responsible for the close interaction of *L. infantum* parasites with the hepatocyte membrane that was observed in the present work.

Urea production is a characteristic of functional hepatocytes. The liver is quantitatively the major organ involved in urea synthesis and it is doubtful whether other cell types, such as enterocytes, can produce significant amounts of urea (Wu, 1995). However, at least some urea cycle enzymes are found in extra-hepatic tissues, where they are involved in providing arginine for nitric oxide (NO) synthesis (Husson *et al.*, 2003). There are two major pathways for ammonia detoxication in the liver: urea and glutamine synthesis. The liver is the most important site of ammonia metabolism; it removes much of the toxic ammonia presented to it by urea and glutamine synthesis. To prevent hyperammonemia and its dangerous effects, liver detoxifies ammonia by ureagenesis in periportal hepatocytes and by glutamine synthesis in perivenous hepatocytes. However, hyperammonemia was observed in experimental rodent models of sepsis suggesting defective hepatic ammonia detoxification (Luiking *et al.*, 2008). According with a recent work from Soria *et al.*, (2014) hepatocyte ureagenesis in LPS-treated rats seemed to be functional but dependent on the specific source of precursors. In the case, ureagenesis from ammonia was decreased but ureagenesis from glutamine was significantly increased. In the present work LPS addition to 2D cultured hepatocytes resulted in an initial burst of urea, probable due to a similar mechanism by which glutamine, present in the medium is being taken up into mitochondria, and metabolized to ammonia and glutamate via glutaminase. However, as the culture medium was not changed over the 72 h incubation, turning glutamine into a limiting substrate together with the establishment of an inflammatory response (elevating levels of TNF-α) lead to a decrease in urea production in LPS exposed hepatocytes. Balancing enzymes from the urea cycle is a key feature in *Leishmania* survival. When infecting macrophages *Leishmania* has to balance two inducible enzymes present in the cell that might kill or allow its survival: nitric oxide synthase (iNOS) and arginase. These two enzymes use a common substrate, L-arginine, and are competitively regulated by cytokines secreted by Th1 and Th2 cells. Arginase is classically considered to be an enzyme of the urea cycle in the liver and *Leishmania* through the modulation of a Th2 polarized response is able to induce arginase activity and urea synthesis, ensuring

parasite survival (Kropf *et al.*, 2005). The initially exposition of hepatocytes to *L. infantum* amastigotes or promastigotes also induced an increase in IL-10 expression concomitant with an increase in urea synthesis, probably induced by the parasite presence in order to control NO synthesis by hepatocytes, which was not different from non-exposed hepatocytes. After that initial burst of urea, exposed hepatocytes produced very little urea throughout the 72 h exposition in both 2D and 3D culture system. It is also described that inflammation can likewise lead to inhibition of urea synthesis in hepatocytes. Cytokines secreted locally by hepatic non-parenchymal cells, including TNF-α, IL-1β and IL-6 have been implicated in the inhibition of a wide range of hepatic functions after systemic injury or local hepatic injury (Luster *et al.*, 1994). Kang *et al.*, (2002) proved that hepatocytes chronically exposed to inflammatory cytokines IL-1 and IL-6 exhibited altered function as suppressed albumin production and reduced urea synthesis. The decreased observed in urea production in hepatocytes exposed to *L. infantum* amastigotes or promastigotes may also reflect the mix immune environment with IL-6 and TNF-α inflammatory cytokines and also IL-10 regulatory and permissive effect.

It is well known that cytotoxic factors, such as lipopolysaccharides, disrupt nitrogen metabolism in hepatocytes and NO is involved among other factors in regulating this metabolic pathway (Tabuchi *et al.*, 2000). NO is a free radical that is involved in many cellular events. Its biosynthesis are mainly performed by converting L-arginine to L-citrulline. Nitric oxide synthase (NOS) is either a constitutive or inducible enzyme. NOS has three distinguishable isoforms: NOS-1 (ncNOS), a constitutive isoform originally isolated from neuronal sources; NOS-2 (iNOS), an inducible isoform that may generate large quantities of NO and may be induced in a variety of cell types by inflammatory stimuli, such as TNF-α, IL-1 and IL-6; and NOS-3 (ecNOS), a constitutive isoform originally located in endothelial cells (Alexander, 1998). Unlike the constitutive form, iNOS is not regulated by the intracellular calcium concentration or by the Ca^{2+}-calmodulin complex. It is also known that iNOS is expressed by many cell types including MØ, smooth muscle cells and hepatocytes (Sesti *et al.*, 2005). Hepatocytes have been shown to express increased levels of NO following exposure to endotoxins, such as bacterial lipopolysaccharide or cytokines, such as TNFα, IL-6 and IL-1 (Saad *et al.*, 1995). NO may possess both cytoprotective and

cytotoxic properties, depending on the amount and on the NOS isoform by which it is produced. In the liver, NO release in small quantities may be beneficial since it has been shown to decrease tumor cell growth and prostaglandin levels (pro-inflammatory products) and to increase protein synthesis and DNA-repair enzymes (Alexander, 1998). 2D- and 3D-cultured hepatocytes exposed to *L. infantum* amastigote or promastigote register small NO increase, more evident in the 3D system. *L. infantum* is known to exhibit the ability to control NO production since it is a potent leishmanicidal molecule. *L. infantum* parasites stimulate the urea production and do not disturb NO pathway, subverting the host immune system to promote a Th2 response. NO production on hepatocytes was accompanied by the increased expression of inflammatory cytokines TNFα, IL-6 and also anti-inflammatory IL-10 probably to control the inflammatory response and avoid cellular damage.

In the present work two culture models of hepatocytes were used to better characterize the interaction between the parasite forms and the hepatocytes. The 2D-culture system consisted in the addition of hepatocytes to flat plates, allowing cells to adhere to a surface in a monolayer. This might be considered an inadequate representation of the cell's original environment and 3D-spheroids culture system were developed in order to overcome this issue. The generation of 3D-multicellular spheroids is an attempt to mimic the *in vivo* liver architecture. 3D-culture model improved cell-to-cell and cell-to-matrix interactions, recapitulating many *in vivo* tissue structures and functions. A 3D-culture system was developed to solve one of the main problems in the pharmaceutical industry: the need for viable and metabolic active hepatocytes to drug screening (Bachmann *et al.*, 2015). In the present work it was observed that hepatocytes recover faster from isolation trauma if cultured in the 3D-spheroid system. The recovery was indicated by the higher CYPs activity after early after hepatocyte isolation (24 h). However, 2D-cultured hepatocytes also recovered, but take longer time (48 h). Similar urea production rate indicates that hepatocytes of both culture systems were at similar conditions. However, in the present work there were differences in the immune response magnitude to *L. infantum* promastigotes between hepatocytes cultured in 2D- and 3D-system. In 2D-culture system the interaction between the parasites (in both forms) was more intense and persistent than in the 3D-culture system. In the 3D-system due to the nature of the spinner vessel, hepatocytes had a less intense, more indirect

interaction with the parasites. Although the results indicate that hepatocytes were able to recognize the presence of the pathogen and react by orchestration an inflammatory response. Generation of IL-6 and TNF-α in the 3D-culture system were kept stable compared with non-exposed hepatocytes and did not register the increase of TNF-α genes expression recorded in the 2D-culture system. It seems that, hepatic spheroids induced tolerance to the inflammatory stimulus (presence of promastigote), probably by increased cell-to-cell contact and decreased direct contact between the promastigotes and individual hepatocyte membrane.

By controlling systemic innate immunity via production of secreted PRRs and of the complement components found in plasma, hepatocytes play and essential role in innate immunity. During the acute phase response, a variety of pro-inflammatory cytokines, such as IL-6, IL-1, TNF-α, and IFN-γ, can stimulate hepatocytes to produce high levels of complement and secreted PRRs (Gao *et al.*, 2008). Hepatocytes also express membrane-bound PRRs, such as TLRs and NODs. TLRs are a family of proteins that recognize pathogen associated molecular patterns (PAMPs) and are expressed on all major subsets of liver cells. TLRs can be triggered by exogenous ligands derived from pathogens or by endogenous ligands resulting from cellular injury, activating innate immunity. TLR recognition is often associated with the production of pro-inflammatory cytokines and generation of effector molecules, which promote differentiation of Th1 cells, leading to an inflammatory response. The specific response initiated by individual TLRs depends on the recruitment of a single or a specific combination of TIR-domain-containing adaptor protein (e.g., MyD88, TIRAP, TRIF, or TRAM) (Kawai and Akira, 2010). MyD88, utilized by all TLRs (with the exception of TLR3) and members of the IL-1 receptor family, transmits signals culminating in NF-κB and MAP kinase activation and downstream induction of inflammatory cytokines such as IL-1β, IL-12, TNF-α, and IL-6. TLR3 and TLR4 use TRIF to activate an alternative pathway leading to the activation of NF-κB and IRF3 and the production of type I IFN and of other inflammatory cytokine. TLR2 and TLR4 use TIRAP as an additional adaptor to recruit MyD88. Hepatocytes express mRNAs for all known TLRs, but their role in hepatocyte defense against invading pathogens is less clear. Hepatocyte expresses TLR2 and TLR4 and is responsive to LPS (Szabo *et al.*, 2006; Schwabe *et al.*, 2006). In the present study a tendency to increased TLR2 and TLR4 expression was

observed in LPS stimulated hepatocytes, in accordance to what was previously described. However, the obtained values were not statistically significant, may be due to low LPS concentration or low exposition time. NOD-like receptors (NLRs) comprise a family of intracellular pattern recognition receptors that are important for recognition of the cell damage and of microbial-associated molecular patterns. NOD1 and NOD2 are specialized NLRs that participate in the recognition of a subset of pathogenic microorganisms that are able to invade and multiply intracellularly. Once activated, these molecules trigger intracellular signaling pathways that lead to the activation of transcriptional responses culminating in the expression of a subset of inflammatory genes. The role NOD receptors are not yet well defined in liver immunology (Isogawa *et al.*, 2005; Thomas *et al.*, 2007). In the present work it was assessed, for the first time, the expression pattern of NOD1 and NOD2, as well as TLR2, TLR4 and TLR9 exhibited after hepatocytes were exposed to *L. infantum* promastigotes or amastigotes. The liver is another main target organ in VL. In contrast to the spleen that stays chronically infected, infection in the liver is normally self-containing. Resolution of disease in the liver is associated with granuloma formation which is one of the key features of hepatic resistance (Murray, 2001). However, the role of hepatocytes to control the infection by *Leishmania* is poorly studied. The resolution of the infection in the liver is attributed to the development of a Th-dominated granulomatous response, characterized by high IFN-γ, IL-12 and TNF-α production through $CD4^+$ T cells. IL-12 is an essential cytokine in the development of protective immunity against *L. donovani*, since IL-12 blocking reduce both IFN-γ production and granuloma formation in the liver of infected mice (Murray, 1997). Hepatic resistance in VL is also attributed to the generation of reactive nitrogen and oxygen intermediates, both of which have been shown to play a role in containing parasite growth during the early stages of infection (Murray and Nathan, 1999). In the present study, amastigote and promastigote exposition seem to trigger similar responses from the canine hepatocytes. Although both receptors react to the parasite presence seem to have a specificity for the morphological form of the parasite, amastigote and promastigote parasites induced NOD2 and amastigotes induce NOD1 gene expression. To the best of our knowledge, this is the first time that NOD1 and NOD2 gene expression are assessed in *L. infantum* exposed hepatocytes. Structurally, NLRs are multi-domain proteins containing an N-terminal

Caspase Recruitment Domain (CARD) that associates with downstream signaling molecules, a centrally located nucleotide-binding oligomerization domain (NBD or NACHT), and a C-terminal leucine-rich repeat domain (LRR) or sensor domain. The NLR members NOD1 and NOD2 belong to the NLRC subgroup as they contain an amino-terminal CARD domain and share the two common domains (NBD and LRR). NOD1 contains a single CARD domain, whereas NOD2 has two (Ogura *et al.,* 2001). NOD1 and NOD2 function as intracellular receptors for bacterial peptidoglycan fragments, d-glutamyl-meso-diaminopimelic acid (DAP), which is found in Gram-negative and in a few Gram-positive bacteria, including *Listeria* and *Bacillus* (Chamaillard *et al.*, 2003). In contrast, NOD2 activation was triggered by muramyl dipeptide (MDP), a peptidoglycan motif widely distributed among both Gram-positive and Gram-negative bacteria (Girardin *et al.*, 2003). It also has been shown that NOD2 can act synergistically with TLRs. The addition of MDP in combination with TLR agonists, such as lipoteicoic acid, LPS, and peptidoglycan, triggers a robust inflammatory response, including the release of pro-inflammatory cytokines such as IL-1β and IL-6 (Wolfert *et al.*, 2002). It is possible that TLR stimulation facilitates the internalization of MDP, a process that is required for NOD2 activation under physiological conditions. In the present study, it is possible that the increase expression observed for NOD1 and NOD2 receptors are a result of a synergy with TLRs in order to activate an inflammatory and protective immune response against *L. infantum* parasites. TLR2 also registered, in the present study, a tendency to increase in hepatocytes exposed to *L. infantum* amastigotes or promastigotes. Curiously, in the 3D hepatic spheroids, only TLR2 expression was increased, probably due to the nature of the spinner vessel. In this case hepatic spheroids were exposed to parasites with less intensity and a more indirect interaction was established. Comparing the two cultures system, TLR2 receptor emerges as a possible first innate immune receptor (of the assessed ones) to be triggered by *L. infantum* virulent promastigotes, leading to the hypothesis that this innate immune receptor may play an important role in *L. infantum* detection and orchestrate the immune response against it. TLR2 and TLR4 are extracellular PRRs and TLR9 is an intracellular PRR. The microbial molecules recognized by TLRs are conserved polymers, such as bacterial LPS, peptidoglycans, unmethylated bacterial DNA, and double-strand viral RNA, among others. Since

protozoan lack most of these structures, TLRs also recognize other groups of molecules in order to sense these pathogens. The recognition of *Trypanosoma cruzi* tGPI (glicosylphosphatidylinositol anchor) by TLR2 (Campos *et al.*, 2001) and of glycoinositolphospholipids by TLR4 (Oliveira *et al.*, 2004), of *Plasmodium* hemozoin by TLR9 (Coban *et al.*, 2005), and of the profilin-like molecule of *Toxoplasma gondii* by TLR11 (Yarovinsky *et al.*, 2005) are such well described examples. A few *Leishmania*-derived molecules have been reported to activate TLRs, and the majority of the studies focused on the activation of TLR2, TLR4, and TLR9 in MØ of several mammal host species (Faria *et al.*, 2012). Purified *L. major* lipophosphoglycan (LPG) induced the upregulation and stimulation of TLR2 in human NK cells, with additional enhancement of TNF-α and IFN-γ production (Becker *et al.*, 2003). LPGs of *L. major, L. mexicana, L. aethiopica*, and *L. tropica* were defined as TLR2 ligands in studies using murine MØ, although the stimulation degree was variable. Intriguingly, LPG also induced the expression of suppressors of pro-inflammatory cytokines, the signaling family proteins SOCS-1 and SOCS-3. The finding that negative modulators of cytokine synthesis were also induced by LPG indicated that TLR2 stimulation by *L. major* can lead to both positive and negative inflammatory signals (de Veer *et al.*, 2003). The fact that SOCS1 itself directly downmodulates TLR4 signaling pathways illustrates how the initial stimulation of TLR2 by *L. major* can ultimately lead to the attenuation of further TLR responses (Nakagawa *et al.*, 2002). The potential role played by the innate immune receptors TLR2, TLR4 and TR9 of canine hepatocytes in surveillance and activation against *L. infantum* parasites is not yet described. There are evidences that TLR4 might have a protective role in *L. major* infection. Kropf *et al.* (2004) observed diminished parasite load at the skin lesions of infected mice at the initial stages of infection and found increased parasite survival in host cells from TLR4-deficient mice, which correlates with a higher activity of arginase. However, TLR4 does not seem important to define the range of cytokines produced in the skin or in the draining lymph nodes of mice infected with *L. major* (Antoniazi *et al.*, 2004). It was described in *L. pifanoi* amastigotes a potential ligand for TLR4, the proteoglycolipid complex (P8) composed of a cysteine and serine metalloprotease, host-derived ApoE, and four glycolipids. Stimulation of MØ with complex P8 or its isolated glycolipids provoked the synthesis of pro-inflammatory cytokines, including IL-1β and TNF-α (Whitaker *et al.*, 2008).

However, the exact molecular entity serving as the TLR4 ligand in P8 complex still remains to be determined and the role for TLR4 in *Leishmania* infection is still not fully understood. TLR9 stimulation with CpG motifs was described to induce pro-inflammatory cytokines synthesis, in particular of IL-12, indicating that TLR9 stimulation could lead to protective immunity against the parasite. Liese *et al.* (2007) showed that TLR9 was required to induce IL-12 in bone marrow derived DCs by either *L. major* or its DNA. Furthermore, it was also observed that TLR9$^{-/-}$ mice exhibited more severe skin lesions and higher parasite burdens compared to controls, coupled with a transient increase in IL-4, IL-13, and arginase. In another study, it was reported that plasmacytoid DCs responded to *L. infantum* by secreting IFN-α and β and IL-12 in a TLR9 dependent manner and that NK induced cytotoxicity was abolished in TLR9$^{-/-}$ mice (Schleicher *et al.*, 2007). Those results were the first to link TLR9, IL-12, and DCs to the activation of NK cells during VL. The protective role of NK cells in murine leishmaniasis is now proven to be strictly dependent on IL-12 secreted by myeloid DCs in response to *Leishmania* via TLR9, proven that TLR9 is important in protection against *Leishmania*. The present work constitutes the first report of the innate immune response exhibited by canine hepatocytes in the presence of *L. infantum*. Taking together, the majority of the above described studies were performed in MØ of several host species that makes difficult a comparison with hepatocytes. MØ constitute the final host cell for *Leishmania*, but the parasite or parts of it may yet contact with other non-immune cells, as hepatocytes. Recently in a canine study, increased frequency and expression of TLR9 was associated with a lower parasite load in the jejunum of *L. infantum* infected dogs, whereas the colon showed a higher parasite load along with an increased frequency and expression of TLR2 (Figueiredo *et al.*, 2014). Hosein *et al.* (2015) addressed the role of TLRs in the canine model of VL caused by *L. infantum*. The authors analyzed several tissues, including the liver, where they describe increased TLR2 transcription and TLR9 downregulation. However, the study is not conclusive and the analysis was performed with whole liver tissue and not isolated cells. In the present study, increase gene expression of TLR2 seems to confirm previous findings that in fact TLR2 a have a non negligible role in *Leishmania* infection, and in particular in *L. infantum* infection of the canine liver. TLR9 exhibited an increasing tendency in 2D cultured hepatocytes exposed to *L. infantum*. Nevertheless, the increase TLR2 and

the tendency to increase TLR9 gene expression was concomitant with pro-inflammatory IL-6 and TNF-α increased expression suggesting a synergistic model in hepatocyte activation. Curiously in the 3D culture system only occurred the increase in TLR2 and no major alterations in cytokine profile were observed. From the close interaction of parasites with hepatocyte membrane in the 2D culture system it is possible that a synergistic effect of both NOD1 and NOD2 with TLR2, in conjugation with a possible TLR9 stimulation lead to the activation of hepatocytes to produce inflammatory cytokines IL-6 and TNF-α, breaking the immune tolerance that characterizes the liver tissue and initiating a protective immune response. The results indicate that hepatocytes are active cells in innate immune surveillance and that are able to initiate an immune response. Throughout the present study, several outliers were registered as a reflex of the dog's natural population. The dogs included in the present study were collected from a natural population with no restrictions of breed and sex, in order to reflect the natural diversity of the population. It is interesting to notice, that it is described that some dogs are more resistant to *L. infantum* infection than others and the outliers identified support that hypothesis. However, genetic associations are not yet fully defined.

The liver is also a major metabolic organ and CYP450s constitute the major drug metabolizing enzymes in the liver. These enzymes are responsible for the metabolization of endogenous molecules such as hormones and exogenous and potentially harmful compounds. CYPs are particularly important in the pharmacological industry as induction/inhibition of CYPs is one of the factors that can affect the pharmacokinetics of a drug molecule upon multiple dosing, and it can result in pharmacokinetic drug-drug interactions with co-administered drugs causing potential therapeutic failures. Canine hepatocytes, and thus canine CYP450, have been used by the pharmaceutical industry to study the pharmacokinetics of new drugs in order to predict drug interaction based on enzymes homology between dogs and human. By doing so, canine CYPs are well documented and, as in humans, the canine enzymes typically display high interindividual variability in expression and function, as a consequence of the balance between enzyme induction/inhibition and of genetic polymorphism, thereby predisposing patients to adverse drug reactions or therapeutic failure (Court, 2013). As CYP450 are functionally important for organism survival it is

interesting to investigate their presence in invading pathogens. Curiously, *L. donovani* genome database annotates 3 CYP450s in *Leishmania*, nevertheless the functional role of cytochrome P450 enzymes in *Leishmania* spp. remains elusive. Verma *et al.* (2011) reported the involvement of one effective CYP450s, the CYP5122A1 in the survival and synthesis of ergosterol in *L. donovani* parasite. The importance of CYP5122A1 for parasite survival indicates this enzyme for further evaluation as a possible drug target.

The present work addresses the question whether *L. infantum* amastigotes or promastigotes influence the activity of canine hepatocytes' CYPs (CYP1A1, CYP1A2, CYP2B11, CYP3A12, CYP3A26 and CYP1B) by altering the immune environment. Both culture systems showed a pattern of preserve or reduction of CYPs activity when in the presence of *L. infantum* amastigotes or promastigotes. 2D-cultured hepatocytes incubated with LPS, as a positive control for inflammation, also evidence a similar tendency. It has been described that the immune environment can influence CYPs activity. The present work constitutes the first report on inflammation caused by *L. infantum* presence and influence on canine hepatic CYPs activity. As dogs constitute the peridomestic reservoir of the parasite, understanding how the major metabolic enzymes of the liver react in the presence of the parasite, might be useful to predict drug metabolization during treatment. The first indications of an interaction between the host immune system and hepatic drug metabolizing enzymes arise from studies using modulators of the reticuloendothelial system (RES), which includes KCs. In 1953, Samaras and Dietz reported that rats injected with the RES depressant trypan blue had prolonged pentobarbital sleeping times (Samaras and Dietz, 1953). Due to the clinical importance of understanding the CYP450 mechanisms of inhibition or induction, several studies were performed since then. In humans and animals, infections or inflammatory stimuli cause changes in the expression and activity of various forms of liver CYP450, as well as in extrahepatic tissues such as kidney and brain. However the effects are not uniform for all CYPs. In most cases, CYP450s activities are suppressed, but some are unaffected and others are induced under inflammatory conditions. CYP450 suppression can result in increased clinical toxicity of drugs with a low therapeutic index (Morgan, 1997). Likewise, some drugs must be converted to their pharmacological or toxicological active metabolites by CYP450 enzymes, and suppression of their metabolism during an inflammatory response can lead to a reduced

therapeutic or toxic effect (Morgan, 1997). On the other hand, the CYP4A and CYP2E1 families in Wistar rats are described to be induced by inflammatory stimuli such as LPS (Morgan, 1997). The results of the present study revealed that the presence of *L. infantum* increased the gene expression of the inflammatory cytokine IL-6 and TNF-α by canine hepatocytes, directing hepatic CYPs to a pro-inflammatory immune environment, and as a consequence, decreasing their activity. However, the inflammation is also balance by the expression of IL-10, that promotes tolerance to the parasite and may avoid that CYPs activity decrease, remaining similar to the control (non-exposed hepatocytes). Inhibition of CYPs by inflammation was particularly evident in the case of BROD (CYP2B11, CYP3A12 and CYP3A26) and phase II UGT for hepatocytes cultured in the 2D system and EROD (CYP1A1 and CYP1A2) and MROD (CYP1A2 and CYP1B) for the hepatocytes cultured as spheroids. Interestingly, exposition to *L. infantum*, especially to amastigotes reduced the levels of BROD metabolization, as well as the phase II UGT, deeper than LPS in 2D-cultured hepatocytes. These observations are in concordance with what is described in literature. In a given infection, the innate immune system is activated via TLRs and NODs receptors and, as previously discussed *L. infantum* presence activates NOD1 and TLR2 of individual hepatocytes associated with the generation of IL-10 and TNF-α and TLR2 in aggregated hepatocytes. The liver responds to infection by increasing acute phase proteins and concomitantly downregulating liver specific proteins involved in drug metabolism (Morgan, 1997; Aitken *et al.*, 2006). CYPs are downregulated by infection and inflammatory stimuli and the rapid reduction of CYP mRNAs has led to consider transcription as the primary mechanism (Morgan, 1997). This hypothesis is supported by the magnitude and speed of transcriptional downregulation as demonstrated in rats *in vivo* (Cheng *et al.*, 2003). In humans, this downregulation is associated with decreased drug clearance and can result in increased incidence of drug toxicity (Aitken and Morgan, 2007). It is also important to investigate this possibility in veterinary medicine as dogs are more often treated for canine leishmaniasis. Understand that canine hepatocytes are also influenced by the inflammatory response may help to better direct the treatment. According to Aitken and Morgan (2007), three lines of evidence support a major role of inflammatory cytokines as the mediators of CYP regulation in the liver during inflammation. First, cytokines can deregulate CYP expression in cultures of

rodent and human hepatocytes; secondly, mice with null mutations in cytokine or cytokine receptor genes display less CYP downregulation in response to some inflammatory stimuli and finally CYP dependent drug clearance has been correlated inversely with plasma levels of IL-6 in tumor human patients. However, the reason for the inflammatory process to be able to downregulate CYPs remains in discussion. Despite the possibility that downregulation of P450 constitutes a pathophysiological consequence of inflammatory processes, there are three major properties of CYP450 that offer a possible explanation for the organism find advantageous to suppress hepatic CYP450 enzymes during inflammation. The first came from models of inflammation (such as injection of LPS or a model of bacterial sepsis) that induce iNOS and, by doing so causing reduction of cellular glutathione available in the hepatocyte (Harbrecht *et al.*, 1997). Reaction of NO with superoxide (from CYP450s) generates the highly reactive peroxynitrite, which can result in protein oxidation and nitration reactions, and depletion of reduced glutathione would render the cell more susceptible to oxidative stress. Thus, CYP450 downregulation could be a mechanism to protect the cell from the deleterious effects of oxidizing species (Aitken and Morgan, 2007). Also, the CYPs suppression would allow cells to allocate all the energy resources in the protein producing machinery to the inflammatory response in order to rapidly control the invading pathogen. Another reason for downregulation of CYP450 enzymes in the liver during inflammation could be related to the ability of hepatocytes regulate NO, by producing an extra burst of NO or reducing its production, attenuating the immune response (Aitken and Morgan, 2007). In the present study, the parasite did not stimulate NO production by dog hepatocytes. So, the most probable cause for the observed CYP450 decrease in activity is related to the generated cytokines. Milosevic *et al.* (1999) evaluated the responses of hepatocytes co-cultured with KCs in comparison with hepatocytes cultured alone, showing that suppression of phenobarbital-induced CYP2B1 mRNA by LPS was mediated by TNF-α release from KCs. Therefore, is possible to hypothesize that the response of isolated hepatocytes is limited when compared with the total potential of a complete coordinated organ response. Even so, isolated hepatocytes exhibited a similar response to what would happen in the whole organ. Samant *et al.* (2003) assessed the metabolism of the hamster liver infected with *L. donovani*. A significant decrease in the level of CYP450 was observed concomitantly

with the maximum parasite load in the liver. The decrease in CYP450 level was accompanied by a significant increase of liver enzymes and tissue degeneration. The impairment in CYP450 metabolism was isoezyme-specific and associated with the induction of iNOS. Interestingly, treatment of the infected animals with a combined therapy showed reduction in parasite load and reverted CYP450 impairment closed to normal values. Regarding phase II liver enzymes the assessed UDP-glucuronosyl transferases are the key enzymes in the formation of glucuronide conjugates, constituting the most important detoxification pathway of the Phase II drug metabolism in all vertebrates. In humans, approximately 40–70% of all clinical drugs are subjected to glucuronidation reactions metabolized by UGTs (Wells *et al.*, 2004). UGT enzymes are responsible for the metabolism of many xenobiotics and endobiotics (e.g.: bilirubin, steroid hormones and fat-soluble vitamins) (Jancova *et al.*, 2010). These enzymes can also be induced by several drugs as analgesics, antivirals and anticonvulsants, which may act as putative UDP-glucuronosyltransferase inducers in humans. However, their role in inflammation is not yet completed described and is poorly exploited. In the present work UGT activity was found to be reduced in hepatocytes exposed to inflammatory stimuli such as *L. infantum* amastigotes, promastigotes or LPS. The most probable cause of inflammatory environment reduction is the presence of IL-6 and TNF-α. In order to keep the balance, phase I and phase II enzyme metabolization rate should be coordinated, as imbalance may lead to the accumulation of potentially harmful products. In the present situation, the decrease of CYP450 was accompanied by the metabolization reduction or stabilization by phase II UGT in order to keep the balance in the hepatocytes.

Beyond inflammation, another factor such as age, sex and polymorphisms can also influence CYPs metabolizing rate. The canine population included in this study did not have restriction on sex and age (preference was made for young adults) and polymorphism were not assessed although there are two important CYP polymorphism described in dogs. Sex influences a number of pharmacokinetically important parameters, including body weight, fat distribution, liver blood flow, as well as expression of drug metabolizing enzymes and transporters (Beierle *et al.*, 1999). Sex-specific expression of CYP450 are common in laboratory animals including rats and mice and was shown to be controlled by the different secretion profiles of growth

hormone in female versus male animals (Dhir and Shapiro, 2003). Most clinical studies indicate that women metabolize drugs more quickly than men. This is particularly the case of substrates as erythromycin, midazolam or verapamil for the major drug metabolizing CYP450, the CYP3A4 (Cotreau *et al.*, 2005). Age is also a well-established influential factor for drug metabolism capacity, particularly at the extremes of life, where drug metabolism capacity appears to be substantially lower (Zanger and Schwab, 2013). Polymorphisms have also been identified as to be able to impact in CYPs metabolization. The most comprehensively studied of canine CYP genetic polymorphism is the premature stop codon mutation (c.1117C>T; R373X) located in the coding region of the CYP1A2 gene that results in complete loss of hepatic CYP1A2 protein and associated enzyme activity (Tenmizu *et al.*, 2004). However, animals that had at least one normal CYP1A2 copy (heterozygotes) did not have substantially different drug metabolization from the wild-type dogs. Other canine CYPs with known genetic polymorphisms include CYP2C41 (gene deletion), as well as CYP2D15, CYP2E1, and CYP3A12 (coding SNPs). However, the impact of these variants on drug metabolism *in vitro* or on drug pharmacokinetics is unknown (Court, 2013). Other factors that can influence the CYP450 activities are related to food intake and also to disease state. As discussed above, during infection, inflammation and cancer, circulating pro-inflammatory cytokines such as IL-1β, TNF-α and IL-6, which act as signaling molecules to elicit marked changes in liver gene expression profiles, lead to severe downregulation of many drugs metabolizing enzymes (Aitken *et al.*, 2006).

In the present work were tested three potential new leishmanicidal compounds isolated from natural sources: ursolic acid, chalcone-8 and quercetin. The ursolic acid (URA) used in the present study was extracted from *Petiveria alliaceae* (Phytolaccaceae). URA is a triterpenoid compound that widely occurs in nature in the free acid form or as an aglycone precursor for triterpenoid saponins (Liu, 1995). URA diversity is highly associated with the broad range of pharmacological effects, and different studies already showed that these compounds were effective against *L. major, L. donovani, L. infantum, L. amazonensis, L. mexicana, L. braziliensis* and *L. panamensis,* suggesting that these classes of compounds present strong leishmanicidal activity (da Silva Filho *et al.*, 2009; Inocêncio da Luz *et al.*, 2011; Passero *et al.*, 2011).

However, the mechanism of action of this compound is not yet described, but appears to be directed to amastigote forms of the parasite (Passero *et al.*, 2011). Chalcone is an aromatic ketone and an enone that forms the central core for a variety of important biological compounds, which are known collectively as chalcones or chalconoids. Several chalcones have been described as potential leishmanicidal compounds against promastigotes of *L. braziliensis, L. amazonensis, L. donovani, L. infantum, L. major* and *L. enrietti* (Borges-Argáez *et al.*, 2007; Andrighetti-Fröhner *et al.*, 2009; de Mello *et al.*, 2014). In some cases, the estimate leishmanicidal activity is even superior to the standard drug (Boeck *et al.*, 2006). However, the toxicity is variable between the chalcone analogous. In the present study, a synthetic analogue of a natural occurring chalcone, the chalcone-8, isolated from *Piper aduncum* (Silva, 2014) was used against *L. infantum* amastigotes in culture with hepatocytes. The third compound was quercetin, a flavonoid. This compound is naturally present in several plants and was isolated from *Kalanchoe pinnata* (Muzitano *et al.*, 2006). In nature, quercetin is found in its glycosidated form, quercitrine, and is during digestion that is transformed in quercetin. This flavonoid has been associated with the inhibition of MRP1 transporter in cancer cells (Leslie *et al.*, 2001), efflux pumps in fungus (Roohparvar *et al.* 2007) and recently inhibition of arginase in *L. amazonenis* (Manjolin *et al.*, 2013). Leishmanicidal activity on promastigotes of *L. donovani* (Mittra *et al.*, 2000) and *L. amazonensis* (Montrieux *et al.*, 2014) have recently proved, and its administration in conjugation with other drugs also have been investigated (Sen *et al.*, 2005). Torres-Santos *et al.*, (2003) presented a clinical case where the cutaneous lesion was reverted with the application of quercetin. However, due to some problems with delivery and potential complexation with other compounds, the incorporation of these compounds in nanoparticle formulations has also been investigated (Das *et al.*, 2013). Meglumine antimoniate (MgA) is a classical leishmanicidal drug belonging to the pentavalent antimonial class. In this study, it was used a commercial version, Glucantime®. MgA mechanism of action is still not fully understood. Initial studies suggested that antimonials inhibit the biosynthesis of macromolecules in the amastigotes, possibly by perturbing the energy metabolism caused by the inhibition of glycolysis and fatty acid beta-oxidation. Sodium stibogluconate, another antimonial formulation, specifically inhibits type I DNA topoisomerase, thus reducing unwinding and cleavage. A similar mechanism is also

described for MgA (Lucumi *et al.*, 1998). In the present study these four leishmanicidal compounds were added to hepatocytes exposed to *L. infantum* amastigotes for a long period (72 h). The results corroborate what has been described in literature for the new leishmanicidal compounds. All new drugs (URA, CH-8 and QC) quickly (in 5 h after drug contact) decrease amastigote viability to to non-detectable levels, constituting promising leishmanicidal drugs, probably more effective than MgA. Hepatocyte viability was not affected even after a long period of contact with the drugs, making the new compounds real hopeful drugs. The addition of leishmanicidal drugs impacts the immune response exhibited by hepatocytes at cytokine generation and at innate immune receptor gene expression. Curiously, MgA treatment increases NOD2, TLR2 and TLR4 gene expression and generates IL-10. This coincides with MgA accentuated leishmanicidal activity. These findings may suggest that amastigote killing ensure the exposition of hepatocytes to new antigens, affecting the immune response. In the 3D-culture system also treated with MgA, augments of NOD1, NOD2, TLR2 and TLR9 found, highlighting the importance of innate immune receptors in controlling the immune response. In this case, IL-10 registered increased gene expression. URA, Ch-8 and QC also increase NOD1 and TLR2 gene expression during the treatment, but TLR2 and TLR9 was kept at similar levels as before treatment. The addition of these compounds also changed the cytokine profile, IL-10 gene expression was increased, but IL-6 and TNF-α was kept at similar levels as before treatment. These findings highlight the importance of NOD1 and TLR2 in sensing *L. infantum* in the liver. The role of TLR4 and TLR9, however, seem more difficult to define.

As TNF-α, the major inflammatory cytokine exhibited a tendency to decreased expression over incubation time with leishmanicidal drugs and the generation of IL-10 to control immune homeostasis, produced an immunological environment propitiate to CYPs regain their activity. CYPs appear also to be involved in the metabolization of the added drugs, as enzymes presented an induced activity. Understanding what are the CYPs that metabolize the used drugs are important in order to avoid future drug-to-drug interaction. CYP1A1 and CYP1A2 (measured in EROD assay) and CYP2B11 (measured in PROD) were increased in all the testes drugs in the first 24 h, specially by MgA and URA. CYP1A2 and CYP1B (measured in MROD assay) were highly induced by the administration of MgA, indicating that this compound (or a metabolite derivated

from it) probably is metabolized by these two CYP450. CYP2B11, CYP3A12 and CYP3A26 (measured in BROD assay) were highly induced by MgA. Using the described homology between species, it is possible to infer that the some drugs, including MgA and URA, will be probable metabolized by human CYP3A4. This information is particularly valuable because human CYP3A4 is the CYP450 responsible for the metabolization of the majority of clinically used drugs and by that some drug-to-drug interaction may be observed or impairment of treatment if administered in simultaneous with other drugs (Zanger and Schwab, 2013). However, the products of phase I metabolism are potentially more toxic than the original molecules, which does not present a problem if the phase II enzymes are functioning at a rate to rapidly neutralize the phase I products as they are formed. This was observed as all the added drugs induced phase II UGT enzyme. However, CH-8 and QC induced the higher increase in UGT activity, indicating that these compounds probably during metabolization probable generate some toxic intermediates that induced phase II functioning at a rate to rapidly neutralize them. Factors which increase the ratio of phase I to phase II activity can upset this delicate balance, producing harmful metabolites faster than they can be detoxified, and increasing the risk of cellular damage (Jancova *et al.*, 2010). In the 3D-system the exposition to promastigotes followed by the addition of MgA induced some morphological alteration on hepatocytes, as evidenced by SEM. Hepatocyte membrane became rough and full of vesicles. However, the initially added *L. infantum* promastigotes were found to be transformed into amastigotes and remain permanently attached to the hepatocyte membrane in low concentrations, compared to the 2D-culture system. Barak *et al.* (2005) reported the development of host-free differentiation systems for *L. donovani*. Previous studies indicated that concomitant exposure to elevated temperatures and acidic pH signals promastigotes to differentiate into amastigotes (Saar *et al.*, 1998; Debrabant *et al.*, 2004). In this study, the authors determined the time course of *L. donovani* differentiation and assessed the role of several parameters. The major observations were: (1) morphological transformation to amastigote-shaped cells initiates 5 h after the signal, (2) differentiation occurs synchronously, while cells arrest at G1, (3) heat induces the cell cycle arrest in promastigotes, (4) subsequently, acidic pH resumed growth and route promastigotes to differentiate into amastigotes and (5) modulators of heat shock can replace heat in the

differentiation signal. In the present study, promastigotes added to the hepatocytes were observed to lose their mobility and gain an oval shape characteristic of amastigotes. Taking this together, it is possible that *L. infantum* parasites promastigotes, incubated at 37 ºC in contact with cells and were transformed into amastigotes or amastigote-*like* forms.

Since the liver constitutes a major metabolic organ, impairment of liver functions may have great impact on individual health. Hepatocyte culture, although not represents the full complexity of the liver or of the organism constitutes effective tools contributing to understanding how hepatocytes can activate and orchestrate an immune response and react to *L. infantum* parasites, testing of new leishmanicidal drugs, increasing our knowledge on this complex and amazing cells.

4.6. Conclusion

In summary, the main conclusions raised in this chapter are the following:

- To the best of our knowledge, this constitutes the first study that investigates *in vitro* the immune response exhibited against *L. infantum* parasites by canine hepatocytes;
- Although this study presented some evidences that *L. infantum* parasite can be internalized by canine hepatocytes, they were not enough to be conclusive;
- Dog hepatocyte 2D- and 3D-culture systems evidence similar metabolic and immune parameters when challenged with *L. infantum* parasites, being good *in vitro* models for the evaluation of drug efficacy and toxicity*;*
- Dog hepatocytes react to *L. infantum* by increasing ferritin accumulation, making difficult the acquisition of iron by the parasite;
- Dog hepatocytes recognize *L. infantum* parasites by activation of NOD1 and NOD2 receptors and by a possible synergistic effect with TLR2. Amastigotes seem to induce higher NOD1 expression than promastigotes.
- *L. infantum* direct hepatocytes to generate pro- and anti-inflammatory cytokines ensuring the immune tolerance that characterizes the liver tissue;
- Inflammation caused by the presence of the parasite induces the decrease of phase I CYP450s and phase II enzymes, in accordance with the literature.
- The new leishmanicidal drugs, ursolic acid, chalcone-8 and quercetin, revealed good hepatic tolerance and good leishmanicidal activity, comparable to the commercial meglumine antimoniate;
- The tested drugs induced an increase in phase I CYP450, indicating a possibility of some the drugs being metabolized by the assessed enzymes, as meglumine antimoniate and ursolic acid;
- When in the presence of parasites, all leishmanicidal drugs used in this study stimulates hepatocytes to generate IL-10, preserving immune homeostasis and ensuring parasite tolerance.

4.7. References

Adamec J, Rusnak F, Owen WG, Naylor S, Benson LM, Gacy AM, Isaya G. 2000. Iron-dependent self-assembly of recombinant yeast frataxin: implications for Friedreich ataxia. Am J Hum Genet. 67(3):549-62.

Adamec J, Rusnak F, Owen WG, Naylor S, Benson LM, Gacy AM, Isaya G, Agius L. 2008. Glucokinase and molecular aspects of liver glycogen metabolism. Biochem J. 414(1):1-18.

Agius L. 2008. Glucokinase and molecular aspects of liver glycogen metabolism. Biochem J. 414(1):1-18. doi: 10.1042/BJ20080595.

Aitken AE, Richardson TA, Morgan ET. 2006. Regulation of drug-metabolizing enzymes and transporters in inflammation. Annu Rev Pharmacol Toxicol. 46:123-49.

Aitken AE, Morgan ET. 2007. Gene-specific effects of inflammatory cytokines on cytochrome P450 2C, 2B6 and 3A4 mRNA levels in human hepatocytes. Drug Metab Dispos.35(9):1687-93.

Akira S, Takeda K. 2004. Toll-like receptor signalling. Nat Rev Immunol. 4:499-511.

Alexander B. 1998. The role of nitric oxide in hepatic metabolism. Nutrition. 14(4):376-90.

Andrighetti-Fröhner CR, Oliveira KN, Gaspar-Silva D, Pacheco LK, Joussef AC, Steindel M, Simões CMO, Souza AMT, Magalhães UO, Afonso IF, Rodrigues CR, Nunes RJ, Castro HC. 2009. Synthesis, Biological Evaluation and SAR of Sulfonamide 4-Methoxychalcone Derivatives with Potential Antileishmanial Activity. European Journal of Medicinal Chemistry 44: 755–763.

Antoniazi S, Price HP, Kropf P, Freudenberg MA, Galanos C, Smith DF, Müller I. 2004. Chemokine gene expression in toll-like receptor-competent and -deficient mice infected with *Leishmania major*. Infect Immun. 72(9):5168-74.

Anzenbacher P, Anzenbacherová E. 2001. Cytochromes P450 and metabolism of xenobiotics. Cell Mol Life Sci. 58(5-6):737-47.

Bandmann O, Vaughan J, Holmans P, Marsden CD, Wood NW.1997. Association of slow acetylator genotype for N-acetyltransferase 2 with familial Parkinson's disease. Lancet. 350(9085):1136-9.

Barak E, Amin-Spector S, Gerliak E, Goyard S, Holland N, Zilberstein D. 2005. Differentiation of *Leishmania donovani* in host-free system: analysis of signal perception and response. Mol Biochem Parasitol. 141(1):99-108.

Barch DH, Rundhaugen LM, Pillay NS. 1995. Ellagic acid induces transcription of the rat glutathione S-transferase-Ya gene. Carcinogenesis. 16:665-668.

Becker I, Salaiza N, Aguirre M, Delgado J, Carrillo-Carrasco N, Kobeh LG, Ruiz A, Cervantes R, Torres AP, Cabrera N, González A, Maldonado C, Isibasi A. 2003. *Leishmania l*ipophosphoglycan (LPG) activates NK cells through toll-like receptor-2. Mol Biochem Parasitol. 130(2):65-74.

Beierle I, Meibohm B, Derendorf H. 1999. Gender differences in pharmacokinetics and pharmacodynamics. Int J Clin Pharmacol Ther 37, 529–547.

Ben-Othman R, Flannery AR1, Miguel DC1, Ward DM2, Kaplan J2, Andrews NW1. 2014. *Leishmania*-mediated inhibition of iron export promotes parasite replication in macrophages. PLoS Pathog. 10(1):e1003901. doi: 10.1371/journal.ppat.1003901.

Boeck P, Falcão CAB, Leal PC, Yunes RA, Filho VC, Torres-Santos EC, Rossi-Bergmann AC. 2006. Synthesis of Chalcone Analogues with Increased Antileishmanial Activity. Bioorganic & Medicinal Chemistry 14: 1538–1545.

Borges-Argáez R, Balnbury L, Flowers A, Giménez-Turba A, Ruiz G, Waterman PG, Peña-Rodríguez LM. 2007. Cytotoxic and antiprotozoal activity of flavonoids from *Lonchocarpus* spp. Phytomedicine. 14(7-8):530-3.

Burchmore RJ, Barrett MP. 2001. Life in vacuoles--nutrient acquisition by *Leishmania* amastigotes. Int J Parasitol. 31(12):1311-20.

Cai Y, Baer-Dubowska W, Ashwood-Smith MJ, Ceska O, Tachibana S, DiGiovanni J.1996. Mechanism-based inactivation of hepatic ethoxyresorufin O-dealkylation activity by naturally occurring coumarins. Chem Res Toxicol. 9(4):729-36.

Campos MA1, Almeida IC, Takeuchi O, Akira S, Valente EP, Procópio DO, Travassos LR, Smith JA, Golenbock DT, Gazzinelli RT. 2001. Activation of Toll-like receptor-2 by glycosylphosphatidylinositol anchors from a protozoan parasite. J Immunol. 167(1):416-23.

Celton-Morizur S, Desdouets C. 2010. Polyploidization of liver cells. Adv Exp Med Biol. 676:123-35.

Chakraborty P, Ghosh D, Basu MK. 2001. Modulation of macrophage mannose receptor affects the uptake of virulent and avirulent *Leishmania donovani* promastigotes. J. Parasitol. 87(5): 1023–7.

Charlton MR. 1996. Protein metabolism and liver disease. Baillieres Clin Endocrinol Metab.10(4):617-35.

Chamaillard M, Hashimoto M, Horie Y, Masumoto J, Qiu S, Saab L, Ogura Y, Kawasaki A, Fukase K, Kusumoto S, Valvano MA, Foster SJ, Mak TW, Nuñez G, Inohara N. 2003. An essential role for NOD1 in host recognition of bacterial peptidoglycan containing diaminopimelic acid. Nat Immunol. 4(7):702-7.

Chang CS, Chang KP.1985. Heme requirement and acquisition by extracellular and intracellular stages of *Leishmania mexicana amazonensis*. Mol Biochem Parasitol. 16(3):267-76.

Cheng PY, Wang M, Morgan ET. 2003. Rapid transcriptional suppression of rat cytochrome P450 genes by endotoxin treatment and its inhibition by curcumin. J Pharmacol Exp Ther. 307:1205–1212.

Coban C, Ishii KJ, Kawai T, Hemmi H, Sato S, Uematsu S, Yamamoto M, Takeuchi O, Itagaki S, Kumar N, Horii T, Akira S. 2005. Toll-like receptor 9 mediates innate immune activation by the malaria pigment hemozoin. J Exp Med. 201(1):19-25.

Cotreau M M, Von Moltke L L, Greenblatt D J. 2005. The influence of age and sex on the clearance of cytochrome P450 3A substrates. Clin Pharmacokinet 44, 33–60.

Court H. 2013. Canine cytochrome P450 (CYP) pharmacogenetics. Vet Clin North Am Small Anim Pract. 43(5): 1027–1038. doi: 10.1016/j.cvsm.2013.05.001.

Da Silva Filho AA, Resende DO, Fukui MJ, Santos FF, Pauletti PM, Cunha WR, Silva ML, Gregório LE, Bastos JK, Nanayakkara NP. 2009. In vitro antileishmanial, antiplasmodial and cytotoxic activities of phenolics and triterpenoids from *Baccharis dracunculifolia* D. C. (*Asteraceae*). Fitoterapia. 80(8):478-82.

Daly AK, Cholerton S, Gregory W, Idle JR. 1993. Metabolic polymorphisms. Pharmacol Ther. 57(2-3):129-60.

Das S, Das J, Samadder A, Bhattacharyya SS, Das D, Khuda-Bukhsh AR. 2013. Biosynthesized silver nanoparticles by ethanolic extracts of *Phytolacca decandra, Gelsemium sempervirens, Hydrastis canadensis* and *Thuja occidentalis* induce differential cytotoxicity

through G2/M arrest in A375 cells. Colloids Surf B Biointerfaces. 101:325-36. doi: 10.1016/j.colsurfb.2012.07.008.

Dayal M, Parmar D, Dhawan A, Dwivedi UN, Doehmer J, Seth PK. 1999. Induction of rat brain and liver cytochrome P450 1A1/1A2 and 2B1/2B2 isoenzymes by deltamethrin. Environ Toxicol Pharmacol. 7(3):169-78.

De Mello TFP, Bitencourt HR, Pedroso RB, Aristides SMA, Lonardoni MVC, Silveira TGV. 2014. Leishmanicidal Activity of Synthetic Chalcones in *Leishmania (Viannia) braziliensis*. Experimental Parasitology 136: 27–34.

De Veer MJ, Curtis JM, Baldwin TM, DiDonato JA, Sexton A, McConville MJ, Handman E, Schofield L. 2003. MyD88 is essential for clearance of *Leishmania major*: possible role for lipophosphoglycan and Toll-like receptor 2 signaling. Eur J Immunol. 33(10):2822-31.

Debrabant A, Joshi MB, Pimenta PF, Dwyer DM. Generation of *Leishmania donovani* axenic amastigotes: their growth and biological characteristics. Int J Parasitol 2004;34:205–17.

Denison MS, Whitlock JP Jr. 1995. Xenobiotic-inducible transcription of cytochrome P450 genes. J Biol Chem. 270:18175-18178.

Dhir RN, Shapiro BH. 2003. Interpulse growth hormone secretion in the episodic plasma profile causes the sex reversal of cytochrome P450s in senescent male rats. Proc Natl Acad Sci U S A 100, 15224–15228.

Drakesmith H, Schimanski LM, Ormerod E, Merryweather-Clarke AT, Viprakasit V, Edwards JP, Sweetland E, Bastin JM, Cowley D, Chinthammitr Y, Robson KJ, Townsend AR. 2005. Resistance to hepcidin is conferred by hemochromatosis-associated mutations of ferroportin. Blood. 106(3):1092-7.

Duarte MI, Mariano ON, Corbett CE. 1989. Liver parenchymal cell parasitism in human visceral leishmaniasis. Virchows Arch A Pathol Anat Histopathol. 415(1):1-6.

Elangovan V, Sekar N, Govindasamy S. 1994. Chemopreventive potential of dietary

Ezekowitz RA, Williams DJ, Koziel H, Armstrong MY, Warner A, Richards FF, Rose RM. 1991. Uptake of Pneumocystis carinii mediated by the macrophage mannose receptor. Nature. 351 (6322): 155–8. doi:10.1038/351155a0.

Faria MS, Reis FC, Lima AP. 2012. Toll-like receptors in *Leishmania* infections: guardians or promoters? J Parasitol Res. 2012:930257. doi: 10.1155/2012/930257.

Festa M, Ricciardelli G, Mele G, Pietropaolo C, Ruffo A, Colonna A. 2000. Overexpression of H ferritin and up-regulation of iron regulatory protein genes during differentiation of 3T3-L1 pre-adipocytes. J Biol Chem 275(47):36708 – 12.

Figueiredo MM, Deoti B, Amorim IF, Pinto AJ, Moraes A, Carvalho CS, da Silva SM, de Assis AC, de Faria AM, Tafuri WL. 2014. Expression of regulatory T cells in jejunum, colon, and cervical and mesenteric lymph nodes of dogs naturally infected with *Leishmania infantum*. Infect Immun. 82(9):3704-12. doi: 10.1128/IAI.01862-14.

Flannery AR, Huynh C, Mittra B, Mortara RA, Andrews NW. 2011. LFR1 ferric iron reductase of *Leishmania amazonensis* is essential for the generation of infective parasite forms. J Biol Chem. 286(26):23266-79.

Galbraith RA, McElrath MJ. 1988. Heme binding to *Leishmania mexicanaamazonensis*. Mol Biochem Parasitol. 29(1):47-53.

Gangneux JP, Lemenand O, Reinhard Y, Guiguen C, Guguen-Guillouzo C, Gripon P. 2005. *In vitro* and *ex vivo* permissivity of hepatocytes for *Leishmania donovani*. J Eukaryot Microbiol.52(6):489-91.

Gao B, Jeong WI, Tian Z. 2008. Liver: An organ with predominant innate immunity. Hepatology. 47(2):729-36. doi: 10.1002/hep.22034.

Gazi U, Martinez-Pomares L. 2009. Influence of the mannose receptor in host immune responses. Immunobiology. 214 (7): 554–61. *doi*:*10.1016/j.imbio.2008.11.004*.

Gentric G, Celton-Morizur S, Desdouets C. 2012. Polyploidy and liver proliferation. Clin Res Hepatol Gastroenterol. 36(1):29-34. doi: 10.1016/j.clinre.2011.05.011.

Girardin SE, Boneca IG, Viala J, Chamaillard M, Labigne A, Thomas G, Philpott DJ, Sansonetti PJ. 2003. Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. J Biol Chem. 278(11):8869-72.

Glatt H.2000. Sulfotransferases in the bioactivation of xenobiotics. Chem Biol Interact. 129(1-2):141-70.

Gruys E, Toussaint MJM, Niewold TA, Koopmans SJ. 2005. Acute phase reaction and acute phase proteins. J Zhejiang Univ Sci B. 6(11): 1045–1056.doi: 10.1631/jzus.2005.B1045.

Guengerich FP. 1984. Effects of nutritive factors on metabolic processes involving bioactivation and detoxification of chemicals. Ann Rev Nutr. 4:207-231

Hagemeyer CE, Bürck C, Schwab R, Knoth R, Meyer RP.2010. 7-Benzyloxyresorufin-O-dealkylase activity as a marker for measuring cytochrome P450 CYP3A induction in mouse liver. Anal Biochem. 398(1):104-11. doi: 10.1016/j.ab.2009.11.004.

Harbrecht BG, Taylor BS, Xu Z, Ramalakshmi S, Ganster RW, Geller DA.2001. cAMP inhibits inducible nitric oxide synthase expression and NF-kappaB-binding activity in cultured rat hepatocytes. J Surg Res. 99(2):258-64.

Haüssinger D. 1990. Nitrogen metabolism in liver: structural and functional organization and physiological relevance. Biochem J. 267(2):281-90.

Heinrich PC, Behrmann I, Müller-Newen G, Schaper F, Graeve L. 1998. Interleukin-6-type cytokine signalling through the gp130/Jak/STAT pathway. Biochem J. 334 (Pt 2):297-314.

Hosein S, Rodríguez-Cortés A, Blake DP, Allenspach K, Alberola J, Solano-Gallego L. 2015. Transcription of Toll-Like Receptors 2, 3, 4 and 9, FoxP3 and Th17 Cytokines in a Susceptible Experimental Model of Canine *Leishmania infantum* Infection. PLoS One. 10(10):e0140325. doi: 10.1371/journal.pone.0140325.

Husson A1, Brasse-Lagnel C, Fairand A, Renouf S, Lavoinne A. 2003. Argininosuccinate synthetase from the urea cycle to the citrulline-NO cycle. Eur J Biochem. 270(9):1887-99.

Huynh C, Sacks DL, Andrews NW. 2006. A *Leishmania amazonensis* ZIP family iron transporter is essential for parasite replication within macrophage phagolysosomes. J Exp Med. 203(10):2363-75.

Inocêncio da Luz RA, Vermeersch M, Deschacht M, Hendrickx S, Van Assche T, Cos P, Maes L. 2011. *In vitro* and *in vivo* prophylactic and curative activity of the triterpene saponin PX-6518 against cutaneous *Leishmania* species. J Antimicrob Chemother. 66(2):350-3.

