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Neutrophil-*Leishmania* interaction during the initial phase of *Leishmania infantum* infection in dogs

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Resumo

Interação Neutrófilo-*Leishmania* na fase inicial da infeção por *Leishmania infantum* em cães

Maria de Aires Machado Pereira

PALAVRAS-CHAVE: Leishmaniose canina; neutrófilos, interação neutrófilo-macrófago

A leishmaniose canina (LCan) é uma doença zoonótica causada pelo protozoário *Leishmania infantum* e transmitida por insetos do género *Phlebotomus* e *Lutzomyia*, respetivamente no Velho e Novo Mundos. Trata-se de uma doença grave, sistémica e potencialmente fatal. Embora estejam disponíveis algumas medidas profiláticas e terapêuticas, a sua eficácia é questionável. Apesar dos neutrófilos (PMN) serem a primeira linha de defesa do organismo, fagocitando rapidamente o parasita após inoculação, os macrófagos (MØ) são as células hospedeiras definitivas, na medida em que suportam a sua multiplicação e disseminação. Este trabalho teve como objetivos identificar os mecanismos efetores ativados pelos PMN em resposta à exposição ao parasita e o impacto da interação PMN-MØ na fase inicial da infeção canina, determinando a sua contribuição no controlo da infeção ou, pelo contrário, no estabelecimento da doença clínica. Foram selecionados animais clinica e analiticamente saudáveis que testaram negativamente para as principais patologias infeciosas e parasitárias e estabeleceram-se culturas PMN-*Leishmania*. Verificou-se por citometria e observação microscópica que promastigotas de *L. infantum* foram eficazmente fagocitados por PMN na maioria dos canídeos avaliados, activando os mecanismos oxidativos (produção de superóxido) e não oxidativos (exocitose de elastase neutrofílica), mas prevenindo a libertação de armadilhas extracelulares neutrofílicas (*neutrophil extracelular traps*, NET). Para além disso, promastigotas e sobrenadantes de cultura induziram a migração de PMN, mas o contacto prévio com *Leishmania* inibiu a quimiotaxia, o que contribui para a retenção dos PMN no local da inoculação. A interação com o parasita teve um impacto negativo na viabilidade dos PMN, tendo induzido a necrose secundária. A interação PMN-parasita resultou numa diminuição da viabilidade parasitária, embora alguns promastigotas intracelulares tenham sobrevivido e mantido a capacidade de proliferar, assegurando o estabelecimento da infeção. MØ diferenciados a partir de monócitos sanguíneos sofreram alterações morfológicas importantes com o intuito de conter o parasita e também libertaram Histona H1, cujo efeito leishmanicida não foi ainda provado em promastigotas de *L. infantum*. A transferência do parasita para os MØ foi confirmada por citometria em co-culturas de MØ+PMN infetados aos quais foram removidos os parasitas extracelulares. A observação microscópica destas co-culturas mostrou que a eferocitose de PMN infetados e provavelmente a fagocitose de parasitas libertados por PMN apoptóticos são importantes na transferência do parasita para a célula hospedeira definitiva. Inesperadamente, a interação PMN-MØ ativou a produção de óxido nítrico e induziu a libertação de NET, o que pode contribuir para a contenção do parasita e para o controlo da infeção numa fase precoce. Os resultados obtidos ampliam o conhecimento da infeção por *L. infantum* no cão e podem contribuir para o desenvolvimento de novas ferramentas terapêuticas e profiláticas que conduzam à redução acentuada da LCan.

Abstract

Neutrophil-*Leishmania* interaction during the initial phase of *Leishmania infantum* infection in dogs

Maria de Aires Machado Pereira

KEYWORDS: Canine leishmaniasis; neutrophils; interaction neutrophil-macrophage

Canine leishmaniosis (CanL) is a zoonotic disease caused by the protozoan *Leishmania infantum* transmitted by insects of the genus *Phlebotomus* and *Lutzomyia* in the Old and New Worlds, respectively. It is a severe, systemic and potentially fatal disease. Despite the availability of some prophylactic and therapeutic tools, their effectiveness is questionable. Although neutrophils (PMN) are the first line of defense of the organism, rapidly phagocytizing the parasite after inoculation, macrophages (MØ) are the definitive host cells, supporting their multiplication and spread. This study aimed to identify the effector mechanisms activated by PMN in response to *L. infantum* promastigotes and the impact of PMN-MØ interaction in the initial phase of canine infection by determining its contribution to infection control or, on the contrary, the establishment of clinical disease. Clinically and analytically healthy animals who tested negative for the main infectious and parasitic diseases were selected and PMN-*Leishmania* cultures were established. It was found by flow cytometry and microscopic observation that *L. infantum* promastigotes are efficiently phagocytized by PMN in the majority of the dogs, activating oxidative (superoxide production) and non-oxidative (neutrophil elastase exocytosis) mechanisms, but preventing the release of neutrophil extracellular traps (NET). Furthermore, promastigotes and culture supernatants induced PMN migration, but the prior contact with *Leishmania* inhibited chemotaxis, which contributes to PMN retention at the inoculation site. The interaction with the parasite had a negative impact on PMN viability, promoting secondary necrosis. PMN-parasite interaction resulted in a decrease in parasite viability, although some intracellular promastigotes survived and maintained their proliferative capacity, contributing to the establishment of the infection. MØ differentiated from blood monocytes underwent major morphological changes in order to contain the parasite and also released Histone H1, but its leishmanicidal effect was not yet proven in *L. infantum* promastigotes. Parasite transfer to MØ was confirmed by flow cytometry in co-cultures of MØ-infected PMN whose extracellular parasites were removed. The microscopic observation of these co-cultures showed that infected PMN efferocytosis and probably phagocytosis of parasites released from apoptotic PMN are crucial for parasite transfer to the definitive host cell. Unexpectedly, PMN-MØ interaction activated nitric oxide production and induced NET release, which can contribute to parasite containment and to the early control of the infection. These findings broaden the knowledge of *L. infantum* infection in the dog and can shed light in the design of new therapeutic and prophylactic tools, leading to a marked reduction in CanL.

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Abbreviation List

<	Less than
×*g*	Times gravity
≈	Approximately equal to
2P-IVM	Two-photon intra-vital microscopy
3'NT/NU	3'-nucleotidase/nuclease
Ag	Antigen
AP-1	Activator protein-1
APC	Antigen presenting cells
Arp2/3	Actin-related protein-2/3
Bcl-2	B-cell lymphoma 2
BH_4	Tetrahydrobiopterin
BID	Twice a day
bp	Base pair
BPI	Bactericidal permeability-inducing protein
C1q	Complement component 3 subunit q
C3	Complement component 3
Ca_2^+	Calcium
CaM	Calmodulin
Campto	(S)-(+)-camptothecin
CanL	Canine Leishmaniosis
CARD; NLRC	Caspase activation and recruitment domain
catG	cathepsin G
CCL2; MCP-1	C-C motif ligand 2 chemokine; monocyte chemotactic protein 1
$CCR2^+$	Chemokine receptor 2 positive monocytes
CD	Cluster of differentiation
Cdc42	Cell division control protein 42 homolog
cDNA	Complementary deoxyribonucleic acid

CEDOC	Chronic Diseases Research Center
CIE	Counterimmunoelectrophoresis
CO_2	Carbon dioxide
CP	Cysteine protease
CPB	Cysteine protease B
CPDA-1	Citrate Phosphate Dextrose Adenine-1
CpG	Cytosine-phosphate-guanosine
CR	Complement receptor
CSF	Colony-stimulating factor
CSF-1R	Colony-stimulating factor-1 receptor
CXCL8; IL-8	C-X-C motif chemokine 8; Interleukine-8
Cyt D	Cytochalasin D
DAMP	Damage-associated molecular patterns
DAPI	Fluorescent nuclear dye 4’, 6-diamidino-2-phenylindole
DAT	Direct agglutination test
DC	Dendritic cells
DFMO	Difluoromethyl ornithine
DLA-DRB1	Dog leukocyte antigen-DRB1
DNA	Deoxyribonucleic acid
DTH	Delayed-type hypersensitivity
e.g	*Exempli gratia*
EDTA	Ethylenediamine tetraacetic acid
EEA1	Early endosome antigen 1
eGFP	Expressed green fluorescent protein
ELISA	Enzyme-Linked Immunosorbent Assay
ER	Endoplasmic reticulum
ESL-1	E-selectin ligand-1
ET	Extracellular traps
FAD	Flavin adenine dinucleotide

Fas-FasL	FAS-Fas ligand
FasR	FAS receptor
FBS	Fetal bovine serum
FcR	Fc receptor
FcγR	Fc gamma receptor
Fe_2^+	Iron ion
Fe-SODe	Iron superoxide dismutase excreted
FITC	Fluorescein isothiocyanate
FL1-H	FL1 height channel
FL2-H	FL2 height channel
FML	Fucose mannose ligand
fMLP	N-formyl-methionyl-leucyl-phenylalanine
FMN	Flavin mononucleotide
FMV-UL	Faculdade de Medicina Veterinária-Universidade de Lisboa
FnR	Fibronectin receptors
FOXP3	Forkhead box P3 or scurfin
fPPG	Filamentous proteophosphoglycans
FSC	Forward-scattered light
FSC-A	Forward scatter area
FSC-H	Forward scatter height
Fw	Forward
G-CSF	Granulocyte colony-stimulating factor
GFP	Green Fluorescent Protein
GIPL	Glycosylinositol phospholipids
GM-CSF	Granulocyte/macrophage colony-stimulating factor
gp63	Glycoprotein of 63kDa
$gp91^{phox}$ (*Nox2*)	gp91 phagocytic oxidase
GPcR	G-protein-coupled receptor
GPI	Glycosylphosphatidylinositol

GTP	Guanosine-5'-triphosphate
GTPases	Guanosine triphosphatase
h	Hour
H2A	Histone H2A
H_2O	Water
H_2O_2	Hydrogen peroxide
HBSS	Hanks' Balanced Salt Solution
HCl	Hydrochloric acid
HOCl	Hypochlorous acid
HOP	Heat-shock proteins organizer protein
HSC	Haematopoietic stem cell
HSP	Heat-shock proteins
HSP100	Heat-shock proteins 100 kDa
HVL	Human visceral leishmaniosis
IAP	Inhibitor of apoptosis protein
iC3b	Inactivated C3b
ICAM	Intercellular adhesion molecule
IFAT	Indirect immuno-fluorescent antibody test
IFN	Interferon
IFN-γR	Interferon-gamma receptor
IgG	Immunoglobulin G
IL	Interleukin
IL-10R	Interleukin 10 receptor
IL-12p40	Interleukin-12 subunit p40
IL-2R-α chain (CD25)	Interleukin 2 receptor alpha chain
iNOS	Inducible nitric oxide synthase
IP-10; CXCL10	Interferon gamma-induced protein 10; C-X-C motif chemokine 10
IQR	Interquartile range

IRF	Interferon regulatory factor
JAK	Janus kinase 1
JNK	Jun N-terminal kinase
kDNA	Kinetoplast deoxyribonucleic acid
LAMP	Lysosome-associated membrane protein
L	Leukotriene
LC	Langerhans cells
LCF	*Leishmania* chemotactic factor
LdAAP3	*Leishmania donovani* amino acid permease 3
LJL	Recombinant *Lutzomya longipalpus* salivary protein
LPG	Lipophosphoglycan
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
M1	Classically activated macrophages
M2	Alternatively activated macrophages
M6PR	Mannose-6-phosphate receptor
MAC	Membrane attack complex
MAP	Mitogen-activated protein
MAPK; ERK	Mitogen-activated protein kinase; extracellular signal-regulated kinase
MARCKS	Myristoylated alanine-rich C kinase substrate
MARCO	Macrophage receptor with collagenous structure
MBL	Mannose-binding lectin
mCAT2B	Mouse cationic amino acid transporter 2B
Mcl-1	Myeloid cell leukemia-1
M-CSF	Macrophage colony-stimulating factor
MET	Macrophage extracellular traps
MHCI	Class I molecules of major of histocompatibility complex
MHCII	Class II molecules of major of histocompatibility complex