Inoue H, Ogawa W, Asakawa A, Okamoto Y, Nishizawa A, Matsumoto M, Teshigawara K, Matsuki Y, Watanabe E, Hiramatsu R, Notohara K, Katayose K, Okamura H, Kahn CR, Noda T, Takeda K, Akira S, Inui A, Kasuga M. 2006. Role of hepatic STAT3 in brain-insulin action on hepatic glucose production. Cell Metab. 3(4):267-75.

Isogawa M, Robek MD, Furuichi Y, Chisari FV. 2005. Toll-like receptor signaling inhibits hepatitis B virus replication in vivo. J Virol. 79:7269-7272.

Jacques I, Andrews NW, Huynh C. 2010. Functional characterization of LIT1, the *Leishmania amazonensis* ferrous iron transporter. Mol Biochem Parasitol. 170(1):28-36.

Jancova P, Anzenbacher P, Anzenbacherova E. 2010. Phase II drug metabolizing enzymes. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub. 154(2):103-16.

Johansson S, Forsberg E, Lundgren B.1987. Comparison of fibronectin receptors from rat hepatocytes and fibroblasts. J Biol Chem. 262(16):7819-24.

Johnson RW. 1997. Inhibition of growth by pro-inflammatory cytokines: an integrated view. J Anim Sci. 75(5):1244-55.

Kang YH, Berthiaume F, Yarmush ML. 2002. Long-term stable cultures of rat hepatocytes: an *in vitro* model to study acute and chronic hepatic inflammation. Tissue Eng. 8(4):681-93.

Koorts AM, Viljoen M. 2007. Ferritin and ferritin isoforms I: Structure-function relationships, synthesis, degradation and secretion. Arch Physiol Biochem. 113(1):30-54.

Kovalovich K, DeAngelis RA, Li W, Furth EE, Ciliberto G, Taub R. 2000. Increased toxin-induced liver injury and fibrosis in interleukin-6-deficient mice. Hepatology. 31(1):149-59.

Kropf P, Freudenberg MA, Modolell M, Price HP, Herath S, Antoniazi S, Galanos C, Smith DF, Müller I. 2004. Toll-like receptor 4 contributes to efficient control of infection with the protozoan parasite *Leishmania major*. Infect Immun. 72(4):1920-8.

Lee WM. 1995. Drug-induced hepatotoxicity. N Engl J Med. 333(17):1118-27.

Leslie EM, Mao Q, Oleschuk CJ, Deeley RG, Cole SPC. 2001. Modulation of Multidrug Resistance Protein 1 (MRP1/ABCC1) Transport and ATPase Activities by Interaction with Dietary Flavonoids. Molecular Pharmacology 59:1171–1180.

Liese J, Schleicher U, Bogdan C. 2007. TLR9 signaling is essential for the innate NK cell response in murine cutaneous leishmaniasis. Eur J Immunol. 37(12):3424-34.

Liska DJ. 1998. The detoxification enzyme systems. Altern Med Rev. 3(3):187-98.

Liu J. 1995. Pharmacology of oleanolic acid and ursolic acid. J Ethnopharmacol. 49(2):57-68.

Loyer P, Ilyin G, Abdel Razzak Z, Banchereau J, Dezier JF, Campion JP, Guguen-Guillouzo C, Guillouzo A. 1993. Interleukin 4 inhibits the production of some acute-phase proteins by human hepatocytes in primary culture. FEBS Lett. 336(2):215-20.

Lu Y, Mo C, Zeng Z, Chen S, Xie Y, Peng Q, He Y, Deng Y, Wang J, Xie L, Zeng J, Li S, Qin X. 2013. CYP2D6*4 allele polymorphism increases the risk of Parkinson's disease: evidence from meta-analysis. PLoS One. 8(12):e84413. doi: 10.1371/journal.pone.0084413.

Lucumi A, Robledo S, Gama V, Saravia NG. 1998. Sensitivity of *Leishmania vianniapanamensis* to pentavalent antimony is correlated with the formation of cleavable DNA-protein complexes. Antimicrob Agents Chemother.42(8):1990-5.

Luiking YC, Hallemeesch MM, van de Poll MC, Dejong CH, de Jonge WJ, Lamers WH, Deutz NE. 2008. Reduced citrulline availability by OTC deficiency in mice is related to reduced nitric oxide production. Am J Physiol Endocrinol Metab. 295(6):E1315-22. doi: 10.1152/ajpendo.00055.2008.

Luster MI, Germolec DR, Yoshida T, Kayama F, Thompson M. 1994. Endotoxin-induced cytokine gene expression and excretion in the liver. Hepatology. 19(2):480-8.

Ingelman-SundbergM . 2005. Genetic polymorphisms of cytochrome P450 2D6 (CYP2D6): clinical consequences, evolutionary aspects and functional diversity. The Pharmacogenomics Journal. 5, 6–13. doi:10.1038/sj.tpj.6500285

Manjolin LC, Reis MBG, Maquiaveli CC, Santos-Filho OA, Silva ER. 2013. Dietary Flavonoids Fisetin, Luteolin and Their Derived Compounds Inhibit Arginase, a Central Enzyme in *Leishmania (Leishmania) amazonensis* Infection. Food Chemistry 141: 2253–2262.

Manson MM, Ball HW, Barrett MC, Clark HL, Judah DJ, Williamson G, Neal GE. 1997. Mechanism of action of dietary chemoprotective agents in rat liver: induction of phase I and II drug metabolizing enzymes and aflatoxin B1 metabolism. Carcinogenesis. 18(9):1729-38.

Maródi L, Korchak HM, Johnston RB.1991.Mechanisms of host defense against *Candida* species. I. Phagocytosis by monocytes and monocyte-derived macrophages. J. Immunol. 146 (8): 2783–9.

Martignoni M, Groothuis GM, de Kanter R. 2006. Species differences between mouse, rat, dog, monkey and human CYP-mediated drug metabolism, inhibition and induction. Expert Opin Drug Metab Toxicol. 2(6):875-94.

Meyer UA, Zanger UM, Skoda RC, Grant D, Blum M. 1990. Genetic polymorphisms of drug metabolism. Prog Liver Dis. 9:307-23.

Milosevic N, Schawalder H, Maier P. 1999. Kupffer cell-mediated differential down-regulation of cytochrome P450 metabolism in rat hepatocytes. Eur J Pharmacol. 26;368(1):75-87.

Miranda JP, Leite SB, Muller-Vieira U, Rodrigues A, Carrondo MJ, Alves PM. 2009. Towards an extended functional hepatocyte in vitro culture. Tissue Eng Part C Methods. 15(2):157-67. doi: 10.1089/ten.tec.2008.0352.

Miranda JP, Rodrigues A, Tostões RM, Leite S, Zimmerman H, Carrondo MJ, Alves PM. 2010. Extending hepatocyte functionality for drug-testing applications using high-viscosity alginate-encapsulated three-dimensional cultures in bioreactors. Tissue Eng Part C Methods.16(6):1223-32. doi: 10.1089/ten.TEC.2009.0784.

Mise M, Hashizume T, Komuro S. 2008. Characterization of substrate specificity of dog CYP1A2 using CYP1A2-deficient and wild-type dog liver microsomes. Drug Metab Dispos. 36(9):1903-8.

Mittra B, Andrews NW. 2013. IRONy OF FATE: role of iron-mediated ROS in Leishmania differentiation. Trends Parasitol. 29(10):489-96. doi: 10.1016/j.pt.2013.07.007.

Mittra B, Cortez M, Haydock A, Ramasamy G, Myler PJ, Andrews NW. 2013. Iron uptake controls the generation of *Leishmania* infective forms through regulation of ROS levels. J Exp Med. 210(2):401-16. doi: 10.1084/jem.20121368.

Mittra B, Saha A, Chowdhury AR, Pal C, Mandal S, Mukhopadhyay S, Bandyopadhyay S, Majumder HK. 2000. Luteolin , an Abundant Dietary Component Is a Potent Anti-Leishmanial Agent That Acts by Inducing Topoisomerase II-Mediated Kinetoplast DNA Cleavage Leading to Apoptosis. Molecular Medicine 6: 527–541.

Mizuhara H, O'Neill E, Seki N, Ogawa T, Kusunoki C, Otsuka K, Satoh S, Niwa M, Senoh H, Fujiwara H. 1994. T cell activation-associated hepatic injury: mediation by tumor necrosis factors and protection by interleukin 6. J Exp Med. 179(5):1529-37.

Montrieux E, Perera WH, García M, Maes L, Cos P, Monzote L. 2014. *In vitro* and *in vivo* Activity of Major Constituents from *Pluchea carolinensis* against *Leishmania amazonensis*. Parasitology Research 113: 2925–2932.

Morgan BP, Gasque P. 1997. Extrahepatic complement biosynthesis: where, when and why? Clin Exp Immunol. 107(1):1-7.

Morgan ET. 1997. Regulation of cytochromes P450 during inflammation and infection. Drug Metab Rev. 29(4):1129-88.

Morgan ET. 2001. Regulation of cytochrome p450 by inflammatory mediators: why and how? Drug Metab Dispos. 29(3):207-12.

Mulero V, Brock JH. 1999. Regulation of iron metabolism in murine J774 macrophages: role of nitric oxide-dependent and -independent pathways following activation with gamma interferon and lipopolysaccharide. Blood. 94(7):2383-9.

Murray HW, Nathan CF. 1999. Macrophage microbicidal mechanisms in vivo: reactive nitrogen versus oxygen intermediates in the killing of intracellular visceral *Leishmania donovani*. J Exp Med. 189(4):741-6.

Murray HW. 1997. Endogenous interleukin-12 regulates acquired resistance in experimental visceral leishmaniasis. J Infect Dis. 175(6):1477-9.

Murray HW. 2001. Tissue granuloma structure-function in experimental visceral leishmaniasis. Int J Exp Pathol. 82(5):249-67.

Muzitano MF, Tinoco LW, Guette C, Kaiser CR, Rossi-Bergmann B, Costa SS. 2006. The Antileishmanial Activity Assessment of Unusual Flavonoids from *Kalanchoe pinnata.* Phytochemistry 67: 2071–2077.

Nakagawa R, Naka T, Tsutsui H, Fujimoto M, Kimura A, Abe T, Seki E, Sato S, Takeuchi O, Takeda K, Akira S, Yamanishi K, Kawase I, Nakanishi K, Kishimoto. 2002. SOCS-1 participates in negative regulation of LPS responses. Immunity. 17(5):677-87.

Nairaz M, Theurl I, Ludwiczek S, Theurl M, Mair SM, Fritsche G, Weiss G. 2007. The co-ordinated regulation of iron homeostasis in murine macrophages limits the availability of iron for intracellular*Salmonella typhimurium.* Cell Microbiol. 9:2126–2140.

Nelson DR, Zeldin DC, Hoffman SM, Maltais LJ, Wain HM, Nebert DW. 2004. Comparison of cytochrome P450 (CYP) genes from the mouse and human genomes, including nomenclature recommendations for genes, pseudogenes and alternative-splice variants. Pharmacogenetics. 14(1):1-18.

Ogu CC, Maxa JL.2000. Drug interactions due to cytochrome P450.Proc (Bayl Univ Med Cent). 13(4):421-3.

Ogura Y, Inohara N, Benito A, Chen FF, Yamaoka S, Nunez G. 2001. nod2, a Nod1/Apaf-1 family member that is restricted to monocytes and activates NF-kappaB. J Biol Chem. 276(7):4812-8.

Ohnishi H, Oka K, Mizuno S, Nakamura T. 2012. Identification of mannose receptor as receptor for hepatocyte growth factor β-chain: novel ligand-receptor pathway for enhancing macrophage phagocytosis. J Biol Chem. 287(16):13371-81. doi: 10.1074/jbc.M111.318568.

Oliveira AC, Peixoto JR, de Arruda LB, Campos MA, Gazzinelli RT, Golenbock DT, Akira S, Previato JO, Mendonça-Previato L, Nobrega A, Bellio M. 2004. Expression of functional TLR4 confers proinflammatory responsiveness to *Trypanosoma cruzi* glycoinositolphospholipids and higher resistance to infection with T. cruzi. J Immunol. 173(9):5688-96.

Panesar N, Tolman K, Mazuski JE. 1999. Endotoxin stimulates hepatocyte interleukin-6 production. J Surg Res. 85(2):251-8.

Paramchuk WJ, Ismail SO, Bhatia A, Gedamu L. 1997. Cloning, characterization and overexpression of two iron superoxide dismutase cDNAs from *Leishmania chagasi*: role in pathogenesis. Mol Biochem Parasitol. 90(1):203-21.

Park BK, Kitteringham NR, Pirmohamed M, Tucker GT. 1996. Relevance of induction of human drug-metabolizing enzymes: pharmacological and toxicological implications. Br J Clin Pharmacol. 41(6):477-91.

Passero LF, Bonfim-Melo A, Corbett CE, Laurenti MD, Toyama MH, de Toyama DO, Romoff P, Fávero OA, dos Grecco SS, Zalewsky CA, Lago JH. 2011. Anti-leishmanial effects of purified compounds from aerial parts of *Baccharis uncinella* C. DC. (Asteraceae). Parasitol Res. 108(3):529-36.

Pritchard MT, McMullen MR, Stavitsky AB, Cohen JI, Lin F, Medof ME, Nagy LE. 2007. Differential contributions of C3, C5, and decay-accelerating factor to ethanol-induced fatty liver in mice. Gastroenterology. 132(3):1117-26.

Qin XB, Gao B. 2006. The complement system in liver diseases. Cell Mol Immunol. 3(5):333-40.

Rafael-Fernandes M. 2015. Caracterização da Internalização de fármacos antileishmania e novos compostos por macrófagos parasitados por *Leishmania* spp.. Master Thesis from Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa. 128pp

Roohparvar R, Huser H, Zwiers LH, Waard MA. 2007. Control of Mycosphaerella graminicola on Wheat Seedlings by Medical Drugs Known to Modulate the Activity of ATP-Binding Cassette Transporters. Applied and Environmental Microbiology 73: 5011–5019.

Rui L. 2014. Energy Metabolism in the Liver. Compr Physiol. 4(1): 177–197. doi: 10.1002/cphy.c130024

Saad B, Frei K, Scholl FA, Fontana A, Maier P. 1995. Hepatocyte-derived interleukin-6 and tumor-necrosis factor alpha mediate the lipopolysaccharide-induced acute-phase response and nitric oxide release by cultured rat hepatocytes. Eur J Biochem. 229(2):349-55.

Saar Y, Ransford A, Waldman E, Mazareb S, Amin-Spector S, Plumblee J, Turco SJ, Zilberstein D. 1998. Characterization of developmentally-regulated activities in axenic amastigotes of *Leishmania donovani*. Mol Biochem Parasitol. 95(1):9-20.

Saar Y, Ransford A, Waldman E, Mazareb S, Amin-Spector S, Plumblee J, Turco SJ, Zilberstein D. 1998. Mol Biochem Parasitol. 95(1):9-20.

Samanta TB, Das N, Das M, Marik R. 2003. Mechanism of impairment of cytochrome P450-dependent metabolism in hamster liver during leishmaniasis. Biochem Biophys Res Commun. 312(1):75-9.

Samaras SC, Dietz N. 1953. Jr., Fed. Proc., 12, 122. Cited by Morgan ET. 1997. Regulation of cytochromes P450 during inflammation and infection. Drug Metab Rev. 29(4):1129-88.

Schindler R, Mancilla J, Endres S, Ghorbani R, Clark SC, Dinarello CA. 1990. Correlations and interactions in the production of interleukin-6 (IL-6), IL-1, and tumor necrosis factor (TNF) in human blood mononuclear cells: IL-6 suppresses IL-1 and TNF. Blood. 75(1):40-7.

Schleicher U, Liese J, Knippertz I, Kurzmann C, Hesse A, Heit A, Fischer JA, Weiss S, Kalinke U, Kunz S, Bogdan C. 2007. NK cell activation in visceral leishmaniasis requires TLR9, myeloid DCs, and IL-12, but is independent of plasmacytoid DCs. J Exp Med. 204(4):893-906.

Schwabe RF, Seki E, Brenner DA. 2006. Toll-like receptor signaling in the liver. Gastroenterology. 130:1886-1900

Sen G, Mandal S, Roy SS, Mukhopadhyay S, Biswas T. 2005. Therapeutic Use of Quercetin in the Control of Infection and Anemia Associated with Visceral Leishmaniasis. Free Radical Biology & Medicine 38: 1257–1264.

Sesti S, Martino G, Mazzulla S, Chimenti R. 2005. Effect of bradykinin on nitric oxide production, urea synthesis and viability of rat hepatocyte cultures. BMC Physiol. 5(1):2.

Silva CIM. 2014. Eficácia Da Quercetina Na Leishmaniose Visceral Murina: Associação Com a Chalcona CH8, Formulações Nanoestruturadas E Imunomodulação. Thesis

Universidade Federal do Rio de Janeiro, Centro de Ciências da Saúde Instituto de Biofísica Carlos Chagas Filho, Rio de Janeiro: 1-148.

Silvestrini P, Zoia A, Planellas M, Roura X, Pastor J, Cerón JJ, Caldin M. 2014. Iron status and C-reactive protein in canine leishmaniasis. J Small Anim Pract. 55(2):95-101. doi: 10.1111/jsap.12172.

Slaughter RL, Edwards DJ. 1995. Recent advances: the cytochrome P450 enzymes. Ann Pharmacother. 29(6):619-24.

Soji T, Murata Y, Ohira A, Nishizono H, Tanaka M, Herbert DC. 1992. Evidence that hepatocytes can phagocytize exogenous substances. Anat Rec. 233(4):543-6.

Soria LR, Marrone J, Molinas SM, Lehmann GL, Calamita G, Marinelli RA. 2014. Lipopolysaccharide impairs hepatocyte ureagenesis from ammonia: involvement of mitochondrial aquaporin-8. FEBS Lett. 588(9):1686-91. doi: 10.1016/j.febslet.2014.03.012.

Stanya KJ, Jacobi D, Liu S, Bhargava P, Dai L, Gangl MR, Inouye K, Barlow JL, Ji Y, Mizgerd JP, Qi L, Shi H, McKenzie AN, Lee CH. 2013. Direct control of hepatic glucose production by interleukin-13 in mice. J Clin Invest. 123(1):261-71.

Streetz KL, Luedde T, Manns MP, Trautwein C. 2000. Interleukin 6 and liver regeneration. Gut.47(2):309-12.

Stijlemans B, Vankrunkelsven A, Brys L, Magez S, De P. 2008. Role of iron homeostasis in trypanosomiasis-associated anemia. Immunobiology. 213:823–835.

Sumpter R Jr, Loo YM, Foy E, Li K, Yoneyama M, Fujita T, Lemon SM, Gale M Jr. 2005. Regulating intracellular antiviral defense and permissiveness to hepatitis C virus RNA replication through a cellular RNA helicase, RIG-I. J Virol. 79(5):2689-99.

Sunman J, Hawke R, LeCluyse E, Kashuba A. 2004. Kupffer cell-mediated IL-2 suppression of CYP3A activity in human hepatocytes. Drug Metabolism and Disposition. 32 (3). Pag. 359-363

Szabo G, Dolganiuc A, Mandrekar P. 2006. Pattern recognition receptors: a contemporary view on liver diseases. HEPATOLOGY. 44:287-298

Tabuchi S, Gotoh T, Miyanaka K, Tomita K, Mori M. 2000. Regulation of genes for inducible nitric oxide synthase and urea cycle enzymes in rat liver in endotoxin shock. Biochem Biophys Res Commun. 268: 221-224. 10.1006/bbrc.2000.2105.

Tenmizu D, Endo Y, Noguchi K, Kamimura H. 2004. Identification of the novel canine CYP1A2 1117 C > T SNP causing protein deletion. Xenobiotica. 34(9):835-46.

Theil EC. 2003. Ferritin: at the crossroads of iron and oxygen metabolism. J Nutr. 133(5 Suppl 1):1549S-53S.

Thomas A, Laxton C, Rodman J, Myangar N, Horscroft N, Parkinson T. 2007. Investigating toll-like receptor agonists for potential to treat hepatitis C virus infection. Antimicrob Agents Chemother. 51:2969-2978.

Torres-Santos EC, Da Silva SA, Costa SS, Santos AP, Almeida AP, Rossi-Bergmann B. 2003. Toxicological Analysis and Effectiveness of Oral *Kalanchoe pinnata* on a Human Case of Cutaneous Leishmaniasis. Phytotherapy Research 17: 801–803.

Tran TN, Eubanks SK, Schaffer KJ, Zhou CY, Linder MC. 1997. Secretion of ferritin by rat hepatoma cells and its regulation by inflammatory cytokines and iron. Blood. 90(12):4979-86.

Tsuji Y, Ayaki H, Whitman SP, Morrow CS, Torti SV, Torti FM.2000. Coordinate transcriptional and translational regulation of ferritin in response to oxidative stress. Mol Cell Biol. 20(16):5818-27.

Turpeinen M, Ghiciuc C, Opritoui M, Tursas L, Pelkonen O, Pasanen M. 2007. Predictive value of animal models for human cytochrome P450 (CYP)-mediated metabolism: a comparative study *in vitro*. Xenobiotica 37, 1367-77.

Ueno N, Wilson ME. 2012. Receptor-mediated phagocytosis of *Leishmania*: implications for intracellular survival. Trends Parasitol. 28(8):335-44. doi: 10.1016/j.pt.2012.05.002.

Verma S, Mehta A, Shaha C. 2011. CYP5122A1, a novel cytochrome P450 is essential for survival of *Leishmania donovani*. PLoS One. 6(9):e25273. doi: 10.1371/journal.pone.0025273.

Wells PG, Mackenzie PI, Chowdhury JR, Guillemette C, Gregory PA, Ishii Y, Hansen AJ, Kessler FK, Kim PM, Chowdhury NR, Ritter JK. 2004. Glucuronidation and the UDP-glucuronosyltransferases in health and disease. Drug Metab Dispos. 32(3):281-90.

Whitaker SM, Colmenares M, Pestana KG, McMahon-Pratt D. 2008. *Leishmania pifanoi* proteoglycolipid complex P8 induces macrophage cytokine production through Toll-like receptor 4. Infect Immun. 76(5):2149-56. doi: 10.1128/IAI.01528-07.

Wilkinson GR. 2005. Drug metabolismand variability among patients in drug response. N Engl J Med. 352:2211–21.

Wilson ME, Lewis TS, Miller MA, McCormick ML, Britigan BE. 2002. *Leishmania chagasi:* uptake of iron bound to lactoferrin or transferrin requires an iron reductase. Exp Parasitol.100(3):196-207.

Wilson ME, Vorhies RW, Andersen KA, Britigan BE. 1994. Acquisition of iron from transferrin and lactoferrin by the protozoan *Leishmania chagasi*. Infect Immun. 62(8):3262-9.

Wolfert MA, Murray TF, Boons GJ, Moore JN. 2002. The origin of the synergistic effect of muramyl dipeptide with endotoxin and peptidoglycan. J Biol Chem. 77(42):39179-86.

Wu G. 1995. Urea synthesis in enterocytes of developing pigs. Biochem J. 312, 717–723.

Xu C, Li CY, Kong AN. 2005. Induction of phase I, II and III drug metabolism/transport by xenobiotics. Arch Pharm Res. 28(3):249-68.

Yarovinsky F, Zhang D, Andersen JF, Bannenberg GL, Serhan CN, Hayden MS, Hieny S, Sutterwala FS, Flavell RA, Ghosh S, Sher A. 2005. TLR11 activation of dendritic cells by a protozoan profilin-like protein. Science. 308(5728):1626-9.

Zanger UM, Schwab M. 2013. Cytochrome P450 enzymes in drug metabolism: regulation of gene expression, enzyme activities, and impact of genetic variation. Pharmacol Ther. 138(1):103-41. doi: 10.1016/j.pharmthera.2012.12.007.

Zhou SF. 2009. Polymorphism of human cytochrome P450 2D6 and its clinical significance: Part I. Clin Pharmacokinet. 48(11):689-723. doi: 10.2165/11318030-000000000-00000.

CHAPTER 2

Interaction of canine liver Kupffer cells and *L. infantum*

5 Chapter 2: Interaction of canine liver Kupffer cells and *L. infantum*

5.1. Introduction

5.1.1. Mononuclear phagocytic system: monocytes and macrophages

5.1.1.1. Circulating monocytes

Monocytes are members of the mononuclear phagocytic system, which constitute a crucial defense system for host nonspecific antimicrobial defense and tumor surveillance. These cells are also a critical effector component of the innate immune system, equipped with chemokine receptors and adhesion molecules are easily recruited to sites of infection. Monocytes secrete inflammatory cytokines, take up cells and toxic molecules, thus contributing to the immune defense against bacterial, protozoa, and fungal pathogens. Monocytes can kill bacteria by producing reactive nitrogen intermediates (RNIs), reactive oxygen intermediates (ROIs) and through the action of phagolysosomal enzymes. However, these cells also constitute the host cells for *Leishmania* infection, replication and permanent establishment inside the host. *Leishmania* parasites are well adapted to the aggressive conditions inside phagolysosome, being able to influence and control the immune activation of macrophages (MØs) (Mauël, 1990).

Monocytes are established circulating precursors for tissue macrophages and dendritic cells (DCs) and originate from a common myeloid progenitor cell in the bone marrow that is shared with neutrophils. Monocytes are released into the bloodstream as non-differentiated cells and circulate in the blood for 1 or 3 days in the case of mice and humans, respectively (Tacke and Randolph, 2006). Following tissue recruitment, monocytes can differentiate into tissue MØs or myeloid DCs, replenishing the existing populations and contributing to homeostasis maintenance, host defense and to tissue remodeling and repair (Gordon and Taylor, 2005). Macrophages have been recognized to play a central role in the immune response. They are mononuclear phagocytic cells,

which are involved in pathogen recognition, clearance, antigen processing and presentation, inflammation and tissue repair as well as pro-and anti-tumoral responses. Tissue MØs perform a range of important homeostatic functions and exist in many different tissue-specific forms: microglia in the brain, alveolar MØs in the lung, KCs in the liver, osteoclast in the bone as well as sub-capsular sinusoidal/modular MØs associated with spleen, intestine, bone marrow and lymph node tissues. Thus, MØs are central to innate immune inflammatory mechanisms and in priming the adaptive immune responses to both intracellular and extracellular pathogens as well as participate in the immune regulation and tumor surveillance (Foey, 2014). Circulating monocytes constitute approximately 5–10% of peripheral blood leukocytes and exhibit morphological heterogeneity. The heterogeneity among human monocytes is known since 1989 (Passlick *et al*., 1989). The differential expression of CD14 (part of the receptor for bacterial lipopolysaccharide -LPS) and CD16 (also known as FcγRIII) was initially traced in order to define two major subsets in peripheral blood: the so-called "classical" $CD14^{+}CD16^{-}$ monocytes, typically representing up to 80% of the monocytes in a healthy individual, and the "non-classical" $CD14^{low}CD16^{+}$ monocytes comprising the remaining fraction of monocytes. These subsets differ in many respects, including the adhesion molecules and in the expression of chemokine receptor (CCR). $CD14^{+}CD16^{-}$ monocytes express CCR2, CD62L (L-Selectin) and FCγ RI (CD64) whereas $CD14^{low}CD16^{+}$monocytes lack CCR2 but have higher levels of MHCII and FCγRII (CD32) (Tacke and Randolph, 2006). It is important to refer that considerable heterogeneity is known to exist in the minor $CD16^{+}$ monocyte fraction. An "intermediate" population of monocytes that is $CD14^{+}$ (as opposed to $CD14^{low}$) and $CD16^{+}$ differentiates *in vitro* differently than $CD14^{low}CD16^{+}$ monocytes (Grage-Griebenow *et al*., 2001). The interest in this $CD16^{+}$ monocytes has been driven in part by observations showing that this monocyte subset was elevated in blood during inflammatory conditions, carcinoma, atherosclerosis (Schlitt *et al*., 2004), rheumatoid arthritis (Baeten *et al*., 2000) and cancer (Saleh *et al*., 1995), linking this $CD16^{+}$ monocytes to the context of chronic inflammation. For mouse blood monocytes, a subdivision into three subsets, classical, intermediate and non-classical, similar to humans is also proposed.

5.1.1.2. Kupffer cell – the liver resident macrophages

Kupffer cells (KCs), the resident MØs of the liver, comprise the largest population of resident tissue MØs in the body. First described by Karl Wilhelm von Kupffer in 1876 as "sternzellen" (star cells or stellate cells), KC were first thought to be a part of the endothelium of the liver blood vessels. Only in 1898, Tadeusz Browiecz correctly identified these cells as MØs (Naito *et al*., 1997). KCs are now defined as MØs of the hepatic sinusoid evidencing defined MØs characteristics such as active phagocytosis, reactivity to anti-macrophage monoclonal antibodies and proliferative capacity. As resident MØ, KCs play an essential role in the innate immune response. The preferential localization of KCs in the hepatic sinusoid allows them to efficiently phagocyte pathogens entering from the portal or arterial circulation, functioning as the first line of defense against particulates and immunoreactive material coming from the gastrointestinal tract via the portal circulation. Their location within the hepatic sinusoids enable an intimate contact with circulating blood, including with blood-derived leukocytes, facilitating their role in the clearance of bacterial products and interaction. KCs have been implicated in both immunogenic and tolerogenic immune reactions. These cells eliminate pathogens, including bacteria and parasites and may directly kill tumor cells by receptor-mediated phagocytosis or release tumor necrosis factor-α (TNF-α), oxygen metabolites, and proteinases. As professional antigen-presenting cells (APCs), can also promote adaptive immune responses by processing and presenting antigen via class I and class II of major histocompatibility complex (MHC). In case of pathogenic infection or tissue damage, the release of cytokines from activated MØ recruits other cells of the immune system to control inflammation, infection and to participate in tissue repair (Seki *et al*., 2000). KCs thus comprise the major phagocytic activity of what was classically termed the reticular-endothelial system and now more properly called the mononuclear phagocytic system (Dixon *et al*., 2013).

5.1.1.2.1. KC origin

The origin of KCs and how homeostasis of the cell population is maintained over time still are controversy. Classically, tissue MØs are not self-renewing and are replenished from bone marrow–derived monocytes (Dulk *et al*., 1979). However, it has been suggested that KCs are a self-renewing population that replicate as mature cells or originate from local intrahepatic progenitors (Yamamoto *et al.*, 1996). This issue assumes practical importance in clinical situations such as myeloid diseases treated with bone marrow allografts and also during liver transplantation. In both situations, large self-renewing APC populations that are not readily replaced from bone marrow sources are likely to affect the specificity of immune responses. In adult mammals, monocytes of the peripheral blood originated from precursor cells in the bone marrow and are considered to be immature precursors for tissue MØs. Peripheral blood monocytes can enter the liver and then mature into a phenotype characteristic of tissue MØs. MØs differentiation is regulated by various growth factors, but the role of macrophage colony stimulating factor (M-CSF) appears to be crucial for the development of mature KCs (Naito *et al.*, 1997). Klein *et al*., (2007) found evidence of the presence of two distinct KC populations within the liver, depending on the method of evaluation. Multiparameter flow cytometry of isolated hepatic leukocytes, a method frequently used to analyze the frequency of both intrahepatic and extrahepatic leukocyte populations, indicated a clear predominance of bone marrow–derived KCs that were rapidly replaced after irradiation reconstitution. However, *in situ* analysis by immunohistologic staining revealed a large population of residual non–bone marrow–derived KCs – the sessile KCs. While both the bone marrow–derived and the sessile KCs shared the same morphology and phagocytic capabilities, only the bone marrow–derived KCs engaged in inflammatory responses. Thus, the term “sessile” alludes both to their lack of rapid turnover and to their lack of capacity for local recruitment, inducing tolerance. However, the local precursor for these sessile KCs was not found and the model of a single lineage is still debated. Although it is known that the amount of KCs in the liver is tightly maintained, the mechanisms responsible for this control are not well understood. It is clear that the rate of influx of peripheral monocytes into the liver is higher than in other tissues, such as the lung. But, the KC life span in the liver still is debated. Studies performed in KC depleted animals, either in response to clodronate or

in bone marrow transplants revealed that KC replacement occurs over 14 to 21 days (Naito *et al*., 1997). However, under physiological circumstances the KC fate is not understood. It was hypothesized that the KC turnover may occur due to programmed cell death (apoptosis) and/or migration to other sites, such as lymph nodes. Recent data suggest that in response to Th2 inflammatory signals, such as IL-4, resident MØ, including KCs can proliferate after stimulation (Jenkins *et al*., 2011).

5.1.1.2.2. KC localization within the liver architecture

The liver is a complex organ constituted by several different cell types distribute within the sinusoidal structure of the liver. Hepatocytes, which encompass the majority of the liver are considered the key cells of the liver and carry out a vast array of metabolic, regulatory and toxicological functions. The hepatic sinusoid is lined with a specialized liver sinusoidal endothelial cell characterized by the presence of fenestrae. KCs, as well as other cells of the innate immune system, including NK cells, NKT cells, and dendritic cells reside within the sinusoid (Figure 7). KCs exhibit an amoeboid shape and adhere to the surface of fenestrated sinusoidal endothelial cells. The surface of KCs bears microvilli and lamellipodia, extending their projections in all directions. Cytoplasm is enriched with dense bodies and vacuoles of various sizes. Some of vacuoles are lysosomes staining positive for acid phosphatase. Golgi apparatus, coated vesicles, pinocytic vesicles, ribosomes, centrioles, microfilaments, and microtubules are also present in the cytoplasm. KCs nuclei are ovoid and occasionally lobulated. Peroxidase activity is observed in the rough endoplasmic reticulum (RER), nuclear envelope, and lamellae. Wormlike structures, fuzzy coat, microvilli, and peudopodia are KCs structural components involved in endocytic mechanisms. KCs incorporate large particles, such as erythrocytes and bacteria, by phagocytosis and take up small particles and molecules via pinocytic vesicles (Naito *et al*., 2004).

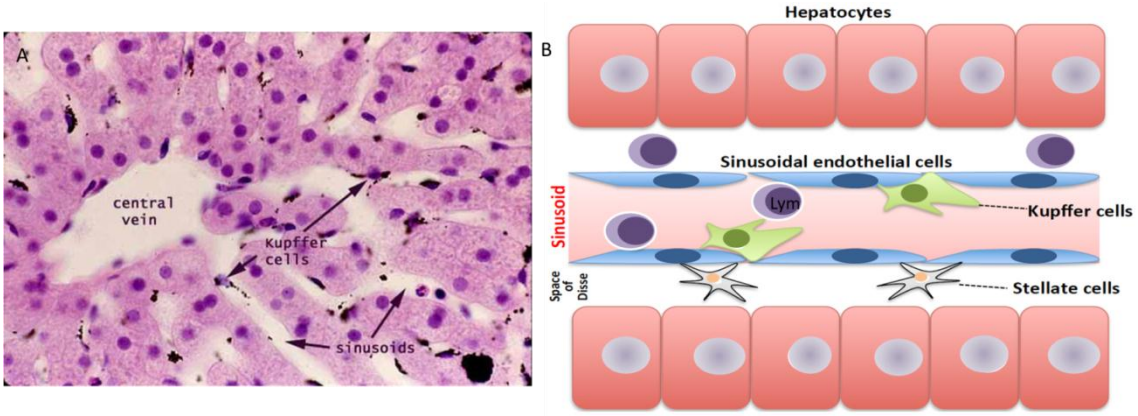


Figure 57: Hepatic localization of KC.
A) Histological preparation of liver tissue from mice which was injected intravenously with a suspension of carbon particles. These particles are actively phagocytized by Kupffer cells (macrophages), whose cytoplasm becomes packed with black carbon particles (1000*x* magnification). B) Schematic structure of the liver. Hepatic sinusoids are constituted by sinusoidal endothelial cells and KCs. Blood circulates within the sinusoids in the liver. Stellate cells have implications on hepatic development, regeneration, xenobiotic responses, intermediary metabolism, and immunoregulation. Lym: lymphocytes, including NK cells, NKT cells, and other lymphocytes. [Adapted from http://www.siumed.edu/~dking2/erg/GI166b.htm and Tsutsui and Nishiguchi, 2014].

The close proximity of KCs to parenchymal and non-parenchymal cells within the liver supports the ability of these cells to regulate hepatic function, both in health and disease. In a healthy liver, KCs exhibit a tolerogenic phenotype, preventing excessive and undesired immune responses when faced with incoming immunoreactive materials, including gut-derived materials and antigens of dead or dying cells as they are cleared from bloodstream by the liver (Thomson and Knolle, 2010). However, under certain conditions as is the case of chronic inflammatory diseases, KCs shift from tolerance to pathologically activation. As these cells are in close proximity with hepatocytes and several non-parenchymal cells, loss of the tolerogenic state can render cellular injury and liver damage. Thus, like many other components of the innate immune system, maintenance of the appropriate balance between activation and tolerance is critical to preserve a healthy organism. Absence or lowered functional activity of KCs can contribute to pathogen invasion and/or systemic inflammation. On the other hand, activation may result in uncontrolled inflammatory state in the liver.

5.1.2. MØ immune activation

MØ and neutrophils are cells of the innate immune system, which provide a first line of defense against many common microorganisms, being essential for the control of common infections. The cells of the innate immune system are equipped with pathogen-recognition receptors (PRRs) are able to recognize both pathogens-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs), which are involved in the activation and control of the innate immune response. PAMPs represent exogenous stimuli arriving from infectious agents, while DAMPs are endogenous indicators of stress. Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) are the two major classes of PRRs that recognize both PAMPs and DAMPs. However, there are some pathogens able to negatively modulate crucial pathways, rending cells unable to control infection. Lymphocytes of the adaptive immune system have evolved to provide a more versatile means of defense which, in addition, provides increased protection against subsequent re-infection with the same pathogen, through the existence of memory cells. Cells of the innate immune system, however, play a crucial role in the initiation and subsequent direction of adaptive immune responses, as well as participating in the removal of pathogens that have been targeted by an adaptive immune response. Moreover, since there is a delay of 4–7 days before the adaptive immune response to be initiated, the innate immune response has a key role in controlling infections (Janeway *et al*., 2001).

There is a growing appreciation that the mature phenotype of resident tissue MØs is very plastic, with the MØs functional activity based on inputs from both the local metabolic and immune environment, superimposed on the MØ intrinsic program. In the liver, plasticity can rend changes in KC activation state and/or a recruitment of new monocytes/ MØs. Much of our understanding of MØ plasticity comes from adipose tissue resident MØs and from non-tissue MØs (Fukushima *et al*., 2009). However, the regulation of functional phenotype of hepatic MØs is associated with the progression of various liver diseases, including non-alcoholic steatohepatitis (NASH), alcoholic liver disease (ALD), fibrosis, and hepatocellular carcinoma (Dixon *et al*., 2013). In an attempt to classify these activation states, two extremes of MØ macrophage polarization have been designated as M1 and M2. M1 or classically MØs are characterized by the increased expression of pro-inflammatory cytokines, such as TNF-α, IL-6, IL-12, and of

inducible NO synthase (iNOS). By other hand, M2 or alternatively activated MØs exhibit low expression of pro-inflammatory cytokines, but increased expression of anti-inflammatory mediators, such as IL-10 and IL-1 decoy receptor expression (Gordon and Taylor, 2005). The M1/M2 classification is a simplified view, with data demonstrating that even within each of these groupings there is significant heterogeneity. For example, the M2 grouping has been further subdivided into M2a, M2b, and M2c. These subclasses exhibit distinct marker proteins on their cell surface and are induced by different regulators, presenting distinct functional activity (Mosser and Edwards, 2008). Although the understanding of the precise mechanism involved in the regulation of MØ polarization is still very rudimentary, recent studies have begun to elucidate the specific transcription factors that regulate the switch, including signal transducers and activator of transcriptions (STATs), peroxisome proliferator-activated receptor (PPAR) family members, hypoxia-inducible factor 2α and Kruppel-like factor 4 (Lawrence and Natoli 2011; Liao *et al*., 2011). Strong M1 polarizing factors include STAT1 and interferon-regulatory factor 5 (IRF), while STAT6, IRF-4, and PPARγ are important transcription factors regulating M2 polarization (Lawrence and Natoli, 2011). MicroRNAs also contribute to the regulation of MØ polarization in cultured MØ cell lines (Graff *et al*., 2012). However, data for an *in vivo* role of nicroRNA in regulating KC phenotype is still lacking. Proliferation of M2 MØs in response to IL-4 has also been reported (Jenkins *et al*., 2011). There is also growing evidence that the MØ metabolic state, in particular whether it utilizes glucose or fatty acids as a primary energy source, influences its phenotypic activity (Chawla *et al*., 2011). Indeed, regulators of lipid metabolism, such as the PPAR family members and adiponectin are important mediators of macrophage polarization. This dual regulation of lipid metabolism and polarization is particularly interesting in the light of the contribution of KC and other hepatic MØs for the progression of metabolic liver diseases, such as ALD and NASH, which are initially characterized by deregulation in hepatic lipid metabolism (Dixon *et al*., 2013).

KCs are active phagocytes, which uptake intravascular debris, dead bacterial cells, and other blood-borne particles, and are able to secrete various inflammatory cytokines, including IL-1, IL-6, tumor necrosis factor (TNF), granulocyte-MØ colony-stimulating factor (GM-CSF), and chemokines such as MØ inflammatory

protein 1 alpha (MIP-1α). However, overproduction of such inflammatory mediators by KCs can rend liver injury (Morita *et al*., 2003). KCs, as MØ, express several cell-surface receptor complexes involved in immune stimulation. Lining in the sinusoids, KCs express a wide range of phagocytics and innate recognition receptors, including CD32, complement receptors, Fc receptors, receptors for lectin-containing opsonins such as plasma mannose-binding lectin, adhesion receptors and those that bind to ICAM-1 and to TLRs (notably TLR 2, 3, 4 and 9) and, receptors for polysaccharides of microbial and host origin. Their avid phagocytic activity has been associated with the clearance of blood borne pathogens and the maintenance of immune homeostasis (Naito *et al*., 2004). The expression of high-affinity Fc receptors facilitates phagocytosis of IgG-coated particles, as well as scavenger receptors and receptors for IgA, galactose, mannose are able of directly binding microbial surface components (Ross and Vetvicka, 1993).

After the exposition to various stimuli, KC activation is characterized by a rapid phenotype change. Phagocytosable particles and several soluble substances are able to activate MØs by binding to specific receptors on the plasma membrane. The most important KC activators are the complement factors C3a and C5a, β-glucans from bacteria and fungi or lipopolysaccharide (LPS) and, the endotoxins of Gram-negative intestinal bacteria (Thornton *et al*., 1996; Schieferdecker *et al*., 2001). The role of LPS, a component of the cell wall of gram-negative bacteria and a ligand of TLR4, in triggering TLR4-downstream pathway has been well described.

In addition, high LPS concentrations can indirectly activated KCs by triggering complement activation either in the portal or in the systemic circulation. After activation of complement cascade, cleavage of C3 and C5 leads to the generation of the potent anaphylatoxins C3a and C5a, and the subsequent stimulation of their specific receptors, RC3a and RC5a (Ember and Hugli, 1997). Thus, KC activation can be best described as a wide spectrum of gradually different alterations of the KC phenotype resulting from a complex interplay of various activators and signaling pathways. However, much less is known about the role of other PRRs in the progression of liver injury. Both TLR2 and TLR9 have also been identified as specific contributors to non-alcoholic fatty liver disease and Non-alcoholic Steatohepatitis (NAFLD/NASH) in mouse models (Miura *et al*., 2010; Rivera *et al*., 2010). Based on these few reports, there is a clear need to

expand our knowledge of the role of PRRs in the progression of liver injury and incorporate the likely complex interactions between KCs and multiple PAMPs and DAMPs in the progression of chronic liver injury. In spite of the importance of KCs in the uptake of pathogens, data on their role in the presentation of pathogen-derived antigens are scarce, with most studies focusing on the role of sinusoidal endothelial cells and hepatocytes in the induction of $CD8^+$ T cell tolerance, or the ability of hepatic stellate cells and dendritic cells to prime $CD4^+$, $CD8^+$ and NKT cells.

KCs can also recognize DAMPs. Initially, damaged cells release cytoplasmic and nuclear components, constituting alarm signals that are able to activate tissue resident macrophages. CLEC4E is a transmembrane C type lectin, which has been reported to be involved in initiating the early inflammatory response after necrotic cell death. The subsequent production of pro-inflammatory cytokines and chemokines, including TNF, IL-6, CXCL1, CXCL2, CXCL3, CXCL8, CCL2, CCL3, and CCL4 can stimulate the recruitment of neutrophils and monocytes. Granule proteins discharged from activated neutrophils anchor on endothelial proteoglycans and are recognized by monocytes promoting their firm adhesion. Moreover, azurocidin, IL-37 and cathepsin G proteases release from activated recruited neutrophils. Neutrophil granule proteins can promote *de novo* synthesis of monocyte-attracting chemokines by endothelial cells and MØs. During inflammation resolution, neutrophil apoptosis holds a central position as it stops the recruitment of neutrophils, while the phagocytic clearance of apoptotic neutrophils reprograms MØs throw an anti-inflammatory phenotype (Liaskou *et al*., 2012).

5.1.2.1. MØs in visceral leishmaniasis

Leishmania parasites are obligatory intracellular pathogens and MØ are indispensable for parasite survival, replication, and differentiation. Both neutrophils and MØs are recruited to the infection site and their interaction with parasites significantly influences the outcome of infection (Ribeiro-Gomes *et al*., 2004). However, recent studies suggest that neutrophils recruited to the site of infection are very efficient in parasite uptake. Since neutrophils are short-lived phagocytes, they are believed to serve as intermediate host cells and have been proposed to act as “Trojan horses” used by parasites to get a lift in order to silently enter MØs, thereby avoiding cell activation

(Laufs *et al*., 2002). In fact, *L. major* infection of polymorphonuclear neutrophils (PMNs) leads to significant delay in cell programmed death and secretion of high levels of MIP-1β, which is known to attract MØs to the site of infection (van Zandbergen *et al*., 2004). Recruited MØs phagocytosis free parasites and infected PMNs, becoming the definitive host cells for parasite replication as well as effector cells responsible for parasite destruction. The uptake of *Leishmania* promastigotes by host cells is a classical receptor-mediated process that initiates phagocytosis. The complement receptors (CR)1, CR3 (Mac-1), fibronectin receptor, and the mannose-fucose receptor (MR) on the MØs surface play import roles in promastigote binding (Kane and Mosser, 2000). Interestingly, an earlier study found that the individual ligation of parasites to these receptors does not trigger MØs activation, suggesting that multiple receptor parasite ligands are needed to initiate the appropriate protective response (Aderem and Underhill, 1999). However, some studies report differences between MØ receptors used by metacyclic promastigotes and avirulent ones. *L. infantum* (*chagasi*) metacyclic promastigotes of were described to use CR3 but not MR to be uptake by MØ, in contrast to avirulent promastigotes, which use both receptors to enter the cells. It is already known that CR3 ligation by itself does not trigger NADPH oxidase activation and subsequently the respiratory burst (Sehgal *et al*., 1993), while MR ligation has been shown to promote inflammatory responses (Linehan *et al*., 2000). Thus metacyclic promastigotes avoid using MR receptor during the MØ invasion in order to enhance their intracellular survival.

MØs are also the major effector cells responsible for parasite destruction. MØs can be activated by different signals leading to their development into functionally distinct subsets with different outcomes. Thus, MØs appropriate activation is crucial for the elimination of this intracellular pathogen. MØ activation is generally divided into two functionally distinct spectra: classical and alternative activation. Classical activation is mediated by Th1 and NK cell immune mediators, in particular IFN-γ, which stimulates MØs to produce inducible nitric oxide synthase (iNOS), an enzyme which catalyzes L-arginine to generate nitric oxide (NO) (Liew *et al*., 1990). NO is a toxic molecule that plays a major role in killing intracellular parasites, including *Leishmania*. In the absence of IFN-γ or microbial products such as LPS, iNOS mRNA and the respective protein are undetectable in MØs. In contrast to classical activation,

alternative MØ activation is induced by the Th2 cytokines interleukin 4 (IL-4) and interleukin 13 (IL-13) (Gordon, 2003). It has been demonstrated that IL-4-induces polyamine biosynthesis (via upregulation of arginase), favoring *L. major* parasite survival inside MØs (Kropf *et al*., 2005). Another cytokine that negatively affects classical MØ activation is interleukin 10 (IL-10). Inhibits the respiratory burst and the release pro-inflammatory cytokines, particularly TNF (Gordon, 2003), affecting leishmanicidal activity of infected cells and promoting parasite survival. To avoid being killed by activating MØs, parasites must manipulate the activation of MØ pathways at his favor, ensuring their survival replication and dissemination in the host. Interleukin 12 (IL-12) is a critical cytokine required for $CD4^+$ Th1 development and for IFN-γ production (Mosmann and Coffman, 1989). IL-12 is mainly produced by antigen-presenting cells (APCs) and MØs were initially proposed as the major source of IL-12 in *L. major*-infected mice. Although MØs efficient phagocytosis *Leishmania* parasites, there is evidence that their ability to release IL-12 is selectively impaired by the parasites (Desjardins and Descoteaux, 1998). In addition, infected MØ also directs the production of immune-regulatory cytokines such as IL-10 and TGF-β, cytokines that are known for their inhibitory effect on MØ functions. Certain parasite-derived molecules have been shown to regulate the outcome of *L. major* infection in mice. It was demonstrated that in the absence of phosphoglycan (PG) *Leishmania* parasites are unable to induce early production of IL-4 in BALB/c mice (Liu *et al*., 2009), suggesting that parasite-derived molecules can modulate host adaptive immune response, favoring parasite survival. Similarly, parasites can also induce the expression of immunomodulatory molecules, such as CD200, to inhibit MØ activation (Cortez *et al*., 2011). Taking these evidences together, the balance between the host and parasite factors that control the activation *vs* deactivation of MØ s determines the parasite fate within infected MØ.

In addition to providing a first line of defense against pathogens, KCs are also believed to be involved in downstream events associated with chronic disease. However, concerning the role played by KC in VL, little is described, apart from their notably role in granulomatous inflammation. Granulomas are well-defined mononuclear cell-rich aggregates that ideally serve to contain and control pathogen spread, but when unregulated may also contribute to disease pathology. Experimental infection with

visceralizing species of *Leishmania* provided along with experimental mycobacterial infection, some of the best characterized models for evaluating granuloma form and function, particularly within the hepatic microenvironment (Murray, 2001; Russell *et al*., 2009). In experimental VL, current models of hepatic granuloma formation, suggests that effector cells within the granuloma are largely tissue MØs and influxing blood monocytes fused together and ultimately attracting lymphocytes and monocytes through chemokine secretion. Activation, primarily by IFN-γ and after by other inflammatory stimuli such as TNF is the final common pathway to the functional antimicrobial state (Murray, 2001). BCG studies have provided some additional information on MØ dynamics and T cell motility within hepatic granulomas (Egen *et al*., 2008) but fail to directly address KC function. In spite of the fact that granuloma MØs harbor much of the hepatic pathogen load during experimental VL, presenting their function as antigen presenting cells (APCs) affected, the role of KCs as APC has yet to be directly addressed (Meier *et al*., 2003). Beattie *et al*. (2010) proven for the first time that within the granuloma microenvironment amastigotes loaded-KCs are the principal target for antigen recognition by effector $CD8^+$ T cells. This evidence suggests that if the recognition by $CD8^+$ T cell is vital to generate an immune response. Understand how infected KCs present antigens to $CD8^+$ T cells, as well as others APCs, responsible for induction of $CD8^+$ T cell response would be useful to generate effective prophylactic or therapeutic vaccination.

.

5.2. Chapter objectives

In the present chapter it is proposed to investigate how liver resident macrophages or Kupffer cells (KC) sense and react to *L. infantum* presence. Since KC constitute the target cell of *L. infantum,* in order to better understand the role played by these cells to control the infection in the canine liver several specific objectives were addressed:

- Phenotype characterization of isolated cells regarding the expression of macrophage key characteristics;
- Infection of isolated KC with *L. infantum* amastigotes or promastigotes in order to investigate the parasite/cell adaptation;
- Assess the impact of a commercial leishmanicidal drug to infected KC;
- Explore the impact of canine hepatocytes in infected KC activity;
- Compare the immune profile generated by two different macrophage lineages, blood macrophage *vs* KC, in the presence of *L. infantum*.

Some techniques used in the present work, mainly confocal microscopy, scan electron microscopy (SEM) and flow cytometry, were the result of a generous collaboration with Professor Graça Alexandre-Pires from Faculdade de Medicina Veterinária, Universidade de Lisboa. Additional processing of the images for artificial coloration was performed by Tiago Pereira (MSc) under my tutorial guidance.

.

5.3. Methods

5.3.1. Kupffer cell isolation

Dog KCs were isolated from a liver lobule using a double Percoll® gradient. The protocol used was adapted from Planagumà *et al.* (2002), Froh *et al.* (2003) and Alabrada *et al.* (2007).

A liver lobule was excised and externally cleaned with a 0.9% NaCl solution supplement with penicillin/streptomycin. Then, the lobule was sliced into small pieces with a scalpel and added to 500 ml of Gey's balanced salt solution (GBSS) (Sigma-Aldrich) supplemented with 0.2% (m/v) pronase (Sigma-Aldrich) and 0.8 $\mu g.ml^{-1}$ DNAse (Roche). The tissue small pieces were then left to incubate in agitation (800 rpm) for 1 h at 37 ºC, in a humidified atmosphere with 5% CO_2 (Figure 588A-B). GBSS buffer was used to ensure red blood cell lysis and DNAse was added to avoid cell aggregation as a consequence of cell death. Finally, the enzyme pronase was used to digest the extracellular matrix and the tissue collagen, releasing KCs from the interstitial spaces. Following digestion, cellular suspension was filtered with a falcon cell strainer with 40 µm pore (BD, Bioscience) and centrifuged at 600 *x*g for 5 min.

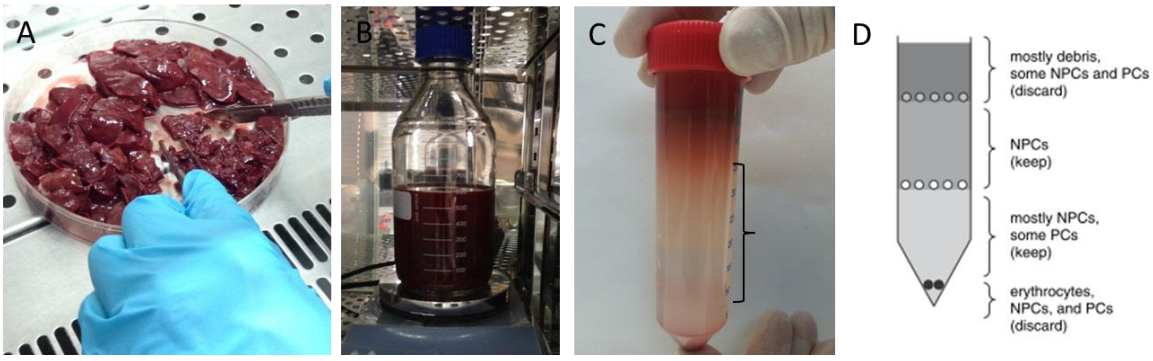


Figure 58: Steps from the KC isolation of dog liver.
A liver lobule was sliced into small pieces (A) and the small pieces were incubated in Gey's balanced salt solution (GBSS) with pronase and DNAse in agitation (B). The cell suspension overlaid on the top of a double gradient of Percoll was centrifuged and the intermediate fraction (indicated by a curly bracket) was collected (C). Schematic representation of the different fractions found in the tube after centrifugation (D). NPCs - non-parenchymal cells; PCs-parenchymal cell [adapted from Froh *et al.* (2003)].

Pellets were resuspended in PBS 1*x* with 0.8 $\mu g.ml^{-1}$ of DNAse and 10 ml of the cell suspension were overlaid into a double Percoll® (GE Healthcare) gradient

constituted by two consecutive 20 ml layers of 25% and 50% Percol. Then, cells were centrifuged at 800 *x*g for 25 min (Figure 588C and D).

After centrifugation, the tube intermediate fraction (see Figure 588D) was collected to new tubes and cells were washed three times with PBS 1*x*. The pellet was resuspended in RPMI (Roswell Park Memorial Institute) medium (BioWhittaker Lonza) supplemented with 10% (v/v) of FBS, L-Glutamine 0.2 mM (Merck) and 100 $U.m^{-1}$ of penicillin and 100 $\mu g.ml^{-1}$ of streptomycin (Sigma-Aldrich). Cell concentration and viability was determinate in a Neubauer's chamber under optical microscopy by using trypan blue exclusion dye. Viable cell concentration was estimated as described in Equation 4 (see Chapter 1 - 4.3.1). This procedure allowed the isolation of cells with approximately 95% of viability.