MIP	Macrophage inflammatory proteins
mM	Milimolar
Mn_2^+	Manganese ion
MØ	Macrophages
MPO	Myeloperoxidase
mPPG	Membrane bound proteophosphoglycans
MR	Mannose receptor
mRNA	Messenger ribonucleic acid
MRP	MARCKS-related proteins
MT-ZVL	Mountain-type-zoonotic visceral leishmaniosis
MyD88	Myeloid differentiation factor 88
N	Normality
n1	Necrotic 1
n2	Necrotic 2
n3	Necrotic 3
NaCl	Sodium chloride
NADPH; PHOX	Nicotinamide adenine dinucleotide phosphate
NBT	Nitroblue tetrazolium
NE	Neutrophilic elastase
NET	Neutrophil extracellular traps
NF-kB	Nuclear factor-κB
NK	Natural killer cells
NLR	Nucleotide-binding and oligomerization domain (NOD)-like receptors
NLRA	Nucleotide-binding and oligomerization domain (NOD)-like receptors acidic transactivation domain
NLRB	Nucleotide-binding and oligomerization domain (NOD)-like receptors baculoviral inhibitory repeat-like domain
NLRP	Nucleotide-binding and oligomerization domain (NOD)-like receptors pyrin domain
NO	Nitric oxide

NOD	Nucleotide-binding and oligomerization domain
NOD1	Nucleotide-binding and oligomerization domain-containing protein 1
NOD2	Nucleotide-binding and oligomerization domain-containing protein 2
nor-NOHA	N^{ω}-hydroxy-nor-L-arginine
O_2^-	Superoxide anion
OAT	Ornithine aminotransferase
ºC	degree Celsius
ODC	Ornithine decarboxylaseTh2
OH-arg	Nω-hydroxy-arginine
OM	Optical microscopy
$ONOO^-$	Peroxynitrite
p	Probability
$p22^{phox}$ (*CYBA*)	p22 phagocytic oxidase
p38 MAPK	p38 Mitogen-activated protein kinase
p38MAP	p38 mitogen-activated protein
$p40^{phox}$	p40 phagocytic oxidase
$p47^{phox}$	p47 phagocytic oxidase
$p67^{phox}$	p67 phagocytic oxidase
PAD4	Peptidylarginine deiminase 4
PAMP	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PFR	Paraflagellar rod
PG	Prostaglandin
pH	Potential of hydrogen
PI	Propidium iodide
PI3P	Phosphatidylinositol 3-phosphate

PICD	Phagocytosis-induced cell death
PIK	Phosphatidyl inositol kinases
PK	Protein kinase
PM	Peritrophic matrix
PMA	Phorbol myristate acetate
PMN	Polymorphonuclear leukocytes
PO	*Per os*, by mouth
PPARγ	Peroxisome proliferator-activated receptor-γ
PPG	Proteophosphoglycans
PpGalec	*Phlebotomus papatasi* galactose binding protein
PpSP15	*Phlebotomus papatasi* salivary protein 15
PRR	Pattern recognition receptors
PS	Phosphatidylserine
PSG	Promastigote secretory gel
PSGL-1	P-selectin glycoprotein ligand-1
PTX3	Pentraxin 3
q-PCR	Quantitative- polymerase chain reaction
QuilA	Quillaja saponin
Rac1	Ras-related C3 botulinum toxin substrate 1
RANTES	Regulated on activation, normal T cell expressed and secreted
RFP	Red fluorescent protein
Rho	Ras homolog family
RhoA	Ras homolog gene family, member A
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxigen species
RPMI	Roswell Park Memorial Institute medium
RV	Reverse
SAP	Secreted acid phosphatases

SBE	STAT-binding elements
SC	Subcutaneous
SCHN	Schneider medium
SDS	Sodium dodecyl sulfate
SEM	Scanning electron microscopy
SID	Once a day
Slc11a1; N-RAMP1	Solute carrier family 11 member 1
SNAP	Semi-HMM-based Nucleic Acid Parser
SNARE	Soluble-N-ethylmaleimide sensitive-factor accessory-protein (SNAP) receptor
SNP	Single nucleotide polymorphism
sPPG	Secreted proteophosphoglycans
SSC	Side scatter
SSC-A	Side scatter-area
SSC-H	Side scatter-height
STAT	Signal transducer and activator of transcription
STI1	Stress-inducible protein 1
Syt	Synaptotagmin
T $CD8^+$	Cytotoxic T cell
T-bet	Type 1 associated transcription factor
TEM	Transmission electron microscopy
TGF	Transforming growth factor
Th1	T helper cells type 1
Th2	T helper cells type 2
TLR	Toll-like receptors
TNF	Tumor necrosis factor
TNT	Tunneling nanotubes
TRAIL	TNF-related apoptosis-inducing ligand
Treg	Regulatory T cells

TRIF	Toll-interleukin 1 receptor domain-containing adaptor inducing interferon-β
Tris-HCl	Tris hydrochloride
Tyk2	Tyrosine kinase 2
U	Unit
u-PA	Urokinase-type plasminogen activator
USA	United States of America
v/v; w/v	Volume/volume; weight/volume
VAMP8	Vesicle-associated membrane protein 8
v-ATPase	vesicular ATPase
VCAM	Vascular cell adhesion molecule
VL	Visceral leishmaniosis
vs	*Versus*
WASP	Wiskott-Aldrich syndrome protein
WHO	World Health Organization
Y-NBT	Yellow-colored nitroblue tetrazolium
ZVL	Zoonotic visceral leishmaniosis

1 GENERAL INTRODUCTION

1.1 CANINE LEISHMANIOSIS

1.1.1 Etiology and geographical distribution

Canine leishmaniosis (CanL) is a parasitic disease caused by a protozoan of the genus *Leishmania* transmitted to the domestic dogs (*Canis familiaris*) through the bite of a phlebotomine sand fly (Alvar et al, 2004; Dantas-Torres, 2009).

Leishmania belongs to the order Kinetoplastida, family Trypanosomatidae and genus *Leishmania*, which comprises the subgenera *Leishmania* and *Viannia* (Lainson et al, 1987) and several complexes. With some exceptions, all species of the subgenus *Viannia* are found in the New World, while those belonging to the subgenus *Leishmania* are isolated in the Old World. Species of *L. mexicana* complex are located in the New World and *L. infantum* (syn. *L. chagasi*) is present in both the New and Old Worlds (Maurício et al, 2000; Alvar et al, 2004; Bañuls et al, 2007; Baneth et al, 2008). Recent studies based on multilocus microsatellite typing and genomic studies support the hypothesis that *L. infantum* was introduced into the Americas by infected dogs of European settlers during the colonization of South America in the 16th century and that *L. infantum* and *L. chagasi* are indistinguishable (Maurício et al, 2000; Kuhls et al, 2011; Leblois et al, 2011).

CanL is endemic in more than 70 countries from South and Central America, the Mediterranean region, Africa and Asia (Baneth et al, 2008; Solano-Gallego et al, 2009, 2011; Dantas-Torres et al, 2009, 2012).

At least 14 *Leishmania* species infect the dog (Saridomichelakis, 2009; Solano-Gallego et al, 2009; Cantacessi et al 2015) (Table 1). Presenting a wide geographical distribution in both the Old and New World, high prevalence of infection and disease in endemic areas, increased severity and life-threatening character associated with a zoonotic nature, *L. infantum* is probably the most representative species (Gramiccia and Gradoni, 2005; Saridomichelakis, 2009; Solano-Gallego et al, 2009; Saridomichelakis and Koutinas, 2014). However, at least 11 isoenzymatic variants (zymodemes) of *L. infantum* have been isolated in the dog (Gramiccia et al, 1992; Dereure et al, 1999; Gaskin et al, 2002).

Table 1: Taxonomic classification of *Leishmania* species that infect dogs based on multilocus enzyme electrophoresis, its geographical distribution and vectors involved in transmission. All species listed belong to the section Euleishmania, except *L. colombiensis* that belongs to Paraleishmania section. Adapted from Solano-Gallego et al (2009) and Cantacessi et al (2015).

Subgenus	Species complex	Species	Geographical distribution	Proven or suspected sand fly vectors
Leishmania	*L. donovani*	*L. infantum*	Europe, America, Asia, Africa	*L. longipalpus, L. evansi, P. neglectus, P. perniciosus,* others
		L. donovani	East Africa	*P. orientalis* , P. *martini* , *P. rodhaini*
		L. archibaldi	Sudan	
	L. tropica	*L. tropica*	India, Iran, Israel, Morocco, Syria	*P. sargenti*
	L. major	*L. major*	Egypt, Saudi Arabia	*P. papatasi*
		L. arabica	Saudi Arabia	*P. papatasi*
	L. mexicana	*L. amazonensis*	Brazil	*L. flaviscutellata* , *L. nociva* , *L. whitmani*
		L. mexicana	Ecuador, USA	*L. ayacuchensis* , *L. olmeca*
		L. pifanoi	Ecuador	*L. flaviscutellata* , *L. youngi*
Viannia	*L. braziliensis*	*L. braziliensis*	Argentine, Bolivia, Brazil, Colombia, Peru, Venezuela	*L. intermedia* , *L. migonei* , *L. wellcomei* , *L. whitmani* , others
		L. peruviana	Peru	*L. peruensis* , *L. verrucarum*
	L. guyanensis	*L. panamensis*	Colombia, Ecuador, Panama	*L. hartmanni* , *L. gomezi* , *L. panamensis, L. trapidoi*
		L. guyanensis	Colombia	*L. anduzei* , *L. umbratilis* , *L. whitmani*
		L. colombiensis	Venezuela	*L. hartmanni*

1.1.2 *Leishmania infantum* infection

In the Mediterranean, seroprevalence of *L. infantum* infection varies from 5% to 30%, depending on the region and its ecological aspects (Solano-Gallego et al, 2009). It has been estimated that at least 2.5 million dogs are infected in Southwest Europe (Moreno and Alvar, 2002). However, surveys employing other detection methods, such as amplification of *Leishmania* deoxyribonucleic acid (DNA) or the detection of specific anti-*Leishmania* cellular immunity have revealed even higher infection rates, reaching 70% in some foci (Baneth et al, 2008).

The endemicity of the infection in Portugal was confirmed by a national survey carried out in January 2009 with the screening of 3974 dogs from all the 18 districts of mainland Portugal. Using the direct agglutination test (DAT), an overall prevalence of 6.31%, ranging from 0.88% to 16.16% was observed and the emergency of Beja, Castelo Branco and Portalegre districts as new foci was noticed. According to this study, the oldest foci of Trás-os-Montes and Alto Douro, Lisbon and Algarve regions lost their importance (Cortes et al, 2012). A seroprevalence of 21.3% was obtained in the northeastern Portugal, in the years 2008 and 2009, assessed by DAT and Enzyme-Linked Immunosorbent Assay (ELISA) (Sousa et al, 2011). In the municipality of Évora (southern Portugal) the seroprevalence evaluated by DAT in 2010 was 5.6% (Schallig et al, 2013). Recent studies realized in the south of Portugal revealed that 60.4% of dogs were quantitative polymerase chain reaction (q-PCR) positive for *L. infantum* (Maia et al, 2016) and that 16.06% were seropositive (Maia et al, 2013).

The distribution of CanL in Europe has been changing, probably due to socioeconomic and possibly to climate factors (Solano-Gallego et al, 2011). In endemic countries, *L. infantum* infection has spread northward, reaching the foothills of the Alps in northern Italy (Morosetti et al, 2009; Otranto et al, 2009; Baldelli et al, 2011), the Pyrenees in France (Chamaille et al, 2010) and northern Spain (Amusategui et al, 2004). The disease is also an important problem in non-endemic countries. Recently, numerous cases of CanL have been reported in northern countries, such as Germany (Menn et al, 2010), the Netherlands, United Kingdom (Shaw et al, 2009) and Poland (Kaszak et al, 2015) where the disease is considered as emergent. Dogs imported from or travelling to the Mediterranean region have increased the number of CanL cases in central and northern Europe (Solano-Gallego et al, 2011).

A survey done in a new focus of zoonotic visceral leishmaniosis (ZVL) in Northeastern Turkey showed a seroprevalence rate of infection of 7.2% (Sari et al, 2015). Several studies have described the presence of CanL in Israel and Palestine (Nasereddin et al, 2009). In Palestine 11.5% of dogs analyzed were positive for *Leishmania* DNA, whereas ELISA and culture revealed 7.5% and 1.5% of infection, respectively (Hamarsheh et al, 2012). A seroprevalence of 12.2% was described in Iran, where *L. infantum* is the principal causative agent of CanL (Mohebali, 2013). A recent review (Strelkova et al, 2015) summarized the available information about CanL in Southern Caucasus, Central

Asia, Crimean Peninsula and Russia. In Azerbaijan (Southern Caucasus), the seroprevalence assessed by IFAT ranged between 17.3% and 70.4%, depending on the region and in Georgia (Southern Caucasus), the seroprevalence assessed by DetectTM rK39 rapid test ranged between 8% and 28.1%. In Uzbekistan (Central Asia), a study carried out in 2007 and 2008 showed that 31.5% of symptomatic and 20.2% of asymptomatic dogs living in areas where visceral leishmaniosis (VL) had been previously diagnosed were seropositive when assayed by ELISA. In Crimean Peninsula, the parasite was not isolated from the dog and in Russia this possibility has not yet been investigated. In China the implementation of control programs resulted in the elimination of the disease from most endemic areas. Dogs are reservoirs of the mountain-type-ZVL (MT-ZVL) and canine infection has been reported at high rates in the western region (Lun et al, 2015), with a prevalence of 59.43% in the Sichuan province (Wang et al, 2011) and 77.21% in the Gansu province (Wang et al, 2006).

Epidemiological surveys in dogs and clinical cases of CanL have been reported in North Africa, namely Egypt with a seroprevalence of 10% (Morsy et al, 1983; Rosypal et al, 2013), Tunisia (Ben Said et al, 1992), Algeria (Harrat et al,1996), but also in countries of West Africa, namely Gambia (Desjeux et al, 1983) and Senegal (Faye et al, 2011) and of East Africa, like Kenya (Mutinga et al, 1980), Sudan (Dereure et al, 2000), Ethiopia (Kalayou et al, 2011) and Uganda (Millán et al, 2013). Even so, data from southern Africa are still scarce. Few cases of CanL have been reported in Angola and some of them were imported. A recent study established a frequency of infection of 1.0% by polymerase chain reaction (PCR) and 1.9% by serology (Vilhena et al, 2014).

The disease has been identified in the United States of America (USA) where it is considered an emerging problem in some dog breeds, with an estimated annual PCR prevalence greater than 20% on the Foxhound population (Petersen and Barr, 2009) and also identified in some provinces of southern Canada (Duprey et al, 2006). In South America, the infection prevalence was estimated in millions (Moreno and Alvar, 2002; Baneth et al, 2008). High seroprevalence rates were observed in Northern Brazil. For instance, in the state of Ceará 21.4% of stray dogs and 26.2% of domestic dogs are infected (Rondon et al, 2008) and in the state of Bahia 21.7% of stray dogs (Julião et al, 2007) and 23.4% of domestic dogs also have the parasite (Paranhos-Silva et al, 1996). In Margarita Island, Venezuela, 33.1% of dogs are seropositive (Zerpa et al, 2003). The

emergence of CanL in southern Brazil and northern Argentina has been associated with modifications in the ecology and distribution of the principal vector species, *L. longipalpis* (Salomon et al, 2008; Souza et al, 2009; Bravo et al, 2013).

1.1.3 Infection by “exotic” *Leishmania* species

Several *Leishmania* species responsible for the American Cutaneous Leishmaniosis also infect dogs. A high prevalence of *L. braziliensis* infection has been reported in Brazil, mainly in rural areas, where about 55% of dogs tested PCR positive in the blood (Dantas-Torres et al, 2010, Carvalho et al, 2015). One outbreak of cutaneous leishmaniosis caused by *L. braziliensis* and *L. panamensis* was observed between 2005-2009 in soldiers of the Colombian Army and in their dogs (Vélez et al, 2012). *L. guyanensis* and *L. braziliensis* were isolated from dogs living in Colombia (Santaella et al, 2011). Cases of dog natural infection caused by *L. mexicana* (Velasco-Castrejón et al, 2009; Arjona-Jiménez et al, 2012), *L. amazonensis* (Dias et al, 2011) and *L. peruviana* (Llanos-Cuentas et al, 1999) were also reported.

L. donovani, a primarily viscerotropic species present in the Indian subcontinent and East Africa was rarely identified in dogs living in eastern Sudan (Dereure et al, 2003), Sri Lanka (Nawaratna et al, 2009), India (Sharma et al, 2009), Bangladesh (Alam et al, 2013) and in Cyprus, where one dog was found to be co-infected with both *L. infantum* and *L. donovani* (Antoniou et al, 2008).

L. major, the main agent of the human cutaneous leishmaniosis in the Old World, widespread from West Africa to the Middle East and India can also infect dogs (Elbihari et al, 1987; Morsy et al, 1987; Pratlong et al, 2009; Baneth et al, 2016). Sporadic cases of canine infection by *L. tropica* were identified in Morocco, Iran, Crete, Greece and Turkey (Dereure et al, 1991; Guessous-Idrissi et al, 1997; Lemrani et al, 2002; Mohebali et al, 2005, 2011; Pratlong et al, 2009; Hajjaran et al, 2013; Toz et al, 2013; Ntais et al, 2013, 2014; Baneth et al, 2014). *L. tropica* produces human cutaneous disease in the Middle East, North Africa, Central Asia and some parts of southern Europe (Ionian Islands and Crete) and rarely human visceral leishmaniosis (HVL) (Sacks et al, 1995; Christodoulou

et al, 2012). Natural infection by *L. arabica* was also reported in the dog (Peters et al, 1986).

1.1.4 Dog as *Leishmania* host and reservoir

Canine *L. infantum* infection constitutes a veterinary and public health problem. From a public health point of view, the dog is considered the main peridomestic reservoir of the parasite in endemic areas (Ashford, 1996; Moreno and Alvar, 2002; Alvar et al, 2004; Gramiccia and Gradoni, 2005; Dantas-Torres and Brandão-Filho, 2006; Dantas-Torres, 2007). This statement is supported by the following observations. (i) Parasites isolated from dogs are indistinguishable from those obtained from humans; for example, *L. infantum* zymodeme MON-1, which is responsible for most of HVL cases in the Mediterranean basin is also the predominant zymodeme isolated from dogs (Pratlong et al, 2004). (ii) Dogs are highly susceptible to the parasite and the prevalence of infection is often high in endemic areas, with a large proportion of asymptomatic cases (Ashford, 1996; Quinnell et al, 1997; Dantas-Torres and Brandão-Filho, 2006). (iii) Dogs usually live in or next to human houses, which favors the maintenance of the domestic transmission cycle (Dantas-Torres and Brandão-Filho, 2006). (iv) Symptomatic dogs are infectious to the vector due to their high skin parasitism and even asymptomatic dogs can present the parasite (Gradoni et al, 1987; Molina et al, 1994; Travi et al, 2001; Michalsky et al, 2007; Laurenti et al, 2013) to sand fly, ensuring the parasite life cycle.

Furthermore, infected dogs are a source of parasite for phlebotomine sand flies of species *L. longipalpis* (Lainson et al, 1985) and *P. perniciosus* (Molina et al, 1994), the main vectors of *L. infantum* in the New and Old Worlds, respectively. These phlebotomine sand flies can easily adapt to the peridomestic environment or human dwellings and feed frequently on dogs (De Colmenares et al, 1995; Killick-Kendrick, 1999; Feliciangeli, 2004; Lainson and Rangel, 2005).

Despite these observations, only one study carried out in Iran has shown that child seropositivity increases significantly with village dog density and that dog ownership is a significant risk factor for children (Gavgani et al, 2002b). The presence of infected dogs in the vicinity of humans is certainly associated with an increase in the prevalence of

infection among sand flies, enhancing the entomologic inoculation rate and parasite transmission (Gavgani et al, 2002b; Solano-Gallego et al, 2009). However, the presence of an infected dog in the household does not appear to greatly increase the risk of infection to the family when transmission is already taking place in the region (Solano-Gallego et al, 2009).

For the “exotic” *Leishmania* species, dogs do not appear to have a significant role as a reservoir or have a particular importance as a source of parasites to humans (Ashford, 1996; Gramiccia and Gradoni, 2005; Dantas-Torres, 2007). Even so, the possibility of the dog being the reservoir has been discussed for some species, namely *L. donovani* (Alam et al, 2013; Rohousová et al, 2015), *L. braziliensis* (Dantas-Torres, 2007) and *L. peruviana* (Llanos-Cuentas et al, 1999).

1.1.5 Dynamics of dog infection

In endemic areas, when favorable conditions for disease transmission are present, the infection spreads rapidly and widely among the dog population (Baneth et al, 2008). Oliva et al (2006) demonstrated that all naïve Beagle dogs introduced in a hyperendemic area of southern Italy become infected after three transmission seasons. However, a large proportion of animals living in endemic areas present neither clinical signs nor clinicopathological abnormalities related to CanL and the presence of the parasite cannot be demonstrated, but seropositivity with basal antibody levels is common (Roura et al, 2013). In endemic areas, some dogs present a subclinical infection. They do not present neither clinical signs nor laboratory abnormalities compatible with CanL, but the presence of the parasite is confirmed (Solano-Gallego et al, 2009). Nevertheless, these healthy carriers contribute to the infection of sand flies, although to a lesser extent than the clinically diseased dogs (Baneth et al, 2008; Laurenti et al, 2013). The active disease (CanL) is present in a minority of dogs living in an endemic area and is characterized by clinical signs and/or clinicopathological abnormalities compatible with CanL and the infection is confirmed. Indeed, except in hyperendemic areas, CanL prevalence is lower than 10%, because the majority of infected dogs do not develop clinical disease (Solano-Gallego et al, 2009).

The balance between humoral and cellular immune response determine the clinical status of the dog. Delayed-type hypersensitivity (DTH) positive animals usually control the infection due to the presence of a demonstrable cellular immune response against *L. infantum*. A study carried out in a highly endemic area of Sicily (Italy) showed that 73.8% of outdoor dogs have a positive DTH reaction to leishmanin antigen (Lombardo et al, 2012). Antibody positive and DTH negative dogs are considered susceptible due to the suppressed cellular immune response and typically develop clinical disease in a short or middle term. However, factors such as immunosuppression and concomitant diseases can disrupt the immunological equilibrium of resistant dogs and lead to the development of leishmaniosis (Solano-Gallego et al, 2009).

1.1.6 Pathology and clinical manifestations

CanL is a chronic disease characterized by a wide range of clinical signs, consequence of the numerous pathogenic mechanisms that are involved in the disease, the different organs affected and the diversity of immune strategies mounted by the dog in response to infection (Koutinas and Koutinas, 2014). CanL is also a systemic disease, affecting potentially any organ, tissue or body fluid (Solano-Gallego et al, 2009; Koutinas and Koutinas, 2014). The involvement of the skin and other organs results from hematogenous dissemination of the parasite (Solano-Gallego et al, 2004).

Dogs suffering from CanL show depletion of T cells in lymphoid organs, which is compensated by an exuberant proliferation of B cells, plasma cells, histiocytes and macrophages (MØ), responsible for the generalized lymphadenomegaly and splenomegaly characteristics of the disease. Excessive granulomatous inflammation, for instance liver granulomas, is an attempt to counteract the cellular immune deficiency typical of the disease (Koutinas and Koutinas, 2014). The persistent antigenemia induce a polyclonal B cell response, leading to specific and non-specific antibody production that instead of giving protection causes organ damaging. Immune mediated pathology can be associated with the circulating antibodies, autoantibodies, namely anti-histone antibodies and immune complexes, which are deposited in the renal glomeruli, vascular endothelium, anterior uvea, and synovial membrane or linked to blood cells, such as platelets and red blood cells (Lopez et al, 1996; Terrazzano et al, 2006; Ginel et al, 2008;

Cortese et al, 2009; Paltrinieri et al, 2010; Koutinas and Koutinas, 2014). In cold weather, the production and precipitation of cryoglobulins in the blood vessel walls results in ischemic necrosis (Baneth and Solano-Gallego, 2012).

Common clinical signs include weight loss (Fig. 1A) and lymphadenopathy that are observed in 65.1% to 90% of dogs. However, lymph nodes may show normal size or even hypoplasia in advanced disease, particularly in nephritic dogs. Although splenomegaly is not a common clinical finding, since spleen usually does not present a high enlargement to be palpated on physical exam (Slappendel, 1988; Denerolle, 1996; Ciaramella et al, 1997; Koutinas et al, 1999; Saridomichelakis, 2009) it can be detected by imaging techniques (Paltrinieri et al, 2010) (Fig. 1G). The high parasite load frequently observed in the spleen seems to be related to the ineffective immune response mounted by the organ (Engwerda and Kaye, 2000; Lima et al, 2007). The pathological features observed in the bone marrow may explain the anemia, thrombocytopenia and others hematological abnormalities related to CanL. This organ usually shows high parasite load (Foglia et al, 2006; Koutinas and Koutinas, 2014; Momo et al, 2014).