5.3.1.1. Kupffer cell culture

To ensure maximum differentiation, KCs were cultured for 7 days in 6 well plate (VWR) with supplemented RPMI as described in section 5.3.1.. An additional supplementation with M-CSF (macrophage colony stimulating factor) produced by L929 cell line (see subsection 5.3.1.1.1) was added. The supplementation was performed adding 10% (v/v) of the L929 centrifuged supernatant to the RPMI medium added to the cells. Cells were incubated at 37 ºC, in a humidified atmosphere with 5% CO_2. After 24 h and 48 h of incubation the existing medium was discharged, removing non-attached cells and fresh medium was added to cells. Cells were followed by optical microscopy observation under an inverted microscope (Olympus, CKX41) and photographs were taken with an Olympus CS30 camera.

5.3.1.1.1. M-CSF production

L929 cell line, which presents characteristics of murine fibroblast, secretes a factor that promotes the differentiation of peripheral blood mononuclear cells into MØ and monocytes. This factor is identical to murine M-CSF. The conditioned medium of L929 cells is frequently used as a crude source of murine M-CSF (Fisher *et al.*, 1988). L929 cells also secrete a chemotactic factor specific for monocytes and an uncharacterized factor that promotes the growth of a large variety of cell lines. M-CSF used in the present study was produced using the L929 cell line.

L929 cell line at a concentration of $1x10^6$ cells.ml^{-1} was maintained for 3 days in supplemented RPMI at 37 ºC, in a humidified atmosphere with 5% CO_2 and regularly observed under OM. Then, supernatants were collected, centrifuged at 500 *x*g for 10 min and stored at -20 ºC until use. After trypsinization, cells were detached, counted in a Neubauer's chamber using trypan blue dye and inoculated in new t-flasks, at the same cell concentration, repeating the production cycle.

5.3.2. *Leishmania infantum* infected Kupffer cells

After 7 days in culture, the KC concentration was estimated and cells were infected with *L. infantum* virulent promastigotes and axenic amastigotes at the proportion of parasite to cell of 3:1. To avoid/minimize cell stress and cell damage, cells were not detached from the plate well sand concentration was estimated by the number of cells observed in three random microscope fields, under an inverted microscope (Olympus, CKX41) at 400*x* magnification. A direct proportion between the average of counting cell number and the area observed under the microscope was established, taking into account the total area of each well of the plate (see the following equation, Equation 7).

Equation 7:

$$[EC] = \frac{average\ cell\ number\ (in\ 3\ OM\ fields) \times\ 961.6\ (well\ diameter\ in\ mm^2)}{0.24\ (observed\ area\ in\ mm^2)}$$

EC= estimated number of cells (total cells per well)
OM = Optical microscopy

L. infantum virulent promastigotes and axenic amastigotes obtained as previously described in General Methods (section 3.3.1 and 3.3.2) were used to infect the cells. Parasite concentration was determinate and the correct volume of culture was centrifuged at 1800 *x*g for 10 min at room temperature. Pelleted parasites were then resuspended in supplemented RPMI medium and added to KC culture. Cells were incubated in a cell incubator at 37 ºC, in a humidified atmosphere with 5% CO_2. Samples of cells and supernatants were collected at 1.5 h, 3 h, 5 h and 24 h post-infection. Cells were followed by optical microscopy observation under an inverted

microscope (Olympus, CKX41) and photographs were taken with an Olympus CS30 camera.

5.3.2.1. Positive control

To assure that cells were in functional conditions a positive control of inflammation was included. Lipopolysaccharide (LPS, 1µg.ml^{-1}) from *E. coli* (Sigma-Aldrich) was added to RPMI medium and left in contact with cells for 1.5 h, 3 h, 5 h and 24 h. Samples of cells and supernatants were periodically collected. Cells were maintained in a cell incubator at 37 ºC, in a humidified atmosphere with 5% CO_2.

5.3.3. Leishmanicidal drug

To test the effect of a leishmanicidal drug in the immune activation of KC, antimoniate meglumine at a final concentration of 100 µM was added to KC with 24 h of infection. The leishmanicidal drug was in contact with cells for 1.5 h, 3 h, 5 h and 24 h. Samples of cells and supernatants were collected at described time points. Incubation took place in a cell incubator at 37 ºC, in a humidified atmosphere with 5% CO_2.

5.3.4. Kupffer cells and hepatocyte co-cultures

After 24 h in culture infected KC were detached from the well plate and added to hepatocytes, generating a co-culture. RPMI medium was removed and PBS 1*x* was added to clean non-internalized parasites. Cells were detached by a thermal shock of 30 min on ice followed by a gently scrape will a cell scraper (VWR). Collected cells were centrifuged at 500 *x*g, for 10 min at 4 ºC and pellets resuspended in complete Wiliam's E medium. Cell viability and concentration was determinate by counting in trypan blue dye in a Neubauer's chamber. Co-cultures were maintained in a cell incubator for 24 h at 37 ºC in a humidified atmosphere with 5% CO_2. Samples of cells and supernatants were collected at 1.5 h, 3 h, 5 h and 24 h of incubation. Cells were followed by optical microscopy observation under an inverted microscope (Olympus, CKX41) and photographs were taken with an Olympus CS30 camera.

5.3.5. Isolation of peripheral blood monocytes

Peripheral blood (20 to 30 ml) of the selected dogs was collected, under veterinary supervision, to a tube with anticoagulant CPDA (Citrate Phosphate Dextrose Adenine Solution) and used for the isolation of blood monocytes. The procedure was done as described by Strasser *et al.* (1998) with some alterations. A double gradient of Hystopaque® density 1119 (Sigma-Aldrich) and Hystopaque® density 1077 (Sigma-Aldrich) was prepared and 2 ml of the peripheral blood carefully overlaid on the top. Gradients were centrifuged at 340 *x*g during 30 min at room temperature and a rich monocyte cell ring was formed at the interface of the two gradients. The ring was carefully collected and washed with NaCl 0.9% (m/v) at 300 *x*g, for 10 min at room temperature. To lyse the contaminant red blood cells, a swift passage through deionized sterile water for 1 min was followed by the addition of a solution of NaCl 1.8% (m/v). Cells were again centrifuged at 300 *x*g for 10 min at room temperature. This procedure allowed the isolation of monocytes with approximately 95% viability. To induce the monocyte differentiation into MØs, cells were resuspended in complete RPMI medium supplemented with 10% (v/v) of M-CSF. Cell viability and concentration was determinate in trypan blue exclusion dye in a Neubauer's chamber.

5.3.5.1. Macrophage infection with *L. infantum*

Monocytes were fully differentiated into MØs after 3 days in culture in RPMI medium supplemented with 10% (v/v) M-CSF. Medium was changed 24 h after inoculation to remove all non-attached cells and again at 72 h. *L. infantum* virulent promastigotes were obtained as described in General Methods (section 3.3.1 and 3.3.2). For MØ infection, a proportion of five parasites per cell were used. Adherent MØs were counted as described in section 5.3.2.. Parasite concentration was determined and adjusted to the 5:1 proportion in supplemented RPMI medium and added to the MØ cell culture. Cells were incubated in a cell incubator at 37 ºC, in a humidified atmosphere with 5% CO_2. Samples of cells and supernatants were collected at 1.5 h, 3 h, 5 h and 24 h post-infection.

5.3.6. Confocal microscopy

Dog KC cultured in eight well chamber slide with cover (Lab-Tek II, Nunc) were incubated at 37 ºC in a humidified atmosphere with 5% CO_2. *L. infantum* GFP-amastigotes or GFP-promastigotes were added to cultured cells in proportion of 3:1. Cell suspensions were incubated for 1.5 h min, 3 h, 5 h, and 24 h. Adherent cells were washed with PBS 1*x* and fixed with 2% paraformaldeyde in PBS for 20 min in ice. Slides were maintained in sterile PBS 1*x* at 4 ºC until further processing. For immunostaining, cells were permeabilized overnight at 4 ºC in a solution of PBS 1% (v/v) Tween-20 (Sigma) and 0.2% (v/v) FBS. Antibodies were incubated in PBS 0.125% (v/v), FBS and 1% (v/v) Tween-20 buffer for 1.5 h at room temperature and kept in the dark.

Antibodies anti-CD68 and anti-lysozyme were used. CD68 is found in the cytoplasmic granules of a range of different blood cells and myocytes. It is particularly useful as a marker for the various cells of the MØ lineage, including monocytes, histiocytes, giant cells, Kupffer cells, and osteoclasts. Lysozymes, also known as muramidase or N-acetylmuramide glycanhydrolase are glycoside hydrolases. These enzymes can damage the bacteria walls by catalyzing and attacking peptidoglycans. Lysozyme is abundant in a number of secretions and it is also present in the cytoplasmic granules of MØ. To mark CD68 on monocyte differentiate MØs, a polyclonal FICT conjugated antibody was used (sc-7083, Santa-Cruz) at a dilution of 1:250. For lysozyme staining, a primary rabbit polyclonal antibody (ab74666, Abcam) at a dilution of 1:10 and a secondary goat anti-rabbit polyclonal Alexa fluor 647 (ab150091, Abcam) antibody at 1:1000 dilution was used. Between primary and secondary antibodies, cells were washed three times with the buffer PBS 0.08% tween-20 (v/v) followed by three more washes in PBS 1*x*. Control slides were also prepared with only one antibody staining. For fluorescent microscopy, slides were covered with TO-PRO®-3 or Vectashield® antifade mounting medium with a red-fluorescent compound and DAPI to localize nucleus and chromosomes, respectively. Slides were observed under a Leica TCS SP2 Laser Scanning Confocal Microscope and digital images were acquired

5.3.7. Scan microscopy

After 7 days in culture, KCs were detached from the wells by thermal shock (30 minutes on ice), and incubated with *L. infantum* parasites during 1.5 h min, 3 h, 5 h, and 24 h. Cells were washed with cold PBS 1x and fixed with 2% paraformaldeyde (m/v) in PBS for 20 min on ice. Cells were then washed again with cold PBS 1*x* by centrifugation at 500 *x*g for 10 min, filtrated through a Millipore mesh and subsequently dehydrated in a graded ethanol series [30, 50, 70, 80, 90 and 100% (v/v)]. Samples were dried using the critical point drying method and coated with gold palladium and mounted on stubs. Observations were made under a scanning electron microscope (JEOL5200-LV). Digital images were acquired at high, medium and low magnification.

5.3.8. Flow cytometry

5.3.8.1. Determination of KC differentiation

To determine the time most suitable for KCs complete differentiation and proliferation, a flow cytometry analysis of cells was performed throughout 12 days of culture. Cells were cultured with supplemented RPMI as described in section 1 and an additional supplementation with M-CSF produced by L929 cell line (see 5.3.1.1.1) was added. Cells were incubated in a cell incubator at 37 ºC, in a humidified atmosphere with 5% CO_2. For flow cytometry analysis cells were detached by a thermal shock of 30 min on ice followed by a gently scrape will a cell scraper (VWR) and centrifuged. Next cells were fixed with a 2% (v/v) paraformaldehyde in PBS 1*x*. After fixation cells were washed in PBS and stored at 4 ºC in PBS until flow cytometer analysis. Cell acquisition was performed on a FACSCalibur cell analyzer (BD Biosciences). The analysis of forward scatter (FSC) *vs* side scatter (SSC) allowed the distinction between several cell types presented in the culture based on their size (FSC) and granular content (SSC).

5.3.8.2. KC dynamic of infection with GFP-*L. infantum*

After 7 days in culture, KCs were detached from the plate wells as described above (see subsection 5.3.1.1), washed with PBS 2% FBS and the concentration determined under optical microscopy (see Chapter 1 - 4.3.1). Cells were resuspended in RPMI with 10% (v/v) FBS and infected with *L. infantum* GFP-promastigotes or *L.*

infantum GFP-axenic amastigotes at parasite to cell proportion of 3:1. Cells were kept in suspension and were incubated in a cell incubator at 37 ºC in a humidified atmosphere with 5% CO_2 for 1.5 h, 3 h, 5 h and 24 h. At the same time, non-infected KCs were incubated for 30 min at 4 ºC with a polyclonal mouse anti-human monocyte/macrophages FITC antibody (AbD Serotec clone MAC387) that recognizes the L1 or Calprotectin molecule, an intracytoplasmic antigen expressed by granulocytes, monocytes and by tissue macrophages. The antibody presented a cross reactivity with dog and allowed the identification of KC population. Non-infected and non-stained cells and parasites were also used as assay controls. After incubation with the parasites, cells were centrifuged and fixed with a 2% (v/v) paraformaldehyde in PBS 1*x*. After fixation cells were washed in PBS and stored at 4 ºC in PBS until flow cytometer analysis. Cell acquisition was performed on a FACSCalibur cell analyzer (BD Biosciences).

5.3.9. Urea quantification

In this study urea production was assessed in the supernatant of KC cultures throughout the experimental period. Samples were analyzed by a commercial kit QuantiChrom™ Urea Assay Kit-DIUR-500 (BioAssay System) for quantitative colorimetric urea determination accordingly with the manufacture's recommendations. The urea assay kit is designed to measure urea directly in biological samples without any pretreatment. The intensity of the color, measured at 450 nm (TRIADTM 1065, DYNEX Technologies), is directly proportional to the urea concentration in the sample. Supernatants of KC cultures were collected from duplicated wells and centrifuged at 500 *x*g, 10 min and 4 ºC. Samples were stored at -20 ºC until further analysis. After thawed, samples were vigorously vortex and, 50 µl of each sample were plated in duplicate in a sterile 96 transparent well plate (Nunc). Standard urea concentration and blank (water) was included and calculations were performed accordingly to manufacturer's instructions as indicate in Equation 8. For posterior result analysis RPMI medium values were subtracted to the samples.

Equation 8:

$$[C](mg/ml) = \frac{OD\ sample - OD\ blank}{OD\ standard - OD\ blank} \times n \times standard\ concentration\ (mg/ml)$$

OD= optical density
n= dilution (in the present study n=1)

5.3.10. Nitric oxide quantification

Nitric oxide production is related to the immune activation state of KCs. In the present study it was used a commercial kit Nitrate/Nitrite Colorimetric Assay kit (Abnova) accordingly with the manufacture's recommendation to quantify the nitrate/nitrite production in cell supernatants. This kit provides a precise and convenient method to measure total nitrate/nitrite production in a two-step protocol. In the first step there is the conversion of nitrate to nitrite by the nitrate reductase enzyme. In the second step, the addition of Griess reagent converts the nitrite into a deep purple azo compound that can be measured at 550 nm (BioRad-680, microplate reader) and accurately determines the NO_2^- concentration. Supernatants of KC cultures were collected from duplicated wells and centrifuged at 500 *x*g, 10 min and 4 ºC. Samples were stored at -20 ºC until further analysis. After thawing samples, were vigorously vortex and, 80 µl of each sample were plated in duplicate in a sterile, transparent 96 well plate (provide by the manufactures). A standard curve was performed with nitrate 1:5 serial dilutions and blank (RPMI medium) was included. Calculations were performed accordingly to manufacturer's instructions (Equation 9). For posterior result analysis RPMI medium values were subtracted to the samples.

Equation 9:

$$[Nitrate + Nitrite](\mu M) = (OD550nm - slope\ standard\ curve) \times SV \times DF$$

OD= optical density
SV= sample volume (µl)
DF= dilution factor

5.3.11. Real-time PCR analysis

Samples of non-infected KCs (negative controls), KCs infected by *L. infantum* promastigotes or axenic amastigotes, infected KCs exposed to leishmanicidal drug and KCs stimulated by inflammatory mediators (positive controls) were collected at previously established time points and processed for RNA extraction, cDNA synthesis and real-time PCR analysis (as described in the chapter General Methods, section 3.4). β-actin was used as normalizing gene and the gene expression of the innate immune receptors NOD1, NOD2, TLR2, TLR4 and TLR9 were quantified. Gene expression of the cytokines IL-12, IL-10, IL-4, TGF-β and TNF-α were also analyzed.

5.3.12. Statistical analysis

The non-parametric Wilcoxon test for two related samples was used to compare differences between time points and different experimental conditions. This statistical test is the equivalent of parametric paired samples T-test and should be used when the sample data do not exhibit a normally distribution, as in the present study. All the data analysis was performed using the software IBM SPSS Statistics version 16.0 (IBM, USA). A significance level of 5% ($p \leq 0.05$) was used as indicative of statistical significance.

5.4. Results

5.4.1. Kupffer cell's identity was confirmed

In order to ensure that hepatic recovered cells were in fact liver resident MØs (KCs), the morphology of isolated cells was regularly observed under optical microscopy, evidencing characteristics of macrophages. KCs as MØs, are cells with phagocytic properties, containing abundant lysosomes required for the breakdown of phagocytic materials. Active MØs, have numerous phagocytic vesicles (or phagosomes) for the transient storage of ingested materials. Nucleus evidence an irregular outline and the cytoplasm may vary in shape (Figure 60A).

To determine the time most suitable for KCs complete differentiation and proliferation, a flow cytometry analysis of cells was performed throughout 12 days of culture (Figure 5959). The analysis of forward scatter (FSC) *vs* side scatter (SSC) allowed the distinction between several cell types presented in the culture based on their size (FSC) and granular content (SSC). These two parameters together were able to identify three distinct cell populations at the initial culture: one population with small size and low granular content identified as lymphocytes, other population with bigger size and higher granular content related to granulocytes, and a third cell population with the highest size and low granular content, associated with MØs. FSC and SCC analysis also allowed distinguishing between cellular debris and living cells. After 6 days in culture, MØs were the dominant cell type presented in the culture and after 9 days the unique cell type existent.

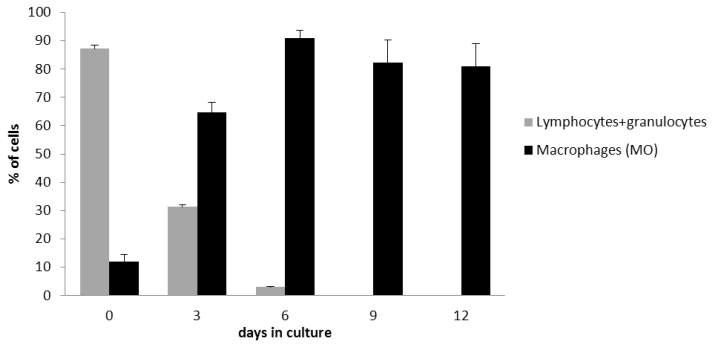


Figure 59: Liver MØ differentiation and proliferation in culture medium.
Identification of cell subsets by flow cytometry analysis of forward scatter (FSC) *vs* side scatter (SSC) during 12 days of culture After 6 days in culture, macrophages were the dominant cell type present in the culture and after 9 days the unique cell type present. Results expressed by mean and standard deviation.

Taking these evidences, isolated cells were maintained in culture for 7 days to ensure differentiation and proliferation. After that period adherent cells were prepared and staining for CD68 and lysozyme detection. Isolated and cultured cells that presented the typical MØ morphology were also $CD68^{+}$ (green) and $lysozyme^{+}$ (red) cells (Figure 600). CD68 is particularly useful as a marker for the various cells of the MØ lineage, being predominantly localized in lysosomes and endosomes with a smaller fraction circulating to the cell surface. Lysozyme is mainly present in the granules of the MØs and the polymorphonuclear neutrophils. Taking into account that cells were isolated from dog livers and that after 6 days in culture were differentiated into $CD68^{+}lysozyme^{+}$ positive cells, it is possible to assure that the isolated cultured cells are in fact canine KCs (liver resident MØs).

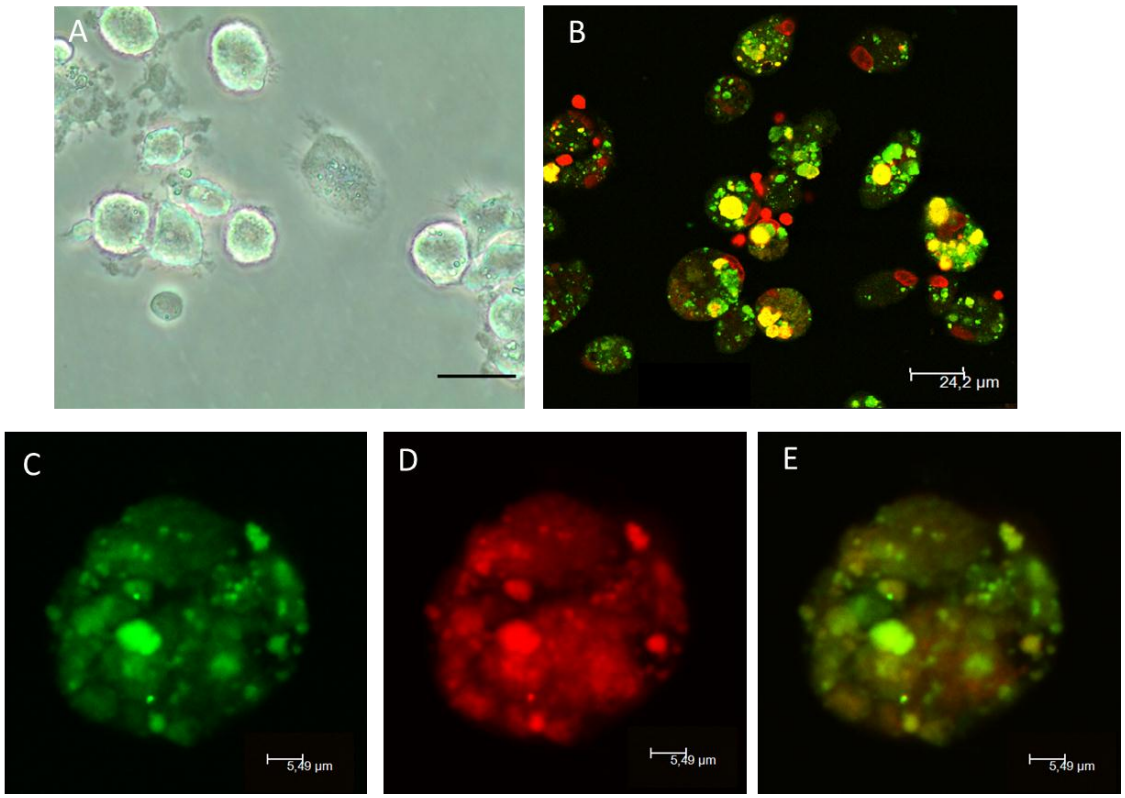


Figure 60: Identification of dog KCs.
Cells cultured for 7 days were observed under inverted optical microscope (A) and stained for CD68 and lysozyme (B, C, D, E). Cells evidencing MØ morphological characteristics, such as numerous phagocytic vesicles, and irregular outline. (Scale bar 20 µm, 400*x* magnification) (A).Image overlay of the double positive staining for CD68 (green) and lysozyme (red). CD68 and lysozyme co-localization is visible in yellow. Nucleus stained with To-pro®-3 (red) are of oval shape and located in periphery of the cell (400*x* magnification) (B). Single cell showing CD68 (green) localization in endocytic compartments (lysosomes and endosomes) (C) and lysozyme (red) in cytoplasmic granules (D). Merging of images C and D images show the co-localization of CD68 and lysozyme in cytoplasmic vesicles (E).

5.4.2. Kupffer cells recognize and internalize *Leishmania* parasites

Infection dynamics of KCs when exposed to *Leishmania* morphological forms, amastigotes and promastigotes, was investigated. Axenic amastigotes *in vitro* differentiated and virulent promastigotes with less than five passages in the culture medium were added to KCs in a proportion of 3 parasites: 1 cell. Infections were followed for 24 h.

After 1.5 h of incubation, KCs were surrounded by promastigotes (Figure 611) showing parasite tropism for these cells. After 5 h of incubation, promastigotes were fully adherent to the cell membrane and at 24 h of incubation KCs exhibit the cytoplasm with numerous vacuoles, probable with parasite internalized, and KCs were increased in size. External parasites were motionless and adherent to the KCs membrane.

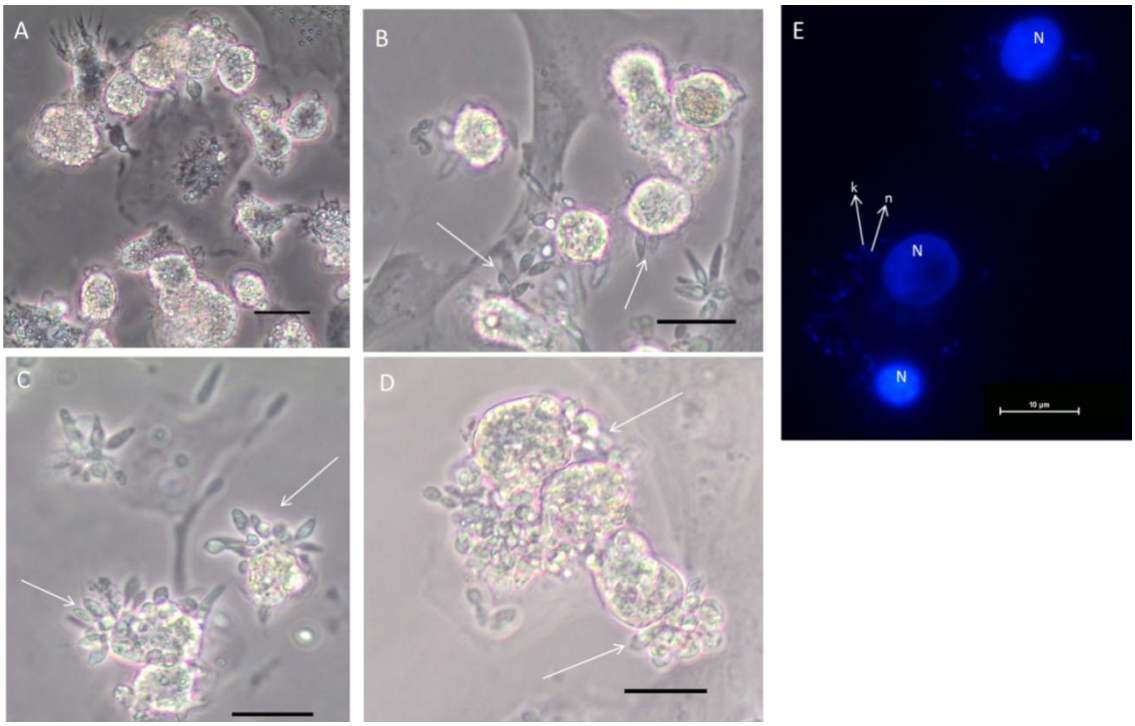


Figure 61: Dynamics of KC infection by *L. infantum* virulent promastigotes.
KCs after 7 days in culture (A) and KCs incubated with promastigotes for 1.5 h (B), 5 h (C) and 24 h (D) were observed under inverted optical microscope (Scale bar 20 µm, 400*x* magnification) and images were acquired. KCs incubated with promastigotes for 5 h stained for nucleic acids (blue)with DAPI were observed under fluorescence microscope and images were acquired (scale bar 10 µm, 1000*x* magnification) (E). Promastigotes interacting with KC membrane are indicated by arrows (B, C). Parasite accumulation on KCs membrane are indicated by arrows (D). KC nucleus (N), parasite nucleus (n) and kinetoplast (k) (E).

KCs surface was also analyzed by scanning electron microscopy (Figure 622). The cells exhibited irregular membrane, characteristic of MØs, and some cytoplasmic extensions, constituting bridges and agglomerates (Figure 622A).

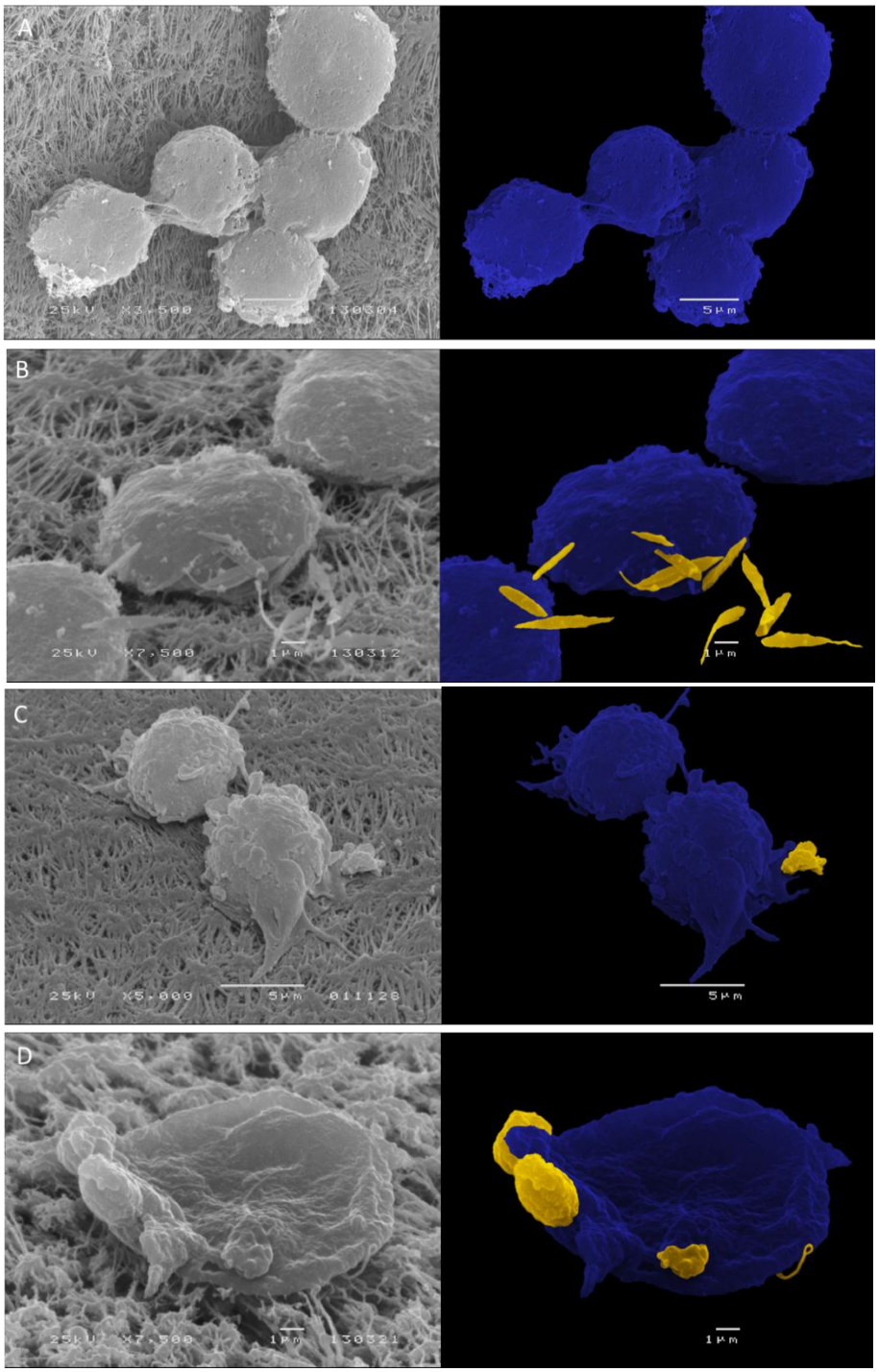


Figure 62: KCs exposed to *L. infantum* virulent promastigotes.
KCs after 7 days in culture (A) and KCs incubated with promastigotes for 3 h (B) and 5 h (C, D) were observed under SEM. Images directly acquired and artificially colored (Blue – KCs; Yellow- *L. infantum* promastigotes) are shown. Non infected KCs evidence irregular cell membrane and some membrane extensions, originating bridges and cell agglomerates (A). Promastigotes on KC membrane (B). KCs phagocyting one (C) or more promastigotes (D) after 5 h of infection.

L. infantum exhibited an evident tropism to KCs (Figure 622B). Infection of KCs by *L. infantum* virulent promastigotes were demonstrated through the phagocytosis of several parasites by a single cell (Figure 622C and D).

To confirm parasite internalization, KCs was incubated with GFP-transformed *L. infantum* promastigotes (Figure 633A) and axenic amastigotes (Figure 633B), in the same proportion and incubation time referred above and cells were observed under fluorescent microscopy. This technique allowed for a better characterization of the parasite behavior and cell performance.

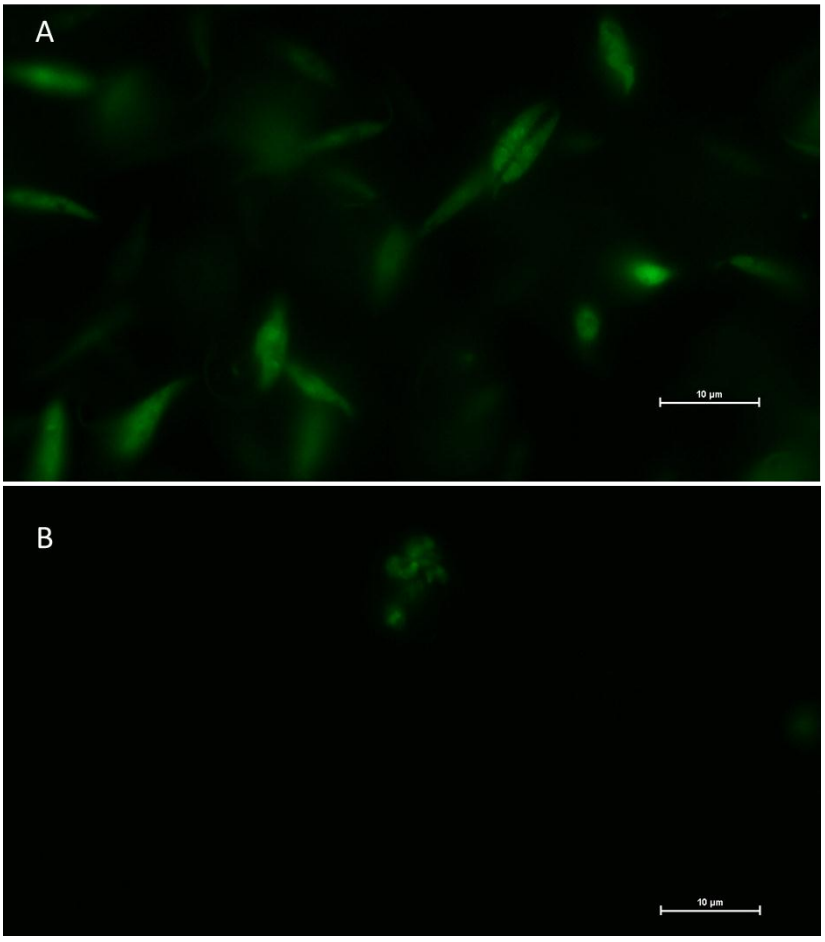


Figure 63: GFP-*L. infantum*.
GFP-promastigotes (A) cultured at 24 ºC and GFP-axenic amastigotes (B) differentiated at 37 ºC were fixed and observed under fluorescent microscope (1000*x* magnification) and images were acquired. Promastigote (A) form exhibits an elongated shape and a flagellum and the axenic amastigotes (B) present the characteristic round body shape without free flagellum.

After 1.5 h of incubation, KCs were rapidly surrounded by parasites, evidencing once again the promastigote preference for these cells (Figure 644). After 5 h of incubation, promastigotes were uptake by KCs, indicating that parasites were

recognized by cell receptors and easily phagocytized. After 24 h of incubation, all the promastigotes were internalized and it was possible to observe the loss of the flagellum and a change into an ovoid body characteristic of the amastigote form. In some cases there was observed morphological alteration on KC morphology with evidences of cell lysis. Nevertheless, the presence of the parasite did not influence the expression of CD68 and lysozyme in KCs, since they were continuously detected throughout the infection.

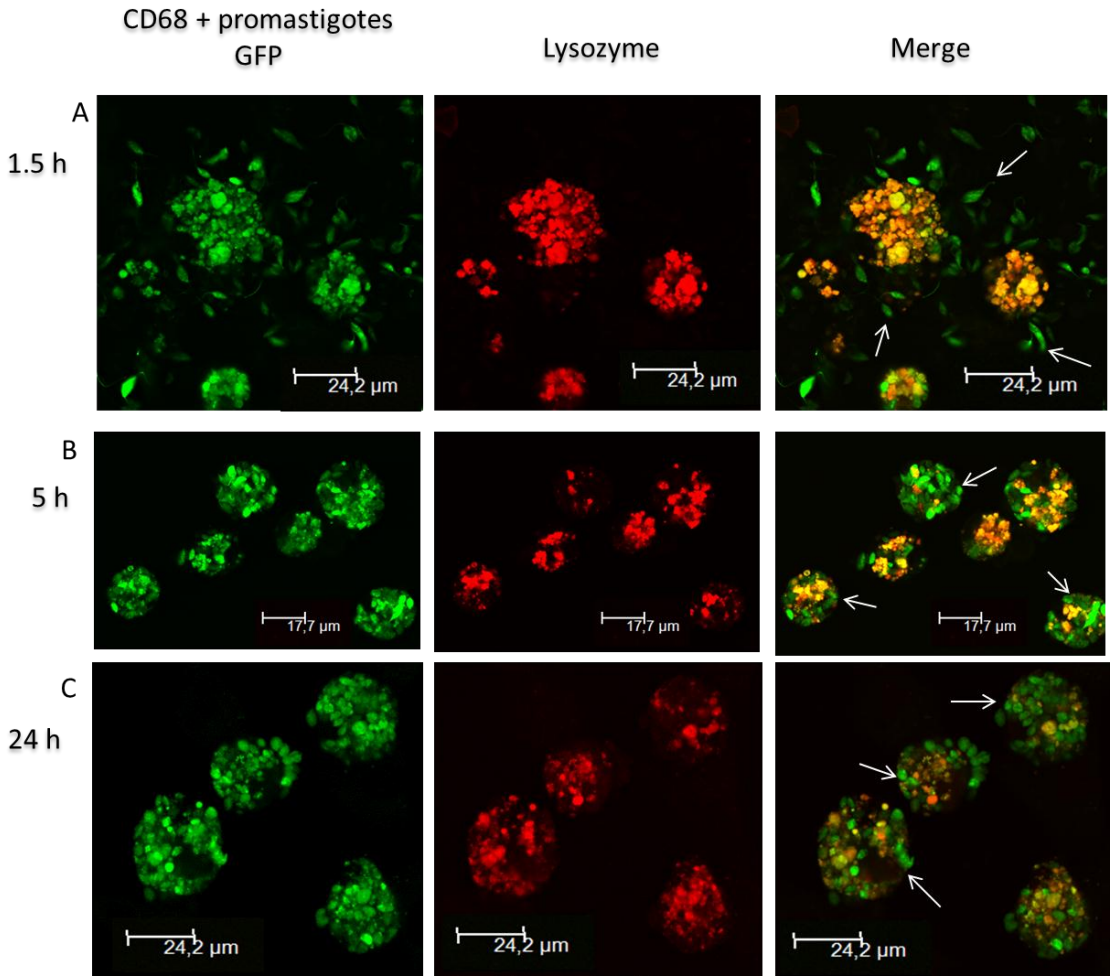


Figure 64: CD68$^+$ lysozyme$^+$ KCs incubated with *L. infantum*.
Cells incubated with GFP-promastigotes (green) for 1.5 h (A), 5 h (B) and 24 h (C) were stained with anti-CD68 antibody conjugated with FICT (green) and anti-lysozyme antibody (red) conjugated with Alexa fluor 647. Cells were then observed under confocal fluorescent microscope and images were acquired. After 1.5 h KCs were surrounded by parasites (A) and after 5 h of incubation (B) promastigotes were internalized by KCs. At 24 h (C) the promastigotes appear to be located inside the KC cytoplasm and exhibit an ovoid body form characteristic of amastigotes. Arrows identify *L. infantum* parasites located extracellular (A) and intracellular (B-C).

As for infection with amastigote form of the parasite, earlier, after amastigote exposure (1.5 h) it was possible to identify some parasites attached to the KC membrane (Figure 655A) showing the clear parasite tropism to the cells. After 5 h, (Figure 655B) the parasite appears to be phagocyted by the cells. After 24 h of incubation, almost no amastigotes were visible in culture medium, indicating that parasites were easily phagocytized, and some cellular debris appeared, suggesting that cell lysis may occur in some cells.

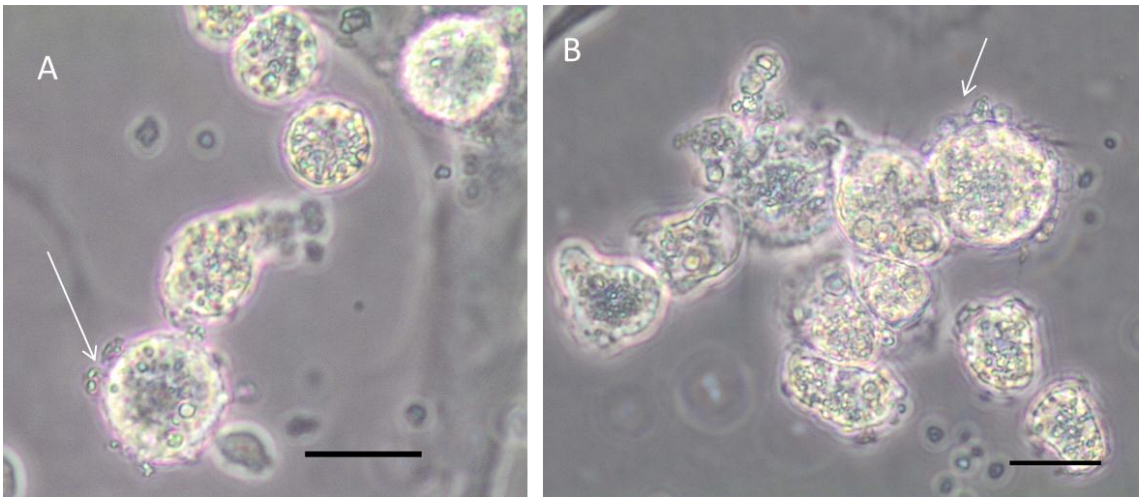


Figure 65: Interaction of dog's KC with *L. infantum* axenic amastigotes.
Cells incubated with axenic amastigotes for 1.5 h (A) and 5 h (B) were observed under inverted optical microscope (Scale bar 20 µm, 400*x* magnification) and images were acquired. Arrows indicate amastigotes close to the surface of KCs (A) and 5 h incubation parasites are being internalized KCs (B). Arrow indicates points the cell membrane appear to be phagocyting amastigotes.

The observation of GFP-amastigote infection under fluorescence microscopy revealed an aggressive infection (Figure 666). At 3 h post-incubation, *L. infantum* axenic amastigotes were completed phagocytized and internalized by KCs and at 5 h incubation are perfectly observed in the KCs cytoplasm. At 24 h of incubation, KCs lost the membrane integrity and cellular lysis occurred. Compared with non-infected KCs, the amastigote infection seems to diminish the expression of CD68 and lysozyme, possible compromising the immune activation of these cells.

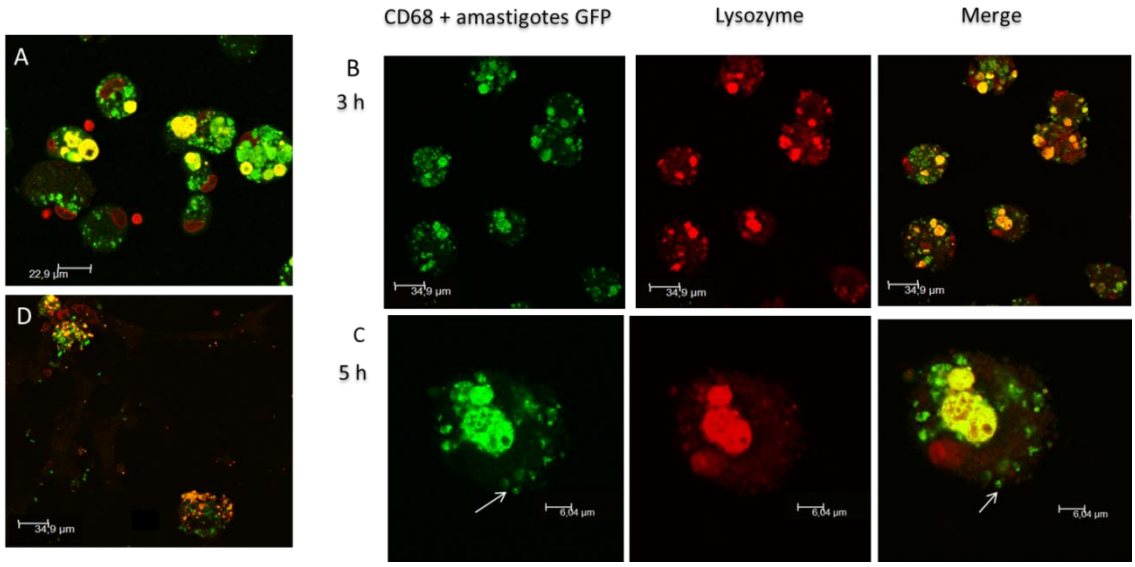


Figure 66: Interrelation of dog's KCs with *L. infantum* amastigotes.
Non infected cells (A) and cells incubated with GFP-axenic amastigotes (green) for 3 h (B), 5 h (C) and 24 h (D) were stained with anti-CD68 antibody conjugated with FICT (green) and anti-lysozyme antibody conjugated with Alexafluor® 647(red). Cells were then observed under confocal fluorescent microscope and images were acquired. Nucleus stained with To-pro®-3 (red) appears as a large regular red bodies. A) Non-infected cells exhibiting high content of CD68 protein and co-localization with lysozyme. B)3 h of incubation amastigotes appear in the to be phagocytized and at 5 h incubation (C) amastigotes were perfectly internalized, as indicate by arrows (D) Later on, KCs loss membrane integrity and cell lysis are observed.

To better characterize the apparent difference of amastigotes and promastigotes infectious potential, the analysis of infected cells was performed by flow cytometry. KCs incubated with GFP-promastigotes or with GFP-amastigotes and KCs not exposed to parasites were positively marked with anti-MØ antibody (Figure 677A). KC gating was defined on uninfected cells.

L. infantum axenic amastigotes were more infectious than promastigotes, achieving near 100% of infection at 1.5 h of incubation (Figure 677G) and cellular lysis after 24 h. Promastigotes required 24 h to achieve maximum values of infected KCs. These results indicate that the fully differentiated intracellular form of the parasite, the amastigote, is able to induce a faster phagocytic response from KCs than the promastigote form. The tested dogs appear to have a similar degree of susceptibility to amastigote infection. But regarding promastigote, some dogs exhibit low infection rate, suggesting some kind of resistance, at least in the first incubation hours. Due to the cellular lysis observed in KCs amastigote infection, the following comparison on KCs infection by amastigotes and promastigotes was analyzed during the first 5 h incubation.

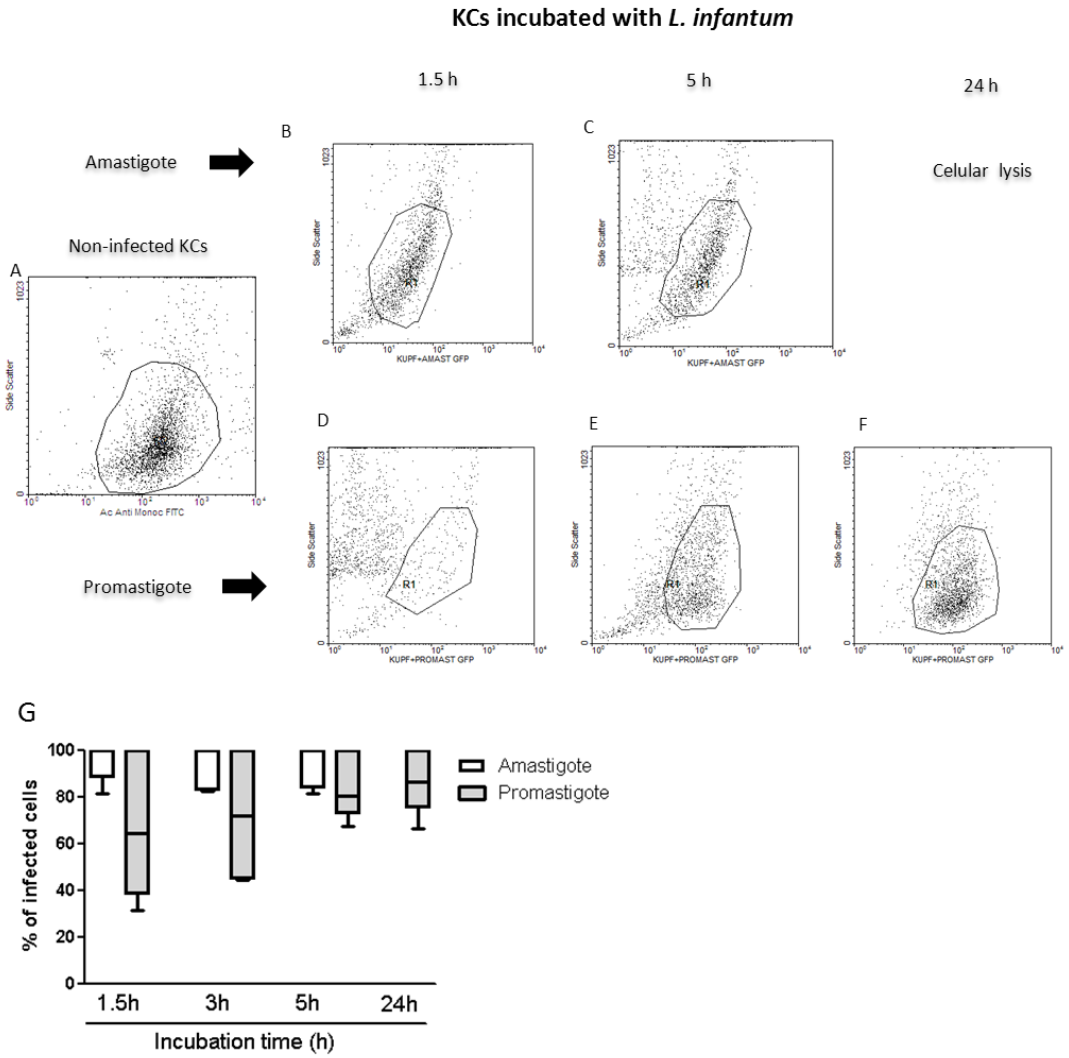


Figure 67: Dynamics of KCs infection with *L. infantum* promastigotes and amastigotes evaluated by flow cytometry.
Dog KCs were incubated with GFP parasites for 1.5 h, 3 h, 5 h and 24 h and marked with anti-MØ antibody. KCs gate was determined using marked cells unexposed to the parasite (A) and the frequency of KCs infected after incubation with axenic GFP-amastigotes (B-C) or promastigotes (D-F) were estimated. The frequency of infected cells is shown by whisker box-plot (minimum to maximum) (G).

5.4.2.1. *L. infantum* parasites mainly direct dog's KCs to produce urea

Nitric oxide (NO) is produced by immune activated MØs and is a potent leishmanicidal agent. Urea can also be produced by MØs, but is not leishmanicidal and promotes parasite survival. To investigate the activation status of infected KCs, the production levels of urea and NO was assessed.

Early in amastigote infection (1.5 h), KCs produced high levels of NO (P=0.0313). (Figure 688A). However, *L. infantum* amastigote hastily diminish the NO production to undetectable levels within 3 h post-infection (P=0.0156), probably to

assure the establishment of infection. Decrease of NO production was also verified in promastigote infected-KCs, but in slower way. This faster immune modulation, leading to NO decrease performed by the parasite intracellular form demonstrates how well *Leishmania* is adapted to intra-KC life. Urea production was higher in amastigote infected KCs when compared to non-infected cells that present very low levels of urea (P=0.0313) (Figure 688B). This production was kept elevated throughout the infection period. Promastigote parasites also caused a burst of urea production by KCs (P=0.0313), but at statistically smaller level than amastigote infection (P=0.0469). Also the addition of lipopolysaccharide (LPS) to KCs, as an inflammation positive control, induced an initial burst of urea (P=0.0313), rapidly decreased and at 3 h incubation LPS induced the higher NO production pick. Some values distant from the majority of measurements found at different time points identified as outliers probably reflect different genetic backgrounds and different degrees of susceptibility to *L. infantum* infection of a natural dog population.

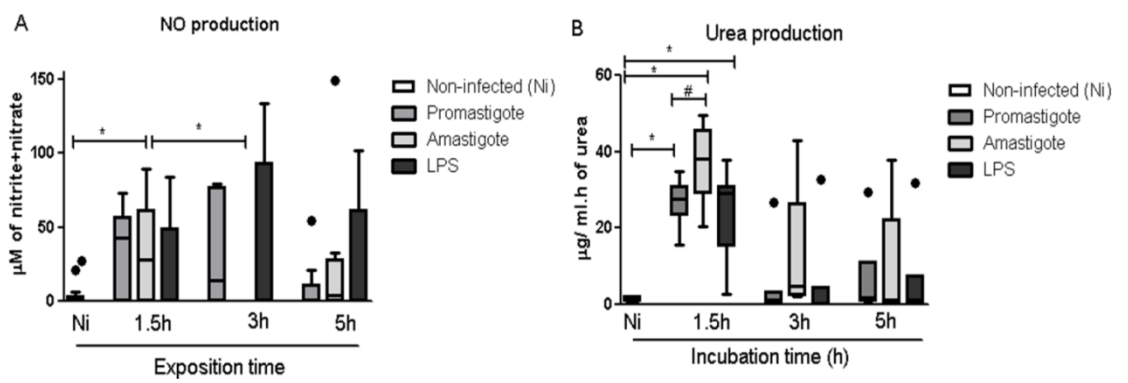


Figure 68: Nitric oxide (NO) and urea production by canine KC exposed to *L. infantum* parasites and LPS.
A) Nitric oxide production. B) Urea production. The black dots represent outlier values. Results performed in triplicate are expressed by whisker box-plot (minimum to maximum). Outlier values are indicated by black dots. The non-parametric Wilcoxon test was used for statistical comparisons. * and # ($p<0.05$) indicate significant differences.

5.4.3. Kupffer cell immune response to *L. infantum* infection

5.4.3.1. *L. infantum* does not seem to be recognized by KC's innate receptors

To characterize the role played by *L. infantum* in activating or silencing KC immune response, the gene expression of several innate immune receptors were accessed by real-time PCR. Since previous results indicated that at 24 h post-infection the membrane integrity of KCs was compromised and cell lysis occurred, real-time PCR analysis was performed only until 5 h post-infection.

NOD1 and NOD2 expression levels important gene expression differences were not found between infections by amastigote or promastigote (Figure 6969). Amastigote infection led to a transient decline of NOD1 mRNA accumulation after 3 h of incubation (P=0.0094). However, comparing the initial copy number and the gene expression observed at 5 h of incubation, there was a significant increase (P=0.0079). This indicates that the amastigote infection did not promote important changes in the gene expression of NOD1 probably avoiding tackling the activation NOD1 downstream pathways in order to fully establish an intracellular infection. *L. infantum* promastigote did not affect the gene expression of NOD1 until 5 h post-infection (Figure 6969A). At this time point, a significant decrease in the copy number was observed when compared with non-infected KCs (P=0.0391). The incubation of KCs with LPS exhibited some increase in NOD1 expression after 5 h incubation, although not statistically significant. KC exposition to *L. infantum* parasites did not exert significant changes in NOD2 gene expression (Figure 6969B). Taking this together it seem that *L. infantum* does not trigger NOD1 or NOD2 expression during the first hours of infection on KCs. However, the presence of LPS increases NOD2 expression significantly more compared to amastigotes (P= 0.0013).