Skin lesions are perhaps the most common clinical signs, observed in 81% to 90% of the cases. Common dermatological entities are non-pruritic exfoliative dermatitis (Fig. 1B, D and E), ulcerative dermatitis, focal or multifocal nodular dermatitis, onychogryphosis (Fig. 1C), hyperkeratosis (Fig. 1F) (Denerolle, 1996; Ciaramella et al, 1997; Koutinas et al, 1999), mucocutaneous proliferative dermatitis and papular dermatitis (Ordeix et al, 2005; Lombardo et al, 2014). Interestingly, nodular and papular dermatitis are considered markers of high and low susceptibility to CanL, respectively. Nodular dermatitis seems to be more prevalent in boxers and has been associated with severely compromised parasite specific cellular immunity (Saridomichelakis and Koutinas, 2014). Conversely, papular dermatitis has been associated with low antibody levels and positive DTH, suggesting a specific immunocompetence and a favorable prognosis (Ordeix et al, 2005; Lombardo et al, 2014).

Leishmania amastigotes are present in both diseased and normal-looking skin of CanL dogs (Solano-Gallego et al, 2004). However, in clinically healthy infected dogs, parasite density in the skin seems to be related to its serologic state. Seronegative dogs do not have histological alterations and the parasite is only detected by PCR, suggesting that

such animals may not be able to transmit the parasite. On the contrary, histological alterations and *Leishmania* amastigotes are present in seropositive animals, although to a lesser degree compared with CanL dogs (Saridomichelakis and Koutinas, 2014).

The kidney is affected in virtually all dogs with CanL (Costa et al, 2003). However, azotemia is a less frequent laboratory finding (Baneth et al, 2008). Renal disease can progress from asymptomatic proteinuria to nephrotic syndrome and/or end-stage kidney disease (uremic syndrome) (Poli et al, 1991; Plevraki et al, 2006; Solano-Gallego et al, 2009). Renal lesions have been associated with high levels of circulating immune complex (Lopez et al, 1996) and high levels of anti-histone antibodies (Ginel et al, 2008). Occasionally, renal disease is the only CanL manifestation (Ciaramella et al, 1997).

Ocular disease is quite common in dogs (Solano-Gallego et al, 2009; Koutinas and Koutinas, 2014) and can be the only clinical sign. Anterior uveitis (Fig 1H), blepharitis and keratoconjunctivitis are the most frequent manifestations of ocular disease (Peña et al, 2000).

Bleeding disorders are frequently reported, but its pathogenesis has not been completely elucidated. Epistaxis can be the only clinical sign and the cause of death if profuse and uncontrolled (Ciaranmella et al, 1997; Jüttner et al, 2001; Petanides et al, 2008; Koutinas and Koutinas, 2014).

Arthritis is present in some animals (Blavier et al, 2001; Agut et al, 2003; Koutinas and Koutinas, 2014; Sbrana et al, 2014). Osteomyelitis characterized by osteolytic (de Souza et al, 2005) and/or osteoproliferative lesions can also occur. An increased intramedullary opacity on radiography can be seen and corresponds to bone sclerosis (Agut et al, 2003). Muscular involvement can be observed as a masticatory muscle myositis or skeletal muscle polymyositis usually with subclinical evolution (Vamvakidis et al, 2000; Paciello et al, 2009).

Digestive and neurologic signs are less frequent. Lesions of the digestive system can involve the oral cavity (Parpaglia et al, 2007; Viegas et al, 2012), liver (Rallis et al, 2005) or large intestine (Adamama-Moraitou et al, 2007). Various neurological signs have been

associated to CanL, namely seizures, painful and rigid neck, and paraplegia (Márquez et al, 2013; Maia et al, 2015).

The variable clinical presentation of the disease is the result of complex interaction between the parasite, the immune competence and the genetic background of the host. Although it is well known that different *Leishmania* species cause different diseases in the dog, there is no clear evidence that the various *L. infantum* strains (zymodemes) can explain the subtle clinical diversity seen between the Mediterranean and Brazilian CanL (Koutinas and Koutinas, 2014).

L. braziliensis infection is generally subclinical or asymptomatic and only 7.7% of dogs show clinical disease (Figueredo et al, 2012; Carvalho et al, 2015). Skin lesions (ulceration, desquamation, hyperpigmentation, onychogryphosis) and weight loss are the main clinical signs. Hematological abnormalities include thrombocytopenia, anemia and eosinophilia (Pirmez et al, 1988; Figueredo et al, 2012). *L. tropica* infection can produce subclinical disease or systemic, cutaneous or mucocutaneous involvement (Baneth et al, 2014; Bamorovat et al, 2015).

1.1.7 Predisposing factors

Several risk factors have been described, including breed, age and genetic background. In Europe, the seroprevalence exhibits a bimodal pattern, with higher rates in dogs younger than 3 and older than 8 years old (Abranches et al, 1991; Cardoso et al, 2004), whereas in South America, the prevailing pattern of dog age appears not to be well-defined (Dantas-Torres, 2009), probably because the populations of phlebotomine sand flies do not suffer important changes throughout the year, remaining constantly present (Dantas-Torres et al, 2012) and ensuring parasite continuous transmission.

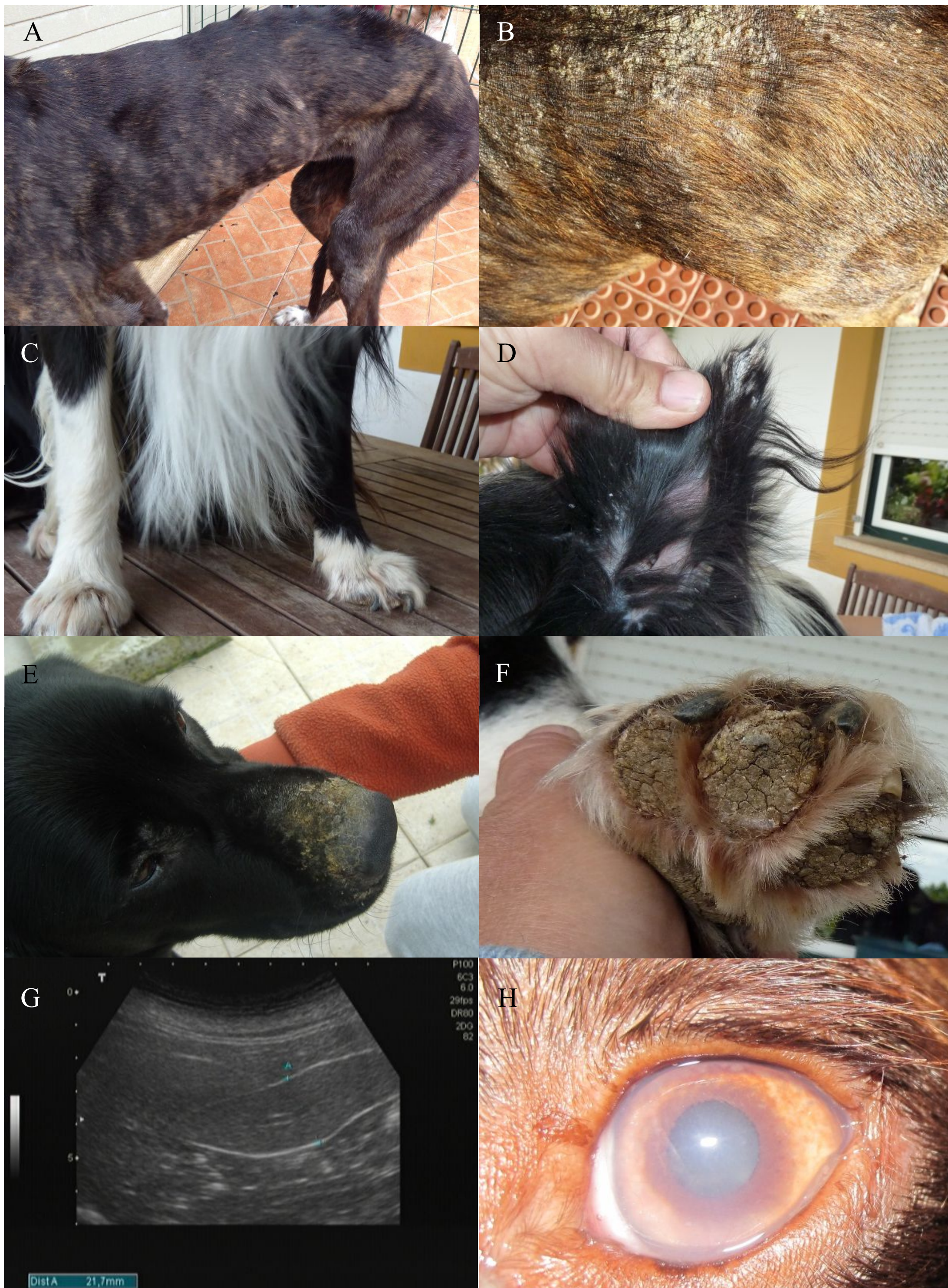

Figure 1: Clinical signs produced by *L. infantum* infection in the dog. Weight loss (A); Generalized exfoliative dermatitis (B); Onychogryphosis (C); Localized exfoliative dermatitis affecting the pinnae (D); Localized exfoliative dermatitis affecting the nose (E); Digital hyperkeratosis (F); Assessment of spleen enlargement by abdominal ultrasound (G); Uveitis (H).

Some studies report no gender predisposition (Abranches et al, 1991; Miró et al, 2007b) while others point towards high risk for the disease in male dogs (Zaffaroni et al, 1999; Zivicnjak et al, 2005), indicating some controversy. A number of studies revealed a high prevalence of infection in some dog breeds such as the Boxer, Cocker Spaniel, Rottweiler and German Shepherd, Doberman Pinscher, American and English Foxhound (Sideris et al, 1999; Gaskin et al, 2002; Franca-Silva et al, 2003; Sanchez-Robert et al, 2005; Duprey et al, 2006). Conversely, other breeds such as the Mediterranean Ibizian Hound, an autochthonous breed from the Balearic Island of Ibiza rarely develop clinical signs of CanL, pointing towards some degree of resistance associated with a strong immune response (Solano-Gallego et al, 2000).

1.1.8 Treatment

Currently, some treatment options are available in different countries. The objectives of treatment are the following: clinical improvement, general reduction of parasite load, handling of organ damage and restoration of an efficient immune response (Oliva et al, 2010). Drugs used to treat the disease are able to temporarily improve the clinical signs or induce the clinical cure, but none eliminates the infection, which can result in clinical relapses (Oliva et al, 1998; Koutinas et al, 2001; Guarga et al, 2002; Noli and Auxilia, 2005; Ikeda-Garcia et al, 2007; Manna et al, 2008, 2009). Furthermore, treated animals may remain infectious for phlebotomine sand flies but to a lesser extent than non-treated ones (Gradoni et al, 1987; Guarga et al, 2002; Ribeiro et al, 2008; Miró et al, 2011).

Meglumine antimoniate has a parasiticidal activity, selectively inhibiting the glycolysis and also potentiating the phagocytic capacity of MØ (Muniz-Junqueira et al, 2008; Oliva et al, 2010). The drug has a short half-life in dogs and is rapidly eliminated by the kidneys (Tassi et al, 1994; Valladares et al, 1996, 1998). Therefore, the risk of toxicity may increase in dogs with reduced glomerular rate (Solano-Gallego et al, 2011).

Allopurinol acts as a purine analogue of adenosine nucleotides and blocks ribonucleic acid (RNA) synthesis by the parasite, acting as a leishmaniostatic drug (Martinez and Marr, 1992). When administered as a single drug for a minimum period of 2 to 3 months leads to moderate clinical improvement and partial restoration of some

clinicopathological parameters (Oliva et al, 2010). However, a long-term administration seems to be crucial to maintain a low parasite load and prevent possible relapses (Koutinas et al, 2001; Miró et al, 2011; Manna et al, 2015).

Miltefosine is a phospholipid (hexadecyl-phosphocholine) registered for oral administration in dogs with CanL in several European countries. Its anti-*Leishmania* activity is attributed to the impairment of signaling pathways and cell membrane synthesis that lead to parasite dead (Croft et al, 1987).

The combination of meglumine antimoniate with allopurinol is considered as the most effective therapy, showing a lower incidence of clinical recurrence than the miltefosine-allopurinol combination (Manna et al, 2015). The length of allopurinol treatment depends on the severity of the disease, the clinical and parasitological response to treatment and the individual tolerance to the drug. Allopurinol can be discontinued when the patient presents a complete physical and clinicopathological recovery and the antibody falls to negative or borderline levels (Solano-Gallego et al, 2009, 2011).

The efficacy of amphotericin B was demonstrated by several clinical trials. Amphotericin B acts by binding to ergosterol of parasite cell membrane, changing its permeability (Miró et al, 2008). However, the intravenous route of administration and its nephrotoxicity are important disadvantages (Miró et al, 2008; Solano-Gallego et al, 2009). Furthermore, amphoterecin B is the first line drug for HVL in Europe and is not recommended by the World Health Organization (WHO) for veterinary use to avoid parasite resistance.

Aminosidine (paramomycin) has severe side effects such as nephrotoxicity and ototoxicity and its use as first line therapy is not recommended (Solano-Gallego et al, 2009). Several other drugs, including pentamidine, ketoconazole, metronidazole, spiramycin and marbofloxacin have been used against CanL, but are currently considered to be second-line drugs, and more extensive clinical studies are necessary to verify their therapeutic effectiveness (Noli and Auxilia, 2005; Miró et al, 2008; Solano-Gallego et al, 2009).

The most common therapeutic protocols used for the treatment of CanL are allopurinol in monotherapy or in association with meglumine antimoniate or miltefosine. Urinary

clinical signs due to xanthinuria, renal mineralisation and urolithiasis were observed in 45.2% of dogs receiving allopurinol (Torres et al, 2016). Dosages and side effects of current therapeutic protocols are presented in Table 2 and Fig. 2.

Table 2: Current therapeutic protocols for canine leishmaniosis. Adapted from Solano-Gallego et al (2011).

Drugs	Dosages	Side effects
Meglumine antimoniate[a]	100 mg/kg SID or 50 mg/kg BID, SC for 4 weeks	Potential nefrotoxicity Cutaneous abscesses
plus		
Allopurinol	10 mg/kg BID PO	Xanthine urolithiasis
Miltefosine[a]	2 mg/kg SID, PO for 28 days	Vomiting, diarrhea
plus		
Allopurinol	10 mg/kg BID PO for at least 6-12 months	Xanthine urolithiasis
Allopurinol	10 mg/kg BID PO for at least 6-12 months	Xanthine urolithiasis

[a] Licenced for veterinary use in Europe; SID - once a day; BID - twice a day; PO - *per os*, by mouth; SC - subcutaneous.

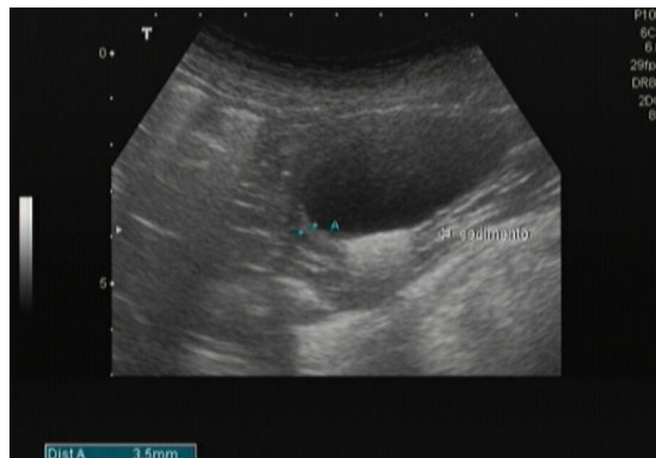

Figure 2: Side effects of long term allopurinol administration. Urinary sediment observed under abdominal ultrasound exam.

1.1.9 Prevention

Environmental control of sand flies immature forms (larvae and pupae) can be achieved, reducing the microhabitats favorable to their development in the vicinity of the dog kennel. Adults control can be done through the use of insecticide spraying, although this

is a temporary measure associated with environmental toxicity and risk of insect resistance (Solano-Gallego et al, 2009; Otranto and Dantas-Torres, 2013).

Synthetic pyrethroides are currently used to prevent sand fly bites. These products have a toxic and irritant effect, preventing blood feeding and causing sand fly dead (Otranto and Dantas-Torres, 2013). Its efficacy was demonstrated both experimentally (Killick-Kendrick et al, 1997; Mencke et al, 2003; Miró et al, 2007a) and in the field (Maroli et al, 2001; Gavgani et al, 2002a; Otranto et al, 2007, 2010). Long-acting topical insecticides are available in spot on formulation or in a collar form and should be used in non-infected and in infected dogs living in or travelling to endemic areas throughout the period of sand fly activity that in the Mediterranean basin is between April and November (Solano-Gallego et al, 2011).

Currently, purified *Leishmania* fraction antigens appear to be the most effective and promising vaccines for dogs. The fucose and mannose containing glycoprotein-enriched fraction of *L. donovani* also known as the "fucose mannose ligand" (FML)-based vaccine, adjuvated with Quillaja saponin (QuilA) was licensed and commercialized in Brazil as Leishmune® (Borja-Cabrera et al, 2002). This vaccine was also proposed for immune therapy in sick dogs (Borja-Cabrera et al, 2004) and considered a transmission blocking vaccine, since antibodies produced by the vaccinated dog prevent the development of the parasite in the insect vector, interrupting the epidemiological cycle (Saraiva et al, 2006).

A second vaccine based on excreted/secreted antigen purified from specific-medium culture supernatant of *L. infantum* with muramyl dipeptide as adjuvant has tested in Europe (Lemesre et al, 2007) and commercialized with a purified fraction of QuilA as adjuvant (CaniLeish®). Another vaccine (Leish-Tec®), which contains the recombinant A2 protein and saponin as adjuvant has also been approved in Brazil (Fernandes et al, 2008).

1.2 THE PARASITE

1.2.1 Morphology

Leishmania is a digenetic or heteroxeno protozoan. The promastigote form localizes in the insect vector and the amastigote form in the mammalian host. These forms are morphologically distinct but show some common characteristics. The promastigote is a spindle-shaped cell, presenting a single flagellum at the anterior extremity, that emerges from a specialized invagination of the plasma membrane known as the flagellar pocket. The flagellum can reach 20 µm and confers parasite mobility. The flagellar cytoskeleton is formed by a canonical 9+2 microtubular axoneme that is assembled on a basal body. In addition, a filamentous structure called paraflagellar rod (PFR) is present and runs parallel to the axoneme. Promastigote have about 10-20 µm in length and 1.5-3.0 µm in width. The nucleus is central and the kinetoplast, that contains an important amount of DNA (kDNA) is located between the nucleus and the anterior extremity of the parasite.

In contrast to the promastigote forms, *Leishmania* amastigotes display a spherical/oval body shape with a short flagellum, which extends from the basal body to the opening of the flagellar pocket. The 9+2 microtubular pattern of amastigote flagellum is present for only a short distance beyond the transitional zone. The PFR is absent in the amastigote flagellum of *Leishmania*. The amastigote measures 2.5-6.8 µm in diameter and the nucleus is located in the posterior pole of the cell (Tomás and Romão, 2008; Gadelha et al, 2013).

Both parasite forms have a single layer microtubule arrangement located immediately below the plasma membrane, named subpellicular microtubules. This structure maintains cell shape, confers some plasma membrane rigidity and prevents the direct interaction of the organelles with the plasma membrane, except the endoplasmic reticulum (ER) that are occasionally seen penetrating between the microtubules. In trypanosomatids, the endocytic and exocytic activities only take place in the flagellar pocket, whose membranes are not associated with subpellicular microtubules (Wanderley de Souza and Attias, 2010).

1.2.2 Virulence factors

Leishmania is exposed to hard environments, namely the sand fly midgut and the MØ phagolysosome. The different morphological forms of *Leishmania* represent an adaptation to the changing environmental conditions faced by the parasite within their two hosts, which requires the expression of stage-specific virulence determinants (Corrales et al, 2009). Several *Leishmania*-related molecules secreted and/or expressed at the parasite surface have been identified as virulence factors. Lipophosphoglycan (LPG), glycosylinositol phospholipids (GIPL), cysteine protease B (CPB) and the zinc-metalloprotease gp63 (gp63) are included in this category (Olivier et al, 2012).

Extracellular components produced by *Leishmania* have been extensively studied, demonstrating its involvement in the parasite virulence in the insect vector and mammalian host. These extracellular components have proved to be highly immunogenic, eliciting an important immune response and protection against infection in mice and dogs. Furthermore, they actively regulate host immune response (Corrales et al, 2009).

The expression 'excreted/secreted factors' is used to include all the molecules found outside the cell, including bona fide secreted proteins. *Leishmania* secretome refers to proteins that are actively secreted from the cell. Finally, the term 'exoproteome' is defined as the set of *Leishmania* proteins present in the extracellular space and thus includes non-secreted proteins (*e.g.* extracellular proteins originating from cell lysis) and those secreted actively (the secretome) (Corrales et al, 2009).

In general, two major secretion mechanisms have been characterized in eukaryotes: classical or ER/Golgi-dependent secretion and non-classical or ER/Golgi-independent secretion. The classical secretory pathway is based on the presence of an N-terminal secretion signal peptide on the soluble secretory protein. This signal peptide directs the protein to the ER, where the signal peptide is cleaved from the mature protein. Then, the protein is transported to the Golgi apparatus and ultimately released into the extracellular space by fusion of Golgi-derived secretory vesicles with the plasma membrane. This mechanism is used by *Leishmania* to secrete surface proteins, namely the members of the proteophosphoglycan family. A variety of mechanisms have been proposed to participate in the non-classical protein secretion of eukaryotic cells, including lysosomal secretion,

plasma membrane shedding and release of exosomes. *Leishmania* mainly uses non-classical mechanisms for protein export and secretion, showing a polarized delivery of secretory material to the flagellar pocket prior to release (Corrales et al, 2009; Lambertz et al, 2012).

LPG is one of the major surface glycoconjugate of promastigote and covers the entire surface of the parasite to produce a dense glycocalyx. However, its expression is down-regulated in the amastigote by at least three orders of magnitude. LPG is a large molecule containing the repeating disaccharide-phosphate Galβ1,4Man-PO4 units attached to a glycan core that is inserted into the membrane *via* a 1-O-alkyl-2-lyso-phosphatidyl(myo)inositol anchor (GPI). The molecule is capped by a small oligosaccharide structure that varies among species. Although the backbone of the repeating units, the glycan core and the lipid anchor are conserved among *Leishmania* species, they differ in the additional oligosaccharide chains branching off the backbone. LPG undergoes several important modifications during the life cycle of the parasite that are characteristic for each *Leishmania* species (Lodge and Descoteaux, 2005; Olivier et al, 2012).

The surface of *Leishmania* promastigotes and amastigotes is also coated with an abundant class of small and structurally related GIPL. GIPL are in fact the major surface components of promastigotes and amastigotes, but their small size keeps them close to the parasite membrane. GIPL vary in sugar and lipid compositions. Some of them are precursors of LPG or protein GPI anchors, whereas others are distinct surface entities (Lodge and Descoteaux, 2005; Olivier et al, 2005).

Proteophosphoglycans (PPG) and secreted acid phosphatases (SAP) contain the repeating disaccharide-phosphate units that are O-linked to phosphoserine residues. PPG are produced by amastigotes and promastigotes of several *Leishmania* species and can be found under various forms, including filamentous (fPPG), secreted (sPPG) and membrane bound (mPPG) components. SAP are present on the surface of *Leishmania* and are continuously secreted by all *Leishmania* species studied to date, although the rate of their secretion appears to largely vary among species (Lodge and Descoteaux, 2005; Olivier et al, 2012).

The zinc-dependent metalloprotease gp63 (or leishmanolysin) is found on the surface of promastigote, although about 10-fold less abundant than LPG. However, its shorter length means that it is essentially buried under the glycocalyx of LPG. It is down-regulated in amastigotes, although detectable in all *Leishmania* species studied so far. This reduced expression may be counteracted by the absence of LPG on the amastigote surface, meaning that gp63 is no longer masked and may therefore play an important role in amastigote survival and modulation of the host response. Biochemical analysis of the extracellular gp63 of *Leishmania* has revealed two forms of the protein: one is a glycosylphosphatidylinositol-anchored form that can be released from the cell surface and another that is apparently secreted (Ellis et al, 2002; McGwire et al, 2002; Olivier et al, 2005).

Exosomes are vesicles of 40-100 nm that are released from a variety of eukaryotic cells, including parasitic protozoa. The generation of exosomes is achieved by invagination of the endosomal membrane, and its release into the extracellular space occurs upon fusion of the vesicle membrane with the plasma membrane (Silverman et al, 2010; Lambertz et al, 2012). Following internalization, *L. donovani* releases vesicles in the cytoplasm of infected MØ and in the extracellular medium that could be taken up by naïve cells. Exosome release by *Leishmania* serves as a general mechanism for protein secretion and for the delivery of cargo to the cytosol of the host cell (Silverman et al, 2010). An increase in protein and exovesicle release occurs following the elevation of ambient temperature to 37 ºC, mimicking the entrance of *Leishmania* promastigotes into the mammalian host (Hassani et al, 2011; Olivier et al, 2012) (Fig. 3).