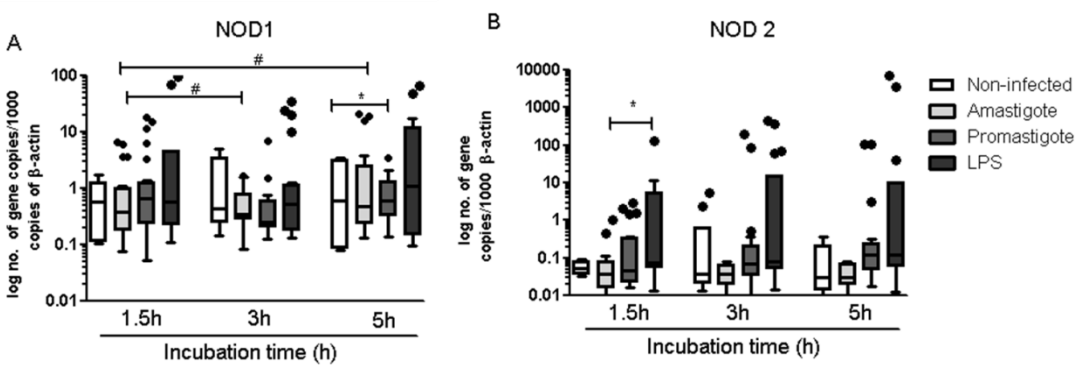


Figure 69: Gene expression of innate immune receptor by *L. infantum*-infected.
KCs exposed to amastigotes or promastigotes for 1.5 h, 3 h and 5 h were used to extract RNA. Uninfected KCs and LPS stimulated-KCs were also used. NOD1 (A) and NOD2 (B) gene expression was quantified by real time PCR Results performed in triplicate are represented by whisker box-plot (Tukey). Outlier values are indicated by black dots. The non-parametric Wilcoxon test was used for statistical comparisons. * and # ($p<0.05$) indicate significant differences.

Gene expression of TLR2, TLR4 and TLR9 were also accessed in KCs exposed to promastigotes and amastigotes parasite forms (Figure 700). After 5 h of incubation TLR2 gene expression was increased in both promastigote (P=0.0097) and amastigote (P=0.0075) infected KCs when compared with earlier infection time point (1.5 h) However this increase is not statistically different from uninfected KCs (Figure 700A). Taken together, these results indicate that both morphological forms of the parasite seem to be recognized by TLR2, possible inducing a slight KC activation. LPS incubation with KCs revealed an increase in TLR2 levels similar to what was observed with *L. infantum* forms. However, gene expression of TLR4 (Figure 700B) and TLR9 (Figure 700C) was constant during the entire period of infection regardless the morphological form of the parasite used in the infection, suggesting that these KC innate immune receptors do not sense *L. infantum* parasites, at least during an initial infection. LPS did not shown great induction of TLR4 or TLR9 expression.

Several outliers representing higher values were identified when evaluating the gene expression of internal and external KC sensors, probably reflecting the diverse genetic background of the tested dogs.

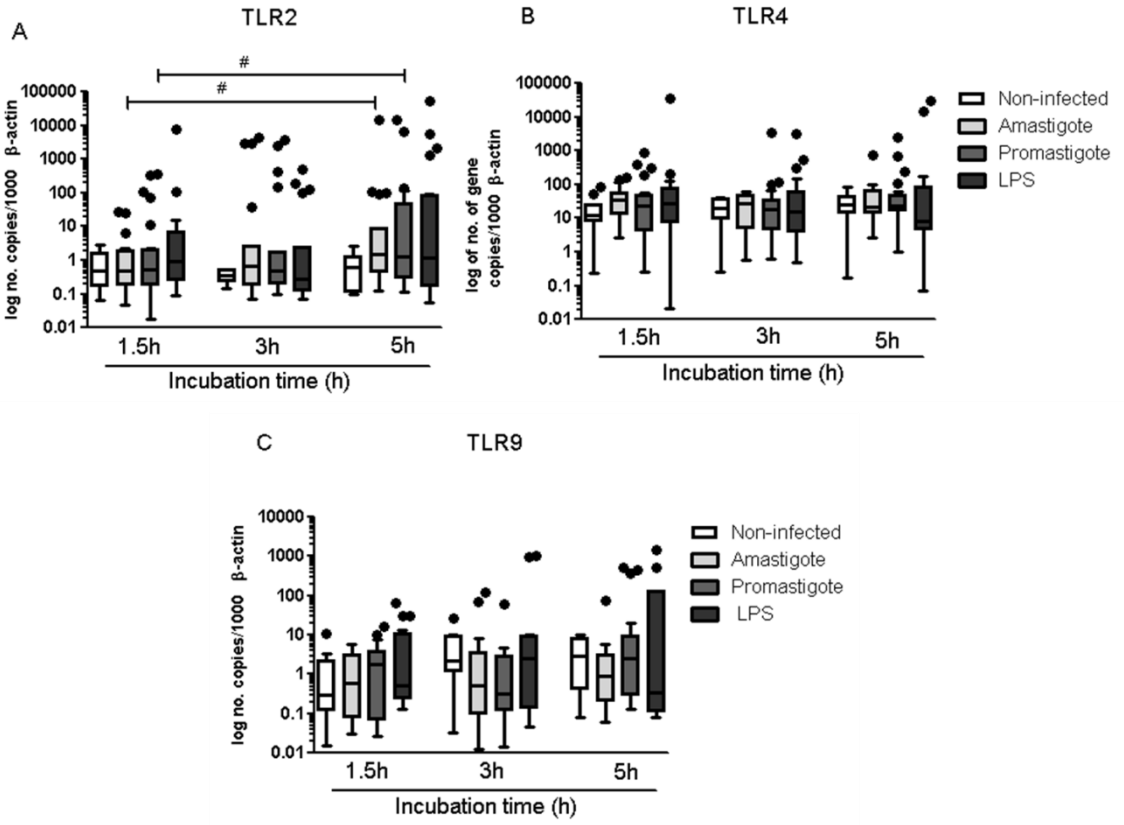


Figure 70: Gene expression of TLR2, TLR4 and TLR9 by *L. infantum*-infected KCs.
KCs exposed to amastigote and promastigote for 1.5 h, 3 h and 5 h were used to extract RNA. Uninfected KCs and LPS stimulated-KCs were also used as negative control. TLR2 (A), TLR4 (B) and TLR9 (C) gene expression was quantified by real time PCR. Results performed in triplicate are represented by whisker box-plot (Tukey). Outlier values are indicated by black dots. The non-parametric Wilcoxon test was used for statistical comparisons. * and # ($p<0.05$) indicate significant differences.

5.4.3.2. *L. infantum* parasites do not induce the generation of pro- and anti-inflammatory cytokines by dog KCs

In order to better characterize the immune response generated by KCs facing the infection by *L. infantum*, gene expression of pro- (IL-12 and TNF-α) and anti-inflammatory (IL-10, IL-4 and TGF-β), cytokines were evaluated by real-time PCR. Promastigote infected-KCs revealed slight variations of IL-12 gene expression over *i*ncubation time (Figure 711A). After 3 h of incubation, KC expressed more IL-12 (P=0.0012) and at 5 h post-infection the amount of mRNA accumulation was more evident (P=0.0382). However, these increases were not enough to present significant differences when compared with non-infected KCs. On the other hand, amastigotes did not seem to induce IL-12 expression, which remained at low levels during the observation period. LPS exposed KCs exhibited an earlier increase in IL-12 expression

(1.5 h incubation) that was maintained through the incubation time, being indicative of activation of a pro-inflammatory response. After 3 h of incubation, amastigote infected-KCs evidenced a transient increase of TNF-α gene expression (P=0.0263) although not significantly different of uninfected KCs (Figure 711B). LPS incubated KCs react early and strongly with increased TNF-α expression, compared to non-infected KCs (P= 0.0059) or amastigote infection (P<0.0001). This increase was sustained through the incubation time. At 3 h incubation the observed TNF-α increase was statistically different from amastigote (P=0.0329) and promastigote infection (P=0.0178), which in their turn were not different from non-exposed KCs. Taken together, these results indicate that during an initial period of infection the amastigote parasite form, which is quickly internalized by KCs, is able to direct these cells into a non-inflammatory state of activation.

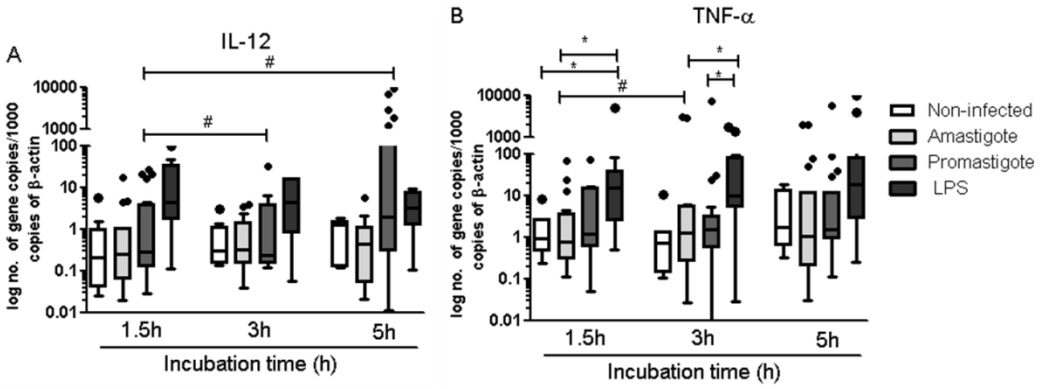


Figure 71: Gene expression of pro-inflammatory cytokines by dog KCs exposed to *L. infantum* virulent promastigotes, axenic amastigotes or LPS.
A) IL- 12. B) TNF-α. Uninfected KCs were also used as negative control. Gene expression was quantified by real time PCR at 1.5 h, 3 h and 5 h of cells incubation. Results performed in triplicate are represented by whisker box-plot (Tukey). Outlier values are indicated by black dots. The non-parametric Wilcoxon test was used for statistical comparisons. * and # (p<0.05) indicate significant differences.

In promastigote infected-KCs, a transient slight increase of IL-10 gene expression was observed early during infection (1.5 h) followed by a rapid decreased to levels similar of non-infected KC. After 3 h of incubation, amastigote infected-KCs exhibited a significant transient increase of IL-10 gene expression (P=0.0214), then returning to normal values (Figure 722A). LPS exposed KCs induce more IL-10 expression than *L. infantum* presence, probably to balance the immune environment, as LPS actives inflammatory cytokine expression. Promastigote promoted KCs to increased IL-4 gene expression, this enhance was significant at 5 h of incubation

(P=0.0022). This increase was statistically different from LPS incubated KCs (P=0.0160). Although amastigote infection maintained IL-4 levels similar to non-infected KCs statistical differences were observed between incubation time points showing an increasing tendency (Figure 722B). After 3 h of incubation, IL-4 gene expression was slightly increased in amastigotes infected KCs (P=0.0014). The IL-4 increased accumulation was maintained until 5 h of incubation (P=0.0353). In amastigote infected-KCs TGF-β gene was kept at low levels (Figure 722C). At 3 h post-infection, there was a significant increase of TGF-β gene expression (P=0.0102). However the values were not different from non-infected KCs.

Several outliers were evident as possible reflection of a diverse genetic background of the tested dogs.

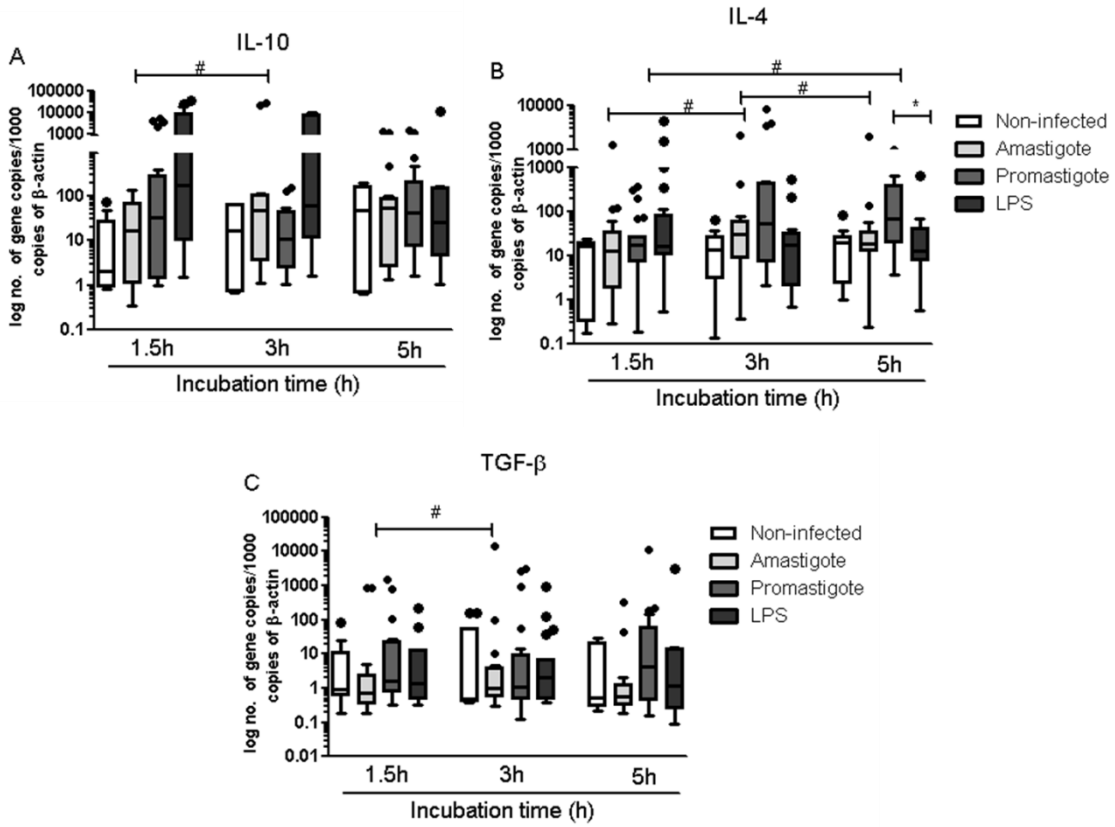


Figure 72: Gene expression of anti-inflammatory cytokines by KC exposed to *L. infantum* virulent promastigotes or axenic amastigotes.
A) IL- 10. B) IL- 4. C) TGF-β. Uninfected KC were also used as negative control. Results performed in triplicate are represented by whisker box-plot (Tukey). Outlier values are indicated by black dots. The non-parametric Wilcoxon was used for statistical comparisons. * and # ($p<0.05$) indicate significant differences.

Taken together, these results indicate that although the presence of the parasite induces some changes in gene expression of anti-inflammatory cytokines by KCs, the effective anti-inflammatory activation of these cells is not much evident regardless the parasite morphological form used for infection.

5.4.3.3. Treatment with Meglumine antimoniate trigger the expression of innate KC sensors and generate pro- and anti-inflammatory cytokines

In order to investigate the effect of treatment in restoring KC activation, breaking the anergic effect imposed by the parasite, meglumine antimoniate (MgA) was added to KC after 24 h of promastigote infection. After this period of time, promastigote had already transformed into amastigote inside KC, as demonstrated in the fluorescence microscope images (Figure 64). NO and urea production were assessed after the addition of MgA.

MgA treatment of infected KC did not lead to increase of NO production (Figure 733A). However, an early transient burst of urea, (Figure 733B) significantly different from non-treated infected-KCs (P=0.0391) was detected. Afterwards, the addition of MgA led to a significant decrease of urea (P_{3h}= 0.0078; P_{24h} = 0.0416) production when compared with the production estimated at 1.5 h.

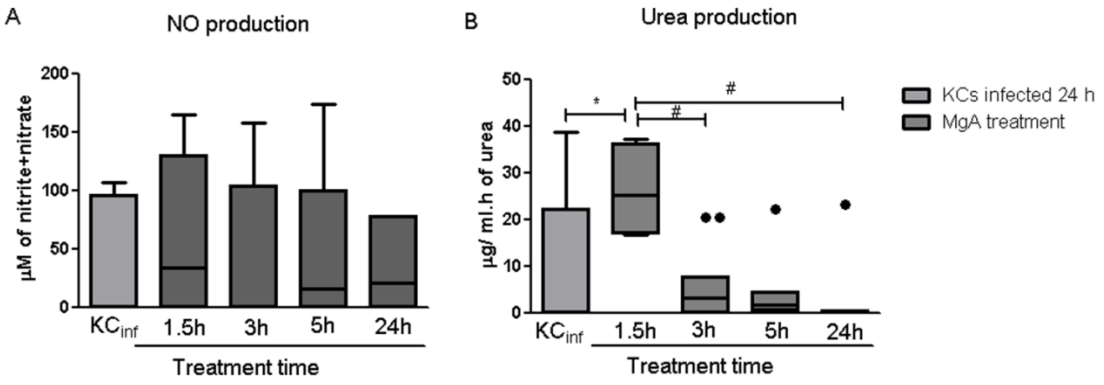


Figure 73: Production of NO and urea by *L. infantum* infected-KC treated with MgA.
KC infected with virulent promastigotes for 24 hand treated with MgA were used to evaluate NO (A) and urea (B) production at 1.5 h, 3 h and 5 h and 24 h after treatment. Untreated infected-KCs (KC_{inf}) were also evaluated. Results evaluated in triplicate are represented by whisker box-plot (minimum to maximum). Outlier values are indicated by black dots. The non-parametric Wilcoxon test was used for statistical comparisons. * and # ($p<0.05$) indicate significant differences.

Gene expression of innate immune receptors NOD1, NOD2, TLR2, TLR4 and TLR9 by *L. infantum* infected-KC that were under MgA treatment were measured by real-time PCR. Gene expression of pro- and anti-inflammatory key cytokines were also accessed.

Treatment with MgA induced an initial increase of NOD1 and NOD2 gene expression by KC after 1.5 h, however, not statistical significant (Figure 744A). NOD2 mRNA in MgA treated KC decreased with the time of exposition to treatment, compared to infected KC and LPS exposed cells (P= 0.0039) (Figure 744B).

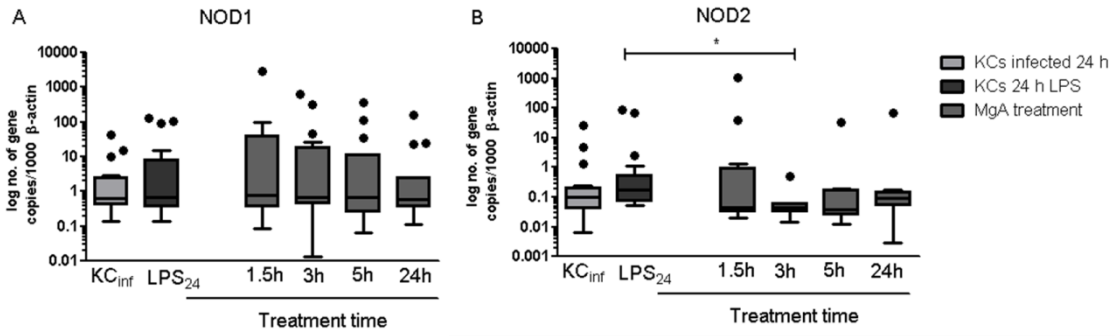


Figure 74: Gene expression of innate sensors by *L. infantum* infected-KC treated with MgA.
KC infected with virulent promastigotes for 24 hand treated with MgA were used to evaluate the number of copies of NOD1 (A) and NOD2 (B) by real time PCR at 1.5 h, 3 h, 5 h and 24 h after treatment. NOD1 and NOD2 of untreated infected KCs and LPS-stimulated KCs were also evaluated. Results evaluated in triplicate are represented by whisker box-plot (Tukey). The non-parametric Wilcoxon test was used for statistical comparisons. Outlier values are indicated by black dots. * and # ($p<0.05$) indicate significant differences.

After MgA addition, it was verified an increase in gene expression of TLR2 and TLR4 indicating a possible involvement of these receptors in the immune activation against *L. infantum* amastigotes (Figure 75). Early after MgA addition (1.5 h incubation) TLR2 expression registered a significant increase compared with not treated infected-KC (P= 0.0070). Significant increases were also observed at 3 h (P=0.0080) and 5 h (P=0.0105) after treatment. TLR4 registered significant increases at 1.5 h (P=0.0067) and 3 h (P= 0.0151) of treatment. TLR9 registered an increase in expression levels after 5 h incubation, however, not statistically significant.

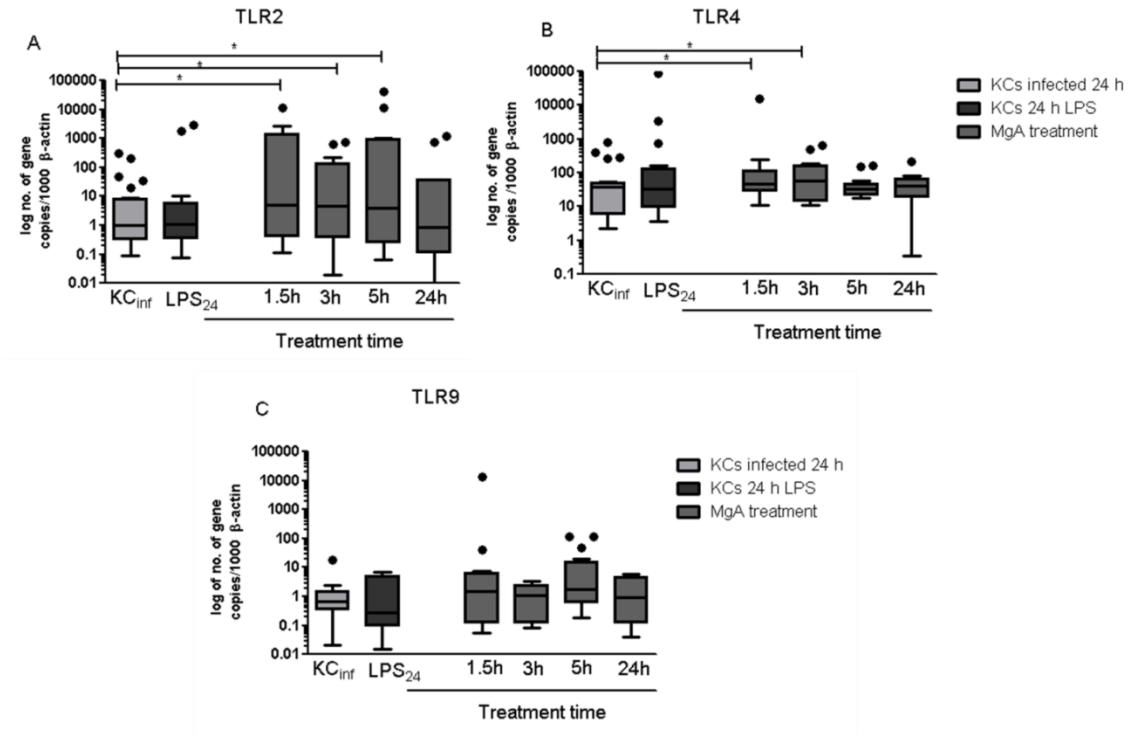


Figure 75: Gene expression of TLRs in KCs infected by *L. infantum* and treated with MgA.
KC infected with virulent promastigotes for 24 hand treated with MgA were used to evaluate the e number of copies of TLR2(A), TLR4 (B) and TLR9 (C) by real time PCR at 1.5 h, 3 h, 5 h and 24 h after treatment. TLR2, TLR4 and TLR9 of untreated infected KCs and LPS-stimulated KCs were also evaluated. Results evaluated in triplicate are represented by whisker box-plot (Tukey). Outlier values are indicated by black dots. * and # ($p<0.05$) indicate significant differences.

After MgA treatment, increased accumulation of TNF-α mRNA by infected KCs was observed, indicating that treatment overcome the silencing effect imposed by the parasite (Figure 766). IL-12 registered an evident increase in expression after 1.5 h incubation with meglumine antimoniate, which decreased with incubation time. Even so, these increases did not present statistical relevance, However, TNF-α presented a significant augment after 1.5 h of treatment (P=0.0033) that was maitainded until 5 h (P_{3h} = 0.0053, P_{5h} = 0.0302) treatment time. At this point some dogs evidence an activation and increased expression and others exibited an decreased in TNF-α expression.

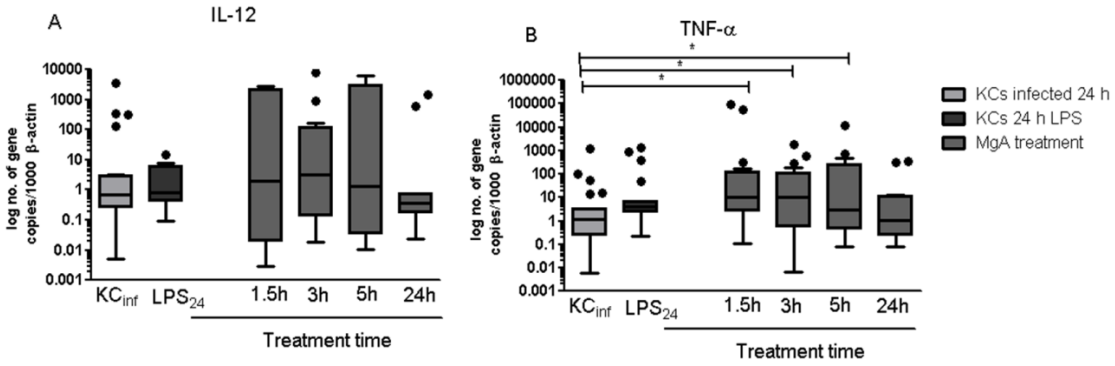


Figure 76: Gene expression pro-inflammatory cytokines by *L. infantum*-infected KC treated with MgA.
KC infected with virulent promastigotes for 24 h and treated with MgA was used to evaluate the number of IL-12 copies (A) and TNF-α (B) by real time PCR at 1.5 h, 3 h, 5 h and 24 h of treatment. IL-12 and TNF-α of untreated infected KCs and of LPS stimulated KCs were also evaluated. Results evaluated in triplicate are represented by whisker box-plot (Tukey). Outlier values are indicated by black dots. The non-parametric Wilcoxon test was used for statistical comparisons * and # ($p<0.05$) indicate significant differences.

Th2 cytokines analyzed also revealed some differences when infected KC were exposed to a leishmanicidal drug (Figure 777). When compared with untreated infected KCs, IL-10 gene expression was significantly increased after 1.5 h exposition to MgA (P=0.0413) and decreased with treatment time, indicating that exposition to MgA may influence IL-10 expression of infected KC. On the other hand, IL-4 gene expression revealed a decrease after 3 h treatment with MgA, when compared to infected-KC (P=0.0465). Non treated infected-KC exhibited the higher accumulation of IL-4 mRNA. LPS-stimulated KC also showed a significant IL-4 decrease when compared with untreated infected KC (P= 0.0043) and with treated cells ($P_{1.5h}$=0.0215 and P_{3h}=0.0342). As for TGF-β expression, an increase was observed after 1.5 h of incubation with MgA, although not statistically significant. TGF-β expression showed a decreasing tendency with MgA treatment. All together, these results indicate that the addition of MgA to infected KCs influences the immune response exhibited by KCs.

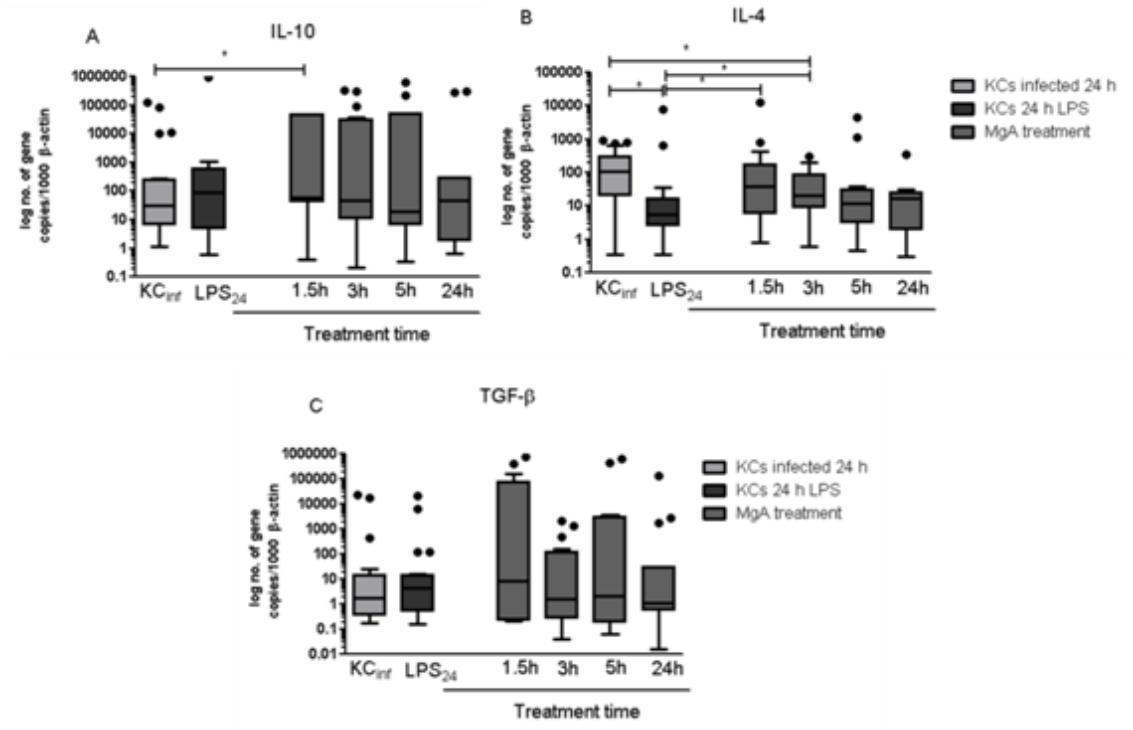


Figure 77: Expression anti-inflammatory cytokines in *L. infantum*-infected KC treated with MgA. KC infected with virulent promastigotes for 24 hand treated with MgA were used to evaluate the number of copies of IL-10 (A), IL-4 (B) and TGF-β (C) by real time PCR at 1.5 h, 3 h, 5 h and 24 h of treatment. IL-10, IL-4 and TGF-β of untreated infected KCs and LPS stimulated KCs were also evaluated. Results evaluated in triplicate are represented by whisker box-plot (Tukey). Outlier values are indicated by black dots. The non-parametric Wilcoxon test was used for statistical comparisons * and # ($p<0.05$) indicate significant differences.

5.4.4. Hepatocytes induce TLR2 gene expression in co-culture with *L. infantum*-infected Kupffer cells and generate a mix profile of cytokines

To investigate if the presence of hepatocytes could increase immune activation signals, co-cultures of KC-infected by *L. infantum* and canine hepatocytes were performed. KC were infected with *L. infantum* virulent promastigotes for 24 h. During this period of time, parasites were internalized and differentiated into amastigotes. Gene expression of innate immune sensors NOD1, NOD2, TLR2, TLR4 and TLR9 of KCs were accessed after 1.5 h, 3 h, and 5 h of culture with hepatocytes and compared with just infected KCs.

NOD1 gene expression registered an increased after 1.5 h, although not statistically important (Figure 788A). At 3 h of incubation cells registered a decrease in

NOD1 mRNA accumulation (P=0.0066). However, it is still considered an increase in NOD1 expression when compared to amastigote-infected KC (P=0.451). Curiously, NOD2 expression levels (Figure 788B) in co-culture did not differ from NOD2 expression levels in KC infected with amastigote.

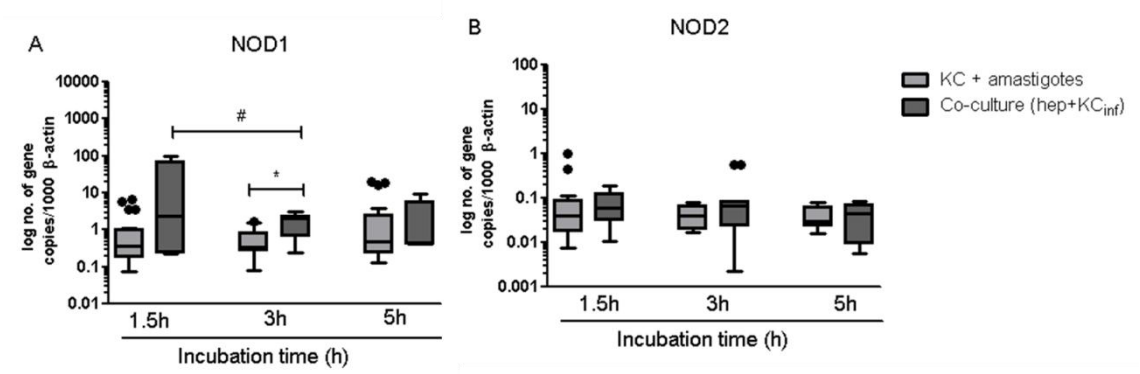


Figure 78: Gene expression of NOD1 and NOD2by infected-KC in co-culture with hepatocytes.
KCs infected with amastigotes for 24 h co-cultured with hepatocytes were used to evaluate the number of copies of NOD1(A) and NOD2 (B). In parallel NOD1 and NOD2 of just infected KCs were also evaluated. The number of copies was determined by real time PCR at 1.5 h, 3 h, and 5 h. Results evaluated in triplicate are represented by whisker box-plot (Tukey). Outlier values are indicated by black dots. The non-parametric Wilcoxon test was used for statistical comparisons. * and # ($p<0.05$) indicate significant differences.

As for TLR2 gene expression, the presence of hepatocyte was able to induce a transient increse in this receptor after 1.5 h incubation in co-culture (P=0.0020) when compared with just infected KCs (Figure 7979). In KC infected with amastigotes there was a significant increse in TLR2 gene expression from 1.5 h to 5 h (P=0.0131). TLR4 gene expression in co-cultures exhibited a significant decrease after 3 h in culture (P=0.0156) and were kept lower than in infected KCs. TLR9 gene expression appeared to increase in infected KCs after 3 h in co-culture, but transiently and without statistically significance.

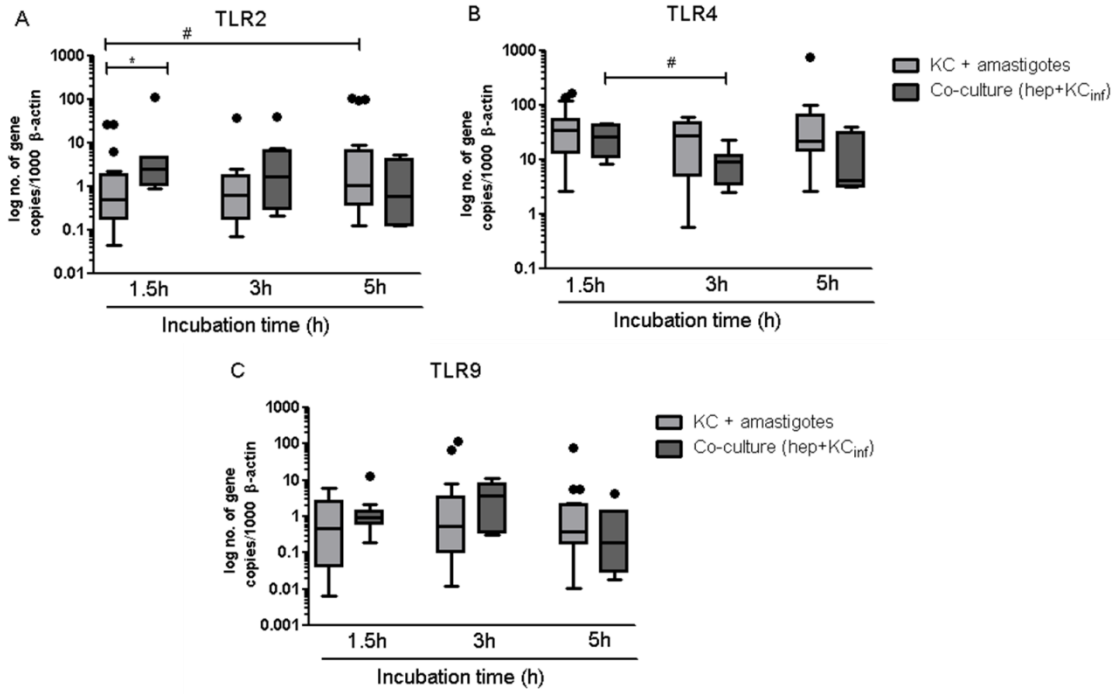


Figure 79: Gene expression of TLR2, TLR4 and TLR9 by infected KC in co-culture with hepatocytes.
KCs infected with amastigotes for 24 h co-cultured with hepatocytes were used to evaluate the number of copies of TLR2 (A). TLR4(B) and TLR9(C). In parallel TLR2, TLR-4 and TLR9of just infected KCs were also evaluated parallel. The number of copies of was determined by real time PCR at 1.5 h, 3 h, and 5 h. Results evaluated in triplicate are represented by whisker box-plot (Tukey). The non-parametric Wilcoxon test was used for statistical comparisons. Outlier values are indicated by black dots. * and # ($p<0.05$) indicate significant differences.

Cytokine gene expression of infected KCs experienced slight changes when in the presence of hepatocytes. IL-12 registered an increase in gene expression after 1.5 h in co-culture, although not statistically significant (Figure 800A). The levels of TNF-α mRNA accumulation in infected-KC co-cultured with hepatocytes for 1.5 h registered a significant increase when compared with amastigote-infected KC (P=0.0098). KC infected with amastigotes significantly decreased TNF-α expression after 3 h post-infection (P=0.0263) and also when in co-culture (P= 0.0137) (Figure 800B). These indicate that hepatocytes were able to induce infected KCs to generate TNF-α, evading parasite imposed silencing.

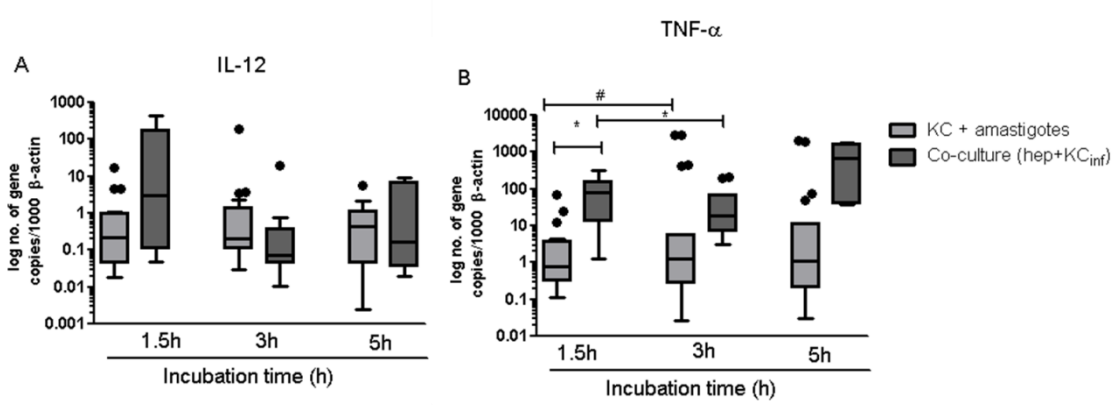


Figure 80: Gene expression of pro-inflammatory cytokines by infected KC co-cultured with hepatocytes.
KCs infected with amastigotes for 24 h co-cultured with hepatocytes were used to evaluate the number of copies of IL-12 (A) TNF-α (B). In parallel IL-12 and TNF- α of just infected KCs were also evaluated. The number of copies was determined by real time PCR at 1.5 h, 3 h, and 5 h. Results evaluated in triplicate are represented by whisker box-plot (Tukey). Outlier values are indicated by black dots. The non-parametric Wilcoxon test was used for statistical comparisons. * and # ($p<0.05$) indicate significant differences.

Anti-inflammatory cytokines IL-4 and TGF-β exhibit major increases in their gene expression in infected-KCs co-cultured with hepatocytes (Figure 811). IL-10 expression levels were increased after 1.5 h in co-culture and remain raised. The increase verified at 5 h in co-culture was statistically important (P=0.0078) and corresponded to the higher expression value observed. KCs infected with amastigote revealed a increase in IL-10 gene expression after 3 h post-infection (P=0.0214). IL-4 gene expression were increased in infected KCs co-cultured with hepatocytes during the entire experimental period when compared with infected-KC ($P_{1.5h}$=0.039, P_{3h}=0.0195, P_{5h} =0.0156). TGF-β gene expression were also greatly impacted by the presence of hepatocyte in a KC-infected co-culture. This cytokine also revealed a major increase after 1.5 h in co-culture (P=0.0039) when compared with just infected KCs. A light increase in TGF-β in KC amastigote infection was observed after 3 h post-infection (P=0.0102).

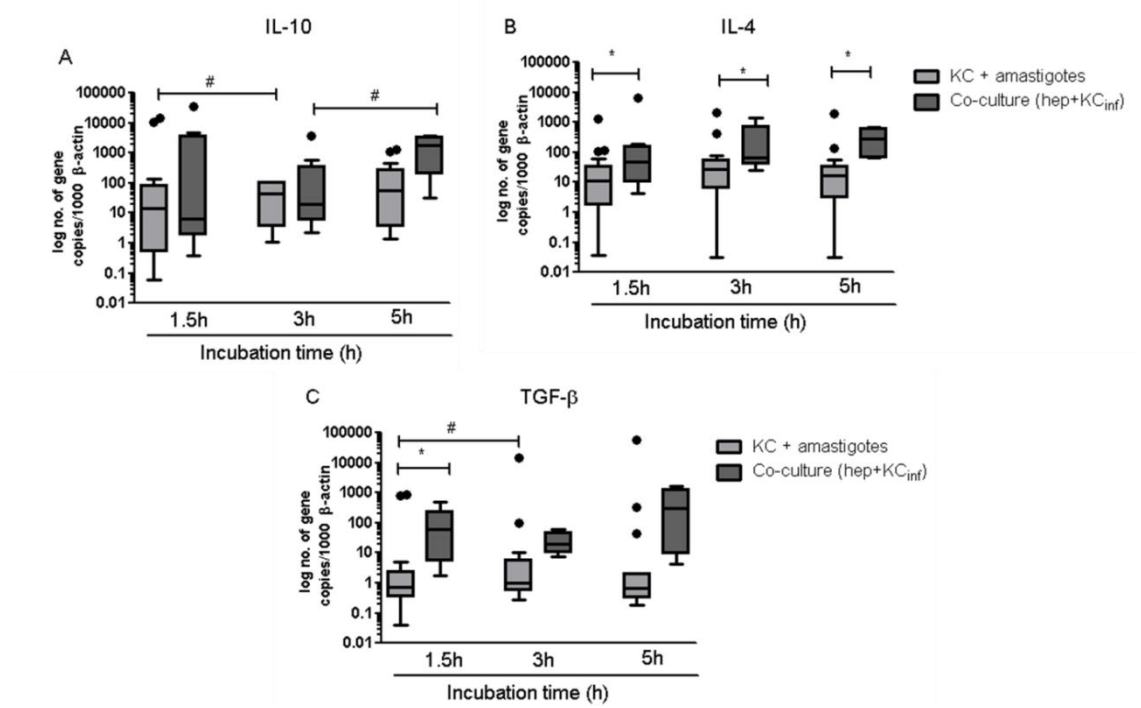


Figure 81: Gene expression of anti-inflammatory cytokines by infected KC co-cultured with hepatocytes.
KCs infected with amastigotes for 24 h
co-cultured with hepatocytes were used to evaluate the number of copies of IL-10 (A), IL-4 (B) and TGF-β (C).In parallel IL-10, IL-4 and TGF-β of just infected KCs were also evaluated. The number of copies was determined by real time PCR at 1.5 h, 3 h, and 5 h. Results evaluated in triplicate are represented by whisker box-plot (Tukey). Outlier values are indicated by black dots. The non-parametric Wilcoxon test was used for statistical comparisons. * and # ($p<0.05$) indicate significant differences.

5.4.5. Kupffer cells are immune tolerant cells in comparison with blood macrophages when facing a *L. infantum* infection

The immune profile evidenced by circulating blood MØs when exposed to *L. infantum* promastigotes and by KCs when exposed to *L. infantum* amastigotes was compared. In this particular case, MØs and KCs were infected with different parasite forms, mimicking the natural progression of the parasite infection. Promastigotes are inoculated by the sand fly in the dog and they are the most likely form able to induce blood monocyte differentiation into MØ in association with parasite phagocytosis. When arriving to the liver, the parasite was already up taking by the host cell, acquiring the intracellular amastigote form. Although, it is possible that blood-MØs transport amastigotes the presence of promastigote forms in internal compartments, such as the liver, is not usually described. Lipopolysaccharide (LPS) was used in KCs as a positive control of inflammation, since it is a classical inducer of MØ inflammation.

5.4.5.1. Gene expression of cell sensors

Gene expression of the innate immune receptors NOD1 and NOD2 revealed a similar tendency in promastigote infected-blood MØs and in amastigote infected-KCs. During the first 3 h of infection, blood-MØs evidenced significant higher levels of expression that were reduced at 5 h of infection (Figure 822). After 1.5 h and 3 h of exposition to promastigotes, there was a significant accumulation of NOD1 ($P_{1.5h}$<0.0001, P_{3h}=0.0145) and NOD2 ($P_{1.5h}$<0.0001, P_{3h} =0.0156) mRNA when compared with KCs. At early infection (1.5 h), the NOD1 gene expression observed in KCs was significantly lower than by LPS stimulated-KCs (Figure 822A). After 5 h of infection, MØs decreased the gene expression of both sensors (P<0.0001) in comparison to the previous time point (3 h). When compared with blood-MØs, KCs exhibited a significant increase of NOD1 copy number (P=0.0303) at 5 h of infection, reaching levels similar to LPS stimulated-KCs However, the accumulation of NOD2 mRNA in blood-MØ (Figure 822B) still is more elevated that in KCs (P=0.0129). LPS-stimulated KCs exhibited higher NOD1 gene expression at 1.5 h of incubation (P=0.0095) and, NOD2 at 1.5 h (P=0.0015) and 3 h (P=0.0067) of incubation.

Taken together, these results indicate that MØs from two different lineages seem to differ in *L. infantum* recognition through NOD1 and NOD2 sensors. Also, the stimulation induced by LPS had different effects in the two sensors investigated.

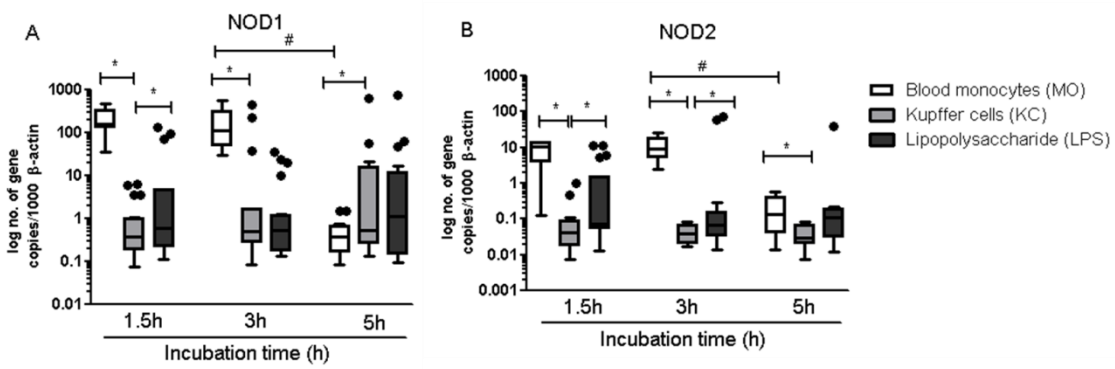


Figure 82: Gene expression of NOD1and NOD2 sensors by *L. infantum* infected KCs and blood-MØs.
KCs were exposed to amastigotes and blood-MØs to promastigotes for 5 h. The number of copies of NOD1(A) and NOD2 (B) was determined by real time PCR at 1.5 h, 3 h and 5 h. In parallel, NOD1 and NOD2 gene expression of LPS-stimulated-KCs was also evaluated. Results expressed by whisker box-plot (Tukey). Outlier values are indicated by black dots. The non-parametric Wilcoxon test was used for statistical comparisons. * and # (p<0.05) indicate significant differences.

Other accessed innate immune receptors included TLR2, TLR4 and TLR9. For these receptors also major differences were registered between blood-MØ sand KCs (Figure 833). After 1.5 h of infection, it was verified a significant increase of TLR2 (Figure 833A) and TLR4 mRNA ($P<0.0001$) (Figure 833B) in comparison with KCs. These higher values persisted until 3 h post-infection, being still statistically significant for TLR4 ($P=0.0020$). After 5 h of infection, the number of copies of both sensors decreased to lower levels ($P_{TLR2}<0.0001$, $P_{TLR4}=0.0010$) when compared with the previous time point. In a similar way, the TLR9 expression levels significantly increase in infected blood MØs when compared with infected KCs at both 1.5 h ($P<0.0001$) and 3 h ($P=0.0195$) time points (Figure 833C). After 5 h of infection, the levels of TLR9 mRNA significantly decrease when compared with 3 h time point ($P<0.0001$) and with KCs ($P=0.0228$).

Taken together, these results indicate that MØs from two different lineages seem to differ in *L. infantum* recognition through cell innate sensors. The reduction of sensor's gene expression observed 5 h post-infection could be a consequence of a possible silencing effect exerted by the parasite. Also, the stimulation induced by LPS had different effects in the sensors investigated. It is also apparent the presence of several outliers as a probable evidence of the different genetic background of the canine population.

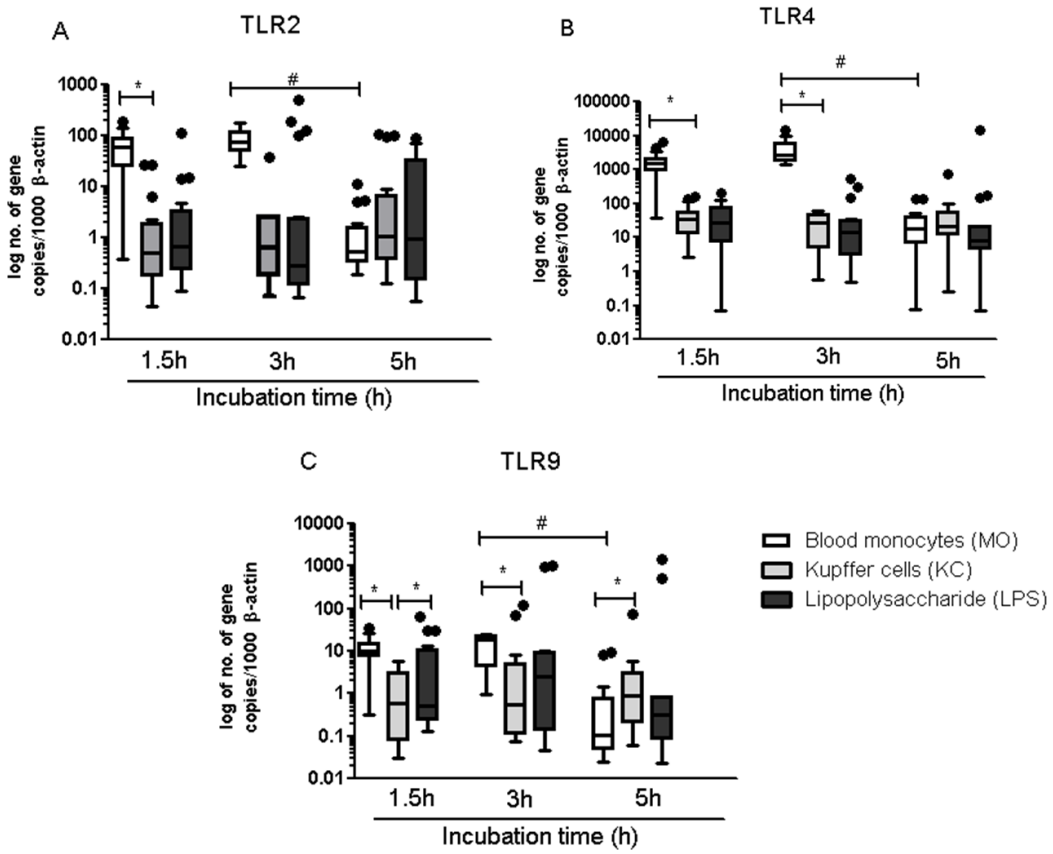


Figure 83: Gene expression of TLR2, TLR4 and TLR9 sensors by *L. infantum* infected blood MØs and KCs.
Blood MØs were exposed to promastigotes and KCs to amastigotes for 5 h. The number of copies of TLR2 (A), TLR4 (B) and TLR9 (C) was determined by real time PCR at 1.5 h, 3 h and 5 h. In parallel TLR2 and TLR4 gene expression of LPS-stimulated KCs was also evaluated. Results expressed by a whiskers box-plot (Tukey). Outlier values are indicated by black dots. The non-parametric Wilcoxon test was used for statistical comparisons.* and # ($p<0.05$) indicate significant differences.

5.4.5.2. Pro- and anti-inflammatory cytokine gene expression

Until 3 h of infection, blood-MØs exhibited significant increase of IL-12 ($P_{1.5h}<0.0001$, $P_{3h}=0.0010$) and TNF-α ($P_{1.5h}<0.0001$) gene expression when compared with KCs (Figure 844). Nevertheless, after 5 h post-infection the expression significantly decays when compared with the previous time point (3 h) as a result of a possible silencing effect exerted by the parasite. Expression levels of IL-12 ($P_{1.5h}=$ 0.0066,

$P_{3h}=$ 0.0052, $P_{5h}=$ 0.0425) and TNF-α ($P_{1.5h}<$ 0.0001, P_{3h} = 0.0329) by infected-KCs is lower than by LPS stimulated-KCs.

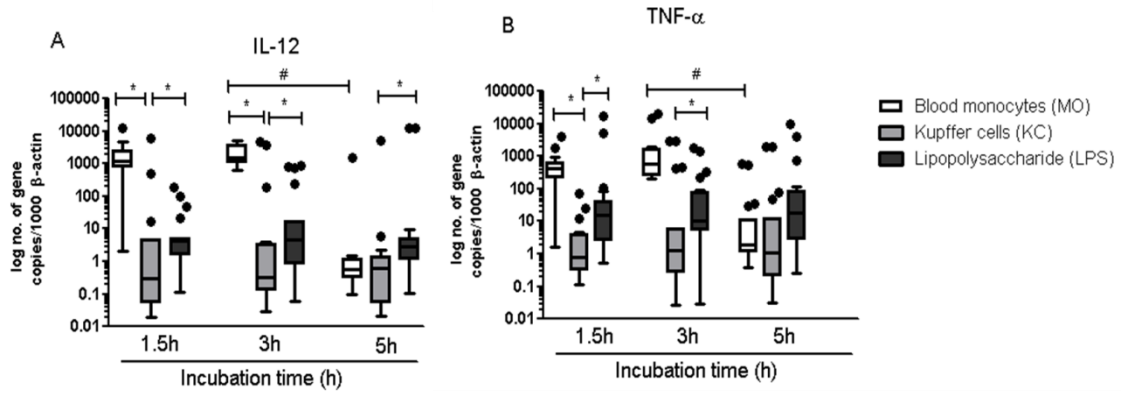


Figure 84: Gene expression of pro-inflammatory cytokines IL-12 and TNF-α by *L. infantum* infected KCs and blood-MØs.
Blood-MØs were exposed to promastigotes and KCs to amastigotes for 5 h. The number of copies ofIL-12 (A) and TNF-α (B) was determined by real time PCR at 1.5 h, 3 h and 5 h. In parallel IL-12 and TNF-α gene expression of LPS-stimulated KCs was also evaluated. Results expressed by a whiskers box-plot (Tukey). Outlier values are indicated by black dots. The non-parametric Wilcoxon test was used for statistical comparisons. * and # ($p<0.05$) indicate significant differences.

Infected blood-MØs presented significant increased IL-10 (Figure 855A) and IL-4 (Figure 855B) gene expression from 1.5 h ($P<0.0001$) until 3 h ($P_{IL-10}=0.0411$, $P_{IL-4}=0.0313$) post-infection when compared with infected KCs. After 5 h in contact with the parasite it was observed a considerable reduction in the copy number of IL-10 ($P_{IL-10}<0.0001$) and IL-4 when compared with the previous time point (3 h) ($P_{IL-4}=0.0003$). At 1.5 h of parasite exposure, KCs evidenced low accumulation of IL-10 ($P=0.0280$) and IL-4 mRNA ($P=0.0318$) in comparison to LPS stimulated-KCs.

TGF-β gene expression followed a similar pattern (Figure 855C). Significant high expression was observed in blood-MØs at 1.5 h ($P<0.0001$), 3 h ($P=0.0039$) and 5 h ($P=0.0240$) of incubation with promastigotes when compared with infected KCs. However, at 5 h of incubation was verified a significant decrease in the accumulation of TGF-β mRNA by blood-MØs when compared with the previous time point (3 h) ($P<0.0001$). At 1.5 h, TGF-β copy number of infected KCs was significant reduced in comparison to LPS stimulated-KCs ($P=0.0294$).