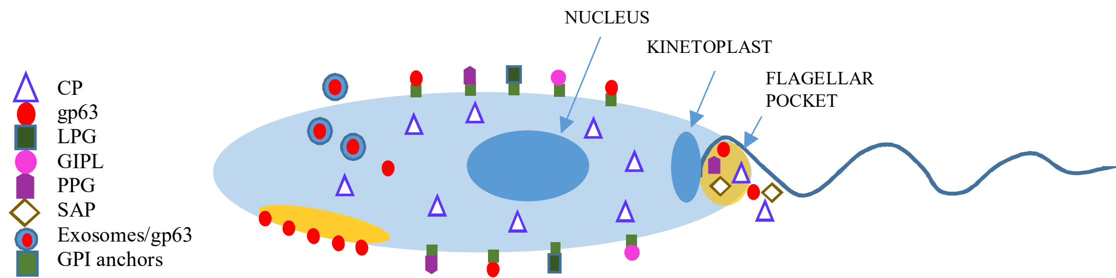

Figure 3: *Leishmania* virulence factors. Schematic representation of *Leishmania* promastigote form showing the glycosylphosphatidylinositol (GPI)-anchored surface molecules: zinc-metalloprotease gp63, lipophosphoglycan (LPG), proteophosphoglycans (PPG) and glycosylinositol phospholipids (GIPL), which are mostly associated to membrane microdomains. Virulence factors that are in the intracellular milieu can be released *via* exosome (gp63) or *via* classical secretion through the flagellar pocket [gp63, PPG, secreted acid phosphatases (SAP) and cysteine protease (CP)]. Adapted from Olivier et al (2012).

1.3 PARASITE-HOST-VECTOR TRIANGLE

In the dog, the evolution of *Leishmania* infection depends on many factors related to the vector (repeated bites, the amount and quality of saliva inoculated), the parasite (species, inocula size, virulence of the strains), and the host (genetic background, immune response, co-existing diseases) (Koutinas and Koutinas, 2014).

In the sand fly midgut, *Leishmania* parasites undergo a complex developmental cycle culminating in the generation of infectious metacyclic promastigotes (vector-parasite interface). During transmission, sand flies release an array of pharmacological, immunomodulatory and immunogenic molecules at the bite site that have immediate and long-lasting effects on the host (the vector-host interface) (Oliveira et al, 2009). The infected host develops an innate and adaptive immune responses that the parasite intents to subvert (parasite-host interface) (De Morais et al, 2015) (Fig. 4).

Thus, the understanding of the triad of complex interactions between *Leishmania*, the sand fly and the mammalian host at a molecular level is crucial to design new therapeutic approach and control measures (Oliveira et al, 2009; Cantacessi et al, 2015).

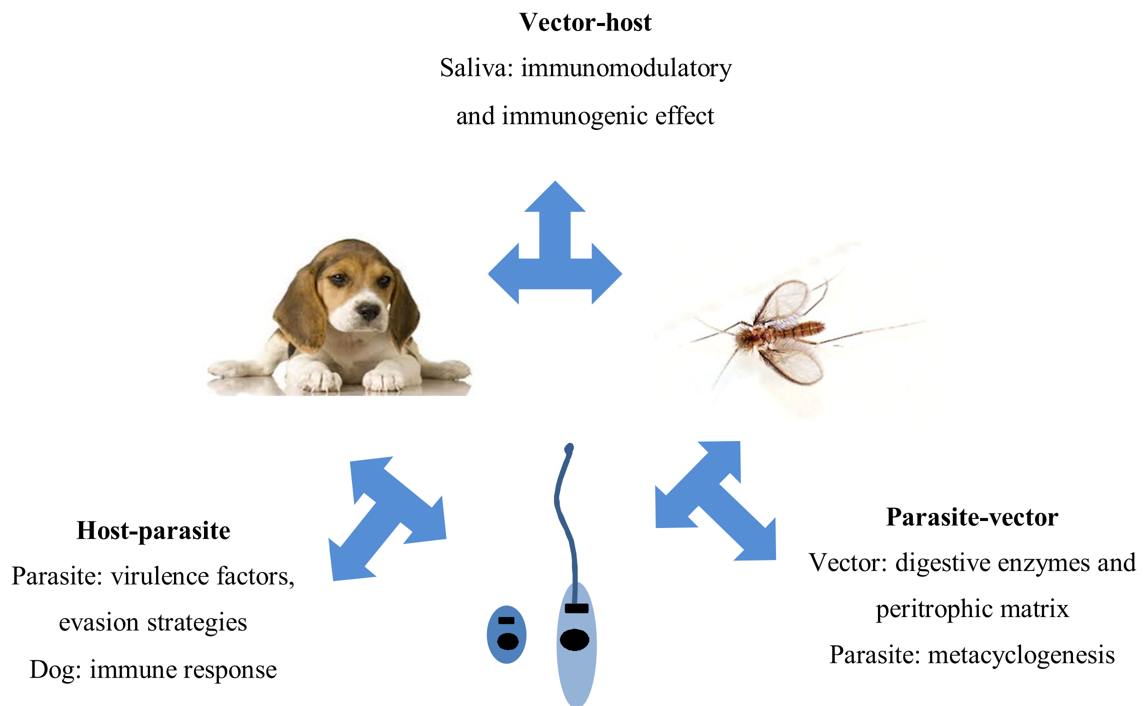

Figure 4: Parasite-host-vector triangle. The interaction parasite-vector, vector-host and host-parasite dictates the outcome of the dog infection.

1.3.1 Parasite-Vector interface

In the sand fly, *Leishmania* life cycle is confined to the digestive tract. Most Old World *Leishmania* species (subgenus *Leishmania*) are suprapylarian parasites, having its development restricted to the midgut. Members of the New World *Viannia* subgenus, such as *L. braziliensis* are peripylarian parasites, because they enter the hindgut before migrating forward into the midgut (Kamhawi, 2006).

Some species of sand flies are specific vectors, meaning that they can support the growth of only one *Leishmania* species, while others are permissive vectors, as they can support the growth of more than one species (Volf et al, 2008). This specificity is caused in part by the presence of appropriate binding sites on the sand fly gut that attach specific ligands expressed by promastigotes (Volf et al, 2008; Dobson et al, 2010; Svárovská et al, 2010). The strong association between the sand flies and the *Leishmania* species that they naturally transmit seems to be a direct consequence of a co-evolutionary process (Andrade et al, 2007; Ramalho-Ortigão et al, 2010; Maroli et al, 2013). Therefore, natural

transmission occurs only in those areas where vectors and the respective adapted species are present (Killick-Kendrick, 1999; Volf et al, 2008).

As far as the interactions between sand fly and *Leishmania* are concerned, Ramalho-Ortigão et al (2010) highlighted that “long relationships are not necessarily easy”. Indeed, blood ingestion induces physiological responses in the sand fly midgut, including the secretion of digestive enzymes and the synthesis of a peritrophic matrix (PM), which represent natural barriers to parasite development (Kamhawi, 2006; Oliveira et al, 2009; Ramalho-Ortigão et al, 2010).

1.3.1.1 *Leishmania* life cycle in the phlebotomine sand flies

Phlebotomine infection is initiated when the sand fly ingests a blood meal containing *Leishmania*-infected MØ. The bloodmeal reaches the posterior abdominal midgut and is fully contained by the PM. Amastigotes are released after MØ rupture and differentiate into procyclic promastigotes, which rapidly enter into a period of intense replication. Procyclic promastigotes transform into nectomonads. The degeneration of the PM enables nectomonads to escape and migrate forward to attach along the midgut epithelium. Then nectomonads give rise to leptomonads, which undergo the second multiplication cycle in the sand fly, resulting in a massive infection at the anterior midgut. Leptomonads then differentiate into numerous non-dividing infectious metacyclic promastigotes that accumulate at the stomodeal valve (Rogers et al, 2002; Gossage et al, 2003; Bates and Rogers, 2004). Metacyclics are rapid, free-swimming forms, highly adapted for successful transmission to the mammalian host. Haptomonads, presumably differentiated from leptomonads, appear as static parasites attached to the cuticular lining of the stomodeal valve and to each other, forming concentric rings of parasites that plug the opening of the valve. The approximate time needed for the parasites to complete their development in the sand fly is about 6-9 days, depending on the species (Kamhawi, 2006; Oliveira et al, 2009) and the environmental conditions (Sharma and Singh, 2008). Although the steps involved in promastigote differentiation are well characterized morphologically, the signals and molecular events resulting in differentiation remain to be established (Cohen-Freue et al, 2007).

1.3.1.2 Evasion strategies developed by *Leishmania*

Midgut proteases facilitate blood-meal digestion, but at the same time create a hostile environment for the parasite (Sacks and Kamhawi, 2001; Volf et al, 2001; Ramalho-Ortigão et al, 2003). Parasites in the transitional stage between amastigotes and promastigotes (procyclic) are more vulnerable to this proteolytic attack and a high level of parasite killing is observed, even in a compatible *Leishmania*-sand fly relationship (Pimenta et al, 1997; Schlein and Jacobson, 1998). The earliest determinant of vector competence is the parasite ability to overcome proteases attack (Kamhawi, 2006), which is attributed to phosphoglycan containing molecules (Sacks and Kamhawi, 2001). Comparison between sand fly blood-fed and *Leishmania*-infected cDNA libraries allow the identification of midgut digestive enzymes that are modulated by the presence of *Leishmania* parasites (Ramalho-Ortigão et al, 2007; Jochim et al, 2008).

PM surrounds the blood meal and protects the epithelium from abrasive food particles and at the same time constitutes the second barrier to *Leishmania* development. However, during the first hours PM protects transitional forms that have resisted to digestive enzyme attack, allowing its differentiation into resistant flagellated promastigotes (Pimenta et al, 1997).

To continue their development, parasites that survive to the proteolytic attack must escape from the endoperitrophic space before defecation. The PM is degraded by chitinases secreted by the sand fly midgut and probably also by the parasite (Schlein et al, 1991; Ramalho-Ortigão et al, 2001, 2003, 2005; Kamhawi, 2006), and nectomonads are free to adhere to the midgut epithelium. Midgut attachment can be mediated by LPG, the largest and most abundant surface glycoconjugate of promastigotes, as in the case of specific vectors (Sacks et al, 2000; Ilg, 2001; Sacks and Kamhawi, 2001; Soares et al, 2002) or *via* a LPG-independent mechanism (Myskova et al, 2007). LPG polymorphism among *Leishmania* species and strains is considered the major determinant of vectorial competence. The restricted vector competence of *P. papatasi* to *L. major* is associated with the presence of galactose side chains on its promastigote LPG that are specific for the sand fly galactose binding protein, PpGalec (Sacks and Kamhawi, 2001; Kamhawi et al, 2004). Furthermore, it was demonstrated that the presence of FML-specific receptors

in *L. longipalpis* midgut is involved in the binding of *L. donovani* or *L. chagasi* promastigotes to the midgut epithelium (Saraiva et al, 2006).

Leptomonads produce a secretory gel (PSG) composed mainly by fPPG that fills the lumen of the thoracic midgut of the sand fly and contributes to the 'blocked fly' effect (Stierhof et al, 1999; Rogers et al, 2002; Bates and Rogers, 2004; Rogers et al, 2004) and *Leishmania* chitinases degenerate the chitinous lining of the stomodeal valve (Schlein et al, 1992; Volf et al, 2004). Both mechanisms impair feeding dynamics and promote an efficient parasite transmission (Kamhawi, 2006).

Metacyclogenesis ensure parasite survival inside the host. In the vector, this process is associated with morphological changes of the parasite and also biochemical transformations in the LPG. During the acquisition of virulence features in the sand fly midgut, LPG elongates the number of repeating units, doubling from about 15 in procyclic promastigotes to about 30 in metacyclic forms. In addition, substitutions to the repeating units occur in several *Leishmania* species. These structural changes are required for the detachment of infectious, metacyclic promastigotes from the sand fly midgut and contribute to the ability of metacyclic promastigote to resist complement-mediated lysis in the mammalian host (Soares et al, 2002; Lodge and Descoteaux, 2005).

Little is known about the effect of *Leishmania* infection on the longevity and fecundity of the sand fly (Kamhawi, 2006). It is probable that sand fly recognizes the presence of the parasite and mounts an immune response. Some reports identify transcripts up-regulated by blood meal and/or *Leishmania* infection that were associated with insect immunity (Ramalho-Ortigão et al, 2001; Boulanger et al, 2004; Pitaluga et al, 2009).