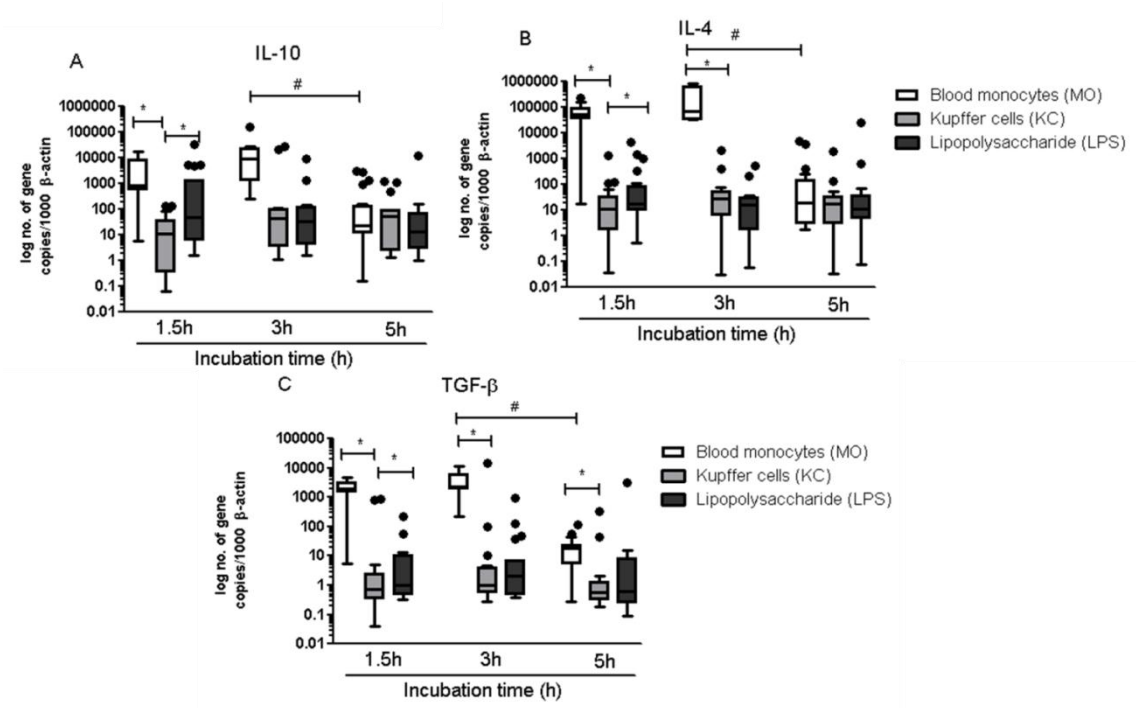


Figure 85: Gene expression of anti-inflammatory cytokines IL-10, IL-4 and TGF-β by *L. infantum* infected KCs and blood-MØs.
Blood MØs were exposed to promastigotes and KCs to amastigotes for 5 h. The number of copies of IL-10 (A), IL-4 (B) and TGF-β (C) was determined by real time PCR at 1.5 h, 3 h and 5 h. In parallel IL-10, IL-4 and TGF-β gene expression of LPS-stimulated KCs was also evaluated. Results expressed by whisker box-plot (Tukey). The non-parametric Wilcoxon test was used for statistical comparisons. Outlier values are indicated by black dots. * and # ($p<0.05$) indicate significant differences.

Taken together, these results put in evidence that the initial contact (≈3 h) of blood-MØs with promastigotes induce host cell activation, leading the simultaneous generation of pro- and anti-inflammatory cytokines. However, a prolonged time of contact with the parasite (5 h) has an opposite effect in the host cell, reducing cell activity and the cytokine gene expression. At this time, is probable that phagocytosis has occurred, at least in same extension and a few parasites have differentiated into amastigotes. On the other hand, KCs harboring internalized amastigotes were unable to trigger immune activation, at least during the period of time considered in this study.

.

5.5. Discussion

Kupffer cells (KCs) constitute the liver resident MØs localized within the lumen of the liver sinusoids, adherent to the endothelial cells that compose the blood vessel walls. KCs are of particular importance as they constitute the first MØ population of the body to come in contact with bacteria, bacterial endotoxins and microbial debris derived from the gastrointestinal tract and transported to the liver via the portal vein. Together with natural killer cells (NKs), dendritic cells (DCs) and soluble compounds, such as complement factors and acute phase proteins, KCs represent an important component of innate immunity, the initial rapid response to a potentially dangerous or, by the contrary have a key role in directing immune tolerance. In the present study, KCs were isolated from livers of apparent healthy dogs and their immune response displayed against *L. infantum* was accessed for the first time. Since dog constitutes the peridomestic reservoir of this parasite, it is of major importance to understand how reacts to parasite and why allows parasite establishment. Understanding how KCs, the host cells of *L. infantum* in the liver, sense and react to the parasite presence, may result in a better understanding of the mechanisms elicited by the parasite to control the host immune response.

Isolated cells revealed to be positive for CD68 receptor and for lysozyme presence, two characteristics of macrophages (MØ). CD68 is glycoprotein highly expressed by monocytes and tissue macrophages, including KC, microglia, histiocytes and osteoclasts. Being classified as a member of the scavenger receptor family, typical functions of CD68 include clearance of cellular debris, promotion of phagocytosis, and the mediation, recruitment and activation of MØs. CD68 is also a member of the lysosome associated membrane protein (LAMP) family, where CD68 predominantly localizes to lysosomes and endosomes with a smaller fraction circulating to the cell surface (Saito *et al.*, 1991). Isolated cells were CD68 positive, with receptor localization in several intracellular vesicles throughout the cells, coinciding with what was described. MØs are also an important source of physiologically active secretory products such as cytokines, growth factors, complement and clotting proteins, proteases and other enzymes. Lysozyme, also known as muramidase or N-acetylmuramide glycanhydrolase are glycoside hydrolases, present in several body secretions, and

exhibit an enzymatic activity that damage bacterial cell walls. Lysozyme is found in the cytoplasm of MØs and is generally regarded as a constitutive marker of MØs biosynthetic activity (Keshav *et al.*, 1991). KCs contain lysozyme in their cytoplasmic granules, vacuoles and phagosomes and some cells show a positive reaction in the rough endoplasmic reticulum, perinuclear cisternae and the Golgi zone (Miyauchi *et al*., 1985).Isolated cells from the canine livers were also positive lysozyme, which together with the presence of CD68, confirm their identity as liver resident MØ or KCs. As for intracellular localization, lysozyme was detected in abundance in several cytoplasmic granules. In human MØs, it was reported co-localization of CD68 with lysozyme (Saito *et al*., 1991). A similar situation was observed with canine KCs, with co-localization of CD68 with lysozyme in several cytoplasmic granules and vacuoles.

Different KC infection patterns were found between amastigotes and promastigotes of *L. infantum*. Amastigotes constitute the natural form of parasite infecting KCs, as it comprises the form of the parasite that arrives to the liver. Promastigotes are the form of the parasite that infects circulating MØs recruited to the site of infection. It is widely known that there are major differences between promastigotes and amastigotes membrane proteins, which confer higher adaptability to its specific environment. *Leishmania* experiences extreme environmental changes as it alternates between the insect gut as a free motile form (promastigote) and the fagolisossome of mammalian host cell as the intracellular non-flagellate form (amastigote). In addition to morphological characteristics, the two life stages also have differences in the surface molecular constitution. While infectious metacyclic promastigotes have a thick glycocalyx over the entire body this cover is almost absent in amastigotes (Pimenta *et al*., 1991). The glycocalyx is mainly constituted by glycoproteins and glycosylated molecules anchored to the surface membrane by a glycosylphosphatidylinositol (GPI) linkage (Ferguson *et al*., 1997). The promastigote surface is predominantly covered by lipophosphoglycan (LPG), a GPI-anchored molecule made of repeating units of a disaccharide and a phosphate. Promastigotes have another important GPI-anchored molecule, the surface protease gp63. By the contrary, amastigotes have been shown to produce very little LPG (McConville and Blackwell, 1991) and have less gp63 at the surface (Souza *et al*., 1992). These differences in the

membrane constitution as a consequence of the adaptation to different micro-environments are important in the immune regulation of the host.

However, the signaling events controlling amastigote differentiation remain poorly understood. In some *Leishmania* species, differentiation of promastigotes into amastigotes is induced by elevated temperature and low pH, conditions found within MØ parasitophorous vacuoles. It is described that the link of *L. mexicana* to the human C-reactive protein present in the serum greatly stimulates the transformation of promastigotes into amastigotes (Bee *et al*., 2001). Recently, Mittra *et al.* (2013) revealed a novel role for iron uptake in orchestrating the differentiation of amastigotes, through a mechanism that involves production of reactive oxygen species (ROS). Function as signaling molecules, ROS regulate *Leishmania* differentiation in a process that is tightly controlled by iron availability and is independent of pH and temperature changes. Thus, the signaling process that triggers parasite differentiation is complex and multifactorial, culminating in the relative promastigote susceptibility and amastigote resistance to MØ-mediated killing and in the apparent inability of susceptible hosts to orchestrate the necessary immune response to activate MØs and destroy intracellular amastigotes (Sadick and Raff, 1985). In the present work, amastigotes were found to be more efficient and apt to infect KCs. This result might be expected as amastigotes constitute the liver infective form. However, promastigotes can also be taken in by KCs, pointing out the susceptibility of this cell lineage to this parasite form, with which the natural contact is unusual, but in this case the immunological "takeover" of host cell takes longer time.

Amastigotes and promastigotes of *L. infantum* exhibit an evident tropism to KCs, with more than one parasite being phagocytized by a single cell. But the rate and efficiency of the infection were different between amastigote and promastigote. Amastigotes quickly achieve 100% infection while promastigotes required prolonged incubation to achieve identical infection levels. Therefore, amastigotes seem to be more fit to infect KCs than promastigotes. Amastigotes uses different phagocytosis receptors than promastigotes to be recognized by MØs, enabling a fast phagocytosis and a rapid control of innate immune activation of host cells. Given that, promastigotes have to avoid MØ microbicidal activity to establish themselves in the host and amastigotes have to suppress MØ killing abilities when try to invade new MØs in the course of a

persistent *Leishmania* infection, it is not surprising that both forms of the parasite might modulate key MØ signaling pathways (Olivier *et al*., 2005). Several studies have previously demonstrated that promastigotes and amastigotes or derived molecules are able to block nitric oxide (NO) production by host MØs in response to activating stimuli, such as gamma interferon (IFN-γ) or bacterial lipopolysaccharide (LPS). As indicated above, promastigote surface contains several glycoconjugates favoring the interaction with MØs and internalization via several types of receptors, such as complement receptors 1, 3, and 4, the mannose fucose receptor, the C-reactive protein receptor and the fibronectin receptor (Guy and Belosevic, 1993; Abu-Dayyeh *et al*., 2010). On the other hand, amastigotes lack many of those glycoconjugates and seem to interact with MØs mainly through glycosylinositol phospholipids (GIPLs) and to be phagocytized via the Fc receptors following opsonization by antibodies or via complement receptors (Guy and Belosevic, 1993). Although redundancies might exist in the way promastigotes and amastigotes interact with MØs and with KCs, both modulate signaling to block MØs killing functions. The differences between both forms, whether at the gene expression, metabolic or at a surface molecule level suggests that some differences ought to exist, so that both forms can modulate MØ signaling to their own favor. Promastigote need to block immune activation to successfully infect MØ for the first time, and the amastigote form in order to be able to continue the ongoing infection without activating MØs. So, both parasitic forms can modulate the immune activation of the host cells, but amastigotes are more efficient in doing so. This form of *L. infantum* is perfectly adapted to keep infection ongoing, by infecting new MØs (or KCs), using different phagocytic receptors and strategies, as the antigen release through the parasitophorus vacuole, downregulating MØ activation and avoiding parasite destruction.

MØs constitute a decisive effector cell in *Leishmania* infection. Depending on the balance between the inducible enzymes nitric oxide synthase (iNOS) and arginase, these cells may kill the parasite or host it, allowing parasite replication and dispersion. Both enzymes use L-arginine as a substrate and are competitively regulated by cytokines secreted by Th1 and Th2 cells. Th1 cytokines induce MØ classical activation, leading iNOS activation that oxidizes L-arginine in a two-step process and release nitric oxide (NO), a metabolite responsible for the parasite killing. Th2 cytokines result in the

MØ alternative activation with the induction of arginase, which hydrolyzes L-arginine into ornithine, an amino acid that is the main intracellular source for the synthesis of polyamines that are important for the parasite survival. Arginase is also classically considered to be an enzyme of the urea cycle in the liver (Hofmann *et al*., 1978). In mammals, have been identified two distinct arginase isoforms encoded by different genes that also differ in the cellular localization and in the mode of regulation. Type 1 arginase is a cytosolic enzyme expressed at high levels in the liver as a component of the urea cycle, and type 2 arginase is a mitochondrial enzyme found in a variety of tissues. It is also known that MØs up-regulate arginase 1 upon activation by Th2 cytokines, expressing at the same time low levels of arginase 2 (Munder *et al*., 1998). Kropf *et al*. (2005) demonstrated a direct correlation between the unrestricted replication of *L. major* parasites in susceptible hosts and the increasing levels of arginase at the site of lesion. In contrast, parasite load is detectable but controlled in genetically resistant hosts, which also resolve the cutaneous lesions and acquire immunity to reinfection accompanied by arginase down-regulation. It is generally accepted that IFN-γ produced by polarized Th1 cells of healer strains of mice, direct the induction of iNOS by MØs and the subsequent synthesis of NO, promoting parasite killing (Bogdan, 2001). In the present study, the initial contact of KCs with *L. infantum,* regardless the parasite form used, lead to NO production and to a burst of urea, indicating the activation of both enzymes iNOS and arginase. However, with the increase to parasite exposure becomes apparent the immune modulator effect exerted by the parasite in KCs activation through the accentuated reduction of available NO, as a probable consequence of iNOS downregulation. This immune modulator effect was much more evident in amastigote infected-KCs, where it was noted a faster decrease of NO and urea, pointing for the silencing of both arginine pathways associated with a possible induction of a KC tolerant stage.

The importance of careful regulation of urea synthesis may also be addressed observing the effect of polyamines production on the host. Polyamines are not only beneficial for the growth of *Leishmania* they also have wider effects by modulating immune functions and affecting signaling transduction pathways. For example, spermine and spermidine have been shown to inhibit the secretion of pro-inflammatory cytokines and spermine counter-regulates innate immune response induced by the TLR4

ligand lipopolysaccharide (LPS) (Perez-Cano *et al*., 2003). Therefore, increased polyamine levels in *Leishmania* infections could contribute to the inefficient host defense, at least in BALB/c mice.

To exert control over NO production, *Leishmania* exploits the host immune response. Interestingly, *Trypanosoma cruzi*, another member of the *Trypanosomatidae* family do not express their own arginase, inducing host MØ arginase via the parasite derived molecule cruzipain (Stempin *et al*., 2002). By capturing host arginase, this parasite reduces the availability of L-arginine for NO synthesis, avoiding parasite killing (Gobert *et al*., 2000). In contrast, *Leishmania* parasites express their own originals and do not directly induce arginase expression in host cells. Instead, exploit the Th2 immune response of susceptible hosts to induce arginase activity and the consequent production of ornithine/polyamines for parasite multiplication, resulting in a devastating circle of uncontrolled parasite growth and pathology. This particular strategy contributes for a successful infection, since the inactivation of both arginase alleles results in non-viable parasites (Roberts *et al*., 2004).

Although this study present indirect evidence of the rapid action of *L. infantum* amastigotes over iNOS and arginase of KCs this modulation is not associated with an imbalance in the generation of pro- and anti-inflammatory cytokines. In fact, the contact with amastigotes or promastigotes is not related to cytokine generation by KCs, pointing for an unresponsiveness state characteristic of immune tolerance as consequence of parasite manipulation.

The majority of the studies is focused in MØ signaling and activation by promastigotes and fewer reports investigated signaling alterations mediated by amastigotes, having a lack of comparative studies. The cytokine profile obtained for KCs in this study indicates an incomplete activation and gives evidence of a rapid suppressive effect of amastigotes and promastigotes over KCs that can outline the infection outcome. For instance, the pro-inflammatory cytokine TNF-α is the key to initiate granuloma formation, which is associated with host resistance to *L. infantum* infection in the mouse model of VL as well as in canine and human host (Pearson and Sousa, 1996; Murray, 2001; Sanchez *et al*., 2004). TNF-α gene expression was kept low, similar to non-infected cells, as well as pro-inflammatory IL-12, demonstrating the

immune modulating activity of *L. infantum*. Curiously, TNF-α gene expression is rescued if a leishmanicidal drug is added or in a co-culture with hepatocytes.

There are plenty of studies on the innate immune response in animal leishmaniasis models, only a few studies are completed in human leishmaniasis and, to the best of our knowledge the present work constitutes the first study of innate immune receptors in dog KCs in the context of a *L. infantum* infection. Toll-like receptors (TLRs) are a well-characterized class of PRRs dependent on the recruitment of a single or a specific combination of TIR-domain-containing adaptor protein (e.g., MyD88, TIRAP, TRIF, or TRAM) (Kawai and Akira, 2010). Myeloid differentiation factor 88 (MyD88) is used by the majority of the TLRs, constituting a protein that interacts with several other molecules in signaling a downstream cascade that leads production of pro-inflammatory cytokines. The activation of MØ functions is first mediate by TLRs that play important roles in the control of infection (Gazzinelli and Denkers, 2006). TLRs are essential in connecting the innate and adaptive immune systems, in enhancing phagocytosis and promoting parasite killing. Signaling TLR downstream pathways is often associated with the production of effector molecules, such as pro-inflammatory cytokines that promote the differentiation of Th1 cells, leading to a resistant response of the host. There are limited studies on the characterization of TLR2 and TLR4 possible role in *Leishmania* infections, even in animal models. One of these studies performed in *L. infantum* infected BALB/c mice demonstrated an induction of TLR2 and TLR4 mRNA throughout the course of infection and a higher expression of TLR4 and TLR2 recorded at the peak of parasitic load. Further evidences suggest that the interaction of parasite with TLR2/TLR4 promotes cytokine modulation during the acute and chronic phases of the disease (Cezario *et al*., 2011). Studies using $TLR4^{-/-}$ mutant mice indicate a protective role in *L. major* infection, clearly showing a less efficient parasite control at the site of infection when compared with wild-type mice (Kropf *et al*., 2004). More recently, it was reported that $TLR2^{-/-}$ mutant mice were less susceptible to the infection with *L. amazonensis* than the wild-type C57BL6 mice, presenting a lower parasite burden and showing less inflammatory cells in the first weeks of infection (Guerra *et al*., 2010). Together, these observations indicate that depending upon *Leishmania* species TLR2 might play a role in disease establishment or resistance. In humans, it was demonstrated the relation of TLR2 and TLR4 with *Leishmania* protection and with the

differentiation of a Th1 immune response, in particular under the administration of a leishmanicidal drug (Tolouei *et al*., 2013). However, in dogs little is known about the role of TLRs during *Leishmania* infection.

De Veer *et al*. (2003) demonstrated that TLR2 is essential for the protective immune response against intracellular pathogens including *L. major* and *Toxoplasma gondii*. TLR2 seems to be able to recognize lipophosphoglycan (LPG), a major surface promastigote phosphoglycan, and be up-regulated by LPG purified from *L. major* metacyclic promastigotes at least in human NKs (Becker *et al*., 2003). Bazzocchi *et al*. (2005) demonstrated TLR2 expression in canine granulocytes, monocytes and less markedly in lymphocytes from peripheral blood. Flandin *et al*. (2006) demonstrated in mouse MØ that TLR2 can act synergistically with TLR3 in recognizing *L. donovani* promastigotes. These authors also verified that when TLR2 or TLR3 were silenced using RNA interference, internalization of *L. donovani* promastigotes and NO and TNF-α secretion were reduced. Kavoosi *et al*. (2010) demonstrated that NO production in response to *L. major* lipophosphoglycan was significantly decreased in macrophage cell line cultures pretreated with anti-TLR2, highlighting the importance of TLRs in *Leishmania* infection by inducing NO production via the TLR2 signaling pathway. Therefore, TLR2 recognizes a numerous of unrelated molecules, and their role in infection by *Leishmania* has been evaluated but is still controversial. In the present work it was observed a slight increase in KC sensor gene expression after 5 h post-infection with both amastigotes and promastigotes. TLR2 constitute a transmembrane receptor expressed on the cell surface of immune cells, as MØ and KCs in particular. The interaction of LPG with TLR2 leads to the production of inflammatory mediators as IL-12 and in a lesser extent of TNF-α, suggesting that signalization through TLR2 can have a potential anti-parasite effector role. However, according to Chandra and Naik (2008), TLR2 could be down-regulated by LPG from parasites of the *L. donovani* complex with an increasing IL-10 production by monocytes-MØs. In dog KCs infected by *L. infantum* parasites, cells seem to be unable of generating TLR2. It is also interesting to note that even the virulent promastigote form, which expresses high levels of LPG were not able to increase TLR2 accumulation. TLR4 polymorphisms have been linked to increased susceptibility and severity of *L. major* infection in human patients (Ajdary *et al*., 2011). In the present

work, KCs infection by amastigote or promastigote did not lead to an increase in the expression of this innate immune receptor, suggesting that *L. infantum* does not activate TLR4. However, TLR2 and TLR4 levels were in fact increased in blood derived MØ, pointing out the differences between tissue MØs (KCs) and circulating MØs.

TLR9 has been associated with host protective anti-leishmanial immune response, as activation of TLR9 associated pathway by its ligand cytosine-phosphate-guanosine (CpG) elicits an anti-*Leishmania* immune response (Rhee *et al*., 2002). TLR9$^{-/-}$ C57BL/6 mutant mice were transiently susceptible to *L. major*. The CpG motif containing *L. major* DNA was suggested signalize TLR9 pathway, inducing a host-protective effect (Fakher *et al*., 2009). In a more recent work, Srivastava *et al*. (2013) investigated the interaction of *L. major* LPG with TLR2 and TLR9 of mouse MØs. Curiously, LPG seems to activate TLR2, reducing host anti-leishmanial response via cytokine-mediated decrease of TLR-9 expression. In the present study, no significant alterations were observed in the levels of TLR9 expression in KCs infected with *L. infantum* amastigotes or promastigotes. This indicates that *L. infantum* does not activate TLR9 in canine KCs. Regarding the role of canine TLR2, TLR4 and TLR9 in KCs anti-*Leishmania* response more studies are needed in order to better understand the role of these receptors and clarify possible interactions between them.

NOD1 and NOD2 gene expression in KCs are well described, but its role in *Leishmania* infection has been poorly explored. Turchetti *et al*. (2015) first investigated NOD1 and NOD2 in *L. infantum* infection in canine blood derived MØ. In the present work, KCs infected by *L. infantum* amastigotes or promastigotes did not evidence increased levels NOD1 or NOD2 mRNA, suggesting that *L. infantum* is not recognized by these immune receptors. According Turchetti's work, NOD1 and NOD2 were found to have lower expression. The present results are in some accordance, as expression levels of both receptors did not increase with parasite presence. However, the levels of gene expression of both sensors were different in blood derived MØ.

In the current study it was found that canine KCs infected by *L. infantum* amastigotes and exposed to an leishmanicidal drug – meglumine antimoniate (MgA) – result in the activation of the cells with increasing expression of specific innate immune receptors and pro-inflammatory cytokines IL-12 and TNF-α. However, to balance the

pro-inflammatory response and avoid possible damage to the host an increase in IL-10 and TGF-β was also verified. MgA is a pentavalent antimonial, which mechanism of action is still not fully understood. It is considered a pro-dug, that need cellular metabolization to achieve its active form and initial studies suggested that antimonials inhibited macromolecular biosynthesis in amastigotes, possibly via perturbation of energy metabolism due to inhibition of glycolysis and fatty acid beta-oxidation. It is also attributed to MgA an immunomodulator role. MgA decreases parasite load in human patients with CL caused by *L. tropica,* with considerable effect on up-regulation of T cells, demonstrating the parasitocidal and immunomodulator effects of MgA (Meymandi *et al*., 2011). As MgA is a leishmanicidal drug the killing of amastigotes may lead to the exposition of parasite antigens otherwise hidden and lead to the activation of the KCs. Amastigotes regulates very carefully the antigens released from the parasitophorous vacuole to avoid MØ activation. In addition to repressing the microbicidal activities, such as NØ and pro-inflammatory cytokine production by host MØ, *Leishmania* also inhibits the ability of the host cell to display parasite antigens to other components of the immune system. The regulation of pro-inflammatory cytokine production is particularly evident in the case of IL-12 and TNF-α inhibition, which prevent MØ from be activate. Some studies have shown that *L. donovani* inhibits antigen presentation by repressing major histocompatibility complex (MHC) class II gene expression (Reiner *et al*., 1987). Antigen presentation may be inhibited by interfering with the loading of antigens onto MHC class II molecules (Fruth *et al*., 1993) or by sequestration of the MHC class II molecule and/or antigens within the phagolysosome (Kima *et al*., 1996). A third mechanism of inhibition was described, at least in *L. amazonensis*, where it was observed direct endocytosis of MHC class II molecules by amastigotes themselves, followed by cysteine peptidase-dependent degradation (De Souza Leao *et al*., 1995). The present results support the idea of an immunoregulator role associated with the administration of MgA. KCs infected with *L. infantum* amastigotes exhibit signals of silencing imposed by the parasite on MØ, with low NO production and anergic cytokine production. The addition of a leishmanicidal drug allows KC to break this imposed silence and activating IL-12 and TNF-α expression orchestrating an inflammatory immune response. Th2 cytokines were also increased in treated KCs, probably due to a balanced immune response, as an

uncontrolled inflammatory immune response may lead to serious consequences in the host, especially in the liver. Urea production was decreasing with treatment time to basal levels, and NO increase production, by the addition of MgA, reflecting the cell activation of iNOS. The addition of leishmanicidal drug suppresses the *Leishmania* immune silencing and TLR2 increased its expression in MgA treated KCs, accompanying the increase verified in IL-12 and TNF-α expression. Also TLR4 showed an increasing expression on KCs treated with MgA, attributing to these receptors a possible relevant role in activating KCs immune response in *L. infantum* infection. It is described the role of TLR4 in shaping the host immune response during *Leishmania* infection. Studies with $TLR4^{-/-}$ mice demonstrate the role of TLR4 in the control of *L. major* infection (Kropf *et al*., 2004). Glyco-sphingophospholipid (GSPL) and proteoglycolipid complex (PGLC) from *Leishmania* are able to induce TLR4, promoting a strong antiparasitic immune response (Karmakar *et al*., 2012). As for TLR2, *Leishmania* has also devised mechanisms to alter TLR4 signaling pathways to favor the establishment of infection. During *L. donovani* infection, TLR4 mediated MØ activation is suppressed through the release of TGF-β that activates the ubiquitin editing enzyme A20 and Src homology 2 domain phosphotyrosine phosphatase 1 (SHP-1) (Das *et al*., 2012). *L. major* utilizes its inhibitors of serine protease (ISP) to prevent TLR4 activation, inhibiting *Leishmania* uptake and killing by host MØs (Faria *et al*., 2011). As for TLR9, its role in *Leishmania* infection is poorly explored. TLR9 recognizes unmethylated CpG sequences in DNA molecules. CpG sites are relatively rare on vertebrate genomes in comparison to bacterial genomes or viral DNA. TLR9 is expressed intracellularly, within the endosomal compartments and is activated by the binding to DNA rich in CpG motifs, being essential in response against virus and bacteria (Krieg, 2008). As other TLRs, TLR9 signals lead to cell activation, initiating pro-inflammatory reactions that result in the production of cytokines such as TNF-α, IFN-γ and IL-12. In KCs treated with the leishmanicidal drug TLR9 does not register a significant increase, leading to the possibility of non-interaction of amastigotes antigens with this receptor.

Although the role of these receptors in *Leishmania* infection is poorly known, it seems that NOD1 has a possible role in MØ activation. NODs can cooperate with TLRs and regulate inflammatory and apoptotic responses. NOD1 and NOD2 recognize

peptidoglycan motifs from bacterial cell which consists of N-acetylglucosamine and N-acetylmuramic acid. These sugar chains are cross-linked by peptide chains that can be sensed by NODs. NOD1 recognizes meso-diaminopimelic acid (meso-DAP) mostly found in Gram-negative bacteria. NOD2 can sense intracellular muramyl dipeptide (MDP) typical of bacteria, such as *Streptococcus pneumoniae* and *Mycobacterium tuberculosis* (Chen *et al*., 2009). The recognition of these ligands induced oligomerization of NACHT domain and CARD-CARD interaction with CARD-containing serine-threonin kinase RIP2, which leads to the activation of RIP2 and a cascade of signaling proteins, culminating in the activation of the transcription factor NF-κB and expression of inflammatory cytokines (Hasegawa *et al*., 2008). In the present work, the increase in NOD1 expression after MgA treatment was transient and for NOD2 no major alterations were observed, leading to non-conclusive results on the implications of NODs receptors in *L. infantum* infected KCs. Further investigations are needed to clarify the implications of NODs and TLRs in *Leishmania* infection and to better understand how to explore these receptors for better therapeutic and prophylactic uses.

The data presented suggest that MgA might exert an immune modulator effect and lead to the killing of intracellular amastigotes and to the activation of KCs. The activation of KCs in the liver is of critical importance to the resolution of the infection and granuloma formation, as this cellular structure is key to control the liver parasite progression. Although meglumine antimoniate is a drug with severe side effects its efficacy is high as demonstrated by several studies and also for the present data. A liposomic formulation is probably a good strategy to continue to use this drug and several authors have already described the use of liposomic formulations of meglumine antimoniate in VL and CL animal models, but not yet in human practice (da Silva *et al*., 2012; Kalat *et al*., 2014).

The co-culture of infected KCs with hepatocytes, revealed a vital role of these cells in the activation of KCs in response to *L. infantum* parasites. Hepatocytes play a key role in controlling systemic innate immunity via production of secreted PRRs and complement components found in plasma. Expression of the genes encoding these proteins is controlled by liver-specific transcription factors, such as hepatocyte nuclear factors, nuclear factor-1, and CCAAT-enhancer-binding protein, which account for their

liver-specific expression. During an acute phase or systemic inflammatory response, a variety of pro-inflammatory cytokines, such as IL-6, IL-1, TNF-α, and IFN-γ, can stimulate hepatocytes to produce high levels of complements and secreted PRRs. After activation, the complement system generates a wide range of biologic activities such as opsonic, inflammatory, and cytotoxic functions. The liver, and hepatocytes in particular, is a major site of complement components biosynthesis. These include C1r/s, C2, C4, and Cbp of the classical pathway, C3 and factor B of the alternative pathway, mannan-binding lectin, mannan-binding lectin-associated serine proteases 1-3, and mannan-binding lectin-associated protein 19 of the lectin pathway, and terminal components C5, C6, C8, and C9 (Qin and Gao 2006; Gao *et al*., 2008). Hepatocytes, not only are the major source of secreted PRRs, but also expresses membrane-bound PRRs, such as TLRs and NODs. Hepatocytes were found to express messenger RNAs for all other TLRs, but the functions of which on hepatocytes remain to be determined and the role of PRRs in host defenses against invading pathogens are less clear (Gustot *et al*., 2006). As discussed previously, TLR2 functional expression has been reported in KCs, stellate cells, and sinusoidal endothelial cells, and activation of TLR2 leads to the production of pro-inflammatory cytokines, but is repressed in *L. infantum* KCs infection. In co-culture with hepatocytes it was verified a transient increase in TLR2 expression, probably from hepatocytes activation, as KCs were previously infected with *L. infantum* amastigotes that repress TLR2 activation. Curiously, this transient increase was coincident with an increase in TNF-α expression. Suggesting that hepatocytes were able to sense KCs infection and react to it leading to the activation of pro-inflammatory cytokines. However, hepatocytes activation was progressively diminished with incubation time, probably related to an increase in anti-inflammatory cytokine gene expression that reduce TLRs and the generation of pro-inflammatory cytokines. The liver is the major metabolic organ and an extensive inflammatory response may have serious consequences to the host. So, hepatocytes, although transiently activate rapidly return to a more permissive state, inducing immune tolerance in order to avoid major damage to the organ and to the host.

Recently, several other cytoplasmic PRRs have been identified in hepatocytes, including NOD-like receptors and the RIG-like helicases (Meylan *et al*., 2006). NOD1 and NOD2 expression were assessed in the co-culture and interestingly, NOD1 did

register an increase in co-culture. NODs activation also leads to the production of pro-inflammatory cytokines, using a different activation mechanism from TLRs. So hepatocytes are able to sense the infection on KCs and to be activated (by innate immune receptors TLR2 and NOD1), producing a burst of Th1 inflammatory cytokines, but the strong immune modulator effected exerted by *L. infantum* parasite conjugated with the tendency of liver cells to avoid inflammation, rapidly down regulate the inflammatory signals to induce immunological permissiveness/tolerance. However, the initial inflammatory burst might be enough to call neutrophils, lymphocytes and other MØs from bloodstream into the liver and resolve the infection and in the case of *Leishmania* infection, create the resistance-associated liver structure: the granuloma. Hepatocytes might start to reveal their potential in immune regulatory mechanisms. Since KCs are silenced by the parasite, hepatocytes are able to be activated and induce the recruitment of other circulating cells to contain and resolve the infection.

Regarding the comparison of the immune response exhibited against *L. infantum* by two different types of MØs, the blood derived MØs and the liver resident MØs (KCs), some interesting differences were found. Blood MØs are the primary host cells of *L. infantum* and is inside the circulating MØs that the parasite is transported into the inner organs, as is the case of the spleen and liver. Tissues MØs, as KCs, have their precursors in non-differentiated monocytes circulating in the bloodstream after be recruited to the tissues, maturing as tissue MØs (Gordon and Taylor, 2005). The constant exposure of liver cells to various gut-derived commensal microbes and food-borne antigens from the portal circulation has led to the concept that the liver is immunologically tolerant under steady-state conditions in the absence of pathogenic challenge (Gorczynski, 1992). To maintain this tolerance, KCs are considered to play an important role by creating an immunosuppressive local environment in the liver. Under steady-state conditions, KCs can produce IL-10, TGF-β and prostaglandins in spontaneous or induced ways, promoting a micro environment of immune suppression (Knolle *et al*., 1995; Roth *et al*., 1998). In addition to their scavenger function, MØs *in vitro* differentiated from bone marrow or obtained from the mouse peritoneal cavity have been shown to be highly responsive to pathogens or pathogen-derived products, such as the TLR ligands (Kawai and Akira 2010). However, in an apparent contrast to their supposed tolerogenic features KCs express distinct TLRs and upon TLR

triggering, have been reported to produce pro-inflammatory mediators (Wu *et al*., 2010). TLR4 signaling by LPS renders stimulated KCs, producing the pro-inflammatory cytokines TNF, IL-1β, IL-6, IL-12 and IL-18 and, the anti-inflammatory cytokine IL-10. Besides, it was described that KCs express TLR 1–9 and are highly responsive to all TLR ligands, inducing the production of pro-inflammatory cytokines (Kinoshita *et al*., 2010) they seem to be weak producers of cytokines in contrast to peritoneal MØs (Movita *et al*., 2012). However, KCs exhibit potent phagocytic activity, whereas TLR-induced cytokine production is minimal despite the TLR gene expression and display relatively high basal levels of ROS compared with splenic and peritoneal MØs, demonstrating that the function of murine KCs is highly specialized and distinct from that of MØs from other anatomical sites. Although these findings indicate that KCs exert equilibrium, favor non-specific endocytic activity and eliminate foreign bodies and debris from blood circulation can also be activated, became highly immunoactive, eliciting potent pro-inflammatory responses. Blood MØs result from the monocyte immune activation and constitute immune active cells. KCs and MØs present different reactions to infection with *L. infantum*, reflecting the cytokine and cellular environment. MØs are rapidly activated by the parasite, with increased expression of NODs and TLRs innate immune receptors, accompanied by the generation of pro- and anti-inflammatory cytokines. However, this burst of activity associated with amastigote early infection (until ≈3 h) immediately (5 h) decay to low values. As discussed previously, *Leishmania* can exert immunomodulator functions that led to the deactivation of pro-inflammatory activated MØs and of parasite killing mechanisms. TLR2, TLR4 and TLR9 and also NOD1 and NOD2 registered augments of gene expression, indicating parasite sensing and MØs activation accompanied by increases in cytokine gene expression. However, *L. infantum* promastigotes rapidly subvert MØs activation by decreasing PRRs and cytokine levels. KCs are immunologically more permissive, probably favoring amastigotes phagocytosis. Being less activated, since the micro-environment of the liver favors the immune tolerance, KCs express lower cytokine levels compared to MØs. However, is curious to notice that even after be isolated from the liver and be maintained in culture, KCs retained the immune tolerance primed by their location.

In addition, the present study also evidences that there were some dogs registering values distant from the majority of the other animals throughout the several assessed parameters. These atypical values, always reflecting elevate amounts were indicated as outliers. In a small sample, like the sample used in this study constituted by 12 dogs, the possible explanation for these outliers might be related to the genetic diversity of a natural canine population. For the present study, the dogs were collected from a dog population with no restrictions on breed and sex, in order to reflect the natural diversity of the population. It is interesting to notice, that it is well described that some dogs are more resistant to *L. infantum* infection than others. Still, the genetic associations are not yet defined. Altet *et al*. (2002) suggested that similar to the mouse model, a mutation on the canine NRAMP1 gene might be linked to increased susceptibility. Polymorphism associated with the MHC II was also investigated and some alleles were related to increase susceptibility to canine VL (Quinnell *et al*., 2003). As the dog constitutes the main reservoir for human infection, the identification of genetic factors influencing canine resistance or susceptibility to VL may provide insights into the immune response and potential control through vaccination.

5.6. Conclusion

The objective of this chapter was to investigate how liver resident macrophages – Kupffer cells (KCs) – sense and react to L. *infantum* presence. Taking together the results obtained in this chapter, the main conclusions are:

- To the best of our knowledge, it is the first time that canine KCs are investigated for their role in *L. infantum* infection. It is also the first report of innate immune receptors NOD1 NOD2, TLR2, TLR4 and TLR9 gene expression on KCs in the context of *L. infantum* infection;

- KCs are susceptible to both parasite forms – amastigote and promastigote – and no major differences were found in KC response to both parasite forms. However, amastigotes are more efficient on infecting KCs, reflecting how well the parasite is adapted to intra-macrophage life;

- *L. infantum* is able to control KCs leishmanicidal mechanisms, avoiding cellular activation, by decreasing nitric oxide production and increasing urea levels, promoting its own survival. The presence of *L. infantum* induces an anergic state in KCs, limiting IL-12 and TNF-α pro-inflammatory cytokine expression;

- *L. infantum* parasites seem to interact with KCs innate immune receptors TLR2 and NOD1 expression;

- The addition of a leishmanicidal compound – meglumine antimoniate – is able to break the parasite imposed silencing and activate infected KCs to produce pro-inflammatory cytokines;

- The addition of *L. infantum* infected KCs to canine hepatocytes in a co-culture, is able to activate cells innate immunity. Hepatocyte seems to have an interesting role in orchestrating a synergistic the immune response against *L. infantum* parasites;

- There were found major differences in the immune response exhibited by blood derivate macrophages (MØ) facing and infection by *L. infantum* and liver resident macrophages (Kupffer cells) facing the same parasite. MØ reacted exuberantly to the presence of the parasite, with PRRs and Th1 cytokine expression, but were rapidly silenced by the parasite. KCs were more permissive and tolerant, with less but, more constant Th1 cytokine production.

5.7. References

Abou Fakher FH, Rachinel N, Klimczak M, Louis J, Doyen N. 2009. TLR9-dependent activation of dendritic cells by DNA from *Leishmania major* favors Th1 cell development and the resolution of lesions. J Immunol.182:1386–96.

Abu-Dayyeh I, Hassani K, Westra ER, Mottram JC, Olivier M. 2010. Comparative study of the ability of *Leishmania mexicana* promastigotes and amastigotes to alter macrophage signaling and functions. Infect Immun. 78(6):2438-45. doi: 10.1128/IAI.00812-09.

Aderem A, Underhill DM. 1999. Mechanisms of phagocytosis in macrophages. Annu Rev Immunol.17:593-623.

Ajdary S, Ghamilouie MM, Alimohammadian MH, Riazi-Rad F, Pakzad SR. 2011. Toll-like receptor 4 polymorphisms predispose to cutaneous leishmaniasis. Microbes Infect. 13(3):226-31. doi: 10.1016/j.micinf.2010.10.018.

Alabraba EB, Curbishley SM, Lai WK, Wigmore SJ, Adams DH, Afford SC .2007. A new approach to isolation and culture of human Kupffer cells. J Immunol Methods. 326(1-2):139-44.

Altet L, Francino O, Solano-Gallego L, Renier C, Sánchez A. 2002. Mapping and sequencing of the canine NRAMP1 gene and identification of mutations in leishmaniasis-susceptible dogs. Infect Immun. 70(6):2763-71.

Baeten D, Boots AMH, Steenbakkers PGA, Elewaut D, Bos E, Verheijden GFM, Verbruggen G, Miltenburg AMM, Rijnders AWM, Veys EM, de Keyser F. 2000. Human cartilage gp-39^{+}, CD16^{+} monocytes in peripheral blood and synovium - correlation with joint destruction in rheumatoid arthritis. Arthr. Rheum. 43, 1233-1243.

Bazzocchi C, Mortarino M, Comazzi S, Bandi C, Franceschi A, Genchi C. 2005. Expression and function of Toll-like receptor 2 in canine blood phagocytes. Vet Immunol Immunopathol. 104(1-2):15-9.

Beattie L, Peltan A, Maroof A, Kirby A, Brown N, Coles M, Smith DF, Kaye PM. 2010. Dynamic imaging of experimental *Leishmania donovani*-induced hepatic granulomas detects Kupffer cell-restricted antigen presentation to antigen-specific CD8 T cells. PLoS Pathog. 6(3):e1000805. doi: 10.1371/journal.ppat.1000805.

Becker I, Salaiza N, Aguirre M, Delgado J, Carrillo-Carrasco N, Kobeh LG, Ruiz A, Cervantes R, Torres AP, Cabrera N, González A, Maldonado C, Isibasi A. 2003. *Leishmania*

lipophosphoglycan (LPG) activates NK cells through toll-like receptor-2. Mol Biochem Parasitol. 130(2):65-74.

Bee A, Culley FJ, Alkhalife IS, Bodman-Smith KB, Raynes JG, Bates PA. 2011. Transformation of *Leishmania mexicana* metacyclic promastigotes to amastigote-like forms mediated by binding of human C-reactive protein. Parasitology. 122(Pt 5):521-9.

Bogdan C. 2001. Nitric oxide and the immune response. Nat Immunol. 2, 907-916.

Cezário GA, de Oliveira LR, Peresi E, Nicolete VC, Polettini J, de Lima CR, Gatto M, Calvi SA. 2011. Analysis of the expression of toll-like receptors 2 and 4 and cytokine production during experimental *Leishmania chagasi* infection. Mem Inst Oswaldo Cruz. 106(5):573-83.

Cezário GA, de Oliveira LR, Peresi E, Nicolete VC, Polettini J, de Lima CR, Gatto M, Calvi SA. 2011. Analysis of the expression of toll-like receptors 2 and 4 and cytokine production during experimental *Leishmania chagasi* infection. Mem Inst Oswaldo Cruz. 106(5):573-83.

Chandra D, Naik S. 2008. *Leishmania donovani* infection down-regulates TLR2-stimulated IL-12p40 and activates IL-10 in cells of macrophage/monocytic lineage by modulating MAPK pathways through a contact-dependent mechanism. Clin Exp Immunol. 154: 224–34.

Chawla A, Nguyen KD, Goh YP. 2011. Macrophage-mediated inflammation in metabolic disease. Nat Rev Immunol. 11: 738-749.

Chen G, Shaw MH, Kim YG, Nuñez G. 2009. NOD-like receptors: role in innate immunity and inflammatory disease. Annu Rev Pathol 4: 365–98. doi:10.1146/annurev.pathol.4.110807.092239.

Cortez M, Huynh C, Fernandes MC, Kennedy KA, Aderem A, Andrews NW. 2011. *Leishmania* promotes its own virulence by inducing expression of the host immune inhibitory ligand CD200. Cell Host Microbe. 9(6):463-71.

Da Silva SM, Amorim IF, Ribeiro RR, Azevedo EG, Demicheli C, Melo MN, Tafuri WL, Gontijo NF, Michalick MS, Frézard F. 2012. Efficacy of combined therapy with liposome-encapsulated meglumine antimoniate and allopurinol in treatment of canine visceral leishmaniasis. Antimicrob Agents Chemother. 56(6):2858-67. doi: 10.1128/AAC.00208-12.

Das S, Pandey K, Kumar A, Sardar AH, Purkait B, Kumar M, Kumar S, Ravidas VN, Roy S, Singh D, Das P. 2012. TGF-β1 re-programs TLR4 signaling in *L. donovani* infection: enhancement of SHP-1 and ubiquitin-editing enzyme A20. Immunol Cell Biol. 90(6):640-54.

De Souza Leao S, Lang T, Prina E, Hellio R, Antoine JC. 1995. Intracellular *Leishmania amazonensis* amastigotes internalize and degrade MHC class II molecules of their host cells. J Cell Sci. 108 (Pt 10):3219-31.

De Veer MJ, Curtis JM, Baldwin TM, DiDonato JA, Sexton A, McConville MJ, Handman E, Schofield L. 2003. MyD88 is essential for clearance of *Leishmania major*: possible role for lipophosphoglycan and Toll-like receptor 2 signaling. Eur J Immunol. 33(10):2822-31

Desjardins M, Descoteaux A. 1998. Survival strategies of *Leishmania donovani* in mammalian host macrophages. Res Immunol. 149(7-8):689-92.

Diesselhoff-den Dulk MM, Crofton RW, van Furth R. 1979. Origin and kinetics of Kupffer cells during an acute inflammatory response. Immunology. 37(1):7-14.

Dixon LJ, Barnes M, Tang H, Pritchard MT, Nagy LE. 2013. Kupffer cells in the liver. Compr Physiol. 3(2):785-97. doi: 10.1002/cphy.c120026.

Egen JG, Rothfuchs AG, Feng CG, Winter N, Sher A, Germain RN. 2008. Macrophage and T cell dynamics during the development and disintegration of mycobacterial granulomas. Immunity. 28(2):271-84. doi: 10.1016/j.immuni.2007.12.010.

Ember JA, Hugli TE. 1997. Complement factors and their receptors. Immunopharmacology. 38: 3–15.

Faria MS, Reis FC, Azevedo-Pereira RL, Morrison LS, Mottram JC, Lima AP. 2011. *Leishmania* inhibitor of serine peptidase 2 prevents TLR4 activation by neutrophil elastase promoting parasite survival in murine macrophages. J Immunol. 186(1):411-22.

Ferguson, MA. 1997. The surface glycoconjugates of trypanosomatid parasites. Philos. Trans. R. Soc. Lond. B Biol. Sci. 352**:**1295-*1302.*

Fischer HG, Opel B, Reske K, Reske-Kunz AB. 1988. Granulocyte-macrophage colony-stimulating factor-cultured bone marrow-derived macrophages reveal accessory cell function and synthesis of MHC class II determinants in the absence of external stimuli. Eur J Immunol. 18(8):1151-8.

Flandin JF, Chano F, Descoteaux A. 2006. RNA interference reveals a role for TLR2 and TLR3 in the recognition of *Leishmania donovani* promastigotes by interferon-gamma-primed macrophages. Eur J Immunol. 36(2):411-20.

Foey AD. 2014. Macrophages- Masters of Immune Activation, Suppression and Deviation, Immune Response Activation, Dr. Ht Duc (Ed.), ISBN: 978-953-51-1374-4, InTech, DOI: 10.5772/57541. [Available from: http://www.intechopen.com/books/immune-response-activation/macrophages-masters-of-immune-activation-suppression-and-deviation]

Froh M, Konno A, Thurman R. 2003. Isolation of Liver Kupffer Cells. In Current Protocols in Toxicology. John Wiley & Sons. Chapter 14: Unit14.4.

Fruth U, Solioz N, Louis JA. 1993. *Leishmania major* interferes with antigen presentation by infected macrophages. J Immunol. 150(5):1857-64.

Fukushima J, Kamada Y, Matsumoto H, Yoshida Y, Ezaki H, Takemura T, Saji Y, Igura T, Tsutsui S, Kihara S, Funahashi T, Shimomura I, Tamura S, Kiso S, Hayashi N.2009. Adiponectin prevents progression of steatohepatitis in mice by regulating oxidative stress and Kupffer cell phenotype polarization. Hepatol Res. 39: 724-738.

Gao B, Jeong WI, Tian Z. 2008. Liver: An organ with predominant innate immunity. Hepatology. 47(2):729-36. doi: 10.1002/hep.22034.

Gazzinelli RT, Denkers EY. *2006.* Protozoan encounters with Toll-like receptor signalling pathways: implications for host parasitism. Nat Rev Immunol. 6:895–906.

Gobert AP, Daulouede S, Lepoivre M, Boucher JL, Bouteille B, Buguet A, Cespuglio R, Veyret P, Vincendeau P. 2000. L-arginine availability modulates local nitric oxide production and parasite killing in experimental trypanosomiasis. Infect. Immun. 68:4653-4657.

Gorczynski RM. 1992. Immunosuppression induced by hepatic portal venous immunization spares reactivity in IL-4 producing T lymphocytes. Immunol Lett. 33:67-77.

Gordon S, Taylor PR. 2005. Monocyte and macrophage heterogeneity. Nat Rev Immunol. 5(12):953-64.

Gordon S. 2003. Alternative activation of macrophages. Nat Rev Immunol. 3(1):23-35.

Graff JW, Dickson AM, Clay G, McCaffrey AP, Wilson ME. 2012. Identifying functional microRNAs in macrophages with polarized phenotypes. JBiolChem 287:21816-21825.

Grage-Griebenow E, Zawatzky R, Kahlert H, Brade L, Flad HD, Ernst M. 2001. Identification of a novel dendritic cell-like subset of $CD64^{+}$/$CD16^{+}$ blood monocytes. Eur. J. Immunol. 31, 48–56.

Guerra CS, Silva RM, Carvalho LO, Calabrese KS, Bozza PT, Corte-Real S. *2010.* Histopathological analysis of initial cellular response in TLR-2 deficient mice experimentally infected by *Leishmania (L.) amazonensis.* Int J Exp Pathol. 91:451–9.

Gustot T, Lemmers A, Moreno C, Nagy N, Quertinmont E, Nicaise C, Franchimont D, Louis H, Devière J, Le Moine O. 2006. Differential liver sensitization to toll-like receptor pathways in mice with alcoholic fatty liver. Hepatology. 43(5):989-1000.

Guy RA, Belosevic M. 1993. Comparison of receptors required for entry of *Leishmania major* amastigotes into macrophages. Infect Immun. 61(4):1553-8.

Hasegawa M, Fujimoto Y, Lucas PC, Nakano H, Fukase K, Núñez G, Inohara N. 2008. A critical role of RICK/RIP2 polyubiquitination in Nod-induced NF-kappaB activation. EMBO J. 27(2):373-83.

Hofmann F, Kreusch J, Maier KP, Munder PG, Decker K. 1978. The urea cycle in different types of macrophages. Biochem. Soc. Trans. 6:990-993.

Huber M, Timms E, Mak TW, Röllinghoff M, Lohoff M. 1998. Effective and long-lasting immunity against the parasite *Leishmania major* in CD8-deficient mice. Infect Immun. 66(8):3968-70.

Janeway CA Jr, Travers P, Walport M, *et al*. Immunobiology: The Immune System in Health and Disease. 5th edition. New York: Garland Science. 2001. Principles of innate and adaptive immunity. [Available from: http://www.ncbi.nlm.nih.gov/books/NBK27090/]

Jenkins SJ, Ruckerl D, Cook PC, Jones LH, Finkelman FD, van Rooijen N, MacDonald AS, Allen JE. 2011. Local macrophage proliferation, rather than recruitment from the blood, is a signature of TH2 inflammation. Science 332:1284-88.

Kalat SA, Khamesipour A, Bavarsad N, Fallah M, Khashayarmanesh Z, Feizi E, Neghabi K, Abbasi A, Jaafari MR. 2014. Use of topical liposomes containing meglumine antimoniate (Glucantime) for the treatment of *L. major* lesion in BALB/c mice. Exp Parasitol. 143:5-10. doi: 10.1016/j.exppara.2014.04.013.

Kane MM, Mosser DM. 2000. *Leishmania* parasites and their ploys to disrupt macrophage activation. Curr Opin Hematol. 7(1):26-31.

Karmakar S, Bhaumik SK, Paul J, De T. 2012. TLR4 and NKT cell synergy in immunotherapy against visceral leishmaniasis. PLoS Pathog. 8(4):e1002646.

Kavoosi G, Ardestani SK, Kariminia A, Alimohammadian MH. 2010. *Leishmania major* lipophosphoglycan: Discrepancy in toll-like receptor signaling. Exp Parasitol. 124: 214–218.

Kawai T, Akira S. 2010. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nat. Immunol.11:373–384.

Keshav S, Chung P, Milon G, Gordon S. 1991. Lysozyme is an inducible marker of macrophage activation in murine tissues as demonstrated by in situ hybridization. J Exp Med. 174(5):1049-58.

Kima PE, Soong L, Chicharro C, Ruddle NH, McMahon-Pratt D. 1996. *Leishmania*-infected macrophages sequester endogenously synthesized parasite antigens from presentation to $CD4^+$ T cells. Eur J Immunol. 26(12):3163-9.

Kinoshita M, Uchida T, Sato A, Nakashima M, Nakashima H, Shono S, Habu Y, Miyazaki, H, Hiroi S, Seki S. 2010. Characterization of two F4/80-positive Kupffer cell subsets by their function and phenotype in mice. J. Hepatol. 53:903–910.

Klein I, Cornejo JC, Polakos NK, John B, Wuensch SA, Topham DJ, Pierce RH, Crispe IN. 2007. Kupffer cell heterogeneity: functional properties of bone marrow derived and sessile hepatic macrophages. Blood. 110(12):4077-85.

Knolle P, Schlaak J, Uhrig A, Kempf P, Meyer zum Buschenfelde KH, Gerken G. 1995. Human Kupffer cells secrete IL-10 in response to lipopolysaccharide (LPS) challenge. J. Hepatol. 22:226 –229.

Krieg AM. 2008. Toll-like receptor 9 (TLR9) agonists in the treatment of cancer. Oncogene 27:161–167; doi:10.1038/sj.onc.1210911.

Kropf P, Freudenberg MA, Modolell M, Price HP, Herath S, Antoniazi S, Galanos C, Smith DF, Müller I. 2004. Toll-like receptor 4 contributes to efficient control of infection with the protozoan parasite *Leishmania major*. Infect Immun. 72(4):1920-8.

Kropf P, Fuentes JM, Fähnrich E, Arpa L, Herath S, Weber V, Soler G, Celada A, Modolell M, Müller I. 2005. Arginase and polyamine synthesis are key factors in the regulation of experimental leishmaniasis *in vivo*. FASEB J. 19(8):1000-2.

Laufs H, Müller K, Fleischer J, Reiling N, Jahnke N, Jensenius JC, Solbach W, Laskay T. 2002. Intracellular survival of *Leishmania major* in neutrophil granulocytes after uptake in the absence of heat-labile serum factors. Infect Immun. 70(2):826-35.

Lawrence T, Natoli G. 2011. Transcriptional regulation of macrophage polarization: Enabling diversity with identity. Nat Rev Immunol. 11:750-761.

Liao X, Sharma N, Kapadia F, Zhou G, Lu Y, Hong H, Paruchuri K, Mahabeleshwar GH, Dalmas E, Venteclef N, Flask CA, Kim J, Doreian BW, Lu KQ, Kaestner KH, Hamik A, Clement K, Jain MK. 2011. Kruppel-like factor 4 regulates macrophage polarization. J Clin Invest 121:2736-2749.

Liaskou E, Wilson DV, Oo YH. 2012. Innate immune cells in liver inflammation. Mediators Inflamm. 2012:949157. doi: 10.1155/2012/949157.

Liew FY, Li Y, Millott S. 1990. Tumour necrosis factor (TNF-alpha) in leishmaniasis. II. TNF-alpha-induced macrophage leishmanicidal activity is mediated by nitric oxide from L-arginine. Immunology. 71(4):556-9.