Transcriptomic analysis, providing a descriptive repertoire of sand fly molecules and comparative transcriptomic of unfed, blood-fed and *Leishmania* infected blood revealed the ability of *Leishmania* parasites to modulate vector midgut transcripts (Oliveira et al, 2009). The rapid progress in genomics and the publication of *L. major* (Ivens et al, 2005), *L. braziliensis*, *L. infantum* (Peacock et al, 2007), *L. mexicana* (Rogers, 2011), *L. donovani* (Downing et al, 2011) and *L. amazonensis* (Real et al, 2013) genomes stress the importance of having the genomes of vector species with medical significance sequenced

to access the necessary tools to dissect the sand fly-*Leishmania* interactions at a molecular level.

1.3.2 Vector-Host Interaction

Female phlebotomine sand flies need a blood meal for the maturation of eggs. During this process the sand fly introduces its mouthparts into the skin, lacerates tissue and capillaries and creates hemorrhagic pools upon which it feeds. The salivary gland content is injected at the same time that *Leishmania* promastigotes are introduced into the host skin (Titus et al, 1988; Ribeiro et al, 1995).

It was suggested that some of the variability in the clinical presentations of *L. infantum* infections may be due to the different composition of the sand fly saliva. The significance of these findings in CanL remains to be studied (Lainson and Rangel, 2005).

Despite several pharmacological and immuno-modulatory activities attributed to sand fly saliva, fewer proteins were identified in the past. Rapid advances in transcriptomic and proteomic resulted in the completion of a number of salivary gland transcriptomes (sialomes) and production of several recombinant salivary proteins from several sand fly species. These approaches allowed the identification of markers of exposure and the development of vaccine candidates against leishmaniosis (Abdeladhim et al, 2014).

1.3.2.1 Immunomodulatory effect of sand fly saliva

For a successful blood meal, sand flies must first evade the host hemostatic system, preventing blood coagulation in the bite site. Their salivary secretion possesses potent pharmacological components, such as an inhibitor of the blood coagulation cascade (Charlab et al, 1999), an inhibitor of the classical complement pathway (Cavalcante et al, 2003), an apyrase that works as an inhibitor of platelet aggregation (Charlab et al, 1999; Valenzuela et al, 2001b; Hamasaki et al, 2009), and a vasodilator (Ribeiro et al, 1999). These components assure a favorable microenvironment for adequate blood-feeding (Abdeladhim et al, 2014).

A number of immunomodulatory activities have been attributed to sand flies saliva (Rohousová and Volf, 2006; Andrade et al, 2007; Oliveira et al, 2008; Prates et al, 2012). The immunomodulator effect of *L. longipalpis* saliva was attributed to the vasodilator maxadilan (Rohousová and Volf, 2006). Saliva of *L. longipalpis* exhibits MØ chemotactic activity (Teixeira et al, 2005), but has also an inhibitory effect on the activation of MØ and in the modulation of T cells by reducing the expression of Th1 type cytokines and inducing the production of Th2 cytokines (Theodos and Titus, 1993; Hall and Titus, 1995; Soares et al, 1998). In a similar way, saliva of *P. papatasi* was shown to have an identical effect on T cells, down-regulating the production of Th1 cytokines and up-regulating the release of Th2 cytokines (Mbow et al, 1998; Abdeladhim et al, 2011; Rogers and Titus, 2003).

Dendritic cells (DC) are potent antigen presenting cells (APC) specialized in the initiation of the immune response by direct activation of naïve T lymphocytes and induction of cell differentiation into specific subtypes. Saliva of both *P. papatasi* and *P. duboscqi* induces the sequential production of prostaglandin (PG)E2 and of interleukin (IL)-10 by DC, which in turn act on DC in an autocrine manner, reducing the expression of class II molecules of complex major of histocompatibility (MHCII) and of CD86 co-stimulatory molecules on their surface, inhibiting the ability of these cells to present antigens. Furthermore, the production of PGE2 and IL-10 by DC inhibits the release of neutrophil chemotactic factors MIP-1α and of leukotriene (L)B4, consequently diminishing neutrophil migration (Carregaro et al, 2008). Saliva of *L. longipalpis* increased the apoptosis of neutrophils, directly in a Fas-FasL mediating caspase-dependent manner (Prates et al, 2011).

Therefore, sand fly saliva influences the host hemostatic and immune systems at the feeding site, generating a microenvironment that favors *Leishmania* transmission and the establishment of infection (Rohousová and Volf, 2006). The effect of vector saliva as an enhancing agent of *Leishmania* infection in the dog was not proven, because dogs experimentally infected do not develop clinical disease or the development of clinical signs occurs only after a long period post inoculation (Paranhos et al, 1993; Killick-Kendrick et al, 1994; Paranhos-Silva et al, 2003).

1.3.2.2 Immunogenic effect of sand fly saliva

Studies performed in murine models show that the previous contact with phlebotomine sand fly saliva or the salivary proteins, maxadilan from *L. longipalpis* and PpSP15 from *P. papatasi* induce an adaptive immune response that mostly confers protection against leishmaniosis (Belkaid et al, 1998; Kamhawi et al, 2000; Morris et al, 2001; Valenzuela et al, 2001a; Thiakaki et al, 2005).

Experimental exposure of dogs to *P. perniciosus* and *L. longipalpis* bites elicits the production of specific anti-saliva IgG, which positively correlates with the number of sand fly bites (Hostomska et al, 2008; Vlkova et al, 2011) or the number of exposures (Collin et al, 2009). In endemic areas, specific antibodies against the *P. perniciosus* saliva increased in naturally bitten dogs over the sand fly season and its levels negatively correlate with anti-*Leishmania* seropositivity (Vlkova et al, 2011). Antibody response to sand fly saliva could be used for monitoring dog exposure to sand flies and might be used as a marker of risk for *Leishmania* transmission in endemic areas (Teixeira et al, 2010; Drahota et al, 2014).

However, the significance of anti-saliva antibodies in the outcome of the infection remains unclear. A positive correlation was observed between protection (DTH positivity to leishmanin) to HVL and the levels of *L. longipalpis* salivary antibodies (Gomes et al, 2002). However, in the case of human cutaneous leishmaniosis, high levels of anti-saliva antibodies were associated with disease (Rohousová et al, 2005; de Moura et al, 2007). Evidence from murine models indicates that anti-saliva antibodies are not required for protection (Valenzuela et al, 2001a).

A positive DTH response to sand fly saliva has been documented in humans (Vinhas et al, 2007) and in dogs after repeated exposures. Immunization with recombinant *L. longipalpus* salivary molecules (LJL143 and LJM17) induced a strong adaptive Th1 local and systemic immune response, characterized by marked dermal infiltrates of $CD3^+$ T cells, scattered MØ and the production of interferon (IFN)-γ by peripheral blood mononuclear cells (PBMC). Furthermore, upon challenge with infected sand flies, dogs immunized with recombinant proteins developed a cellular immune response at the bite site characterized by a lymphocytic infiltration and the expression of IFN-γ and IL-12.

Additionally, it was verified *in vitro* that MØ efficiently killed *L. infantum* following the addition of autologous lymphocytes from LJL143- and LJM17-immunized dogs stimulated with salivary gland homogenate (Collin et al, 2009).

This anti-saliva immune response can influence the type and activation state of the host immune cells, resulting in the direct killing of *Leishmania* parasites, thus reducing the infective load. Additionally, a Th1 anti-saliva immunity may create an environment that accelerates the priming of a protective Th1 anti-*Leishmania* immune response. From this point of view, salivary proteins can be considered ‘non-classical natural adjuvants’ (Kamhawi et al, 2000; Valenzuela et al, 2001a; Oliveira et al, 2009; Teixeira et al, 2014). The protective effect of sand fly saliva has also been attributed to the neutralization of the immunomodulators, such as the *L. longipalpis* peptide maxadilan (Belkaid et al, 1998; Morris et al, 2001).

1.3.3 Host-Parasite interaction

It is the host-parasite interaction that ultimately dictates the winner, that is to say the success of infection. Parasite surface and secreted molecules are involved in activating specific signaling pathways, which are essential for parasite internalization and intracellular survival. The recognition of the parasite antigens by host immune cells generates an immune response directed to eliminate the parasite. However, *Leishmania* has a complex repertoire of strategies to evade or subvert the immune system by interfering with a range of signal transduction pathways in host cells, which causes the inhibition of the protective response and contributes to their persistence in the host. The efficacy of the therapeutic strategies for CanL is limited. The knowledge of the host-parasite interaction and of the immune evasion mechanisms can be used as a tool for the development of new drugs (de Morais et al, 2015).

Transcriptomic studies have investigated *Leishmania*-induced regulation of gene expression in infected tissues. However, most of the studies used murine models of infection. Genomics and proteomics technologies may contribute to enhance our knowledge about the molecular interactions that occur between *L. infantum* and the canine host. Monitoring the changes that occur in host gene transcription and protein expression

throughout the course of the canine infection will allow new therapeutic and prophylactic strategies to be designed (Cantacessi et al, 2015).

1.3.3.1 Promastigote differentiation into amastigote

Unlike the promastigote form, there are no clearly defined developmental stages of differentiation in the amastigote form. For a few species, including *L. mexicana*, *L. infantum* and *L. donovani*, the complete life cycle consisting of promastigote differentiating to axenic amastigote can be replicated *in vitro* by enrichment and modification of the culture media to pH 5.5 and by increasing the temperature to 32-37 ºC (Zilberstein and Shapira, 1994; Debrabant et al, 2004; Alcolea et al, 2010).

Although there are evident morphological changes between promastigote and amastigote forms, the study of gene expression demonstrates that the *Leishmania* genome is constitutively expressed with limited stage-specific expression. However, the abundance of a messenger ribonucleic acid (mRNA) transcript does not necessarily correlate with subsequent protein abundance. Regulation seems to occur post-transcriptionally, including translational control, protein stability or post translational modifications (Cohen-Freue et al, 2007).

Although heat-shock proteins (HSP), including HSP70, HSP90 and STI1 (stress-inducible protein 1)/HOP (HSP organizer protein) are constitutively expressed in both life stages, they seem to play a role in coordinating responses to heat shock and to parasite differentiation. These abundant cytoplasmic proteins are extensively phosphorylated and assemble into large protein complexes following promastigote-to-amastigote differentiation. Contrary to other eukaryotes, *Leishmania* heat-shock response may be regulated by heat-induced kinases instead of heat-shock transcriptional factors. Notice that the expression of some stress proteins, including HSP100, a mitochondrial chaperone, and the ER A2 stress protein is up-regulated during promastigote-to-amastigote differentiation and related to parasite survival in the mammalian host (Naderer and McConville, 2011)

After parasite uptake by MØ, the increased temperature and the decreased phagosomal pH provide the signals required for the promastigote-to-amastigote differentiation. Ultimately, infected MØ rupture, releasing the amastigotes into the surrounding environment where they can infect neighboring MØ (Zilberstein and Shapira, 1994) or amastigotes are extruded in a synchronized fashion, through an exocytosis-like process (zeiotic structures, membrane blebs), being then transferred to vicinal MØ (Real et al, 2014).

1.3.3.2 Innate immune response

At skin level, the innate immune response is the first line of defense against *Leishmania* infection. The initial confront between the parasite and the immune system seems to be a crucial step to control parasite growth. Furthermore, it directs cell recruitment and helps develop the cytokine microenvironment in which parasite-specific T cell are primed. These non-specific defense mechanisms include complement activation, professional phagocytic cells and the recruitment and activation of APC (discussed below) (Laskay et al, 1995; Sacks and Sher, 2002; Manna et al, 2010; Papadogiannakis and Koutinas, 2015).

- **Complement system**

Following their inoculation into the dermis by the sand fly, *Leishmania* promastigotes come into contact with the complement system and C3b is deposited on the parasite surface. Complement activation results in the formation of C5 convertase and subsequent assembly of the lytic C5b-C9 membrane attack complex (MAC) that kills most *Leishmania* promastigotes. The remaining parasites survive employing different strategies (Mosser and Brittingham, 1997; Solbach and Laskay, 2000; Domínguez et al, 2002; Papadogiannakis and Koutinas, 2015). LPG modifications during metacyclogenesis prevent the insertion of the lytic C5b-C9 (Puentes et al, 1990; McConville et al, 1992) and gp63 accelerates the conversion of C3b in its inactive form (iC3b), thus preventing MAC formation (Brittingham et al, 1995). Protein kinase C (PKC), which exhibits increased expression in the metacyclics, phosphorylates several components of the complement system, such as C3, C5, and C9, blocking the activation of the complement cascade (Hermoso et al, 1991).