Linehan SA, Martinez-Pomares L, Gordon S. 2000. Mannose receptor and scavenger receptor: two macrophage pattern recognition receptors with diverse functions in tissue homeostasis and host defense. Adv Exp Med Biol. 479:1-14.

Liu D, Kebaier C, Pakpour N, Capul AA, Beverley SM, Scott P, Uzonna JE. 2009. *Leishmania major* phosphoglycans influence the host early immune response by modulating dendritic cell functions. Infect Immun. 77(8):3272-83.

Locksley RM, Reiner SL, Hatam F, Littman DR, Killeen N. 1993. Helper T cells without CD4: control of leishmaniasis in CD4-deficient mice. Science. 261(5127):1448-51.

Lucumi A, Robledo S, Gama V, Saravia NG. 1998. Sensitivity of *Leishmania viannia panamensis* to pentavalent antimony is correlated with the formation of cleavable DNA-protein complexes. Antimicrob Agents Chemother. 42(8):1990-5.

Mauël J. 1990. Macrophage-parasite interactions in *Leishmania* infections. J Leukoc Biol. 47(2):187-93.

McConville MJ, Blackwell JM. 1991. Developmental changes in the glycosylated phosphatidylinositols of *Leishmania donovani*: characterization of the promastigote and amastigote glycolipids. J. Biol. Chem. 266:15170-15179.

Meier CL, Svensson M, Kaye PM. 2003. *Leishmania*-induced inhibition of macrophage antigen presentation analyzed at the single-cell level. J Immunol. 171(12):6706-13.

Melby PC. 2002. *Recent developments in leishmaniasis.* Curr. Opin. Infect. Dis. 15**:***485-490.*

Meylan E, Tschopp J, Karin M.2006. Intracellular pattern recognition receptors in the host response. Nature. 42:39-44.

Mittra B, Andrews NW. 2013. IRONy OF FATE: role of iron-mediated ROS in *Leishmania* differentiation. Trends Parasitol. 29(10):489-96. doi: 10.1016/j.pt.2013.07.007.

Miura K, Kodama Y, Inokuchi S, Schnabl B, Aoyama T, Ohnishi H, Olefsky JM, Brenner DA, Seki E. 2010. Toll-like receptor 9 promotes steatohepatitis by induction of interleukin-1beta in mice. Gastroenterology. 139:323-34.37.

Miyauchi J, Sasadaira H, Watanabe K, Watanabe Y. 1985. Ultra-structural immunocytochemical localization of lysozyme in human monocytes and macrophages. Cell Tissue Res. 242:269-277.

Morita A, Itoh Y, Toyama T, Fujii H, Nishioji K, Kirishima T, Makiyama A, Yamauchi N, Okanoue T. 2003. Activated Kupffer cells play an important role in intra-hepatic Th1-associated necro-inflammation in Concanavalin A-induced hepatic injury in mice. Hepatol Res. 27(2):143-150.

Mosmann TR, Coffman RL. 1989. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. Annu Rev Immunol. 7:145-73.

Mosser DM, Edwards JP. 2008. Exploring the full spectrum of macrophage activation. Nat Rev Immunol. 8:958-969.

Movita D, Kreefft K, Biesta P, van Oudenaren A, Leenen PJ, Janssen HL, Boonstra A. 2012. Kupffer cells express a unique combination of phenotypic and functional characteristics compared with splenic and peritoneal macrophages. J Leukoc Biol. 92(4):723-33. doi: 10.1189/jlb.1111566.

Munder M, Eichmann K, Modolell M. 1998. Alternative metabolic states in murine macrophages reflected by the nitric oxide synthase/arginase balance: competitive regulation by $CD4^+$ T cells correlates with Th1/Th2 phenotype. J Immunol. 160:5347-5354.

Murray HW. 2001. Tissue granuloma structure-function in experimental visceral leishmaniasis. Int J Exp Pathol 82:249–267.

Naito M, Hasegawa G, Ebe Y, Yamamoto T. 2004. Differentiation and function of Kupffer cells. Med Electron Microsc. 37:16–28.

Naito M, Hasegawa G, Takahashi K. 1997. Development, differentiation, and maturation of Kupffer cells. Microsc. Res Tech 39:350-364.

Olivier M, Gregory DJ, Forget g. 2005. Subversion mechanisms by which *Leishmania* parasites can escape the host immune response: a signaling point of view. Clin. Microbiol. Rev. 18:293-305.

Park JH, Kim YG, McDonald C, Kanneganti TD, Hasegawa M, Body-Malapel M, Inohara N, Núñez G. 2007. RICK/RIP2 mediates innate immune responses induced through Nod1 and Nod2 but not TLRs. J Immunol. 178(4):2380-6.

Passlick B, Flieger D, Ziegler-Heitbrock HW. 1989. Identification and characterization of a novel monocyte subpopulation in human peripheral blood. Blood. 74(7):2527-34.

Pearson RD, Sousa AQ. 1996. Clinical spectrum of Leishmaniasis. Clin Infect Dis. 22(1):1-13.

Perez-Cano FJ, Franch A, Castellote C, Castell M. 2003. Immunomodulatory action of spermine and spermidine on NR8383 macrophage line in various culture conditions. Cell. Immunol. 226:86-94.

Pimenta PF, Saraiva EM, Sacks DL. 1991. The comparative fine structure and surface glycoconjugate expression of three life stages of *Leishmania major*. Exp Parasitol. 72(2):191-204.

Planagumà A, Titos E, López-Parra M, Gaya J, Pueyo G, Arroyo V, Clària J. 2002. Aspirin (ASA) regulates 5-lipoxygenase activity and peroxisome proliferator-activated receptor alpha-mediated CINC-1 release in rat liver cells: novel actions of lipoxin A4 (LXA4) and ASA-triggered 15-epi-LXA4. FASEB J. 16(14):1937-9.

Qin XB, Gao B. 2006. The complement system in liver diseases. Cell Mol Immunol. 3:333-340.

Quinnell RJ, Kennedy LJ, Barnes A, Courtenay O, Dye C, Garcez LM, Shaw MA, Carter SD, Thomson W, Ollier WE. 2003. Susceptibility to visceral leishmaniasis in the domestic dog is associated with MHC class II polymorphism. Immunogenetics. 55(1):23-8.

Reiner NE, Ng W, McMaster WR. 1987. Parasite-accessory cell interactions in murine leishmaniasis. II. *Leishmania donovani* suppresses macrophage expression of class I and class II major histocompatibility complex gene products. J Immunol. 138(6):1926-32.

Rhee EG, Mendez S, Shah JA, Wu CY, Kirman JR, Turon TN, Davey DF, Davis H, Klinman DM, Coler RN, Sacks DL, Seder RA. 2002. Vaccination with heat-killed *leishmania* antigen or recombinant leishmanial protein and CpG oligodeoxynucleotides induces long-term memory $CD4^+$ and $CD8^+$ T cell responses and protection against *leishmania major* infection. J Exp Med. 195(12):1565-73.

Ribeiro-Gomes FL, Otero AC, Gomes NA, Moniz-De-Souza MC, Cysne-Finkelstein L, Arnholdt AC, Calich VL, Coutinho SG, Lopes MF, DosReis GA. 2004. Macrophage interactions with neutrophils regulate *Leishmania major* infection. J Immunol. 172(7):4454-62.

Rivera CA, Gaskin L, Allman M, Pang J, Brady K, Adegboyega P, Pruitt K. 2010. Toll-like receptor-2 deficiency enhances non-alcoholic steatohepatitis. BMC Gastroenterol. 10:52.

Roberts SC, Tancer MJ, Polinsky MR, Gibson KM, Heby O, Ullman B. 2004. Arginase plays a pivotal role in polyamine precursor metabolism in *Leishmania.* Characterization of gene deletion mutants. J. Biol. Chem. 279:23668-23678.

Ross GD, Vetvicka V. 1993. CR3 (CD11b, CD18): a phagocyte and NK cell membrane receptor with multiple ligand specificities and functions. Clinical and Experimental Immunology. 92(2):181–184.

Roth S, Gong W, Gressner AM. 1998. Expression of different isoforms of TGF and the latent TGF-binding protein (LTBP) by rat Kupffer cells J. Hepatol. 29:915–922.

Russell DG, Cardona PJ, Kim MJ, Allain S, Altare F. 2009. Foamy macrophages and the progression of the human tuberculosis granuloma. Nat Immunol 10:943–948.

Sadick MD, Raff HV. 1985. Differences in expression and exposure of promastigote and amastigote membrane molecules in *Leishmania tropica*. Infect Immun. 47(2):395-400.

Saito N, Pulford KA, Breton-Gorius J, Masse JM, Mason DY, Cramer EM. 1991. Ultrastructural localization of the CD68 macrophage-associated antigen in human blood neutrophils and monocytes. Am J Pathol. 139:1053-1059.

Saleh MN, Goldman SJ, LoBuglio AF, Beall AC, Sabio H, McCord MC, Minasian L, Alpaugh RK, Weiner LM, Munn DH. 1995. $CD16^+$ monocytes in patients with cancer: spontaneous elevation and pharmacologic induction by recombinant human macrophage colony-stimulating factor. Blood. 85:2910–2917.

Sanchez MA, Diaz NL, Zerpa O, Negron E, Convit J, Tapia FJ. 2004. Organ-specific immunity in canine visceral leishmaniasis: analysis of symptomatic and asymptomatic dogs naturally infected with *Leishmania chagasi*. Am J Trop Med Hyg. 70(6):618-24.

Schieferdecker HL, Schlaf G, Jungermann K, Gotze O. 2001. Functions of anaphylatoxin C5a in rat liver: direct and indirect actions on nonparenchymal and parenchymal cells. Int Immunopharmacol; 1:469–81.

Schlitt A, Heine GH, Blankenberg S, Espinola-Klein C, Dopheide JF, Bickel C, Lackner KJ, Iz M, Meyer J, Darius H, Rupprecht HJ. 2004. $CD14^+$ $CD16^+$ monocytes in coronary artery disease and their relationship to serum TNF-alpha levels. Thromb. Haem. 92, 419–424.

Sehgal G, Zhang K, Todd RF, Boxer LA, Petty HR. 1993. Lectin-like inhibition of immune complex receptor-mediated stimulation of neutrophils. Effects on cytosolic calcium release and superoxide production. J Immunol. 150(10):4571-80.

Seki S, Habu Y, Kawamura T, Takeda K, Dobashi H, Ohkawa T, Hiraide H. 2000. The liver as a crucial organ in the first line of host defense: the roles of Kupffer cells, natural killer (NK) cells and NK1.1 Ag^+ T cells in T helper 1 immune responses. Immunol Rev. 174:35-46.

Shamsi Meymandi S, Javadi A, Dabiri S, Shamsi Meymandi M, Nadji M. 2011. Comparative histological and immunohistochemical changes of dry type cutaneous leishmaniasis after administration of meglumine antimoniate, imiquimod or combination therapy. Arch Iran Med. 14(4):238-43. doi: 0011144/AIM.003.

Souza AE, Waugh S, Coombs GH, Mottram JC. 1992. Characterization of a multi-copy gene for a major stage-specific cysteine proteinase of *Leishmania mexicana*. FEBS Lett. 311(2):124-7.

Srivastava A, Singh N, Mishra M, Kumar V, Gour JK, Bajpai S, Singh S, Pandey HP, Singh RK. 2012. Identification of TLR inducing Th1-responsive *Leishmania donovani* amastigote-specific antigens. Mol Cell Biochem. 359(1-2):359-68.

Srivastava S, Pandey SP, Jha MK, Chandel HS, Saha B. 2013. *Leishmania* expressed lipophosphoglycan interacts with Toll-like receptor (TLR)-2 to decrease TLR-9 expression and reduce anti-leishmanial responses. Clin Exp Immunol. 172(3):403-9. doi: 10.1111/cei.12074.

Stempin C, Giordanengo L, Gea S, Cerbán F. 2002. Alternative activation and increase of Trypanosoma cruzi survival in murine macrophages stimulated by cruzipain, a parasite antigen. J Leukoc Biol. 72(4):727-34.

Su GL. 2002. Lipopylysaccharides in liver injury: molecular mechanisms of Kupffer cell activation. Am J Physiol Gastrointest Liver Physiol; 283:G256–65.

Tacke F, Randolph GJ. 2006. Migratory fate and differentiation of blood monocyte subsets. Immunobiology. 211(6-8):609-18.

Thomson AW, Knolle PA. 2010. Antigen-presenting cell function in the tolerogenic liver environment. Nat Rev Immunol. 10:753-766.

Thornton BP, Větvicka V, Pitman M, Goldman RC, Ross GD. 1996. Analysis of the sugar specificity and molecular location of the beta-glucan-binding lectin site of complement receptor type 3 (CD11b/CD18). J Immunol. 156(3):1235-46.

Tolouei S, Hejazi SH, Ghaedi K, Khamesipour A, Hasheminia SJ. 2013. TLR2 and TLR4 in cutaneous leishmaniasis caused by *Leishmania major*. Scand J Immunol. 78(5):478-84. doi: 10.1111/sji.12105.

Tsutsui H, Nishiguchi S. 2014. Importance of Kupffer Cells in the Development of Acute Liver Injuries in Mice. Int. J. Mol. Sci. 15(5):7711-7730. doi:10.3390/ijms15057711.

Turchetti AP, da Costa LF, Romão Ede L, Fujiwara RT, da Paixão TA, Santos RL. 2015. Transcription of innate immunity genes and cytokine secretion by canine macrophages resistant or susceptible to intracellular survival of *Leishmania infantum*. Vet Immunol Immunopathol. 163(1-2):67-76. doi: 10.1016/j.vetimm.2014.11.010.

Van Zandbergen G, Klinger M, Mueller A, Dannenberg S, Gebert A, Solbach W, Laskay T. 2004. Cutting edge: neutrophil granulocyte serves as a vector for *Leishmania* entry into macrophages. J Immunol. 173(11):6521-5.

Wu J, Meng Z, Jiang M, Zhang E, Trippler M, Broering R, Bucchi A, Krux F, Dittmer U, Yang D, Roggendorf M, Gerken G, Lu M, Schlaak JF. 2010. Toll-like receptor-induced innate immune responses in non-parenchymal liver cells are cell typespecific. Immunology. 129:363–374.

Yamamoto T, Naito M, Moriyama H, Umezu H, Matsuo H, Kiwada H, Arakawa M. 1996. Repopulation of murine Kupffer cells after intravenous administration of liposome-encapsulated dichloromethylene diphosphonate. Am J Pathol. 149(4):1271-86.

CHAPTER 3

Immunological memory in the liver

6 Chapter 3: Immunological memory in the liver

6.1. Introduction

6.1.1. Liver infection in leishmaniasis-murine model

The liver carries out a number of vital functions of metabolism and of innate and adaptive immunity. Contributions to the innate immune system include production of acute phase proteins, nonspecific phagocytosis and pinocytosis, and nonspecific cell killing. Hepatic involvement in innate immunity contributes to the systemic response to major threats and also to local inflammation, clearance of pathogens and of pathogen related molecules. Hepatic involvement in adaptive immunity allows the clearance of activated T cells and of signaling molecules followed by inflammatory reactions, promoting immunologic tolerance towards potentially antigenic proteins (Parker and Picut 2005). In the more recent years, the liver was recognized as an important immunological organ, with major regulatory functions and with a new discovery as a memory organ.

In VL, evidences from experimental infections indicate that immune responses can be organ-specific. The liver is the compartment of acute resolving infection, with minimal tissue damage and resistance to reinfection, whereas the spleen is the compartment of parasite persistence. Control of hepatic infection in mice requires a coordinated immune response that involves the development of cellular infiltrates around infected macrophages known as inflammatory granulomas (Murray et al., 1992). This granulomatous immune response is analogous to the protective granulomatous response seen in the majority of *L. donovani* infected patients that remain subclinically and observed in asymptomatic dogs (el Hag et al., 1994; Sanchez at al., 2004). To elucidate the mechanisms by which immunity develops in the liver, including the granuloma structure and function, many studies using VL murine models were done, using gene-targeted mice or *in vivo* administration of neutralizing or depleting antibodies. In contrast to the self-containing infection in the liver the spleen and bone marrow become chronically infected, involving mechanisms that are less well known. In the mouse, the spleen becomes grossly enlarged within 6–8 weeks post-infection and parasite persistence is accompanied by fail of granulomatous formation, splenomegaly,

disruption of lymphoid tissue microarchitecture, which includes disruption of B-cell follicles and of the marginal zone (Stanley and Engwerda, 2007). In the liver, the process of granuloma formation, crucial to efficient immune responses to infection by *L. donovani* and *L. infantum* (*L. chagasi*) depends on chemokine production, subsequent recruitment of monocytes, neutrophils, $CD4^+$ T cells and $CD8^+$ T cells, production of inflammatory cytokines and activation of infected cells. Kupffer cells (KC) are the major tissue macrophage found in the liver lining the sinusoids, and are a major target for *Leishmania* infection. There are a number of key pro-inflammatory cytokines required for efficient granulomatous formation, including IL-12, IFN-γ, TNF, granulocyte/macrophage colony-stimulating factor (GM-CSF) and IL-2. These pro-inflammatory cytokines are required for chemokine production and for generation of leishmanicidal molecules by infected KC (Kaye et al., 2004). According to Stager et al. (2003), the anti-inflammatory cytokine IL-4 is also required for the resolution of hepatic infection and for the priming of $CD8^+$ T cells that are critical for long-term protection. After infection, all these cytokines are rapidly secreted, possibly by the infected KC, resulting in the initial recruitment of monocytes and neutrophils, both critical for the effective control of parasite growth. Initial chemokine induction occurs in a T-cell independent manner, but studies have shown that sustained chemokine production requires hepatic lymphocytes. It is likely that also other cells of the innate immune system such as NK cells and NKT cells are also important in the early stages of granuloma formation due to their ability to rapidly produce large quantities of pro-inflammatory cytokines such as IFN-γ. Studies in CD1d knockout mice (that lack NKT cell population) have shown the importance of NKT cells in the early control of parasite growth (Amprey et al., 2004). The fusion of some infected KC with other KC to form multinucleate cells is also evident during these early stages of granuloma formation. T cells required for the resolution of *L. donovani* infection in the liver are recruited for the developing granuloma in response to chemokines and cytokine stimuli, following monocyte and neutrophil recruitment. The number of hepatic $CD4^+$ and $CD8^+$ T cells is increased in the first week post-infection, probably reflecting both local expansion and recruitment from the spleen (Kaye et al., 2004). Studies have shown that the development of hepatic immunity can be manipulated by the administration of neutralizing antibodies or recombinant cytokines, or by manipulating co-stimulatory

pathways, indicating that T-cell responses are continually generated during infection (Stanley and Engwerda, 2007). Curiously, B cells appear to have a regulatory role in granuloma formation. Studies using B-cell deficient mice have shown that these mice rapidly clear *L. donovani* parasites from the liver, accelerate granuloma formation and markedly reduce spleen parasite burden. However, they also present signs of destructive pathology associated with increased number of neutrophils (Smelt et al., 2000). The participation of T lymphocytes in the control of *Leishmania* spp. infection has been recognized and many studies with experimental models of cutaneous leishmaniasis and VL have demonstrated the importance of T cells in granuloma formation. In naturally infected and asymptomatic dogs, the presence of well-defined hepatic granulomas with activated effector T cells, macrophages and dendritic cells and, the prominent expression of adhesion molecules and of inflammation mediators indicate the establishment of an efficient cell-mediated immunity, probably allowing the animals to remain chronically infected with a low parasite burden and undetectable clinical sings. In contrast, the lack of mature hepatic granulomas, the marked decreased of infiltrating T cells and of inflammation mediators in symptomatic dogs represents an unstructured and non-functional granuloma, such as those observed in T cell-deficient mice, highlighting the similarities in between dogs and VL murine models (Sanchez et al., 2004).

6.1.2. Liver as immunological memory organ

Immunological memory is commonly believed to be a hallmark of the adaptive immune system, providing the organism with long-lasting and robust protection against reinfection. Memory cells are populations of prolonged life lymphocytes with self-renewing properties capable of Ag-specific recognition. The liver is a solid organ, and immune cells in the liver can be divided into resident cells and passer-by cells. Resident cells, either come from other organs or develop in the liver. They exist in a stable state in the hepatic sinusoid and do not spontaneously leave. Passer-by cells go through the liver via the bloodstream like travelers. Taking together the unique characteristic of the liver environment and also the liver hematopoietic ability, maintained to some extent until adulthood, the developmental pathway for hepatic lymphocytes may be unique (Jiang et al., 2013). The initial recognition of the liver as an important immunological

memory organ was initiated with the Sun et al. (2009), that discovered that NK cells also have memory characteristics and that this particular population resides in the liver. NK cells were considered to have a short lifespan and lack the capacity of self-renewal. The fact that memory NK cells reside in the liver suggests that may be able to recognize certain Ag, which means that the receptor repertoire of hepatic NK cells is different from that of conventional NK cells. It is probably that owing a unique cytokine microenvironment the liver might be a place for cell reprogramming. The importance and role of this memory NK cells are still under investigation. Other evidences of liver importance in the immunological memory are the priming of $CD8^+$ cells by liver sinusoidal endothelial cells (LSECs), during non-inflammatory conditions and the differentiation of liver-primed T cells into effector cells upon Ag re-encounter presented by mature DC, even after prolonged Ag absence (Bottcher et al., 2013). This recent new data are changing the traditional view of the liver as a major metabolic organ to the discovery that the unique immunological environment in the liver has a major contribution to the immune response to Ag and self-protection as well as maintaining immune tolerance.

6.1.3. Immunological memory: effector and central memory cells

Immunity is the balanced state of having adequate protection against infection, while maintaining adequate tolerance to avoid excessive inflammation, allergy, and autoimmune diseases. In most vertebrates, including humans, the immune system is composed of two principal components: innate and adaptive. Innate immune provides immediate protection against invading microbes, although in not specific way. However, a more directed, long-lasting and robust protective response is needed. The adaptive immune system generates immunological memory after an initial response to a pathogen or non-self-molecules (alloantigens). Immunological memory provides the organism with a faster and stronger protective response to an infection with an agent which had previous contact with, avoiding disease. This system is maintained through-out life in some cases, allowing a life-long immunity. Memory lymphocytes confer immediate protection in peripheral tissues and mount recall responses to antigens in secondary lymphoid organs. In the B cell system these functions are carried out by distinct cell types. Protective memory is mediated by plasma cells that secrete

antibodies, whereas reactive memory is mediated by memory B cells that proliferate and differentiate to plasma cells in response to secondary antigenic stimulation. A similar division has recently emerged in the T cell system. According to the model proposed by Lanzavecchia and Sallusto (2000), protective memory is mediated by effector memory T cells (T_{em}) that migrate to inflamed peripheral tissues and display immediate effector function, whereas reactive memory is mediated by central memory T cells (T_{cm}) that home to T cell areas of secondary lymphoid organs, have little or no effector function, but readily proliferate and differentiate to effector cells in response to antigenic stimulation (Figure 86) (Sallusto et al., 2004).

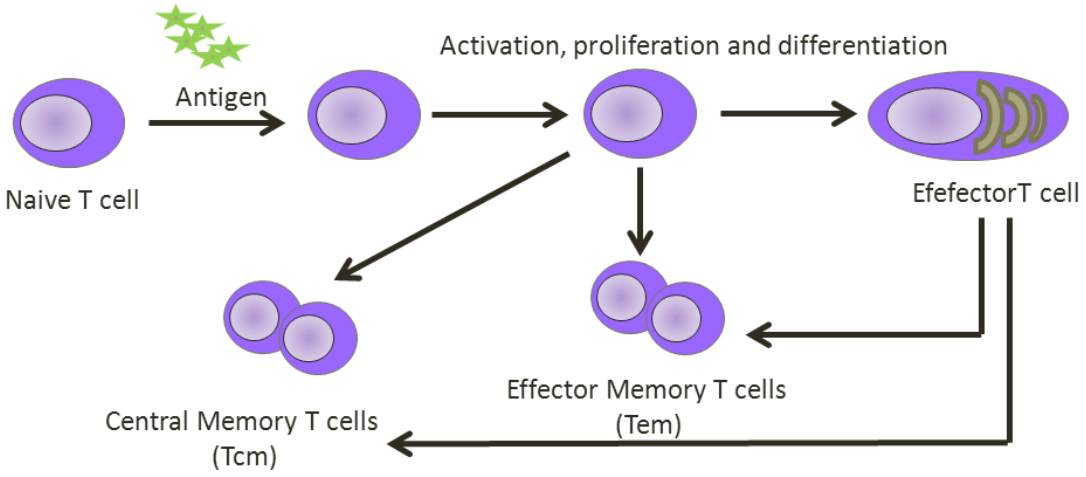


Figure 86: Formation of central (T_{cm}) and effector (T_{em}) memory.
Naïve T cells recognize and activate in the presence of an antigen, originating effector T cells. After the infection is controlled some of the effector T cells generated are transformed into central and effector memory cells

T_{cm} and T_{em} were initially defined in humans based on two distinct characteristics: the presence or absence of immediate effector function and the expression of homing receptors that allow cells to migrate to secondary lymphoid organs versus non-lymphoid tissues. A fraction of primed T lymphocytes persists as circulating memory cells that can confer protection, upon secondary challenge, giving a qualitatively different and quantitatively enhanced response. Sallusto et al., (1999) showed that expression of CCR7, a chemokine receptor that controls homing to secondary lymphoid organs, divides human memory T cells into two functionally distinct subsets. $CCR7^-$ memory cells express receptors for migration to inflamed tissues and display immediate effector function and $CCR7^+$ memory cells expressing the lymph-node homing receptors and lack immediate effector function, but efficiently

stimulate dendritic cells and differentiate into $CCR7^-$ effector cells upon secondary stimulation. The $CCR7^+$ and $CCR7^-$ T cells, which have been named central memory (T_{cm}) and effector memory (T_{em}) respectively, are differentiate from naive T cells and may persist for years after immunization and allow a division of work in the memory response.

Later, human T_{cm} cells were described as $CD45R0^+$ and constitutively express CCR7 and CD62L, two receptors that are also characteristic of naıve T cells, which are required for cell extravasation through endothelial venulesand migration to T cell areas of secondary lymphoid organs (Forster et al., 1999). When compared with naıve T cells, T_{cm} have higher sensitivity to antigenic stimulation, are less dependent on costimulation, and upregulate CD40L to a greater extent, thus providing more effective stimulatory feedback to dendritic cells and B cells. Following activation, T_{cm} produce mainly IL-2, but after proliferation they efficiently differentiate to effector cells and produce large amounts of IFN-γ or IL-4. $CCR7^-$ memory T cells express low levels of the CD62L molecule, migrate to inflamed non-lymphoid tissues, and display immediate effector function such as IFN-γ production. This population of cells constitutes the effector memory (T_{em}). When compared with T_{cm}, T_{em} is characterized by rapid effector function. $CD8^+$ T_{em} cells carry large amounts of perforin, and both $CD4^+$ and $CD8^+$ produce IFN-γ, IL-4, and IL-5 within hours following antigenic stimulation (Sallusto et al., 2004). The relative proportions of T_{cm} and T_{em} in blood vary in the CD4 and CD8 compartments: T_{cm} is predominant in $CD4^+$ and T_{em} in $CD8^+$. Within the tissues, however, T_{cm} and T_{em} show characteristic patterns of distributions. T_{cm} are enriched in lymph nodes and tonsils, whereas lung, liver, and gut contain greater proportions of T_{em} (Campbell et al., 2001). The presence of memory T cells with different migratory capacity and effector function was also documented in mice. Two populations of memory $CD4^+$ T cells survive for months after immunization with antigen in adjuvant. One was primarily found in the lymph nodes, produces IL-2 and the other larger population was found in non-lymphoid tissues produces IFN-γ (Reinhardt et al., 2001). Similarly, two populations of antigen-specific memory $CD8^+$ T cells are present following viral or bacterial infection (Masopust et al., 2001). Whereas $CD8^+$ memory T cells isolated from non-lymphoid tissues exhibit lytic activity directly *ex vivo*, their splenic counterparts do not. These results extended the T_{cm} /T_{em} paradigms to the mouse

system. A more recent study indicated much of the fundamental mechanisms of T cell memory in humans and mice are shared. Thus, significant findings in mouse memory studies may have the potential to be rapid translated to non-human primates and humans, as the knowledge acquired in mouse models studies appears to be interchangeable (Zhang and Lakkis, 2015).

More recently, in-depth studies on local immunity identified a new memory T cell subset, tissue-resident memory (T_{RM}), which resides in peripheral non-lymphoid tissues long after the initial infection has cleared. T_{RM} provide a local protective immunity when the same pathogen is reencountered at the entry site. Although they express low levels of CCR7 and CD62L as do T_{em}, T_{RM} express high levels of local non-lymphoid tissue-homing molecules such as CD103 and CD69, making these cells no-migratory (Gebhardt et al., 2009). CD69 acts to maintain T_{RM} cells at tissue sites by antagonizing the SIPR1 receptor, which promotes the exit of T cells from tissue sites (Ledgerwood et al., 2008). CD103, which is induced by TGF-β, promotes interactions between T_{RM} cells and local epithelial cells, thereby supporting tissue retention (Zhang and Bevan, 2013). T_{RM} do not routinely recirculate and mix with memory subsets in other tissues, being that the probable reason why T_{RM} was initially missed in studies investigating the peripheral blood or secondary lymph organs memory cells populations. In summary, the three described memory subsets (T_{em}, T_{cm}, and T_{RM}) have distinct circulation patterns as a result of their distinct surface chemokine receptor and adhesion molecule expression. T_{cm} mainly circulate in lymphoid tissues (lymph nodes, spleen, and bone marrow) and blood; T_{em} can circulate between lymphoid tissues and non-lymphoid tissues during steady state conditions (although they are largely excluded from lymph nodes) and T_{RM} only reside in peripheral non-lymphoid tissues and do not circulate. Besides, as current methods to dissociate memory T cells in peripheral organs underestimate the numbers of T_{RM} cells, our current knowledge on the presence and activities of these cells is still limited (Steinert et al., 2015).

The heterogeneity of the called T cell memory population was further increases with the description by Gattinoni et al., (2011) of a long-lived human memory T cell population that has an enhanced capacity for self-renewal and a multipotent ability to derive central memory, effector memory and effector T cells (Figure 87).

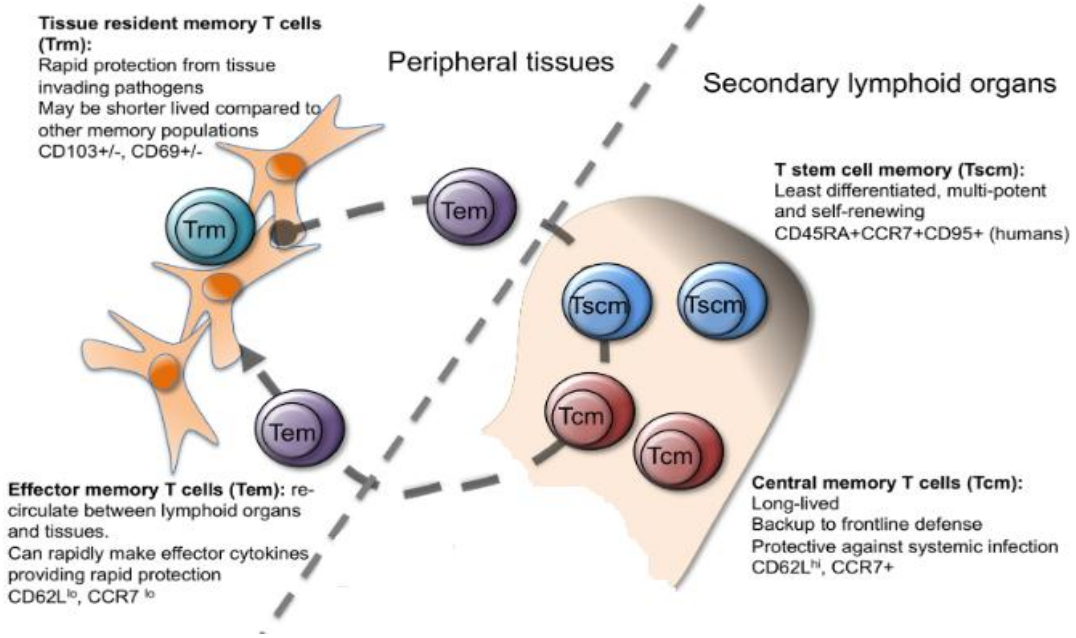


Figure 87: Heterogeneity in memory T cells.
Memory T cells can be found in lymphoid organs, blood, and at tissues. Stem cell memory T cells (Tscm) and central memory T (T_{cm}) cells are found in lymphoid organs and in the blood. Effector memory (T_{em}) and tissue resident memory T (T_{RM}) cells can both be found in peripheral tissues. T_{em} are migratory, passing through tissues and the blood, while T_{RM} are restricted to tissues. Both populations can respond rapidly to tissue invading pathogens (Adapted from Jaigirdar and MacLeod, 2015).

These memory stem T cells (Tscm), specific to multiple viral and self-tumor antigens, were found within a $CD45RO^-$, $CCR7^+$, $CD45RA^+$, $CD62L^+$, $CD27^+$, $CD28^+$ and IL-7Rα^+ T cell compartment characteristic of naive T cells. However, they expressed large amounts of CD95, IL-2Rβ, CXCR3, and LFA-1, and showed numerous functional attributes distinctive of memory cells. Compared with known memory populations, these lymphocytes had increased proliferative capacity and more efficiently reconstituted immunodeficient hosts, and they mediated superior anti-tumor responses in a humanized mouse model. Analysis of antigen-specific CD8 Tscm demonstrates that these cells have previously responded to antigen, can self-renew, and rapidly differentiate into cytokine producing effector cells upon reactivation. Transcriptional analysis of human $CD4^+$ T cell populations identified using CD45RO, CCR7, and CD95, has positioned Tscm as a distinct population with a profile between naive and T_{cm} cells (Takeshita et al., 2015). Memory stem T cells have also been described in non-human primates, where Tscm are found in peripheral blood, secondary lymphoid organs, and the bone marrow (Lugli et al., 2013). Tscm cells in mice have

proven harder to track down, although cells with a similar profile to human Tscm, can be generated *in vitro* or *in vivo* in a model of graft versus host disease.

6.1.4. Guiding immunological memory: the development of vaccines

The ability to induce an immunological response to an Ag during a primary Ag presentation in safe, controlled conditions and allowing the generation of immune memory cells that will ensure the individual immunity in a future encounter with the same Ag is the base of any vaccine. In VL case, the necessity of induction a protective cellular response, able to break the immune silencing imposed by the parasite, is the major challenge in a search for a vaccine for humans and dogs. Host resistance to *Leishmania* spp. infection is mediated by cellular immune responses leading to macrophage activation and parasite killing. Antileishmanial immunity is mediated by both innate and adaptive immune responses which require effective activation of macrophages, dendritic cells, and Ag-specific $CD4^+$ and $CD8^+$ T cells (Stanley and Engwerda, 2007). Effector $CD4^+$ T (T_E) cells are responsible for the production of cytokines critical for the activation of macrophages and are required for optimal host response to infection. Cytotoxic $CD8^+$ T cells also play a role in host protection and are required for the effective clearance of parasites and the generation of memory responses (Kharazmi et al., 1999). Experimental infection models are used to screen and evaluate *Leishmania* vaccines, and several animal species have been used, including mice, hamsters, monkeys, and dogs. Currently there are three available vaccines with different origins and efficacies against CanL, Leishmune® and Leish-Tec® available in Brazil and CaniLeish® accessible in Europe. Although there have been numerous attempts, there is no licensed vaccine against any form of leishmaniasis for human use. Development of vaccines can be divided into five different stages: discovery, pre-clinical development, clinical development, registration, and post-marketing evaluation. For leishmaniasis vaccines, there has been much activity in the discovery area, leading to many candidates of second generation vaccines. The leishmaniasis vaccines in development can be divided into various categories, including: (i) Live vaccine (leishmanization); (ii) Vaccines of first generation consisting of whole killed *Leishmania* or fractions of the parasite; (iii) Vaccines of second generation constituted

of recombinant proteins, DNA vaccines and combinations of both; (iv) Live-attenuated vaccines using molecular engineered parasites (Mutiso et al., 2013). Leishmune® and Leish-Tec® are considered vaccines of second generation constituted of parasite proteins. Leishmune® consists of the fucose mannose ligand isolated from *L. donovani* plus a saponin adjuvant. Leish-Tec® is based on an amastigote specific Ag, the recombinant A2 Ag and CaniLeish®, is constituted by excreted/secreted Ag purified from the culture supernatant of *L. infantum* promastigotes (LiESAP) with muramyl dipeptide (MDP) as adjuvant (LiESP/QA-21). Although several studies on the vaccine efficacy have been done consistent field results are not yet released. In the present study the hepatic lymphocyte memory, recovered by stimulation with three recombinant *L. infantum* proteins, was accessed. The recombinant proteins*Li*rCyP1, *Li*rSOD and *Li*rAMP were obtained of excreted/secreted Ag purified from the culture supernatant of *L. infantum* promastigotes. The levels of *Li*rCyP1 (*L. infantum* recombinant cyclophilin protein 1) immunogenicity in the ZVL murine model were described in Santos-Gomes et al. (2014). Cyclophilins, a class of proteins found in several intracellular compartments and also extracellular, own peptidyl-prolyl cis-trans isomerase activity and can perform different functions. These proteins are well known for operating as cyclosporine receptors, a drug with immune suppression and antimicrobial functions towards different pathogens, including *Leishmania*. *Li*rSOD (*L. infantum* recombinant superoxide dismutase) is an enzyme that detoxifies reactive superoxide radicals produced by activated macrophages essential for parasite survival (Ghosh et al., 2003). The third protein, *Li*rAMP, corresponds to a *L. infantum* recombinant aminopeptidase. These proteins catalyze the removal of N-terminal amino acid residues from peptides and proteins are now being to be exploited as drug targets and vaccine candidates for parasitic diseases, as their potentials important virulence factors is recognized (Morty and Morehead, 2002). Gupta et al., (2012) exploited the immunogenic potential of a recombinant *L. donovani* methionine aminopeptidase, which was able to generate protective immunity by inducing a Th1 stimulatory response against experimental VL in hamsters.

6.2. Chapter objectives

In this chapter, the role of the liver as an immunological memory organ was addressed, particularly in the context of *L. infantum* infection. This study was performed using a murine model mainly for immunoreagents availability reasons. Four groups of mice were established and the population of liver resident lymphocytes was isolated and phenotypic screened.

The specific objectives are:

- Provide evidence of the presence of *L. infantum* in the liver tissue, to ensure that isolated cells had a previous contact with the parasite antigen (Ag) in an *in vivo* infection environment;
- Characterize phenotypically liver T lymphocytes, using markers for effector cells, effector memory cells and central memory cells;
- Evaluate the dynamics of memory T cells in *L. infantum* infected mice *vs* non-infected mice;
- Assess the effect of a leishmanicidal treatment (with meglumine antimoniate) in the differentiation of memory cell subsets;
- Investigate the potential induction of resistance genes in *L. infantum* parasites potentiated by an exposition to the leishmanicidal treatment;
- Explore the effect of an infection with *L. infantum* less susceptible to the leishmanicidal drug in the differentiation of memory cell subsets followed by a treatment with the same leishmanicidal compound;
- Investigate the potential of recognition and activation of effector T cells and of recognition, differentiation and selection of memory T cells by three *L. infantum* recombinant proteins, *Li*rCyP1, *Li*rSOD and *Li*rAMP.

The present results obtained from non-infected (group 1) and infected (group 2) mice were performed by the MSc student Mafalda Claro with my technical and scientific support and are described in her Master thesis "Análise da memória cellular hepatica na infeção por *Leishmania infantum*".

6.3. Methods

6.3.1. Experimental design

The liver is a very complex metabolic organ with immune functions that only now are starting to be explored and understood. The liver-immune memory was also evaluated in the present work. What is the role of the liver-immune memory during *L. infantum* infection and how this cellular compartment is influenced by a treatment with a leishmanicidal drug was addressed in this chapter. This study was performed in BALB/c mice purchased from Harlan laboratories (Horst, Holland) and maintained at the Instituto de Higiene e Medicina Tropical (IHMT) animal facility. All the experiments were conducted according to EU requirements and the Portuguese law (DR DL129/92, Law 1005/92). A total of 27 BALB/c mice were randomly distributed in four groups as follows: non-infected animals (group 1); *L. infantum* infected animals (group 2); *L. infantum* infected animals treated with meglumine antimoniate (MgA, group 3) and, MgA pre-exposed *L. infantum* parasites infected mice treated with MgA (group 4) (Figure 88).

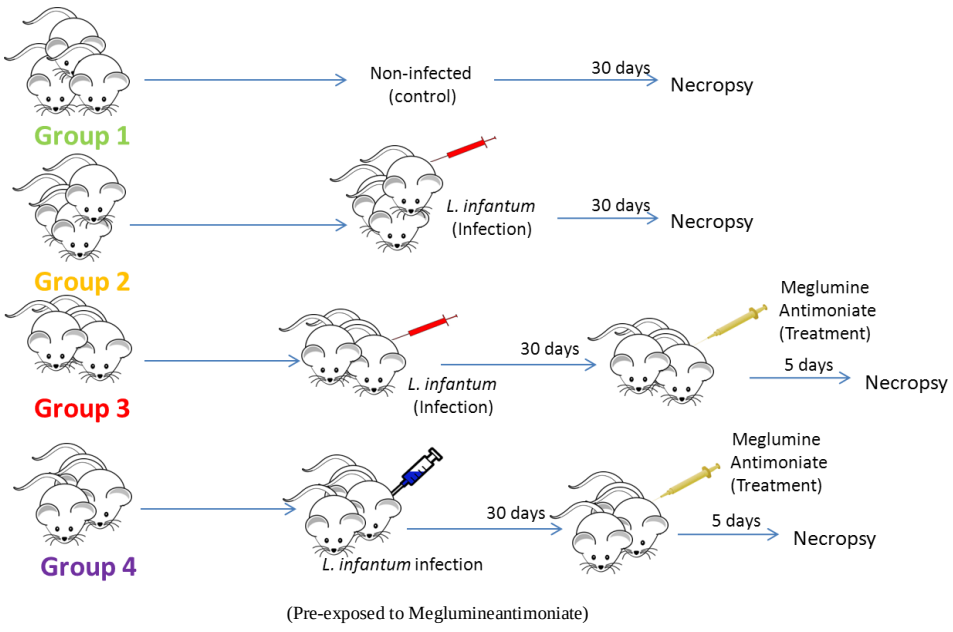


Figure 88: Schematic representation of the experimental design used to evaluate the immune memory cells of the liver.
After necropsy infected, treated and non-infected BALB/c mice were used for the isolation of hepatic resident lymphocytes.

Liver resident leukocytes isolated from mice of the four experimental groups were cultured and stimulated with *L. infantum* recombinant proteins: cyclophilin protein 1 (*Li*rCyP1), superoxide dismutase (*Li*rSOD) and aminopeptidase (*Li*rAMP). A flow cytometry analysis of the expression of memory markers CD127 and CD62L was performed and the frequency of $CD3^+CD4^+$ and $CD3^+CD8^+$ memory cell was accessed.

6.3.2. Mice infection

Each mouse of groups 2 and 3 was intraperitoneally infected with $5x10^6$ virulent promastigotes in a final volume of 100 µl of sterile saline solution [NaCl 0.9% (m/v)]. *L. infantum* virulent promastigotes were obtained as described in General Methods (section 3.3.1) of the general methods chapter. Mice of group 4 were infected with virulent *L. infantum* promastigotes previously isolated from mouse spleen of group 3, which received antileishmanial MgA treatment. To minimize the adaptation of the parasite to culture conditions, the inocula used were obtained from P1 cultures (the first *in vitro* passage after parasite isolation). Thirty days after the beginning of the study, mice of groups 1 and 2 were sacrificed by administration of a lethal dose of anesthetic solution (ketamine/Xylazine). After one month of infection, mice of groups 3 and 4 were treated with a commercial leishmanicidal drug (see section 6.3.3) during five consecutive days and then sacrificed as described above.

6.3.2.1. *L. infantum* genes associated with resistance

The amplification of resistant associated genes in *L. infantum* P1 promastigotes isolated from mice treated with MgA (group 3) and from mice infected with MgA pre-exposed parasites and then treated with the same compound (group 4), were analyzed by real-time PCR. In parallel, wild type *L. infantum* promastigotes were also evaluated.

Promastigotes were collected from the culture medium and washed with PBS 1*x* by centrifugation at 1800 *x*g, 10 min at room temperature. Pellets were processed for DNA extraction using the innuPREP DNA Mini Kit (Analytik Jena AG), according to the manufacturer's protocol and stored at -20 ºC for posterior use. The number of copies of *ABC-thiol transporter MRPA*, *P-glycoprotein MDR1*, *gamma-glutamylcysteine synthase* (GSH1) and *pteridinereductase* (PTR1) were determined by real time PCR.

The design of primers and the optimization of real time PCR conditions are described in Santos-Mateus (2014). The process of plasmid production to obtain the standards used for absolute quantification of the gene copy number is described in General Methods (section 3.4), as well the real-time PCR protocol. Annealing temperature and fragment size for each of the genes assessed is described in Table 8.

Table 8: List of forward (FW) and reverse (RV) primers, length of amplified fragment and primer annealing temperature for each gene associated with *L. infantum* resistance.

Gene	Primers	Fragment size (pb)	Annealing temperature (ºC)
MRPA	FW - 5' CCGCTCGCGGACCACATTGT 3' RV - 5' TCGCCGCAAAGGCAGCGTAA 3'	76	62
MDR1	FW - 5' GGAGGGTGACACAAGCGACACG 3' RV - 5' CGACGCGATCCGCCTTCATGTT 3'	89	62
GSH1	FW - 5' CCCGGCATTTCTGGCTCTCAGC 3' RV - 5' GCGATAGTCAGCCAGCGCACAT 3'	84	63.5
PTR1	FW - 5' ACGTGCTCGTGAACAACGCCTC 3' RV - 5' ATCTCCGACACAGGGCACGTGG 3'	86	65.5

6.3.3. Treatment

Meglumine antimoniate commercially available as glucantime® (Merial) is a drug conventionally used for treating leishmaniasis and formulated with a solution of 81 $mg.ml^{-1}$ of MgA. It belongs to a group of compounds known as the pentavalent antimonials and it is used in human and veterinarian medicine. Thirteen days post-infection, animals of groups 3 and 4 were treated with 15 mg MgA/kg/day, injected subcutaneously for 5 consecutive days. Three days after the ending of the treatment, mice were sacrificed by administration of a lethal dose of anesthetic solution (ketamine/Xylazine).

6.3.4. Isolation of hepatic leukocytes

The protocol used to isolate hepatic leukocytes was adapted from the works of Faure *et al.* (1994) and Gomes-Pereira *et al.* (2004). To isolate hepatic leukocytes, mice were anesthetized with a lethal concentration of a solution of ketamine/Xylazine in phosphate buffer saline (PBS 1*x*) (BioWhittaker, Lonza). Liver portal vein was exposed and perfusion with sterile Hanks Balanced Salt Solution (HBSS) (Sigma-Aldrich) was performed to clean peripheral blood from the liver (Figure 89A).

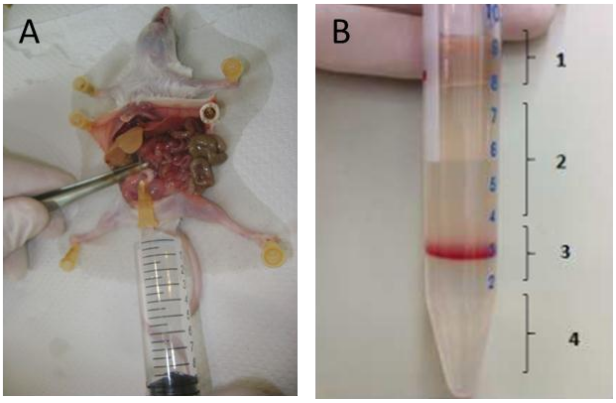


Figure 89: Isolation of liver resident lymphocytes..
After the necropsy, liver perfusion through the portal vein was done to eliminate the peripheral blood (A). The liver was then homogenized and cell suspension overlaid onto a percoll gradient. After centrifugation it is possible to identify: 1 – culture medium; 2 – Percoll (40%); 3 – ring of hepatic leukocytes; 4 – Percoll (70%) (B).

Liver was excised, weighted and homogenized with a tissue disruptor (Medicons, Syntec International) with a 0.5 μm pore in order to obtain a single cell suspension. The cell suspension was washed with cold HBSS and centrifuged at 400*x*g during 10 min at 4 ºC. The pelleted cells were then resuspended in 5 ml of 40% Percoll® (GE Healthcare Life Sciences) in PBS 1*x*, carefully overlaid onto 3 ml of a 70% Percoll® solution in PBS 1*x* and centrifuged at 850 *x*g during 25 min (Figure 89B). Mononuclear cells at the interface of the two gradients were collected, washed with cold PBS 1*x* and counted in a Neubauer chamber with trypan blue exclusion dye to determine viability and concentration as previously described (see Chapter 1 – 4.3.1).

In a sterile 96 well plate (VWR), $1x10^5$cells.ml^{-1} in 150 μl RPMI medium supplemented with 10% (v/v) FBS were plated. Cells were maintained in a cell incubator at 37 ºC in a humidified atmosphere with 5% CO_2.

6.3.5. Hepatic leukocyte stimulation

6.3.5.1. *L. infantum* total antigen

After isolation, hepatic leukocytes were stimulated with 40 μg.ml^{-1} of soluble *L. infantum* Ag for 72 h, at 37 ºC, in a humidified atmosphere with 5% CO_2. Total soluble *L. infantum* Ag was obtained from a *L. infantum* culture with 1*x*10^8 promastigotes/ml. The culture was centrifuged at 1800 *x*g, for 10 min at room temperature and the sediment washed in PBS 1*x* with 2 mM ethylenediaminetetraacetic acid (EDTA) (Sigma-Adrich). Parasites were then subjected to six freeze-thaw cycles (-20 ºC *vs* room temperature) to lyse cells. The obtained parasite extracts were quantified in a Nanodrop 1000 Spectrophotometer (Thermo Fisher Scientific) and the concentration adjusted to 40 μg.ml^{-1} in PBS. Aliquots of this extract were prepared and stored at -20 ºC until use.

6.3.5.2. *L. infantum* recombinant proteins

Three recombinant *L. infantum* proteins, previously obtained as described in Santos-Gomes *et al.* (2014), were used in this study. Proteins were kindly given by Professor Ana Tomás from Instituto de Biologia Molecular e Celular (IBMC), Universidade do Porto, Portugal. Briefly, full length open reading frame of cyclophilin protein 1 (*Li*rCyP1), superoxide dismutase (*Li*rSOD) and aminopeptidase (*Li*rAMP) were amplified. The PCR product was digested and cloned into pET28a plasmid (Novagen). *Escherichia coli* BL21 were transformed with the constructed plasmid and recombinant proteins were obtained from purified bacteria cultures. Bacteria were pelleted, suspended in 500 mM NaCl, 20 mM Tris–HCl buffer and disrupted by sonication. The supernatant was then applied to a His Bin resin (Novagen) column and the recombinant protein eluted trough an imidazole gradient. Fractions confirmed to contain the protein by SDS-PAGE were pooled, applied to PD-10 columns (Amersham Biosciences) and eluted with PBS. Previously to be used in stimulation assays, recombinant proteins were further purified using Detoxi-Gel™ Endotoxin Removing Gel (Pierce Biotechnology), to remove any bacterial contaminant. Isolated hepatic leukocytes were stimulated with each one of the three described recombinant proteins (25 μg.ml^{-1}) for 72 h, at 37 ºC in a humidified atmosphere with 5% CO_2.

6.3.5.3. Limiting dilution Assay (LDA)

To ensure that mice were infected, the viable parasite load was estimated in the liver by limiting dilution assay (LDA). Liver homogenate was diluted at 1:3 followed by a 1:10 dilution in SCH medium. In a sterile 96 well plate, 200 µl of the 1:10 dilution was placed into the first well and a fourfold serial dilution was made. After 15 days at 24 ºC, a sample from each well was examined under light microscopy and presence (positive) or absence (negative) of promastigotes was registered. The final titer was set as the highest dilution for which the well contained at least one parasite and the number of parasites per gram of homogenized organ was calculated. All the samples were evaluated in triplicate.

Viable parasite load (VPL), expressed as the number of promastigotes per gram of homogenized organ, was determined by the following equation (Equation 10).

Equation 10:

$$VPL = \frac{4^{last\ positive\ titer} \times \left(\frac{volume\ of\ homogenized\ organ\ (ml)}{0.2(ml) \times dilution\ factor}\right)}{weight\ of\ the\ homogenized\ organ\ (g)}$$

6.3.6. Flow cytometry

After 72 h in culture, hepatic leukocytes were collected, washed with PBS and divided into two sample groups. One sample was marked for CD4 and the other for CD8. Cells were incubated in PBS with 2% (v/v) FBS, for 30 min at 4 ºC with the following fluorochrome-conjugated anti-mouse monoclonal antibodies: PE anti-mouse CD3 (clone 17A2, Biolegend), Alexa Fluor®647anti-mouse CD62L (clone MEL-14, BioLegend) and PerCP-Cy5.5 anti-mouse CD127 (clone A7R34, eBioscience), FITC anti-mouse CD4 (clone GK1.5, eBioscience) and FITC anti-mouse CD8a (clone 53-6.7, eBioscience). The antibody dilutions used are presented in Table 9. After incubation, cells were fixed with a 2% (m/v) paraformaldehyde in PBS 1*x* and stored at 4 ºC in the dark until further analysis. Hepatic lymphocytes become marked according with their characteristics. Monoclonal antibody CD3 binds to a molecular complex associated with TCR that is exclusive of lymphocytes. The flow cytometry analysis included a first $CD3^+$ cell gating in order to ensure that only lymphocytes were analyzed. Next, $CD4^+$ or

$CD8^+$ T cells were analyzed, allowing the frequencies of $CD4^+$ and $CD8^+$ T cells to be registered. CD62L and CD127 allow the differentiation between central memory and effector cell memory. CD62L is an L-selectin, a cell adhesion molecule found on lymphocytes, favoring the cell retention within the organ. CD127 is the interleukin-7 (IL-7) receptor, normally associated with cell survival. Together, these receptors allow the differentiation between effector cells ($CD62L^{low}$ $CD127^{low}$), central memory ($CD62L^{high}$ $CD127^{high}$) and effector memory ($CD62L^{low}$ $CD127^{high}$). Thus, the frequencies of hepatic memory cell in the four groups of mice were estimated. Cell acquisition was performed on a FACSCalibur flow cytometer (BD Biosciences) and analyzed in triplicate to allow future statistical analysis. The flow cytometry analysis in this study is the fruit of a generous association with Professor Graça Alexandre-Pires of the Faculdade de Medicina Veterinária da Universidade de Lisboa.

Table 9: List of mouse monoclonal antibodies used to analyze hepatic leukocyte memory cell population, respective concentration and the volume applied to each sample.

Monoclonal antibody	Concentration	Volume *per* sample (µl)
FITC Anti-mouse CD8a	0.5 µg.100µl^{-1}	1.0
FITC Anti-mouse CD4	0.25 µg.100µl^{-1}	0.5
PerCPCy5.5 anti-mouse CD127	0.25 µg.100µl^{-1}	0.16
PE anti-mouse CD3	0.25µg.100µl^{-1}	0.42
Alexa Fluor®647 anti-mouse CD62L	0.25µg.100µl^{-1}	0.4

6.3.7. Histological analysis

After liver perfusion, a small portion of the organ was excised and prepared for histological processing in order to evaluate granuloma formation. Granulomas, defensive structures that correspond to alteration on normal tissue morphology, are constituted when the immune system attempts to isolate substances or pathogens that are perceived as foreign but is unable to eliminate them. The excised tissue was conserved in 2% (m/v) paraformaldehyde in PBS 1*x* and stored at 4 ºC until further

processing. Samples were processed at Laboratório de Histopatologia, na Universidade Lusófona de Humanidades e Tecnologias (ULHT), in an automatic tissue sample processor (Spin Tissue Processor 120, Thermo Scientific), under the supervision of Dr. Pedro Faísca and of Dr. Susana Silva. Tissue samples were dehydrated in ethanol gradients [70% (v/v) during 110 min, 96% (v/v) for 110 min and in immersion at 100% during 4 h] and then immersed in a Clear-Rite® solution for 4 h to extract lipids and prepare the tissue to paraffin inclusion (3.5 h). Samples were conserved at 4 ºC until further processing. The sectioning of the paraffin embed tissue blocks were made at the Hospital Santa Cruz (Centro Hospitalar de Lisboa Ocidental, E.P.E), at Anatomia Patológica service under the technical guidance of Dr. Armandina Manuel. The sections were performed in a rotary automatic micrometer Leica DSC-1 (Leica Microsystems GmbH) programmed to execute cuts of 3µm thick. Cuts were unfolded in cold water and adhered to a glass slide and left to rest into a stove at 60 ºC to increase slide adherence. Then, the slides were stained with hematoxylin and eosin (HE). These are the most widely used dyes in medical diagnosis and are often the gold standardin histology. Hematoxylin has a deep blue-purple color and stains nucleic acids and eosin is pink colored and stains nonspecifically proteins. In a typical tissue, nuclei are blue stained, whereas the cytoplasm and the extracellular matrix shown different degrees of pink.

Before starting with coloration it was necessary to remove the paraffin. This was performed in an automatic processor for coloration of slides Sakura tissue-Tek DRS (Sakura Finetek). Slides were immersed in xylol for 10 min to complete remove the paraffin and hydrated by immersion in decreasing ethanol proportions: 96% (v/v) 2 min, 70% (v/v) 2 min and finally washed in water. Slides were immersed in hematoxylin (Merk) and then in 0.5% (v/v) HCL to remove the exceeding dye, stained with 1% (v/v) eosin (Surgipath) for 3 min and washed with water. Tissue samples were again dehydrated in crescent ethanol gradients, to remove all the water from the tissue, and immersed in xylol. Coverslip were added in an automatic coverslip mounter Sakura Tissue-Tek GLC (Sakura Finetek) and then covered with resin to permanently fix the coverslip. Obtained slides were observed under an optical microscopy (Motic Digital Microscope (B1-223 ASC, Motic) and digital image acquisition were performed by a camera and software Motic Images Plus 2.0 M.L and treated with ImageJ free software.

6.3.8. Statistical analysis

To explore the differences between the experimental groups it was used a non-parametrical Mann-Whitney test for two independent samples. To analyze intra-group results with the different stimulations applied to the isolated cells (total *L. infantum*Ag or recombinant protein) it was used a non-parametric Wilcoxon test for two related samples. All the data analysis was performed using the software IBM SPSS Statistics version 16.0 (IBM, USA). A significance level of 5% ($p \leq 0.05$) was used to evaluate the significance of the data analyzed.

6.4. Results

6.4.1. *L. infantum* parasites in the liver trigger a local immune response

In order to ensure that after 30 days post *L. infantum* infection the mice had parasites in the liver tissue, granuloma formation and the viable parasitic load was accessed.

Infected mice presented several granulomas in liver identifiable on liver histological preparations, as a macrophage infiltrate (Figure 90A-D). Granulomas appear well defined with considerable infiltration of macrophages and apparent absence of necrosis. However, granulomas were variable in shape, size and complexity through the liver tissue.

Granuloma formation is a typical marker of an established *L. infantum* liver infection, in which the host intends to circumscribe parasite dissemination. It is also indicative of an inflammatory response, pointing to the development of an unspecific immune response of the liver directed by the parasite

Total viable parasitic load was estimated in the liver of infected mice (groups 2, 3 and 4) through LDA (Figure 90E). *L. infantum* infected animals (group 2) had a parasitic load of $1.3x10^5$ promastigotes/g of liver. However, in both groups of treated mice (groups 3 and 4) it was possible to isolate viable parasites. Treatment with MgA seems to leave residual viable parasites in the liver. The treatment of infected animals with MgA resulted in an 83 % reduction in the liver parasitic load. However the MGA treatment of mice infected with MgA pre-exposed *L. infantum* parasites only caused a reduction of 78% in parasite load.

These results point out that a total *L. infantum* clearing is difficult even with treatment and that a previous contact with the drug, albeit briefly (only five days) has consequences. In fact, by reducing the parasitic sensitivity to MgA the residual parasitic load of the liver had increased.

From the analysis of these results it was concluded that the mice had a well-established *L. infantum* liver infection, which allow continuing to further immune memory studies.

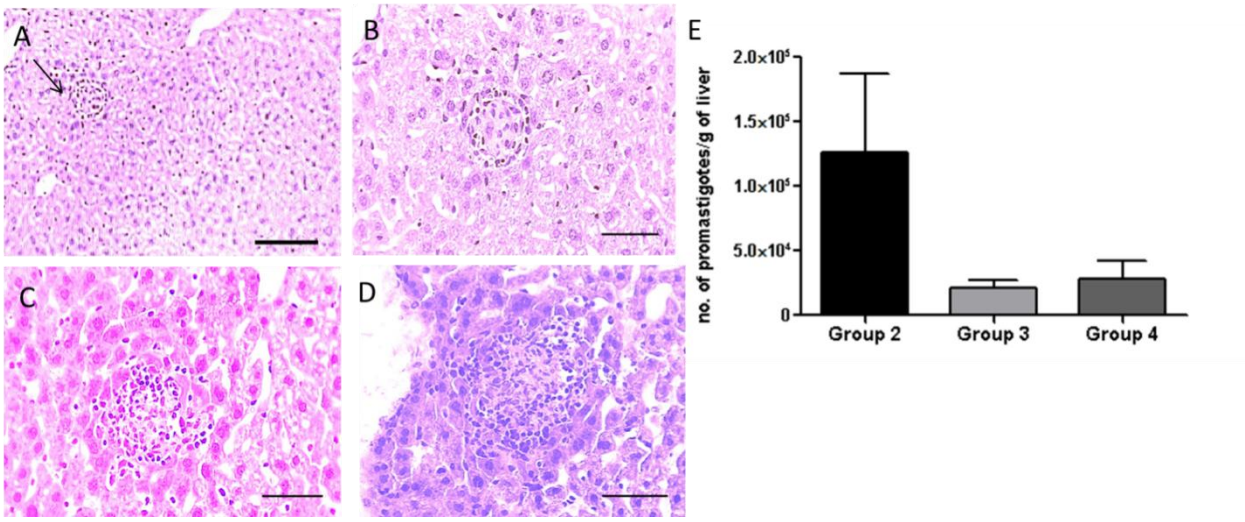


Figure 90: BALB/c mouse liver infected by *L. infantum*.
A) arrow pointing to a granuloma in the normal surrounding liver tissue (black bar 100 µm, 200*x* magnification). B) to D) examples of liver granulomas found in the mice after 30 days post-infection (black bar 50µm, 400*x* magnification). E) Viable parasitic load in the liver of mice infected with *L. infantum* promastigotes (Group 2), infected and treated with MgA (Group 3) and mice infected with MgA pre-exposed *L. infantum* parasites and MgA treated (Group 4). Results are expressed as a mean and standard deviation of three independent experiments.

6.4.2. Phenotypic characterization of liver resident lymphocytes

Liver resident lymphocytes were discriminated into effector T cells (T_E), central (T_{CM}) and effector memory T (T_{EM}) cells according with CD127 and CD62L levels of fluorescence (Figure 91). Using CD3 cellular marker it was possible to isolate lymphocytes from the cell sample, as only these cells express this cell receptor. After a $CD4^+$ or $CD8^+$ gating, it was analyzed CD62L and CD127 expression. The different expression pattern of CD62L and CD127 permit the distinction of T_E cell subset ($CD62^{low}$ $CD127^{low}$), T_{EM}cell subset ($CD62L^{low}$ $CD127^{high}$) and T_{CM} cell subset ($CD62L^{high}$ $CD127^{high}$). These cell populations were present in all four experimental groups with different representation.

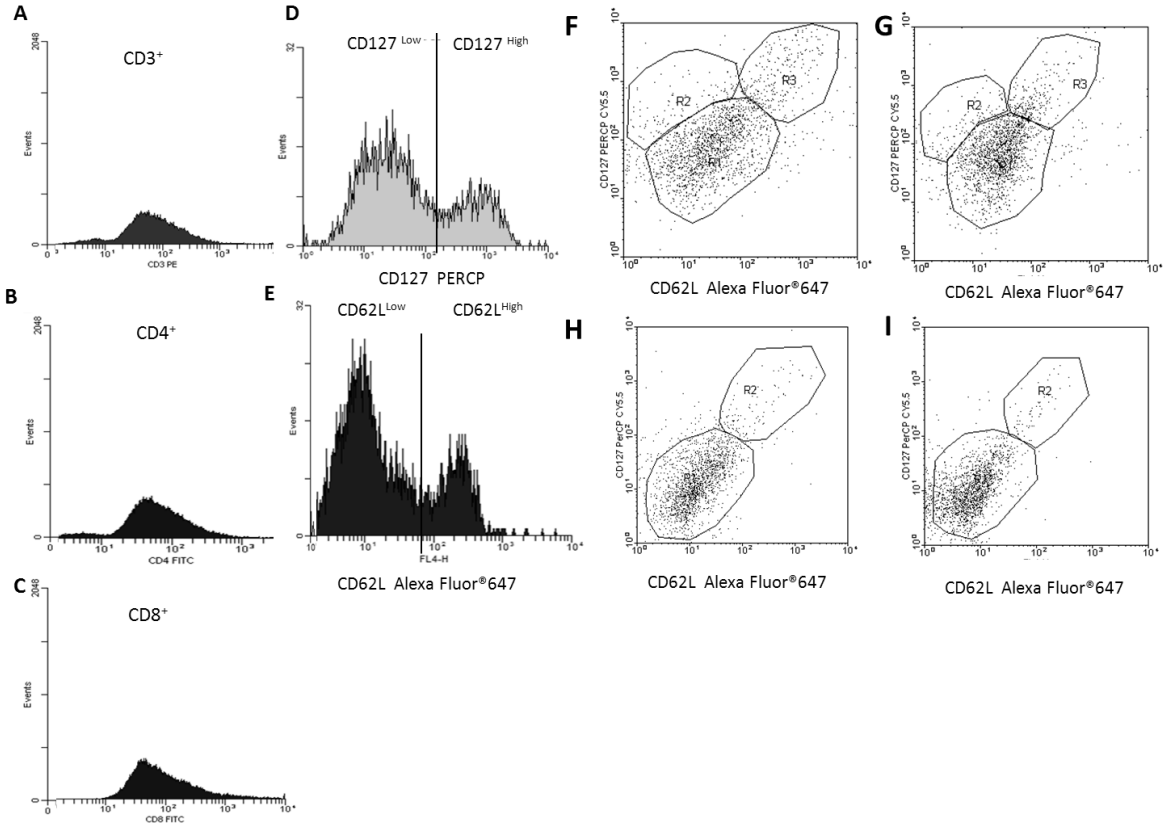


Figure 91: Representative histograms and plots of flow cytometry analysis of liver resident lymphocytes.
A) Histogram representing a $CD3^+$ cell population. B) Histogram showing a $CD3^+CD4^+$ cell subset. C) Histogram presenting a $CD3^+CD8^+$ population. D) Histogram showing a $CD127^+$ cell subset of low fluorescence ($CD127^{low}$ at the left side of the vertical line) and of high fluorescence ($CD127^{high}$ at the right side of the vertical line). E) Histogram presenting a $CD62L^+$ cell subset of reduced fluorescence ($CD62L^{low}$ at the left side of the vertical line) and of high fluorescence ($CD62L^{high}$ at the right side of the vertical line). Dot-plots of liver resident lymphocytes isolated from non-infected mice (group 1, F), *L. infantum* infected mice (group 2, G), *L. infantum* infected mice treated with MgA (group 3, H) and of MgA pre-exposed *L. infantum* infected mice followed by a MgA treatment (group 4, I). R1- $T_E$cells ($CD62^{low}$ $CD127^{low}$); R2-T_{EM} cells ($CD62L^{low}$ $CD127^{high}$); R3-T_{CM}cells ($CD62L^{high}$ $CD127^{high}$).

6.4.2.1. *L. infantum* exert a silencing effect on memory hepatic T cells

The levels of hepatic T cell memory in the liver of non-infected animals (group 1) and of mice infected with *L. infantum* (group 2) was compared to elicit the effect of the presence of the parasite in the differentiation of a subpopulation of resident memory T cells.

Several differences in the levels of $CD4^+$ and $CD8^+$ T_{EM} cell subsets were founded (Figure 92A and B). Infected mice had a significant contraction in $CD4^+$ T_{EM} cell ($CD3^+CD4^+CD62L^{low}CD127^{high}$) population (P=0.0237) comparing with non-infected mice (group 1). Even with the addition of an antigenic stimulus, total

L. infantum Ag, T_{EM} cells were able to expand very little, indicating a suppressive effect caused by the previous exposure to the parasite.

$CD8^+T_{EM}$ cells ($CD3^+CD8^+CD62L^{low}CD127^{high}$) of non-infected animals were able to expand significantly (P=0.0098) when stimulated with total *L. infantum* Ag. However, cells from infected animals did not expand even in the presence of *L. infantum* Ag. $CD4^+$ ($CD3^+CD4^+CD62L^{high}CD127^{high}$) and $CD8^+$ ($CD3^+CD8^+CD62L^{high}CD127^{high}$) T_{CM} cells showed similar tendencies (Figure 92C and D). Although differences were statistically not significant, non-infected mice had higher cell percentages when compared with infected animals. *L. infantum* parasites appear to exert a silencing effect, preventing differentiation and activation of memory cells.

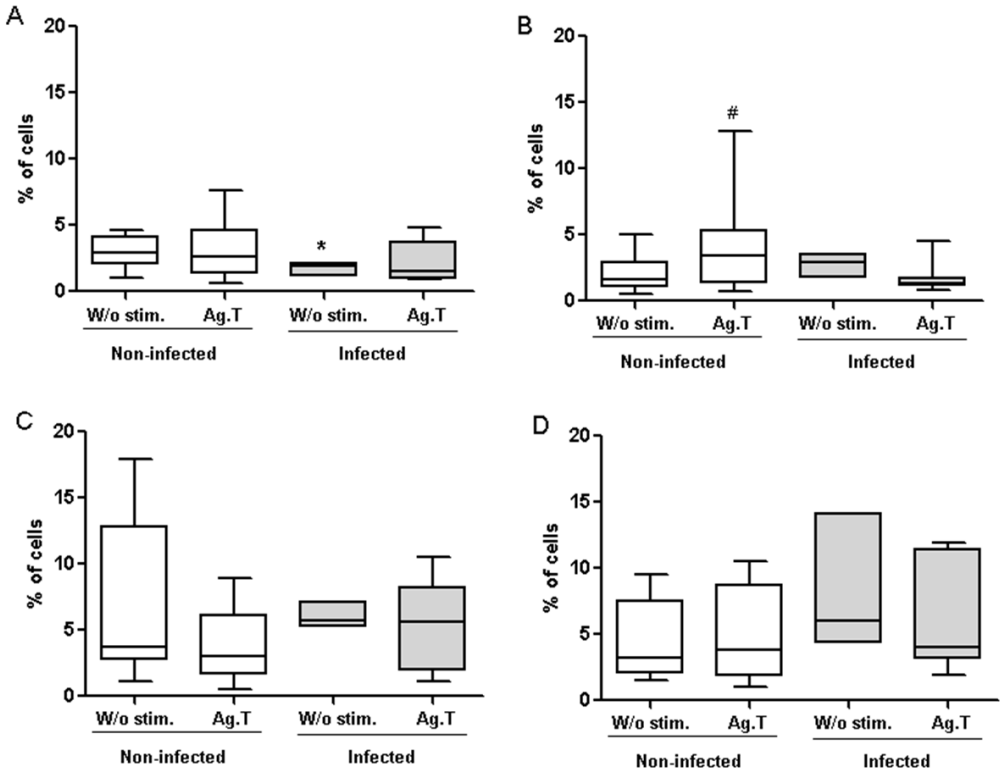


Figure 92: Levels of hepatic memory T cells in non-infected and in *L. infantum* infected mice.
Unexposed (W/o stim) and parasite Ag exposed (Ag.T) leukocytes were evaluated by flow cytometry. A) $CD3^+CD4^+$ effector memory subset. B) $CD3^+CD8^+$ effector memory subset. C) $CD3^+CD4^+$ central memory subset. D) $CD3^+CD8^+$ central memory subset. Results are represented as whisker box-plot and (minimum to maximum) including values from three independent experiments and triplicates per each sample.* represent significant differences (P<0.05), resulting from the comparison of group 1 with group 2 mice. # represent significant differences (P<0.05), resulting from the comparison within an experimental group with different types of stimulation (non-stimulated or Ag.T).

$CD62^{low}CD127^{low}$ T_E cells are relatively short-lived activated cells that display a specific immune response (Figure 93). Activated $CD4^+$ T helper cells and cytotoxic $CD8^+$ T cells are the main responsible for cell-mediated immune responses.

In non-infected animals, the $CD4^+T_E$ cell population has a higher dispersion compared to infected mice. The addition of total *L. infantum* Ag did not induce proliferation of effector cells in infected animals, as the dispersion of the T cell populations is higher than in non-stimulated cells. This indicates a possible silencing effect of parasitic Ag over cell proliferation. A similar effect is observed in $CD8^+$T cells, the stimulation of effector cells from infected mice with total parasite Ag results in a cell population dispersion, indicating poor cell activation and proliferation.

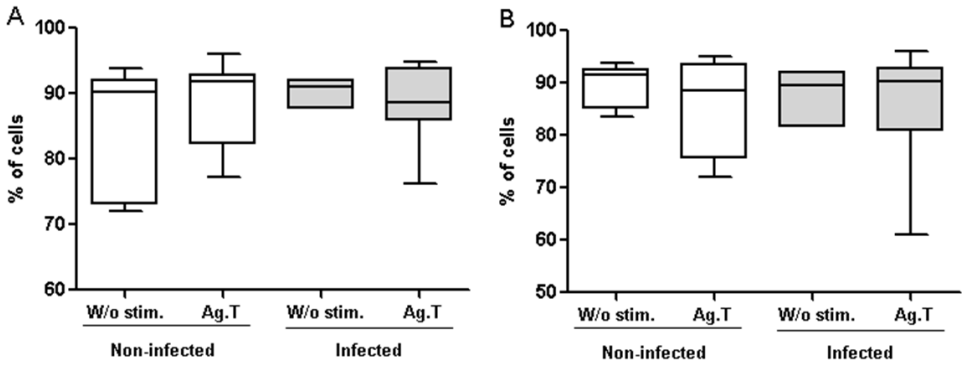


Figure 93: Hepatic T_E cell subsets from non-infected mice and *L. infantum* infected mice.
Leukocytes were stimulated with *L. infantum* total Ag (Ag.T) or unstimulated (W/o stim.) for 72 h and evaluated by flow cytometry. A) $CD4^+$ T_E cells. B) $CD8^+$ T_E cells. Results are represented as whisker box-plot (minimum to maximum) including results from three independent experiments and triplicates per each sample.

6.4.3. Recombinant *L. infantum* proteins do not overcome the silencing imposed by the parasite infection

The capacity of liver isolated leukocytes from non-infected and *L. infantum* infected mice to recognize and expand in the presence of *L. infantum* proteins was accessed for three recombinant proteins, *Li*rCyP1, *Li*rSOD and *Li*rAMP. These are soluble proteins that can be found in the total parasite Ag. Memory cell subpopulations from non-infected (group 1) and from *L. infantum* infected (group 2) mice were cultured with the recombinant proteins (Figure 94).

$CD4^+$ T_{EM} cells ($CD3^+CD4^+CD62L^{low}CD127^{high}$) from non-infected animals were able to expand with the incubation with the three recombinant proteins. There are differences in pattern selection induced by *Lir*AMP (P=0.0137) when comparing with the other two proteins. In this case, *Lir*AMP is the protein that less stimulates the expansion of cell populations. $CD4^+$ T_{EM} cells of infected animals also recognize the tested proteins and stimulation with *Lir*CyP1 is significantly different (P=0.0421) between the two experimental groups.

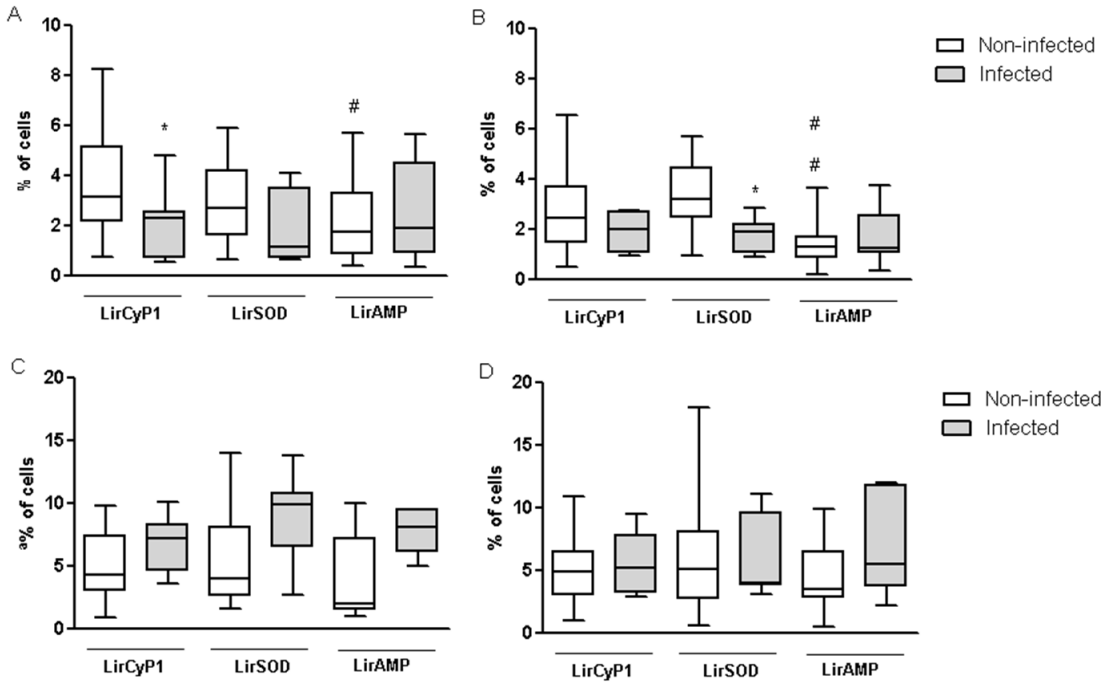


Figure 94: Levels of hepatic memory T cell subsets selected by *L. infantum* recombinant proteins, LirCyP1, LirSOD and LirAMP.
Comparison was performed between infected (group 2) and non-infected mice (group 1). A) $CD4^+$ T_{EM} cells. B) $CD8^+$ T_{EM} cells. C) $CD4^+$ T_{CM} cells. D) $CD8^+$ T_{CM}cells. Results are represented as whisker box-plot (minimum to maximum) comprising results from three independent experiments and triplicates per each sample.* represents statistical significance (P<0.05), resulting of a comparison of group 1 with group 2 mice. # represents statistical significance (P<0.05), resulting from the comparison within an experimental group with different types of stimulation (*Lir*CyP1, *Lir*SOD or*Lir*AMP).

In $CD8^+$T cells from non-infected animals, it is evident a different stimulation pattern for each protein, being again *Lir*AMP the less stimulatory protein (P=0.001, compared to *Lir*SOD and P=0.0244, compared to *Lir*CyP1). Infected animals expand less to the proteins when compared to non-infected animals, especially with *Lir*SOD stimulation (P=0.0096), indicating a possible suppression mechanism from the parasite. $CD4^+$ and $CD8^+T_{CM}$ cells are also able to recognize the recombinant proteins. Infected

animals seem to recognize the recombinant proteins expanding, although without statistics significance.

The effector cell population was also accessed after 72 h of stimulation with the recombinant proteins. In non-infected animals, the $CD4^+T_E$ cells did not proliferate with any of the recombinant proteins (Figure 95). However, *Lir*AMP stimulation of cells from the liver of infected mice presented a contraction of cell dispersion, possible indicating some proliferation, although not statistically significant. As for $CD8^+ T_E$ cells, a similar pattern was obtained with cells from infected and non-infected mice presenting a wide dispersion. Only cells from non-infected mice stimulated with *Lir*AMP showed a proliferation profile different from the stimulation with *Lir*CyP1 or *Lir*SOD (P=0.0244). This is a possible indicator that *Lir*AMP stimulates the proliferation of T_E cells, eventually giving origin to a cytotoxic immune response.

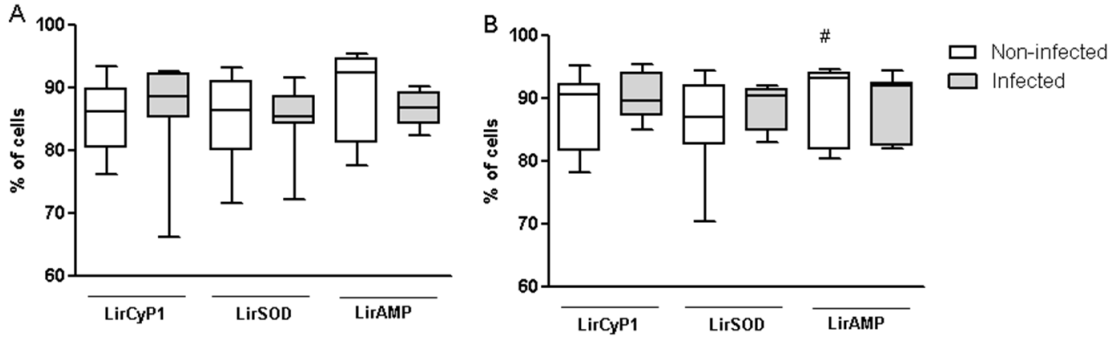


Figure 95: The frequency of hepatic T_E cells stimulated by recombinant *L. infantum* proteins. Leukocytes isolated from non-infected and *L. infantum* infected mice were stimulated with *Lir*CyP1, *Lir*SOD and *Lir*AMP for 72 h and analyzed by flow cytometry. A) $CD4^+ T_E$ cells. B) $CD8^+ T_E$ cells. Results are represented as whisker box-plot (minimum to maximum) comprising results from three independent experiments and triplicates per each sample. # represents statistical significance (P<0.05), resulting from the comparison within an experimental group with different types of stimulation (*Lir*CyP1, *Lir*SOD or*Lir*AMP).

6.4.4. Leishmanicidal treatment favors the differentiation of hepatic $CD4^+T_{EM}$ subset and the proliferation of $CD8^+T_E$

The effect of treatment with a leishmanicidal drug that can kill intracellular amastigotes and possible release parasite Ag in T cell memory was also investigated. The third experimental group was included *L. infantum* infected and treated animals. The treatment was performed with the commercial leishmanicidal drug, MgA, during five consecutive days. When compared with infected mice (group 2), $CD4^+$ T cell

population isolated from the liver of treated mice (group 3) presented higher levels of effector memory cells, particularly when exposed to total *L. infantum* Ag (P=0.0126) (Figure 96A). However, the subset of $CD8^+$ T cells with the effector memory phenotype showed a significant contraction (P=0.0048) (Figure 96B).

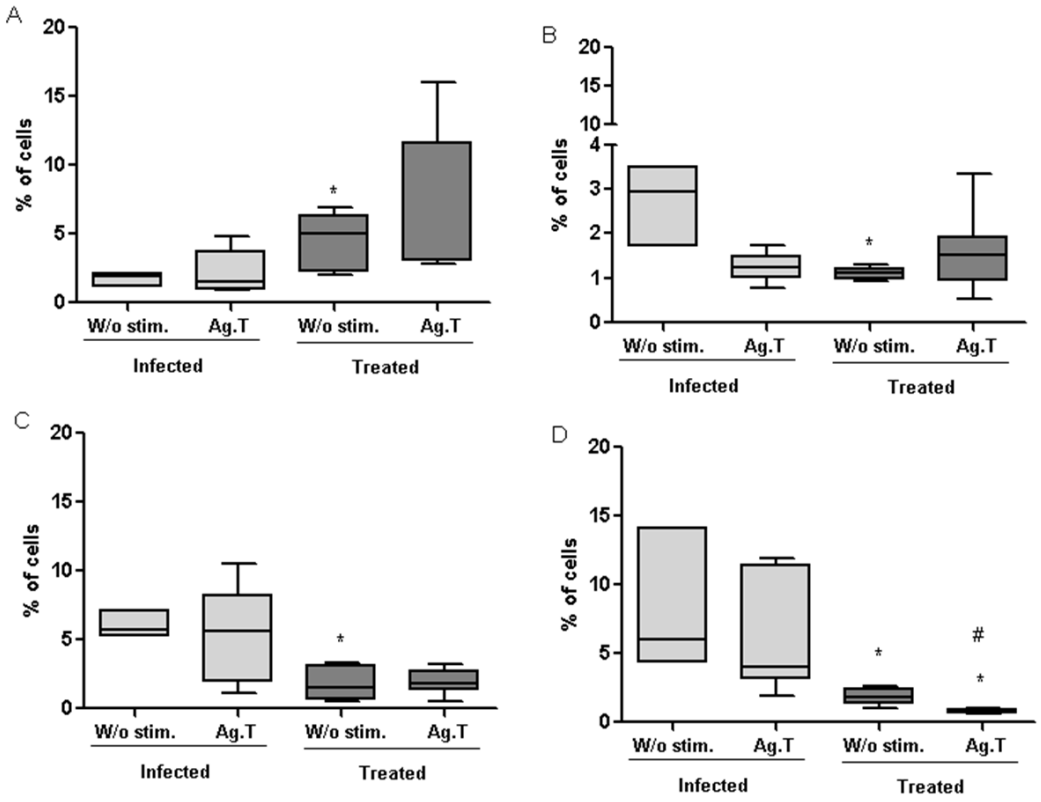


Figure 96: Levels of hepatic memory T cell in infected mice and in treated mice.
Leukocytes isolated from infected (group 2) and treated mice (group 3) were exposed to *L. infantum* total Ag (Ag.T) for 72 h. Parallel unexposed (W/o stim.) cells were also left to incubate for 72 h. Cells were marked and the magnitude of T_{EM} and T_{CM} cell subsets determined by flow cytometry. A) $CD4^+$ T_{EM} cells. B) $CD8^+$ T_{EM} cells. C) $CD4^+$ T_{CM} cells. D) $CD8^+$ T_{CM} cells. Results are represented as whisker box-plot (minimum to maximum) comprising results from three independent experiments and triplicates per each sample. * represents statistical significance (P<0.05), resulting of a comparison of group 2 with group 3 mice. # represents statistical significance (P<0.05), resulting from the comparison within an experimental group with different types of stimulation (non-stimulated or Ag.T).

Exposition of liver leukocytes from treated mice to the parasite total Ag caused a slight increase of $CD8^+$ T cells with effector memory phenotype, although not statistically significant. When compared with infected mice (group 2), central memory $CD4^+$ T cell subset evidence a strong contraction in treated mice (group 3) (P=0.0048). The treated mice group also exhibited lower differentiation of $CD8^+$ T_{CM} cells (P=0.0048) in comparison with infected mice. This cell subset still remained low, even when exposed to the *L. infantum* Ag (P=0.0059). Furthermore, exposition to parasite Ag

caused a strong contraction of $CD8^+$ T_{CM} cell subset when compared with unexposed cells (P=0.0313).

It is interesting to notice that the $CD8^+$ T_E cells originated from treated mice were significantly higher (P=0.0048) when compared with infected mice (Figure 97). This high proliferation stays elevated when stimulated by parasite Ag (P=0.0013), achieving a frequency of near 100%. This elevated frequency might be related to the low memory levels (booth effector and central) observed in the treated animals, suggesting that memory cell subpopulations are a dynamic system with cells being able to be transformed into others cell subset as needed, and in this particular case into effector cells.

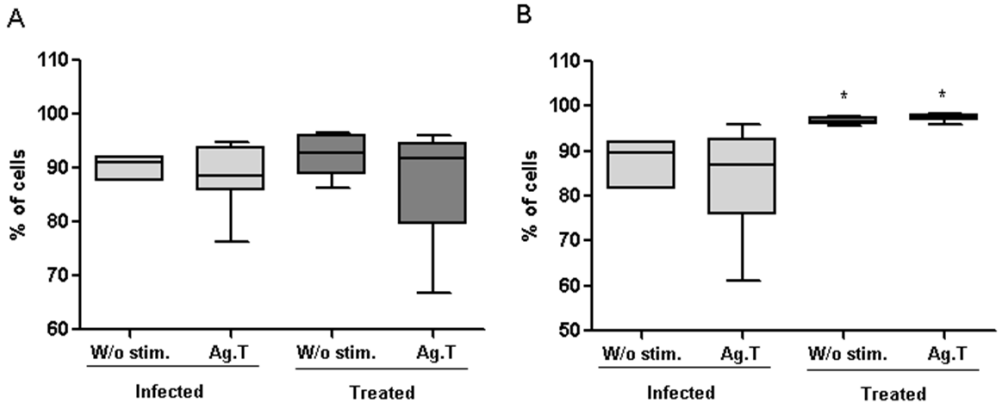


Figure 97: Hepatic T_E cell subsets of infected and treated mice.
Leukocytes isolated from livers of infected (group 2) and treated mice (group 3) were stimulated by *L. infantum* total Ag (Ag.T) for 72 h. Parallel unstimulated cells (W/o stim.) were also left to incubate for 72 h. Cells were marked and the frequency of effector cells determined by flow cytometry. A) $CD4^+$ T_E cells. B) $CD8^+$ T_E cells. Results are represented as whisker box-plot (minimum to maximum) comprising results from three independent experiments and triplicates per each sample. * represents statistical significance (P<0.05), resulting of a comparison of group 2 with group 3 mice.

6.4.4.1. Recombinant *L. infantum* proteins induce the proliferation of hepatic $CD8^+T_E$ cell subset

The ability to recognize and expand in the presence of *L. infantum* recombinant proteins *Li*rCyP1, *Li*rSOD and *Li*rAMP by liver resident lymphocytes of treated mice was also analyzed. The presence of a leishmanicidal drug, MgA, may lead to the killing of intracellular amastigotes and the exposition and presentation of parasite Ag leading to cell activation and proliferation. $CD4^+$ T_{EM} cells from the liver of treated mice (group 3) recognize and expand when exposed to recombinant proteins, particularly in

the presence of *Li*rCyP1 (P=0.0262) and *Li*rSOD (P=0.0101), achieving a higher proliferation than the cells from *L. infantum* infected mice (group 2) (Figure 98A), that probably were subjected to a silencing effect induced by the parasite. $CD8^+$ T_{EM} did not exhibit significant alterations between infected and treated mice groups (Figure 98B). Curiously, $CD4^+$ and $CD8^+$ T_{CM} cell subpopulations of treated mice showed an accentuated reduction when exposed to recombinant proteins (Figure 98C and D). $CD4^+$ central memory T_{CM} cells from treated mice differ significantly from infected mice, presenting a contraction of the subpopulation ($P_{LirCyP1}$=0.0007, P_{LirSOD}=0.0023 and P_{LirAMP}=0.0014). Exposition to *Li*rSOD was significantly different from *Li*rCyP1 (P=0.0313).

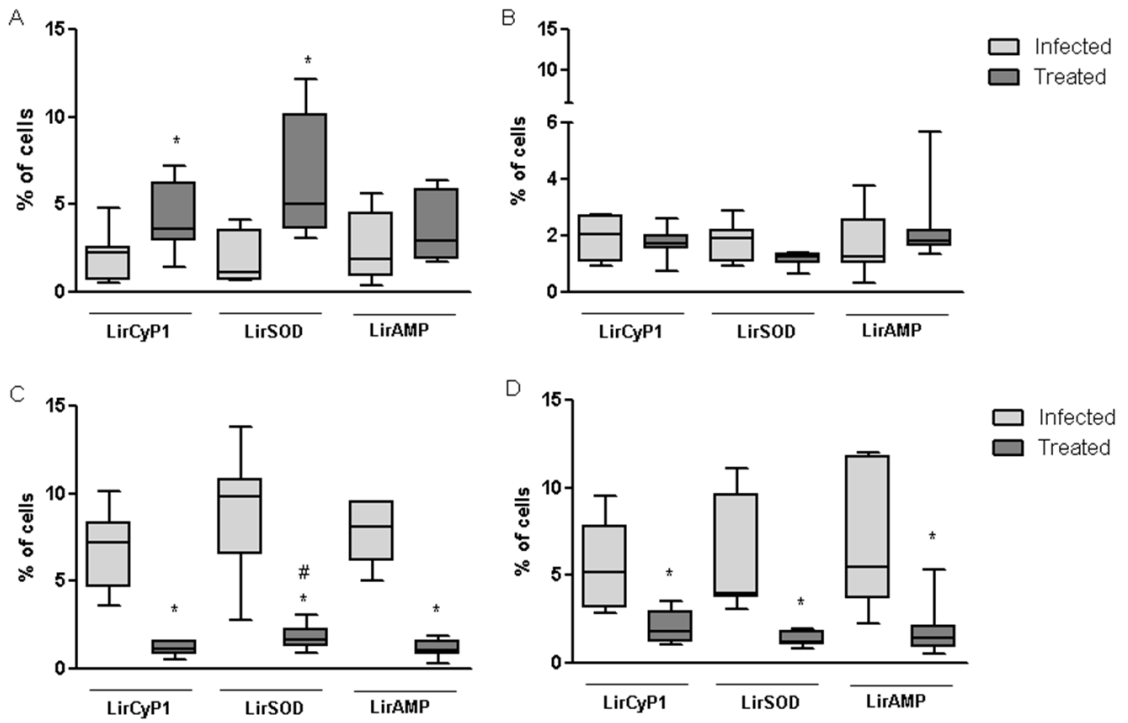


Figure 98: Hepatic memory T cells of infected and treated mice selected by *L. infantum* recombinant proteins.
Leukocytes isolated from infected (group 2) and treated (group 3) mice were exposed to *Li*rCyP1, *Li*rSOD and *Li*rAMP. A) $CD4^+$ T_{EM} cells. B) $CD8^+$ T_{EM} cells. C) $CD4^+$ T_{CM} cells. D) $CD8^+T_{CM}$ cells. Results are represented as whisker box-plot (minimum to maximum) comprising results from three independent experiments and triplicates per each sample. * represents statistical significance (P<0.05), resulting of a comparison of group 2 with group 3 mice. # represents significant differences (P<0.05), when comparing exposition to different recombinant proteins within the same experimental mice group.

The subpopulation of $CD4^+$ T_E cells that comes from the livers of treated mice (group 3) evidenced some proliferation when stimulated with *Li*rAMP (P=0.0003) in comparison to infected mice (group 2). Furthermore, stimulation with *Li*rSOD had a

different effect than *Lir*CyP1 (P=0.0313) (Figure 99A). Stimulation with *Lir*CyP1 (P=0.0027), *Lir*SOD (P=0.0012) and *Lir*AMP (P=0.0262) caused higher proliferation of $CD8^+$ T_E cell subset from treated mice than the cells isolated from infected mice. Also the stimulation with *Lir*SOD has a different effect than the stimulation with *LI*rCyP1 (P=0.0313). The increase of $CD8^+T_E$cell subpopulation derived from liver of treated mice is associated with the significant reduction of both $CD8^+$ memory T cell subsets (effector and central) (Figure 99B), suggesting that memory cell subpopulations are a dynamic system in which the cells are able to exhibit different phenotypes as necessary in order to react to the presence of not self Ag.

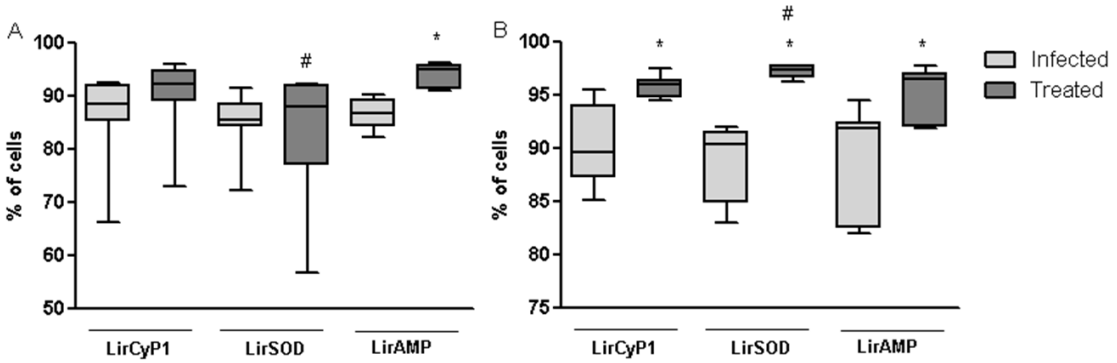


Figure 99: Hepatic T_E cells of infected mice (group 2) and treated mice (group 3) stimulated by *L. infantum* recombinant proteins.
Liver resident leukocyte cells were stimulated by *L*irCyP1, *Lir*SOD and *Lir*AMP, and analyzed in a flow cytometer. A) $CD4^+$ T_E cells. B) $CD8^+$ T_E cells. Results are represented as whisker box-plot (minimum to maximum) comprising results from three independent experiments and triplicates per each sample. * represent statistical significance (P<0.05), resulting of a comparison of infected with treated mice. # represents significant differences (P<0.05), when comparing exposition to different recombinant proteins within the same experimental mice group.

6.4.5. Liver residual *L. infantum* parasites exhibit different sensitivity to MgA

The parasites isolated from the liver of mice infected with *L. infantum* virulent promastigotes and treated with MgA (group 3) were analyzed by real-time PCR to screen for resistance associated gene amplification. The analyzed genes were MRPA, MDR1, GSH1 and PTR1 (Figure 100). All analyzed genes, except MDR1 exhibited an increase in gene copy number when compared with wild type *L. infantum* promastigotes. MRPA in group 3 (treated mice) isolated parasites registered an increase in gene copy numbers equivalent at 6 times the WT (P=0.0044) and group 4 (mice infected with less MgA susceptible *L. infantum* parasites) isolated parasites registered a

10 fold increase compared to WT (P= 0.0012). As for GSH1 a similar tendency was observed. Parasites isolated from group 3 mice increased GSH1 copy number 5 times (P=0.0044) and group 4 isolated parasite revealed an fold increase of 11 times when compared with the WT copy number (P=0.0012). Curiously, MDR1 gene exhibited a decrease in copy numbers in group 3 isolated parasites (P=0.0044) and in group 4 isolated promastigotes the MDR1 copy presented levels similar of WT. PTR1 also presented a major increase in copy number of promastigotes isolated from group 3 mice (P=0.0044) and group 4 mice (P= 0.0009). The increases in PTR1 gene copy numbers observed were 13 and 20 times when compared with the WT promastigotes. These results evidence that parasites twice exposed to MgA had not a yet fully resistant genotype, but had acquired a decreased sensitivity to the leishmanicidal drug.

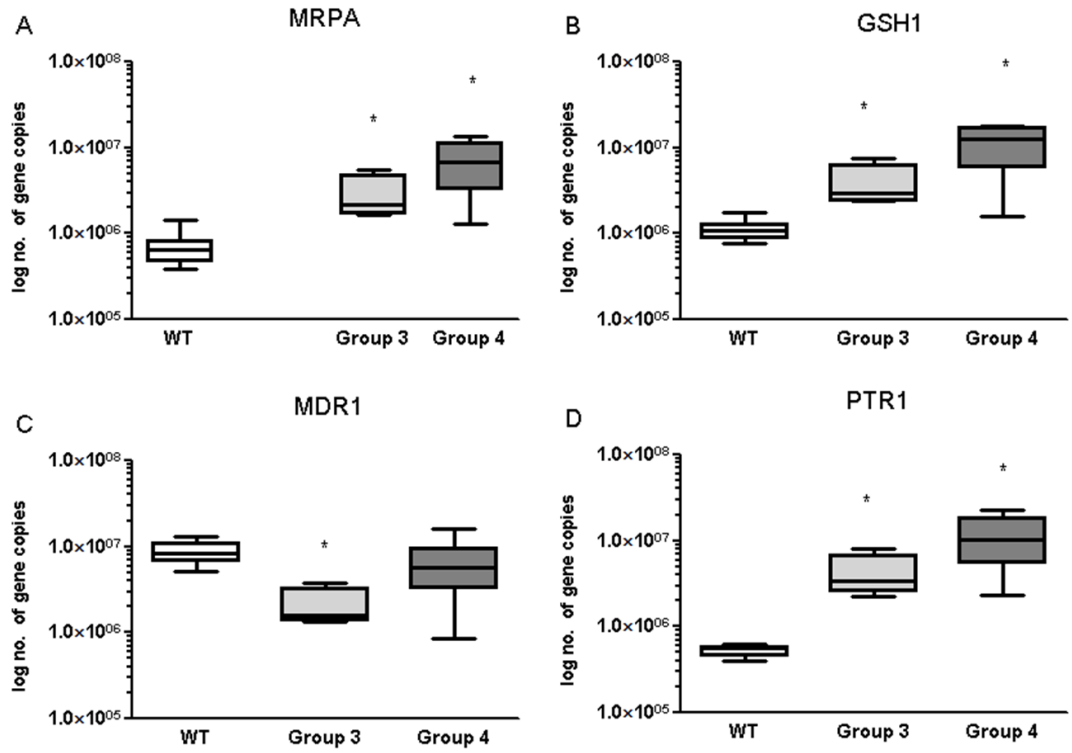


Figure 100: Amplification of MRPA, GSH1, MDR1 and PTR1 from *L. infantum* parasites that were under drug pressure.
Parasite DNA was amplified by real time PCR and the copy number determined. WT- DNA isolated from wild type *L. infantum* parasites were also evaluated. Group 3 - DNA of *L. infantum* parasites isolated from MgA treated mice. Group 4 – DNA of parasites isolated from treated mice previously infected with *L. infantum* parasites pre-exposed to MgA. Results of three independent experiments and triplicates per sample are represented as whisker box-plot (minimum to maximum). * represent statistical significant differences (P<0.05) when comparing WT parasites vs parasites exposed to leishmanicidal drug.

6.4.6. Total parasite Ag favors the expansion of hepatic $CD8^+$T memory cells driving from mice infected with less MgA susceptible parasites

The impact of less susceptible MgA-parasites in the liver memory T cells was analyzed. When compared with group 3 (treated mice), $CD4^+$ T_{EM} cell subset of group 4 (mice infected with less MgA susceptible *L .infantum* parasites) presented a contraction. This contraction is also observed even when exposed to parasite Ag (Figure 101). However, the difference was not statistically significant. $CD8^+$ T_{EM} cell subset originated from group 4 exposed to parasite Ag, expanded significantly more than group 3 (P=0.0037), indicating that liver resident leukocytes, previously sensitized by less MgA susceptible parasites, differentiate a higher subset of $CD8^+$ T cells with effector memory phenotype when exposed to parasite antigen. Also, there is a statistically significant difference when comparing the $CD8^+$ T_{EM} cell subset from group 4 stimulated exposed to total antigen with the non-exposed cells (P=0.0025).

$CD4^+T_{CM}$ cell subset, originating from mice of group 4, exposed to *L. infantum* Ag exhibited an important contraction when compared with unexposed cells (P=0.0313) (Figure 101C). By the contrary, when in contact with *L. infantum* Ag $CD8^+$ T_{CM} cell subset (group 4) significantly expanded when compared with cells isolated from mice of group 3 (P=0.0006) (Figure 101D).

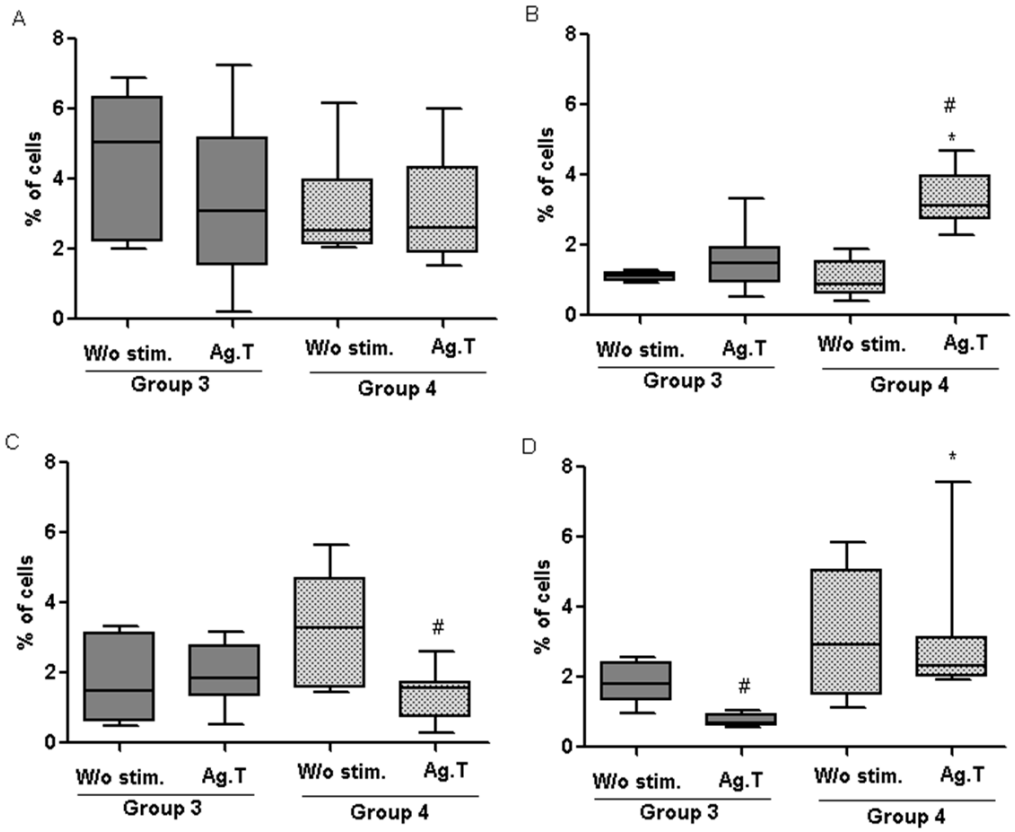


Figure 101: Levels of hepatic memory T cell of mice infected with less MgA susceptible *L. infantum* parasites.
Resident hepatic leukocytes exposed to *L. infantum* total Ag (Ag.T) were evaluated by flow cytometry and the results compared with group 3. Parallel unexposed cells (W/o stim.) were also analyzed. A) $CD4^+$ T_{EM} cells. B) $CD8^+$ T_{EM} cells. C) $CD4^+$ T_{CM} cells. D) $CD8^+$ T_{CM} cells. Results are represented as whisker box-plot (minimum to maximum) comprising results from three independent experiments and triplicates per each sample. * represent significant differences ($P<0.05$), when comparing group 3 with group 4 mice. # represents significant differences ($P<0.05$), when comparing different stimulation (without stimulation or Ag.T) within the same experimental group.

$CD4^+$ T_E cell subpopulation originated from the livers of mice from group 4 exhibited a significant expansion ($P=0.0379$) when compared with animals of group 2 (Figure 102A). Curiously, $CD8^+$ T_{EM} cell subset, in the same experimental conditions, reveals a significant reduction in cell proliferation in comparison with group 3 ($P=0.003$). This reduction was accompanied by a significant increase of $CD8^+$ T_{EM} and T_{CM} cells (Figure 102B), suggesting a differentiation of T_E cells into memory cells.

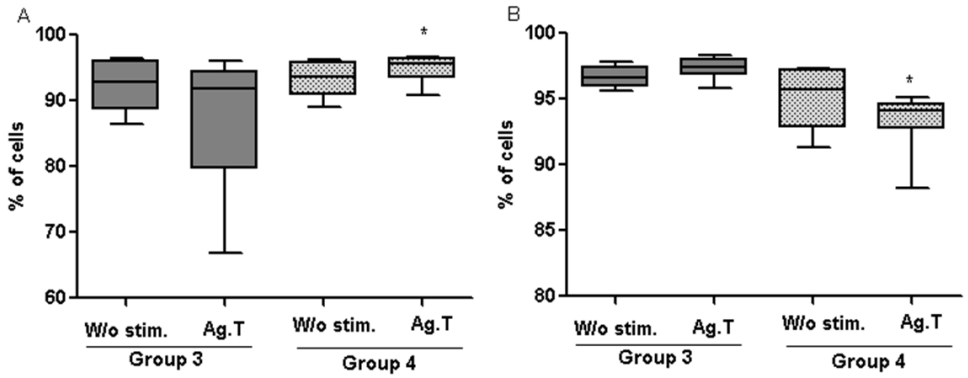


Figure 102: Hepatic T_E cell subsets of mice infected with less MgA susceptible *L. infantum* parasites.
Leukocytes isolated mice of group 3 were stimulated by *L. infantum* total Ag (Ag.T) for 72 h. Parallel unstimulated cells (W/o stim.) were also left to incubate for 72 h. Stimulated and unstimulated (W/o stim.) hepatic leukocytes were evaluated by flow cytometry and the results compared with mice of group 3. A) $CD4^+$ T_E cells. B) Effector $CD8^+$ T_E cells. Results are represented as whisker box-plot (minimum to maximum) comprising results from three independent experiments and triplicates per each sample. * represent statistical significance ($P<0.05$), resulting of a comparison of group 3 with group 4 mice.

6.4.6.1. *Lir*CyP1 triggers the expansion of hepatic $CD8^+T_{EM}$ cells and *Lir*SOD promotes the expansion of $CD4^+T_{CM}$ cells originating in mice infected with less susceptible MgA parasites

Resident hepatic leukocytes isolated from mice of group 4 were exposed to *L. infantum* recombinant proteins, *Lir*CyP1, *Lir*SOD and *Lir*AMP, and memory T cell subsets were assessed and compared with group 3. $CD4^+$ T_{EM} cell subpopulation from experimental group 4 expanded less than group 3 (Figure 103). This retraction is particularly visible in cells exposed to *Lir*SOD ($P=0.0317$). *Lir*CyP1 promoted and accentuated expansion of $CD8^+$ T_{EM} cells ($P=0.0387$) but, the contact with *Lir*AMP leaded to a contraction of the cell sub population ($P=0.0012$). When exposed to *Lir*SOD $CD4^+$ T_{CM} cells from mice of group 4 exhibit an accentuated expansion ($P=0.0087$). Furthermore, the contact with recombinant proteins did not induce significant changes in $CD8^+T_{CM}$ cell subpopulation when compared with group 3.

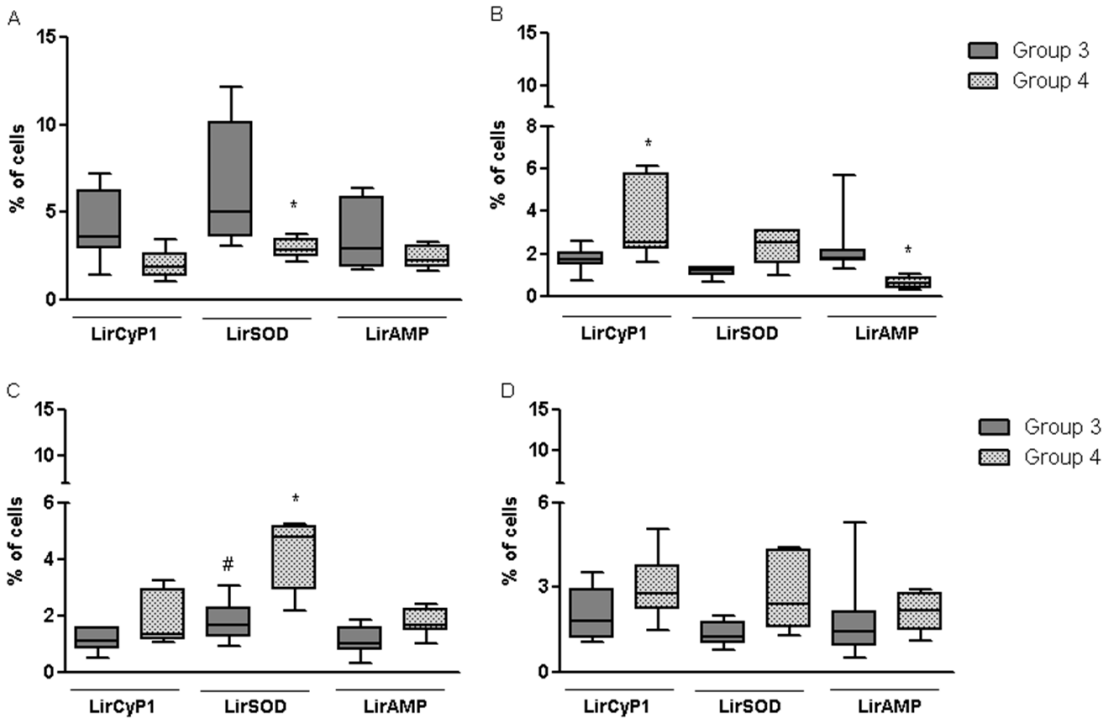


Figure 103: Hepatic memory T cells of mice infected with less MgA susceptible *L. infantum* parasites selected by *L. infantum* recombinant proteins.
Resident hepatic leukocytes isolated mice of group 4were exposed to *Li*rCyP1, *Li*rSOD and *Li*rAMP were evaluated by flow cytometry and the results compared with mice of group 3. A) $CD4^+$ T_{EM} cells. B) $CD8^+$ T_{EM} cells. C) $CD4^+$ T_{CM} cells. D) $CD8^+$ T_{CM} cells. Results are represented as whisker box-plot (minimum to maximum) comprising results from three independent experiments and triplicates per each sample. * represent significance differences ($P<0.05$) when comparing group 3 with group 4 mice. # represents significant differences ($P<0.05$) when comparing different stimulation (*Li*rCyP1, *Li*rSOD and *Li*rAMP) within the same experimental group.

$CD4^+T_E$ cells from mice of group 4 proliferated more than group 3, in particular when stimulated by *Li*rCyP1 (P= 0.0200) (Figure 104). $CD8^+$ T_E cells from mice of group 4 evidenced lower proliferations, above all when stimulated with *Li*rCyP1 (P=0.0237) or *Li*rSOD (P=0.0173). The stimulation with *Li*rAMP is statistically different from *Li*rCyP1 stimulation (P=0.00313). These subset reductions are probably related to the increase of T_{EM} and T_{CM} cells, suggesting that these cell subpopulations are able to interchange as needed.

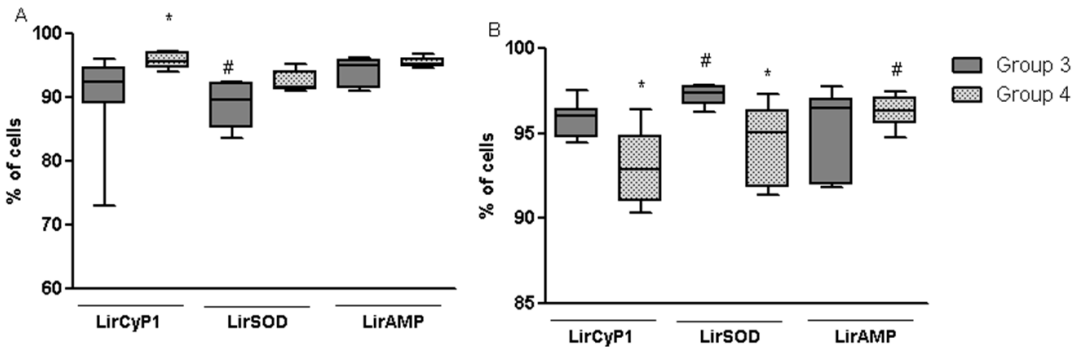


Figure 104: Hepatic T_E cells of mice infected with less MgA susceptible parasites stimulated by *L. infantum* recombinant proteins.
Liver resident leukocytes were stimulated by *L*irCyP1, *Li*rSOD and *Li*rAMP and analyzed by flow cytometry and compared with mice or group 3. A) $CD4^+$ T_E cells. B) $CD8^+$ T_E cells. Results are represented as whisker box-plot (minimum to maximum) comprising results from three independent experiments and triplicates per each sample. * represent statistical differences ($P<0.05$) when comparing group 3 with group 4 mice. # represents significant differences ($P<0.05$), when comparing different stimulation (*L*irCyP1, *Li*rSOD and *Li*rAMP) within the same experimental group.

.

6.5. Discussion

In this chapter, the role of the liver as an immunological memory organ was analyzed during the outcome of *L. infantum* infection. During the past 40 years, murine models of leishmaniasis have been extensively employed to elucidate parasite immune interactions, including cell types, cytokines, signal transduction cascades and receptors involved in infection constrain, in clinical resolution of disease, resistance to a secondary infection and in vaccine development. Although, the domestic dog is the main reservoir of *L. infantum* and is of the highest importance to study their immune response to the parasite, the mouse model has several important practical advantages over the canine model. Mice have long been established as model of the disease. Since different mouse strains are genetically identical it is possible to collect and combine biological data obtained over time from several different sources, resulting in a depth characterization rarely achieved in other mammalian models (Loría-Cervera and Andrade-Narváez, 2014). Another major advantage of working with the murine model when compared with the canine model is related to the simplicity of keeping, breeding and reproducing, as well as the superior availability of commercial reagents for molecular and cellular studies. Taking into account the above considerations, the present study used the susceptible BALB/c mouse strain as a VL mouse model in order to ensure a stable genetic background and eliminate possible genetic influences in the development of immune memory, as well as the use of careful germ-controlled mice reduces the possibility of cross-reactions with other Ag from previous infections. The superior availability of commercial reagents, namely monoclonal antibodies for mouse cell receptors was also taken in consideration in the choice of the murine model.

In mouse models, the infection outcome is genetically determined, being BALB/c mice considered susceptible to VL. Following *Leishmania* infection, BALB/c mice develop an organ-specific immune response. During the first weeks of infection, the parasites multiply rapidly in the liver. But about four weeks later, effective Th1 immune response is developed and parasites are cleared, becoming resistant to re-infection (Murray et al., 1987). Similarly to what is observed in the canine model, the hepatic resistance to *Leishmania* infection is associated with the development of a granulomatous reaction in the liver. Also in human VL, hepatic granulomas appears to

correlate with spontaneous control and maintenance of subclinical infections (Murray, 2001).

While pathology in the liver is limited (although hepatomegalia is verified), parasites persist in the spleen and the infection progresses. Under optimal conditions, granulomas provide anatomically circumscribed, functional structures in which it is possible to limit infection, kill and remove the microbial target, and then repair any associated tissue injury. Intracellular infections associated with the development of granulomatous inflammation include those caused by mycobacteria, bacteria, fungi and certain protozoa such as *Leishmania* (Murray, 2001). In the present work, it was identified several liver granulomas with the typical macrophage infiltrate. As BALB/c mice are susceptible to *L. infantum* infection this observation was expected, given evidence of a well succeeded experimental *L. infantum* infection. The isolation of viable parasites from the liver of infected mice (group 2), infected and treated mice (group 3) and also from treated mice previously infected with less MgA susceptible *L. infantum* (group 4) indicates that despite the granulomatous immune response viable parasite persist, even after treatment with a leishmanicidal drug. This observation may raise the following question: are the granulomas ensuring the persistence of the parasite in the host, difficulty the access of the drug to the parasite? Murray (2001) describes the typical structure of a liver granuloma in *Leishmania i*nfection as a cellular infiltrate rich in macrophages. Monocytes and T lymphocytes from the circulating blood are recruited and move into the mononuclear mantle, which slowly evolves to surround infected macrophages. In some cases, B cells, plasma cells, some neutrophils and eosinophils are also present. The evolution around the core of infected macrophages in the liver was also observed in this experimental work, suggesting mature and active granulomas. According to many authors, granulomas represent a successful T cell-dependent immune response (Sheffield 1990; Murray and Nathan, 1999; Hernandez-Pando et al., 2000). To be assembled, granulomas typically require an Ag-specific immune response mediated by mononuclear cells. Have a relatively slow development and persist for extended periods (McElrath et al., 1988). This is particularly important, as the present study aims to analyses the immune memory generated during *L. infantum* infection in the liver, it is mandatory that the resident hepatic lymphocytes had previous contact with the parasite Ag naturally presented in the context of the organ infection, proven by

the presence of granulomas. Curiously, the overall antimicrobial efficacy of the granulomatous response appears to be variable, depending upon host determinants, the pathogen and the criteria used to define efficacy. Although microbial eradication is the ideal outcome in infections, which trigger granuloma assembly, it is not clear how often this objective is actually achieved. In mice and dog the formation of granulomas, leads to a resistant phenotype and animals are able to control infection progression, at least for a longer period of time, remaining asymptomatic. But, after an initially intense granulomatous response, a number of pathogens, including *L. donovani* and *L. infantum* establish a state of chronic intra-granuloma parasitism and survive at low levels lifelong. Although might allow parasite survival in the host granuloma also requires a constant immune pressure to preserve and renewal the multi-cellular structure, preventing the recrudescence of a latent infection (Murray, 2001). However, this state of concomitant immunity, in which pathogen resistance is dependent on viable pathogen persistence, may be key to delay disease progression. What happens in a lifelong granuloma is still debated, as it may relapse and the parasite may invade out of the granuloma and infect other tissues.

The cellular markers used in the present study for the assessment of memory T cells were the CD127 and CD62L. CD127 is the interleukin-7 receptor (IL7R) and T cells are dependent upon IL-7 for their survival since signaling through the IL7R promotes cell survival via the upregulation of anti-apoptotic proteins, such as Bcl-2, and the glucose transporter, Glut. The IL7R is expressed on naive T cells and is downregulated following TCR engagement. T cells then have the capacity to upregulate the IL7R for their continued preservation (Schluns et al., 2000). Huster et al. (2004) showed for the first time the use of IL7R as a marker for memory T cells in mice, allowing the distinction of effector (T_{EM}) and central memory T (T_{CM}) cells from T_E cells together with the use of CD62L cell marker. CD62L is an L-selectin, which mediates T cell migration into lymphoid organs and has long been used as a marker to distinguish T_{CM} from T_{EM}. In the present study the use of these two cellular markers allowed the identification of three distinct subpopulations: T_E, T_{CM} and T_{EM} in accordance with the previously described.

The infection of an individual with a new pathogenic agent leads to the activation of innate, followed by adaptive immunity and the generation of memory cells

that will persist and assure future immunity and protection. In VL little is described about the formation, activation and maintenance of these cells. Following *L. infantum* infection in the liver, granuloma assembling takes place and an immune active cellular response is generated. The memory levels induced by the infection of mice (group 2) with *L. infantum* virulent promastigotes did not induce major changes compared to the natural memory levels present in the healthy mice (group 1). This situation may be due to the parasite silencing effect and is altered when the mice are treated (group 3) with a commercial leishmanicidal drug, meglumine antimoniate. *Leishmania* has several intrinsic factors described to downregulate the host immune response. The most known and described virulence factors from *Leishmania* include glicoinositolphospholipids (GIPL), lipophosphoglycan (LPG), proteophosphoglycan (PPG), an 11kDa kinetoplastid membrane protein (KMP-11), several proteinases and metalloproteinases. Although the exact impact of these macro molecules in the immune system of mammalian host is not yet totally clarified there is evidence that these macromolecules modulate the interactions between parasite and host immune cells, favoring the establishment of infection (Silva-Almeida et al., 2012). For example, it was shown that LPG inhibit the nuclear translocation of monocytes NF-κB, leading to a subsequent diminution in IL-12 production (Argueta-Donohué et al., 2008) and also influences the host's early immune responses by modulating the activity of dendritic cells, inhibiting Ag presentation and promoting an early IL-4 response (Liu et al., 2009). All together, these actions favor parasite establishment and survival. Also, parasite proteases are important virulence factors, as they are enzymes that hydrolyze peptide bonds and have the potential to degrade peptides and proteins. *Leishmania* has several proteases that participate in host pathogenesis. Proteases are crucial in parasite invasion and migration through host tissues, degradation of immune related proteins, immune evasion and activation of the inflammation pathways (McKerrow et al., 2006). More recently, Marr et al. (2014) suggested a new paradigm in host-pathogen interactions. Upon infection *Leishmania* induces epigenetic changes in the host genome able to direct the downregulation of innate immunity, thereby enabling pathogen survival and replication. The mechanisms by which *Leishmania* spp. evade the host immune response are now to start to be better understood and explored, as the parasite has several strategies to evade the host immune system.

In *L. infantum* infected mice MgA treated (group 3), the effect of the silence imposed by the parasite is overlapped and a more robust memory T cell population is differentiated, in particularly the $CD4^+T_{EM}$ cell subset. $CD4^+$ or $CD8^+$ T_{EM} cells can be recalled when stimulated with *L. infantum* total Ag, which is in fact a typical characteristic of T_{EM} cells, the fast Ag recognition and differentiation in T_E cell followed by proliferation. T_{EM} cells presenting $CD62L^-$ $CD127^+$ phenotype can produce effector cytokines, as IFN-γ but are less proliferative than T_{CM} cells that produce IL-2 and proliferate extensively (Mueller et al., 2012). There was also a major increase in the $CD8^+T_E$ cell population, indicating that $CD8^+$ cytotoxic T cells are highly important in parasite killing and controlling the infection. Interestingly, this increase reflects an accentuated decrease of $CD8^+$ T_{CM} cells, suggesting that central memory is activated in treated mice and transformed into intermediate stage, and from there to T_E cells, capable of delivering cytotoxicity. The differentiation pathways and lineage relationship between naive, T_E, T_{EM}, and T_{CM} cells is still a matter of debate. The more consensual model is a linear differentiation of naive into T_E, T_{EM}, and T_{CM}. According to this model, the majority of Ag-specific T_{CM} and T_{EM} cells derived from one original T cell clone that, upon secondary encounter with Ag, a fraction of T_{CM} cells differentiated into T_{EM} cells. In the absence of a secondary antigenic challenge, some T_{EM} cells re-expressed CD62L (Schluns et al., 2005). Bachmann et al (2005) proposed a model in which naive $CD8^+$ T cells differentiate upon first Ag encounter into a transient intermediate stage ($CD62L^+CD127^–$) and arrest at this stage. If Ag stimulation is not sufficient, an abortive T cell process is initiated. But, if Ag exposure is sufficient, this intermediate stage cell differentiates further to a $CD62L^–CD127^+$ cell (T_{EM} phenotype) with effector function and gain the potential to significantly clonal expansion. Further Ag contact induces CD127 down-regulation (T_E phenotype), and a majority of these cells will not develop into long-lived memory cells, but will eventually die due to activation-induced cell death or to lack of response to homeostatic signals such as IL-7. In Ag absence, at least some T_E cells can re-express CD127, adopting a T_{EM} phenotype, and later on, some cells can re-express CD62L, becoming T_{CM} cells. Such dynamic lineage relationships allow the development of $CD8^+$ T_{EM} cells with different anatomical localization and different recall capabilities. As it is the effector cells that are active and have cytotoxic properties, MgA treatment does increase the immune

activation of the liver resident lymphocytes, probably due to its leishmanicidal effect, providing new antigens for T cell presentation and macrophage activation. This is particular evident in the increase of $CD8^+T_E$cells, resulting from an activation of the $CD8^+T_{CM}$and in the augment of $CD4^+$ T_{EM} cell that represent a potential for posterior differentiation into $CD4^+T_E$cells and in itself constitute a cellular population already with effector potential for secret inflammatory th1 cytokines. This situation is not observed in the infected animals, leading to the hypothesis that treatment increases leishmanicidal immune activity.

These results may reflect the rapid interchange of some memory populations into others and may also reflect the presence of tissue-resident memory cells (T_{RM}). These not so well described cell population is resident in several tissues and does not re-circulate in the blood. It has a similar phenotype with effector cells with low expression of CD62L and CD127. It is also not clear yet in each degree are memory cells resident in the tissue or transiting through it. These non-recirculating memory T cells are found in the brain, in the salivary glands, in the vagina, and in the stomach, lungs, kidney, pancreas, and heart. T_{RM} is maintained within non-lymphoid tissues in the absence of significant external replenishment and with minimal turnover. These memory T cells appear to be terminally differentiated because they do not expand significantly and survive poorly upon introduction into the circulation. Nevertheless, T_{RM} provides site-specific protection against infection, suggesting that when present, representing the first line of defense. T_{RM} expresses different integrins, such as CD103 and CD69 that are involved in the interaction with epithelial cells by binding the ligand E-cadherin, being responsible for the retention of these cells in the tissues (Park and Kupper, 2015). However, T_{RM} cells were only described in the liver following malaria infection (Tse et al., 2013). This cell subset is normally associated with mucosal and frontline tissues, but being the liver the major organ in close contact with the digestive tract Ag it is possible that this resident population is yet to be signalized. However, in the present work, with the used cellular markers, it is not possible to isolate these cells, but they might be present and included in the effector cell phenotype. In future study it would be interesting to include integrins markers, such as CD103 and CD69, to signalize this cell population and understand better its role in the outcome of liver infection by *L. infantum*.

The recognition of *L. infantum* recombinant proteins was achieved in infected mice (group 2) although without particular relevance when compared to non-infected mice (group 1). This situation may be related to the immune modulation that the parasite exert over cells from the host immune system, leading to decrease in recognition of Ag and reaction to them. However, when compared with the expansion observed in cells from *L. infantum* infected and MgA treated mice (group 3), the stimulation with *Li*rSOD leads to the expansion of the $CD4^+$ T_{EM} in a notable way. Superoxide dismutase (SOD) is a detoxify enzyme that removes reactive superoxide radicals produced by activated macrophages. This enzyme, present in the cytosol and in the microsomal fractions of several *Leishmania* species, such as *L. donovani*, *L. infantum* or *L. tropica,* are essential to ensure intra-macrophage *Leishmania* survival (Ghosh et al., 2003). The treatment of mice with a leishmanicidal drug leads to the killing of intracellular amastigotes and to the release of antigens that were previously not accessible due to intracellular localization, may facilitate the recognition of *Li*rSOD by $CD4^+$ T_{EM} cells. Curiously, T_{CM} cells were not particularly stimulated by any of the recombinant proteins, but the $CD8^+$ T_E cells presented higher levels of proliferation when stimulated with *Li*rCyP1 or *Li*rSOD. This might be related to a rapid activation of the $CD8^+$ T_{CM} cells and a differentiation into effector memory and effector cells, illustrating again the flexibility of the memory cell compartment. *Li*rCyp1 is a recombinant *L. infantum* cyclophilin. Cyclophilins are a class of proteins found in several intracellular compartments and also extracellular that possesses peptidylprolylcis–trans isomerase activity and can perform different functions, ranging from cell division, receptor maturation and protein folding (Roy et al., 2014). However, even with at extracellular localization *Li*rCyP1 is not as immunogenic as *Li*rSOD. The ability of memory cells from treated mice recognize recombinant *L. infantum* proteins, with different cellular localizations in the amastigote and probable different abundance, indicates that the leishmanicidal activity of the drug is crucial for antigen presentation process, since memory cells from infected (not treated) mice recognize and react to these proteins in a lesser extent. *Li*rSOD was the more efficient recombinant protein to recall and activate memory cells, making of this protein a potential candidate to be incorporated into a vaccine against VL.

The exposition of parasites to a leishmanicidal drug, MgA (group 3) and a posterior re-exposition to the same drug, in a newer infection followed by a treatment in the same conditions (group 4) decreased the drug sensitivity and induced the amplification of MRPA, GSH1 and PTR1genes that are known to be associated to parasite resistance. ATP-binding cassette (ABC) transporters have been associated with drug resistance in various diseases. In *Leishmania*, the first ABC protein identified was MRPA (Ouellette et al., 1990) which is a member of the ABCC subfamily able to confer resistance to antimonials by sequestering metal-thiol conjugates into an intracellular vesicle (Légaré et al., 2001). The treatment of choice for leishmaniasis are the pentavalent antimony [Sb(V)] drugs such as meglumine antimoniate (Glucantime®) and sodium stibogluconate (Pentostam®). Despite the clinical use of these antileishmanial agents for several years, their mechanism of action and the basis for their selective toxicity remain poorly known. The most accepted hypothesis is that Sb(V) acts as a pro-drug, then converted into the more toxic trivalent antimony [Sb(III)] by cellular enzymes (Goodwin and Page, 1943). This hypothesis was further supported by the observations that Sb(III) is more toxic than Sb(V) against both parasite stages of different *Leishmania* species (Roberts et al., 1995; Sereno and Lemesre, 1997) and that mutants of *L. infantum* amastigotes selected for resistance to Sb(III) were cross-resistant to Sb(V) (Sereno et al., 1998). Frézard et al. (2001) showed that reduced glutathione (GSH) promotes the reduction of Sb(V) to Sb(III) greatly favored by an acidic pH environment and an elevated temperature. Thus, conversion should not occur in the host cell cytosol due to its neutral pH, but in the macrophage acidic organelles, such as lysosomes and endosomes, and also in the parasitophorous vacuole, whose pH lies between 4.7 and 5.2, and in which *Leishmania* resides. The resistance to pentavalent antimonials have been related to antimony conjugation to the parasite-specific glutathione-spermidine conjugate trypanothione [$T(SH)_2$] (Fairlamb and Cerami, 1992). Trypanothione is thought to bind to metals in susceptible isolates and is increased in Sb(III)-resistant parasites (Haimeur et al., 2000). Metal-trypanothione conjugates are either sequestered into an intracellular organelle by the ABC transporter MRPA or extruded outside the cell by an efflux pump (Légaré et al., 2001). Therefore, in the present study it was found increased copy numbers of MRPA gene as part of the response exhibited by *L. infantum* to the stress imposed by the treatment with MgA.

However, other MgA resistance related genes were found with increased copy number in isolated parasites that were under under drug pressure, leading to the hypothesis that MgA perform more than one effect on parasite cell. MgA mechanism of action, although not yet fully understood suggested that antimonials inhibit the biosynthesis of macromolecules in the amastigote, possibly by perturbing the energy metabolism caused by the inhibition of glycolysis and fatty acid beta-oxidation. Sodium stibogluconate, another antimonial formulation, specifically inhibits type I DNA topoisomerase, thus reducing unwinding and cleavage. A similar mechanism is also described for MgA (Lucumi et al., 1998). To counterpart other effects on amastigote it is possible that *L. infantum* increases the expression of other resistance related genes as GSH1 and PTR1. Gamma-glutamylcysteine synthetase (γ-GCS), encoded by the GSH1 gene, is the rate-limiting enzyme in the biosynthesis of glutathione and of trypanothione in *Leishmania*. Mukherjee et al. (2009) assessed the importance of GSH1 in *L. infantum* by generating GSH1 null mutant. Removal of even a single wild-type allelic copy of *GSH1* invariably led to the generation of an extra copy of *GSH1*, maintaining intact wild-type alleles and revealing the crucial importance of this gene for parasite survival. Hydrogen peroxide detoxification in trypanosomatids relies on a cascade of reactions orchestrated by trypanothione [$T(SH)_2$], a bis-glutathione-spermidine conjugate, that consists in a series of reduction reactions culminating on glutathione peroxidase type enzymes and detoxification of reactive hydrogen peroxide. The T(SH)2 system has been implicated in the resistance against antimonial-containing drugs through various mechanisms (Mandal et al., 2007; Mittal et al., 2007;Wyllie et al., 2008). Amplification of *GSH1*or overexpression was observed in *Leishmania* in which resistance to antimonials was induced *in vitro* (Haimeur et al., 2000) or in resistant field isolates (Mukherjee et al., 2007). PTR1 is responsible for pteridines rescue and for the resistance to the antifolate drug methotrexate (Nare et al., 1997). Therefore, the amplification of *PTR1* suggests that less sensitivity to MgA can be also associated with the folate metabolic pathway. *Leishmania* and other trypanosomatid protozoa require reduced pteridines (pterins and folates) for growth, suggesting that inhibition of these pathways could be targeted for effective chemotherapy. Requisition of an exogenous source of folate and of unconjugate pteridines is essential for *Leishmania* survival, replication and, also important in the infection process of the vertebrate host. The

enzyme PTR1 is responsible for the reduction of biopterin into H_4-biopterin, which was shown to play a key role in the biosynthesis of nitric oxide (NO) and to be connected with the *Leishmania* sensitivity to oxidants (Nare et al., 2009). PTR1 amplification may decrease the efficacy of MgA by reducing the reactive oxygen and nitrogen intermediates, making the inhibition of trypanothione reductase less relevant and assuring that folate metabolism is not affected, allowing the parasite survival in the presence of the drug. Curiously, it was observed that MDR1, a gene associated with resistance had decreased the copy number in parasites that were under drug pressure. MDR1-derived permeases belong to the ABC-B sub-family and contribute to the parasite's survival by extruding the drug from its place of action (Katakura et al., 2005), most likely by both decreasing the initial accumulation (Callahan et al., 1994) and increasing the drug efflux. In *Leishmania,* MRP confers multi-drug-resistance phenotype, although this cannot be reversed by conventional MDR modulators. The *Leishmania* MDR1 confers resistance to drugs such as daunorubicin and vinblastine, but there is no evidence of a relationship between the MDR1 protein and antimony resistance (Klokouzas et al., 2003). In the present study no evidence of increased *MDR1* copy number was found, indicating that probably this gene is not related to MgA resistance. Together, these results indicate that *L. infantum* under MgA pressure increases MRPA gene copies to better release the drug from the amastigote cell, increases GSH1 and PTR1 gene copies to improve resistance to the oxidative stress of the host cell. However, amplification of these genes did not reflect a complete resistant phenotype, since parasites under different levels of drug pressure evidence similar values of viable parasitic load on the liver. This situation highlights the parasite easy acquisition of a less sensitive phenotype that in a continued process might lead to the development of fully MgA resistant parasites.

The levels of $CD8^+$ T_{EM} and T_{CM} were found higher in group 4 compared with group 3 and T_E, which are the cells that effective kill the parasite were diminished in group 4. Studies of murine VL infections, in *L. donovani* infected BALB/c mice, have established that an intact T cell population, in particular the Th1 cell subset is required for antimoniates to produce a curative antileishmanial effect (Murray et al., 1991). By itself, MgA has leishmanicidal activity *in vitro* and *in vivo*, however complete cure, *in vivo*, is not achieved without a host concomitant Th1 immune response. Haldar et al.,

(2011) observed that the treatment with sodium antimony gluconate, another antimonial formulation, enhances the expression of MHCI on the macrophage surface, promoting antigen presentation, but not the antigen presentation by MHCII. This might be a mechanism by which the drug can enhance antileishmanial cytotoxic T lymphocyte response. As discussed above, T_E cell proliferation observed in the treated mice (group 3) may in fact be a key activation to enhance Ag presentation, leading to macrophage activation. But in the presence of parasites that are less susceptible to MgA leishmanicidal activity, there is a less efficient immune response with less effector T cells, particularly cytotoxic T cells, easing parasite survival even in the presence of a leishmanicidal drug.

The recognition of recombinant proteins is affected by drug less sensitive parasites (group 4), reducing $CD4^+$ T_{EM} cells and increasing $CD4^+$ T_E cells. Curiously the situation is the inverse of what was observed in treating mice (group 3). In this case, the levels of $CD8^+$ T_{CM} cells were low and $CD8^+$ T_E cells were increased, leading to a crescent cytotoxic immune response. In group 4, the immune response is dominated by $CD4^+$ T cells and in the group 3 a $CD8^+$ T cell predominating response was generated. In group 4, the presence of a parasite that is less sensitive to the drug that was used in the treatment, lead to less parasite killing and less Ag exposure and, in consequence, less immune activation. In this case, parasite seem to be able to silencing the differentiation of $CD8^+$ T_{CM} cells into T_E cells, leading to a decrease in this population to levels similar to what was observed in infected mice (group 2). The effect of an infection with parasites less susceptible to a drug (but yet fully resistant) is seriously as the reduction in parasite killing may delay the activation of a $CD8^+$ T cell mediated immune response, allowing parasite survival. In consequence survival parasites may be able to control the host immune response to a silencing state similar to what was observed in not treated infected mice (group 2), favoring parasite survival and treatment failure.

Only the stimulation with *Lir*CyP1 and *Lir*SOD were able to induce some expansion of memory T cells. As discussed previously, these proteins are located inside the amastigote and the killing of the parasite by MgA addition may lead to exposure of new parasite antigens and making possible recognition. Taking together, these results indicate that *Lir*CyP1 and *Lir*SOD, may be included in future vaccine for VL. The

vaccine might be preventive or therapeutic improving Ag presentation and the consequent cellular immune response.

6.6. Conclusion

In summary, the present study demonstrates that:

- For the first time the generation of memory T cells in the resident liver lymphocytes following an *L. infantum* infection is accessed, as well after treatment;

- The infection with *L. infantum* generates memory T cells, which can be accessed with the cellular markers CD127 and CD62L;

- The treatment with a leishmanicidal drug (antimoniate meglumine) increases the levels of memory and effector T cells, indicating a more robust immune response, possibly due to the exposition to parasite Ag released as a consequence of parasite killing. Thus, the treatment with a leishmanicidal drug enhances the liver immune response;

- The re-exposition of *L. infantum* parasites to a leishmanicidal drug for short periods of time has potential to decrease parasite sensitivity and may lead to the development of resistant phenotypes;

- The recombinant proteins *Lir*Cyp1 and *Lir*SOD are strongly recognized by memory cells from infected and treated mice, indicating these proteins might be used in a prophylactic or therapeutic vaccine formulation;

- The liver has higher levels of immune memory T cells, and the role of the liver as an immunological organ is flourishing, but it still is necessary to perform more studies.

6.7. References

Amprey JL, Im JS, Turco SJ, Murray HW, Illarionov PA, Besra GS, Porcelli SA, Späth GF. 2004. A subset of liver NK T cells is activated during *Leishmania donovani* infection by CD1d-bound lipophosphoglycan. J Exp Med. 200(7):895-904.

Appay V, Dunbar PR, Callan M, Klenerman P, Gillespie GM, Papagno L, Ogg GS, King A, Lechner F, Spina CA, Little S, Havlir DV, Richman DD, Gruener N, Pape G, Waters A, Easterbrook P, Salio M, Cerundolo V, McMichael AJ, Rowland-Jones SL. 2002. Memory $CD8^+$ T cells vary in differentiation phenotype in different persistent virus infections. Nat Med. (4):379-85.

Argueta-Donohué J, Carrillo N, Valdés-Reyes L, Zentella A, Aguirre-García M, Becker I, Gutiérrez-Kobeh L. 2008. *Leishmaniamexicana*: participation of NF-kappaB in the differential production of IL-12 in dendritic cells andmonocytes induced by lipophosphoglycan (LPG). ExpParasitol. 120(1):1–9.

Bachmann MF, Wolint P, Schwarz K, Jäger P, Oxenius A. 2005. Functional properties and lineage relationship of $CD8^+$ T cell subsets identified by expression of IL-7 receptor alpha and CD62L. J Immunol. 175(7):4686-96.

Böttcher JP, Schanz O, Wohlleber D, Abdullah Z, Debey-Pascher S, Staratschek-Jox A, Höchst B, Hegenbarth S, Grell J, Limmer A, Atreya I, Neurath MF, Busch DH, Schmitt E, van Endert P, Kolanus W, Kurts C, Schultze JL, Diehl L, Knolle PA. 2013. Liver-primed memory T cells generated under non-inflammatory conditions provide anti-infectious immunity. Cell Rep. 3(3):779-95. doi: 10.1016/j.celrep.2013.02.008.

Bouneaud C, Garcia Z, Kourilsky P, Pannetier C. 2005. Lineage relationships, homeostasis, and recall capacities of central- and effector-memory CD8 T cells *in vivo*. J. Exp. 201:579-590.

Callahan HL, Roberts WL, Rainey PM, Beverley SM. 1994. The PGPA gene of *Leishmania major* mediates antimony (SbIII) resistance by decreasing influx and not by increasing efflux. Mol Biochem Parasitol. 68(1):145-9.

Campbell JJ, Murphy KE, Kunkel EJ, Brightling CE, Soler D, Shen Z, Boisvert J, Greenberg HB, Vierra MA, Goodman SB, Genovese MC, Wardlaw AJ, Butcher EC, Wu L. 2001. CCR7 expression and memory T cell diversity in humans. J Immunol. 166(2):877-84.

Claro, M. 2015. Análise da memória celular hepática na infeção por *Leishmania infantum*". Marter thesis. Universidade Nova de Lisboa, Instituto de Higiene e Medicina Tropical. 78pp.

el Hag IA, Hashim FA, el Toum IA, Homeida M, el Kalifa M, el Hassan AM.1994. Liver morphology and function in visceral leishmaniasis (Kala-azar). J Clin Pathol. 47(6):547-51.

Fairlamb AH, Cerami A. 1992. Metabolism and functions of trypanothione in the Kinetoplastida. Annu Rev Microbiol. 46:695-729.

Faure M, Marussig P, Goossens F, Miltgen, Mazier D. 1994. Characterization of liver lymphomyeloid cells in mice infected with *Plasmodium yoelii*sporozoites. Immunology. 82(4): 645–650.

Förster R, Schubel A, Breitfeld D, Kremmer E, Renner-Müller I, Wolf E, Lipp M. 1999. CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. Cell. 99(1):23-33.

Frézard F, Demicheli C, Ferreira C, Costa M. 2001. Glutathione-Induced Conversion of Pentavalent Antimony to Trivalent Antimony in Meglumine Antimoniate. Antimicrob Agents Chemother. 45(3):913–916. doi: 10.1128/AAC.45.3.913-916.2001.

Gattinoni L, Lugli E, Ji Y, Pos Z, Paulos CM, Quigley MF, Almeida JR, Gostick E, Yu Z, Carpenito C, Wang E, Douek DC, Price DA, June CH, Marincola FM, Roederer M, Restifo NP. 2011. A human memory T cell subset with stem cell-like properties. Nat Med. 17(10):1290-7. doi: 10.1038/nm.2446.

Gebhardt T, Wakim LM, Eidsmo L, Reading PC, Heath WR, Carbone FR. 2009. Memory T cells in nonlymphoidtissue that provide enhanced local immunity during infection with herpes simplex virus. Nat Immunol. 10(5):524-30. doi: 10.1038/ni.1718.

Ghosh S Goswami S, Adhya S. 2003. Role of superoxide dismutase in survival of *Leishmania* within the macrophage. Biochem J. 369(Pt 3): 447–452. doi: 10.1042/BJ20021684

Gomes-Pereira S, Rodrigues O, Rolão N, Almeida P, Santos-Gomes G. 2004. Hepatic Cellular Immune Responses in Mice with "cure" and "non-cure" phenotype to *Leishmania infantum* Infection: Importance of $CD8^+$ T cells and TGF−β production. FEMS Immunol Med Microbiol. 41(1):59-68.

Goodwin LG, Page JE. 1943. A study of the excretion of organic antimonials using a polarographic procedure. Biochem J. 37(2):198-209.

Haimeur A, Brochu C, Genest P, Papadopoulou B, Ouellette M. 2000. Amplification of the ABC transporter gene PGPA and increased trypanothione levels in potassium antimonyl tartrate (SbIII) resistant *Leishmania tarentolae*. Mol Biochem Parasitol. 108(1):131-5.

Haldar AK, Sen P, Roy S.2011. Use of antimony in the treatment of leishmaniasis: current status and future directions. Mol Biol Int. 2011:571242. doi: 10.4061/2011/571242.

Hernandez-Pando R, Bornstein QL, Aguilar Leon D, Orozco EH, Madrigal VK, Martinez Cordero E. 2000. Inflammatory cytokine production by immunological and foreign body multinucleated giant cells. Immunology. 100(3):352-8.

Jaigirdar S, MacLeod M.2015. Development and Function of Protective and Pathologic Memory CD4 T Cells. Front Immunol. 6:456. doi: 10.3389/fimmu.2015.00456

Jiang X, Chen Y, Peng H, TianZ. 2013. Memory NK cells: why do they reside in the liver? Cell MolImmunol. 10(3):196–201. doi: 10.1038/cmi.2013.8

Katakura K, Fujise H, Takeda K, Kaneko O, Torii M, Suzuki M, Chang KP, Hashiguchi Y. 2004. Overexpression of LaMDR2, a novel multidrug resistance ATP-binding cassette transporter, causes 5-fluorouracil resistance in *Leishmania amazonensis*. FEBS Lett. 561(1-3):207-12.

Kaye PM, Svensson M, Ato M, Maroof A, Polley R, Stager S, Zubairi S, Engwerda CR. 2004. The immunopathology of experimental visceral leishmaniasis.I mmunol Rev. 201:239-53.

Kharazmi A, Kemp K, Ismail A, Gasim S, Gaafar A, Kurtzhals JA, El Hassan AM, Theander TG, Kemp M.1999. T-cell response in human leishmaniasis. Immunol Lett. 65(1-2):105-8.

Kim CH, Kunkel EJ, Boisvert J, Johnston B, Campbell JJ, Genovese MC, Greenberg HB, Butcher EC. 2001. Bonzo/CXCR6 expression defines type 1-polarized T-cell subsets with extralymphoid tissue homing potential. J Clin Invest. 107(5):595-601.

Klokouzas A, Shahi S, Hladky SB, Barrand MA, Van Veen HW. 2003. ABC transporters and drug resistance in parasitic protozoa. Int J Antimicrob Agents. 22:301–317.

Lanzavecchia A, Sallusto F. 2000. Dynamics of Tlymphocyteresponses: intermediates, effectors, and memory cells. Science. 290:92–97

Ledgerwood LG, Lal G, Zhang N, Garin A, Esses SJ, Ginhoux F, Merad M, Peche H, Lira SA, Ding Y, Yang Y, He X, Schuchman EH, Allende ML, Ochando JC, Bromberg JS. 2008. The sphingosine 1-phosphate receptor 1 causes tissue retention by inhibiting the entry of peripheral tissue T lymphocytes into afferent lymphatics. Nat Immunol. 9(1):42-53.

Légaré D, Richard D, Mukhopadhyay R, Stierhof YD, Rosen BP, Haimeur A, Papadopoulou B, Ouellette M. 2001. The *Leishmania* ABC protein PGPA is an intracellular metal-thiol transporter ATPase. J. Biol. Chem. 276:26301–26307.

Liu D, Kebaier C, Pakpour N, Capul A, Beverley S, Scott P, Uzonna J. 2009. *Leishmania major* phosphoglycans influence the host early immune response by modulating dendritic cell functions. Infect Immun. 77(8):3272 – 3283.

Loría-Cervera EN, Andrade-Narváez FJ. 2014. Animal models for the study of leishmaniasis immunology. Rev Inst Med Trop Sao Paulo. 56(1):1-11. doi: 10.1590/S0036-46652014000100001.

Lucumi A. Robledo S, Gama V, Saravia NG.1998. Sensitivity of *Leishmania viannia panamensis* to pentavalent antimony is correlated with the formation of cleavable DNA-protein complexes. Antimicrob Agents Chemother. 42(8):1990-5.

Lugli E, Dominguez MH, Gattinoni L, Chattopadhyay PK, Bolton DL, Song K, Klatt NR, Brenchley JM, Vaccari M, Gostick E, Price DA, Waldmann TA, Restifo NP, Franchini G, Roederer M. 2013. Superior T memory stem cell persistence supports long-lived T cell memory. J Clin Invest. 123(2):594-9. doi: 10.1172/JCI66327.

Mandal G, Wyllie S, Singh N, Sundar S, Fairlamb AH, Chatterjee M. 2007. Increased levels of thiols protect antimony unresponsive *Leishmania donovani* field isolates against reactive oxygen species generated by trivalent antimony. Parasitology. 134(Pt 12):1679-87.

Marr AK, MacIsaac JL, Jiang R, Airo AM, Kobor MS, McMaster WR. 2014. *Leishmania donovani* infection causes distinct epigenetic DNA methylation changes in host macrophages. PLoSPathog. 10(10):e1004419. doi:10.1371/journal.ppat.1004419.

Masopust D, Vezys V, Marzo AL, Lefrancois L. 2001. Preferential localization of effector memory cells in nonlymphoid tissue. Science. 291:2413–17

McElrath M, Murray H, Cohn A.1988. The dynamics of granuloma formation inexperimental visceral leishmaniasis. J ExpMed. 167(6):1927-37.

McKerrow J, Caffrey C, Kelly B, Loke P, Sajid M. 2006. Proteases in parasitic diseases. Ann Rev Pathol. 1:497–536.

Mittal MK, Rai S, Ashutosh, Ravinder, Gupta S, Sundar S, Goyal N. 2007. Characterization of natural antimony resistance in *Leishmania donovani* isolates. Am J Trop Med Hyg. 76(4):681-8.

Morty RE, Morehead J. 2002. Cloning and characterization of a leucylamino peptidase from three pathogenic *Leishmania* species. J Biol Chem. 277(29):26057-65.

Mueller SN, Gebhardt T, Carbone FR, Heath WR. 2012. Memory T cell subsets, migration patterns, and tissue residence. Annu Rev Immunol. 31:137-61. doi: 10.1146/annurev-immunol-032712-095954.

Mukherjee A, Padmanabhan PK, Singh S, Roy G, Girard I, Chatterjee M, Ouellette M, Madhubala R. 2007. Role of ABC transporter MRPA, gamma-glutamyl cysteine synthetase and ornithine decarboxylase in natural antimony-resistant isolates of *Leishmania donovani*. J Antimicrob Chemother. 59(2):204-11.

Mukherjee A, Roy G, Guimond C, Ouellette M. 2009. The γ-glutamyl cysteine synthetase gene of *Leishmania*is essential and involved in response to oxidants. Molecular Microbiology. 74(4):914–927. doi: <10.1111/j.1365-2958.2009.06907.

Murray HW, Granger AM, Mohanty SK., 1991. Response to chemotherapy in experimental visceral leishmaniasis: T cell-dependent but interferon-γ- and interleukin-2-independent.J Infect Dis. 163(3):622-4.

Murray H. 2001. Tissue granuloma structure-function in experimental visceral leishmaniasis. 2001. Int J ExpPathol. 82(5):249–267. doi: 10.1046/j.1365-2613.2001.00199.x

Murray HW, Nathan CF.1999. Macrophage microbicidal mechanisms *in vivo*: reactive nitrogen *vs* oxygen intermediates in the killing of intracellular visceral *Leishmania donovani*. J Exp Med. 189(4):741-6.

Murray HW, Squires KE, Miralles CD, Stoeckle MY, Granger AM, Granelli-Piperno A, Bogdan C. 1992. Acquired resistance and granuloma formation in experimental visceral leishmaniasis. Differential T cell and lymphokine roles in initial versus established immunity. J Immunol. 148(6):1858-63.

Murray HW, Stern JJ, Welte K, Rubin BY, Carriero SM, Nathan CF. 1987. Experimental visceral leishmaniasis: production of interleukin 2 and interferon-gamma, tissue immune reaction, and response to treatment with interleukin 2 and interferon-gamma. J Immunol. 138(7):2290-7.

Mutiso J, Macharia J, Kiio M, Ichagichu J, Rikoi H, Gicherub M. 2013. Development of *Leishmania* vaccines: predicting the future from past and present experience. J Biomed Res. 27(2): 85–102. doi: 10.7555/JBR.27.20120064

Nagata K, Tanaka K, Ogawa K, Kemmotsu K, Imai T, Yoshie O, Abe H, Tada K, Nakamura M, Sugamura K, Takano S. 1999. Selective expression of a novel surface molecule by human Th2 cells *in vivo*. J Immunol. 162(3):1278-86.

Nare B, Garraway L, VickersT, Beverley S. 2009. PTR1-dependent synthesis of tetrahydrobiopterin contributes to oxidant susceptibility in the trypanosomatid protozoan parasite *Leishmania major*. Curr Genet. 55(3): 287–299. doi: 10.1007/s00294-009-0244-z

Nare B, Luba J, Hardy LW, Beverley S. 1997. New approaches to *Leishmania* chemotherapy: pteridinereductase 1 (PTR1) as a target and modulator of antifolate sensitivity. Parasitology. 114 Suppl:S101-10.

Ouellette M, Fase-Fowler F, Borst P. 1990. The amplified H circle of methotrexate-resistant *Leishmania tarentolae* contains a novel P-glycoprotein gene. EMBO J. 9:1027–1033.

Park CO, Kupper TS. 2015. The emerging role of resident memory T cells in protective immunity and inflammatory disease. Nat Med. 21(7):688-97. doi: 10.1038/nm.3883.

Parker GA, Picut CA. 2005. Liver immunobiology. Toxicol Pathol. 33(1):52-62.

Reinhardt RL, Khoruts A, Merica R, Zell T, Jenkins MK. 2001. Visualizing the generation of memory CD4 T cells in the whole body. Nature. 410:101–5

Roberts WL, Berman JD, Rainey PM. 1995. *In vitro* antileishmanial properties of tri- and pentavalent antimonial preparations. Antimicrob Agents Chemother. 39(6):1234-9.

Roy S, Basu S, Datta AK, Bhattacharyya D, Banerjee R, Dasgupta D. 2014. Equilibrium unfolding of cyclophilin from *Leishmania donovani*: characterization of intermediate states. Int J Biol Macromol. 69:353-60. doi: 10.1016/j.ijbiomac.2014.05.063

Rufer N, Zippelius A, Batard P, Pittet MJ, Kurth I, Corthesy P, Cerottini JC, Leyvraz S, Roosnek E, Nabholz M, Romero P. 2003. *Ex vivo* characterization of human $CD8^+$ T subsets with distinct replicative history and partial effector functions.Blood. 102(5):1779-87.

Sallusto F, Lenig D, Forster R, Lipp M, Lanzavecchia A. 1999. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. Nature. 401:708–12

Sallusto F, Geginat J, Lanzavecchia A. 2004. Central memory and effector memory T cell subsets: function, generation, and maintenance. Annu Rev Immunol. 22:745-63.

Sanchez MA, Diaz NL, Zerpa O, Negron E, Convit J, Tapia FJ. 2004. Organ-specific immunity in canine visceral leishmaniasis: analysis of symptomatic and asymptomatic dogs naturally infected with *Leishmania chagasi*. Am J Trop Med Hyg. 70(6):618-24.

Sant'Ana J, Lima W, Oliveira M, Simões L, Michalick M. Melo M, Tafuri W, Tafuri W. 2007. Hepatic granulomas in canine visceral leishmaniasis and clinical status. Arq. Bras. Med. Vet. Zootec. 59(5):1137-1144.

Santos-Gomes G, Rodrigues A, Teixeira F, Alexandre-Pires G, Carvalho S, Santos-Mateus D, Martins C, Vale-Gato I, Marques C, Tomás A. 2014. Immunization with the *Leishmania infantum* Recombinant Cyclophilin Protein 1 Confers Partial Protection to Subsequent Parasite Infection and Generates Specific Memory T Cells. Vaccine. 32(11):1247-53.

Santos-Mateus, D. 2014. Genotypic analysis of the *Leishmania infantum* resistance to conventional and new chemically synthesized compounds. Master thesis. Universidade de Lisboa. 51pp.

Saraiva E, de Figueiredo Barbosa A, Santos F, Borja-Cabrera G, Nico D, Souza L, de Oliveira Mendes-Aguiar C, de Souza E, Fampa P, Parra L, Menz I, Dias J, de Oliveira S, Palatnik-de-Sousa C. 2006. The FML-vaccine (Leishmune) against canine visceral leishmaniasis: a transmission blocking vaccine. Vaccine. 24(13):2423-31.

Schluns KS, Kieper WC, Jameson SC, Lefrancois L. 2000. Interleukin-7 mediates the homeostasis of naïve and memory CD8 T cells *in vivo*. Nat Immunol. 1:426–432.

Sereno D, Cavaleyra M, Zemzoumi K, Maquaire S, Ouaissi A, Lemesre JL. 1998. Axenically grown amastigotes of *Leishmania infantum* used as an *in vitro* model

to investigate the pentavalent antimony mode of action. Antimicrob Agents Chemother. 42(12):3097-102.

Sereno D, Lemesre JL. 1997. Axenically cultured amastigote forms as an *in vitro* model for investigation of antileishmanial agents. Antimicrob Agents Chemother. 41(5):972-6

Sheffield E.A. 1990. The granulomatous inflammatory response. J. Pathol. 160:1-2.

Silva-Almeida M, Pereira B, Ribeiro-Guimarães M, Alves C. 2012. Proteinases as virulence factors in *Leishmania* spp. infection in mammals. Parasit Vectors.5:160. doi: 10.1186/1756-3305-5-160.

Smelt SC, Cotterell SE, Engwerda CR, Kaye PM. 2000. B cell-deficient mice are highly resistant to *Leishmania donovani* infection, but develop neutrophil-mediated tissue pathology. J Immunol. 164(7):3681-8.

Stäger S, Alexander J, Carter KC, Brombacher F, Kaye PM.2003. Both interleukin-4 (IL-4) and IL-4 receptor alpha signaling contribute to the development of hepatic granulomas with optimal antileishmanial activity. Infect Immun. 71(8):4804-7.

Stanley A, Engwerda C. 2007. Balancing immunity and pathology in visceral leishmaniasis. Immunology and Cell Biology. 85:138–147. doi: <10.1038/sj.icb.7100011

Steinert EM, Schenkel JM, Fraser KA, Beura LK, Manlove LS, Igyártó BZ, Southern PJ, Masopust D. 2015. Quantifying Memory CD8 T Cells Reveals Regionalization of Immunosurveillance. Cell. 161(4):737-49. doi: 10.1016/j.cell.2015.03.031.

Sun J, Beilke J, Lanier L. 2009. Adaptive Immune Features of Natural Killer Cells. Nature. 457(7229): 557–561. doi: 10.1038/nature07665

Takeshita M, Suzuki K, Kassai Y, Takiguchi M, Nakayama Y, Otomo Y, Morita R, Miyazaki T, Yoshimura A, Takeuchi T. 2015. Polarization diversity of human $CD4^+$ stem cell memory T cells. Clin Immunol. 159(1):107-17. doi: 10.1016/j.clim.2015.04.010.

Tse S, Cockburn I, Zhang H, Scott A, Zavala F. 2013. Unique transcriptional profile of liver-resident memory $CD8^+$ T cells induced by immunization with malaria sporozoites. Genes Immun. 14(5):302–309. doi: 10.1038/gene.2013.20

Wyllie S, Vickers TJ, Fairlamb AH. 2008. Roles of trypanothione S-transferase and tryparedoxin peroxidase in resistance to antimonials. Antimicrob Agents Chemother. 52(4):1359-65. doi: 10.1128/AAC.01563-07.

Zhang N, Bevan MJ. 2013. Transforming growth factor-β signaling controls the formation and maintenance of gut-resident memory T cells by regulating migration and retention. Immunity. 39(4):687-96. doi: 10.1016/j.immuni.2013.08.019.

Zhang Q, Lakkis F. 2015. Memory T Cell Migration. Front Immunol. 6:504. doi: 10.3389/fimmu.2015.00504

7 Final remarks

This study aimed to elucidate on the natural mechanisms elicited by the liver of the dog, the main reservoir for *L. infantum*, to control the infection. In chapter 2 and 3 of the present dissertation, the role of hepatocytes and KCs isolated from canine livers was assessed in the framework of a *L. infantum* infection. To the best of our knowledge, this report constitutes the first *in vitro* description of immune response exhibited against *L. infantum* parasites by dog hepatocytes as well as the first time that innate immune receptors NOD1, NOD2, TLR2, TLR4 and TLR9 of dog KCs were investigated for their role in *L. infantum* infection.

Hepatocytes constitute rich metabolic cells and their role as immune active cells represent a recent discovery. In the context of *L. infantum* infection, parasites exhibited a strong tropism to cells and a close interaction between hepatocytes and parasites were observed. In some cases, a possible internalization by hepatocytes was considered, however, in a not conclusive way. In culture, the close interaction between hepatocyte's membrane and parasite increased the expression of innate immune receptors NOD1 and NOD2 as well as TLR2, suggesting that these receptors were activated by the parasite antigens, and in the case of NODs, by a close interaction with the hepatocyte membrane. A possible role for TLR9 was also evidenced but not in a conclusive form. The parasite presence induced a mix immune response from hepatocytes. These cells are able to produce several different cytokines and also acute phase proteins that impact on the host immune defense. In contact with *L. infantum*, hepatocytes produced pro-inflammatory TNF-α, the pleiotropic IL-6 and the anti-inflammatory IL-10. Hepatocytes are known for inducing immune tolerance and, naturally do not activate a strong anti-inflammatory Th1 response in order to avoid major damage to the tissue. However, the production of inflammatory cytokines also influenced metabolic pathways. Decrease of CYPs450 and phase II enzyme activities might lead to increased toxicity by the accumulation of non-metabolized drugs or xenobiotics.

The search for new leishmanicidal compounds constitutes an important quest, as many of the used drugs present several side effects. Three new leishmanicidal drugs, ursolic acid, chalcone-8 and quercetin, were tested in hepatocytes exposed to

L. infantum amastigotes in comparison with the classic meglumine antimoniate, all revealing good leishmanicidal activity and low cell toxicity. The addition of drugs induced the CYPs450 metabolism, indicating that these compounds may be metabolized by the assessed CYPs. The knowledge of the metabolization path and the enzyme used for each drug is very useful to predict efficacy and avoid drug-to-drug interaction and should be evaluated since the initial process of drug development.

In the future, it would be of interest to clarify whether *L. infantum* parasites can enter the hepatocyte cell *in vitro* and *in vivo*. The clarification of this point might impact in the role of hepatocytes in canine leishmaniasis from classically considered side-performers to target cells and possible silent reservoir. Also the described evidence that NODs and TLR2 are activated by the parasite could be explored *in vitro* and *in vivo* as therapeutic or vaccine adjuvants to improve response to treatment. Likewise, would be of great interest to progress the evaluation of the new leishmanicidal compounds with studies *in vitro* using canine infection model and *in vivo,* in dogs naturally infected.

KCs, the liver resident macrophages, constitute the main target cells of the liver and form the core of the granulomas in infected animals. Although KCs sense and react to the parasite presence these cells are susceptible to both parasite forms, but amastigotes are more efficient at infecting KCs. *L. infantum* promastigotes and amastigotes exerted immune regulation on infected KCs, avoiding cellular activation, downregulation of NO production. Instead *L. infantum* induces urea production and an anergic response from infected KCs. The innate immune receptors TLR2 and NOD1 seem to be activated on infected KCs, but little effect on Th1 cytokine production was observed. *L. infantum* is able to modulate KCs leishmanicidal mechanisms promoting parasite survival. Curiously, co-culture of infected KCs with hepatocytes was able to create an inflammatory response, activating the innate immune receptors NOD1 and TLR2 and pro-inflammatory TNF-α cytokine expression. Revealing that KCs alone are subverted by the parasite silencing effect, but when in contact with hepatocyte, the cellular crosstalk seems to have an interesting role in orchestrating a synergistic immune response against *L. infantum* parasites. The addition of a leishmanicidal drug to infected KCs obtained a similar effect, with activation of innate immune receptors TLR4 and TLR2 and generation of TNF-α. Again, a role for TLR2 seems evident in canine leishmaniasis, which may be exploited for therapeutic ends. Interestingly, there

were found important differences in the immune response against *L. infantum* displayed by blood derived macrophages (MØs) when compared with liver resident macrophages (KCs). Liver macrophages were anergic and more tolerant to the parasite in comparison with the early active response exhibited by MØs. Even so, *L. infantum* seem to have the ability to exert a negative regulation on MØ activity. The localization of MØs and the respective environment seem to induce a regulation in the potential level of activation.

In chapter 3, the role of the liver as an immunological memory organ was investigated in the framework of an *L. infantum* infection. For this study the model of murine visceral leishmaniasis was used due to practical reasons. To our best knowledge, this chapter also constitutes the first description of local memory generation by liver resident T cells following an *L. infantum* infection and after treatment. Animals treated with antimoniate meglumine increases the levels of memory and of effector T cells, enhancing the liver immune response, highlighting the impact of treatment application on recovery from this parasitic infection. Also was found evidence that re-exposition of *L. infantum* parasites to antimoniate meglumine for short periods of time has potential to decrease parasite sensitivity and may lead to the development of resistant phenotypes, emphasizing the importance of treatment compliance. *Li*rCyp1 and *Li*rSOD, *L. infantum* recombinant proteins were found to be strongly recognized by memory cells from infected and treated mice, indicating that these proteins might be used in a prophylactic or therapeutic vaccine formulation.

In the future, it would be of interest to perform a similar study in dogs naturally infected and after treatment to understand whether parasite specific immune memory can be established and if confers protection against re-infection. Testing the potential for use of the selected recombinant proteins as therapeutic vaccines in naturally infected dogs might also be of interest. Likewise, testing the selected proteins with adjuvants for TLR2 or NOD1 and NOD2 could also lead to interesting results on exploration of the innate immune response.

The liver is, in fact, a very complex organ with unique metabolic functions and newly discovered immune implications. During *L. infantum* infection and visceral disease, the liver executes unique functions on signaling the infection, produce acute phase proteins and inflammatory cytokines initiating a general inflammatory response,

isolate the parasite in a granuloma assembly and ensuring balancing of the immune response to avoid damage to the host. The present work attributes an active role to hepatocytes in an active *L. infantum* infection.