- **Natural killer cells**

Natural killer cells (NK) are known for their ability to directly lyse host cells that express an altered cell surface phenotype. In addition to their cytotoxic activity, NK also produce cytokines such as IFN-γ and tumor necrosis factor (TNF), which activate the antimicrobial mechanisms of MØ and contribute to the differentiation of Th1 cells (Bogdan, 2012).

Following the infiltration of neutrophils that participate in the early defense against *Leishmania* infection in mice and humans, NK accumulate at the inoculation site. These cells play a crucial role in the control of the infection through IFN-γ production, following their activation by *Leishmania* antigens and IL-12 (Laskay et al, 1995; Solbach and Laskay, 2000; Bogdan, 2012). However, there have been no published reports regarding the role of NK in CanL.

- **Dendritic and Langerhans cells**

DC are potent APC and establish the link between innate and adaptive immune responses (Banchereau and Steinman, 1998; Guermonprez et al, 2002). Dermal DC, known as Langerhans cells (LC) are specialized DC that reside in the epidermis as sentinels of the immune system. After the inoculation of promastigotes into the dermis, LC migrate from the epidermis to dermis guided by host-parasite derived cytokines-chemokines (Papadogiannakis and Koutinas, 2015). LC phagocytize the parasite and process and transport parasitic antigens to the regional lymph nodes for T cell priming. Antigen presentation to T lymphocytes is mediated by MHCII molecules (Moll, 1993; Saridomichelakis and Koutinas, 2014).

Studies performed in the *L. major*-mice model have shown that dermal DC preferentially takes up the amastigote form of the parasite *via* FcγRI or FcγRIII and present parasitic antigens *via* MHCII pathway. Infected DC release IL-12 and induce the efficient stimulation of naïve $CD4^+$ and $CD8^+$ T cells (Kautz-Neu et al, 2012).

Dermal DC from *L. infantum* resistant dogs seem to play a major role in the activation of cellular immune response, because the intradermal injection of leishmanin antigen

induced the upregulation of MHCII, associated with progressive ultrastrucutural changes characteristic of DC antigen-specific activation and maturation (Sacchi et al, 2006).

Furthermore, dogs with localized cutaneous lesions presented a moderate dermal infiltrate of LC expressing MHCII molecules, scarce MØ and parasites and a diffuse infiltration of T cells, while those with disseminated nodular lesions presented few LC, which did not express MHCII and the dermis appeared massively infiltrated by heavily parasitized MØ along with a very low number of T cells, suggesting that in the first case dogs control the infection, whereas in the second the immune response is insufficient to restrain the parasite (Fondevila et al, 1997).

- **Toll-like receptors and NOD receptors**

The innate immune response is based on the recognition of pathogen-associated molecular patterns (PAMP) present in invading pathogens that are recognized by pathogen pattern recognition receptors (PRR). There are five classes of PRR, but Toll-like receptors (TLR) and nucleotide-binding and oligomerization domain NOD-like receptors (NLR) are the most studied (Ashour, 2015; de Morais et al, 2015).

TLR are type-1 transmembrane glycoproteins that recognize and interact with PAMP, as well as with damage-associated molecular patterns (DAMP) (Ashour, 2015). These receptors are expressed in various types of cells, including monocytes, MØ, DC and neutrophils (Kawai and Akira, 2011; Prince et al, 2011). TLR types 1, 2, 4, 5, 6 and 10 are expressed on the cell surface and recognize extracellular ligands, whereas TLR types 3, 7, 8 and 9 are expressed in endocytic compartments, such as the ER, endosomes and lysosomes (Takeda and Akira, 2005; Doyle and O'Neil, 2006). TLR can be classified into three groups according to their specific ligands: TLR1, TLR2, TLR4, TLR6 and TLR10 are involved in lipid and lipopeptide recognition; TLR5 and TLR11 recognize proteins and TLR3, TLR7, TLR8 and TLR9 detect nucleic acids (Rakoff-Nahoum and Medzhitov, 2008; Mogensen, 2009). Upon binding of ligands, TLR signal *via* adaptor proteins such as myeloid differentiation factor 88 (MyD88), except for TLR3, which signals through Toll-interleukin 1 receptor domain-containing adaptor inducing interferon-β (TRIF), and for TLR4 that signals through both MyD88 and TRIF pathways (Akira, 2001). TLR initiate inflammation by the activation of nuclear factor-κB (NF-kB) and other

transcription factors, leading to the production of inflammatory cytokines, reactive oxygen (ROS) and nitrogen species (RNS), increasing cellular survival, receptor expression and phagocytosis (Kawai and Akira, 2011; Hayashi et al, 2003). In addition, TLR activate APC and bridge innate and adaptive immunity by coordinating the responses of T cells and B cells (Iwasaki and Medzhitov, 2010).

TLR responses to *Leishmania* ligands play a protective role, but could also serve to promote infection (Faria et al, 2012). Although *Leishmania* species express several PAMP few parasite derived molecules have been reported to activate TLR receptors and the majority of the studies to date focused on the activation of TLR2, TLR3, TLR4 and TLR9 (Hayashi et al, 2003; Faria et al, 2012). Reported *Leishmania* ligands are LPG, cytosine-phosphate-guanosine (CpG), proteo-lipid complex and *Leishmania* RNA virus, which are recognized by TLR2 (Becker et al, 2003), TLR9 (Wu et al, 2006), TLR4 (Whitaker et al, 2008), and TLR3 (Ives et al, 2011), respectively.

NLR are highly conserved cytosolic PRR primarily expressed in immune cells. Mammalian NLR are subdivided into four subfamilies based on the variation in their N-terminal domain: acidic transactivation domain (NLRA), baculoviral inhibitory repeat-like domain (NLRB), caspase activation and recruitment domain (CARD; NLRC), and the pyrin domain (NLRP). NLR that recruit and activate the inflammatory protease caspase-1 are referred to as inflammasome. Caspase 1 is required for the processing and maturation of inflammatory cytokines IL-1β and IL-18. Other NLR, such as NOD1 and NOD2 do not directly engage the inflammatory caspases, but instead activate NF-κB, mitogen-activated protein kinases (MAPK) and IFN regulatory factors (IRF), stimulating innate immunity. NOD1 and NOD2 activation through cytosolic recognition of peptidoglycan ligands triggers the recruitment of mediators needed to form a signaling complex referred to as the nodosome. These receptors are crucial for tissue homeostasis and host defense against bacterial pathogens (Zhong et al, 2013; Saxena and Yeretssian, 2014).

1.3.3.3 Adaptive immune response

The adaptive immune response involves T and B lymphocytes that recognize a large spectrum of antigens using highly specific receptors (de Morais et al, 2015). An effective activation of specific cellular immunity is the basis of resistance against *L. infantum* in the infected dogs (Barbiéri, 2006).

Chemokines are chemotactic factors that coordinate the recruitment of leukocytes, participating in the innate and adaptive immune responses. In the context of CanL, dogs naturally and experimentally infected overexpress IP-10 (CXCL10) and RANTES (CCL5) mRNA in the spleen during both oligosymptomatic and polysymptomatic stages of the disease and treatment induces a decrease in the expression levels of these chemokines and also of MCP-1 (CCL2). Chemokine expression at the skin of naturally infected dogs revealed a positive correlation between parasite density and CCL2, CCL4, CCL5, CCL21 and CXCL8 (IL-8) expression (Menezes-Souza et al, 2012).

Understanding the profile of cytokines expressed in CanL has been a difficult and complex task due to the limited number of studies, the diverse clinical stage investigated, the different tissue analyzed and the type of infection (natural or experimental) (Baneth et al, 2008).

Resistance to CanL has been associated with activation of Th1 cells producing IFN-γ, IL-2 and TNF-α (Pinelli et al, 1994, 1995), which in turn activates infected MØ to kill the parasite *via* the L-arginine-nitric oxide (NO) pathway, down regulating at the same time the Th2 response (Pinelli et al, 1999; Diaz et al, 2012; Papadogiannakis and Koutinas, 2015). *In vitro* studies showed that these mechanisms are deactivated in MØ of infected dogs (Pinelli et al, 1999; Diaz et al, 2012). However, NO production and anti-leishmanial activity were detected in a canine MØ cell line infected with *L. infantum* after incubation with IFN-γ, TNF-α and IL-2 (Pinelli et al, 2000), as well as in dog MØ immunized with killed *L. infantum* promastigotes (Panaro et al, 2001).

IL-12 is produced mostly by inflammatory cells (monocytes, MØ, PMN and DC) in response to intracellular pathogens. IL-12 induces the production of IFN-γ from NK and T-cells and is important in inducing and maintaining a Th1 type response (Hsieh et al,

1993; Seder et al, 1993). A low proportion of experimentally infected beagle dogs showing evidence of parasite dispersion and no clinical signs of disease presented non-stimulated or specifically stimulated PBMC expressing IL-12 p40, IFN-γ or IL-2 mRNA during an extensive time period (8 months), suggesting the ''silent establishment'' of the parasite without induction of the host-cell-mediated immunity nor associated pathology (Santos-Gomes et al, 2002). Strauss-Ayali et al (2005) observed that IL-12 increased IFN-γ production by PBMC from experimentally or naturally infected symptomatic dogs.

Susceptibility to infection has been associated with high anti-*Leishmania* antibody titers and a Th2 immune response with the production of IL-4 and down regulation of cellular immunity. Accumulation of IL-4 mRNA was found in the bone marrow of some naturally infected symptomatic dogs (Quinnell et al, 2001), and significantly higher IL-4 expression was detected in skin lesions from naturally infected dogs (Brachelente et al, 2005). Recently, immunostimulation of $CD4^+$ and $CD8^+$ blood lymphocytes of naturally infected dogs revealed higher percentage of $CD4^+IL\text{-}4^+$ and $CD8^+IL\text{-}4^+$ lymphocytes in sick animals when compared with clinically healthy and subclinically infected dogs (Matralis et al, 2016).

L. infantum appears to induce a mixed Th1 and Th2 response in which the control of parasite replication, disease progression or cure is determined by the balance between these two types of response. A longitudinal study evaluating PBMC cytokine expression in experimentally infected dogs showed an asymptomatic phase with an absence or low expression during a period of "silent establishment of parasite" followed by a short term expression of Th1-type cytokines before the appearance of clinical signs (Santos-Gomes et al, 2002). Cytokine expression in the spleen of infected dogs showed a mixed type-1 and type-2 cytokine profile, characterized by the initial increase in the IL-4 mRNA one month post infection followed by the elevation of the expression of IFN-γ and T-bet (type 1 associated transcription factor) (Strauss-Ayali et al, 2007).

The involvement of the regulatory IL-10 and transforming growth factor (TGF)-β cytokines have been investigated in CanL. The expression of IL-10 in PBMC and in the bone marrow of experimentally and naturally-infected dogs, respectively was not elevated in the course of the *L. infantum* infection (Quinnell et al, 2001; Santos-Gomes et al, 2002). The expression levels of IL-10 and TGF-β was studied in the spleen of naturally

and experimentally infected dogs, but also without changes during the course of the infection (Strauss-Ayali et al, 2007). However, in naturally infected dogs the expression of IL-10 in the spleen was associated with parasitic load increase and disease progression (Lage et al, 2007). Also in whole blood the increase in IL-10 expression was associated with the detection of parasitic DNA (Boggiatto et al, 2010). The analysis of the spleen and liver of *L. infantum*-infected asymptomatic dogs showed higher TGF-β1 levels when compared with symptomatic dogs and elevated concentration of IL-10 was found in both asymptomatic and symptomatic dogs (Corrêa et al, 2007).

These cytokines, which can be produced by many cell types, including B lymphocytes, MØ and $CD4^+$ T cells, act mainly in the deactivation of other cells, such as DC and MØ (Gesser et al, 1997). The most well characterized subpopulation of regulatory T (Treg) cells is the subset of $CD4^+$ T cells that is defined by the expression of the constitutive IL-2R-α chain (CD25) (Sakaguchi et al, 1995) and more specifically by the expression of transcriptional factor FOXP3 (Ramsdell, 2003). The regulatory role of $CD4^+FOXP3^+$ T cells in dogs with CanL has not been fully elucidated. A reduction in the percentage of Treg cells was observed in peripheral blood (Cortese et al, 2013) and an increase in IL-10 producing Treg cells was verified by flow cytometry in the spleen of dogs naturally infected with *Leishmania* (Silva et al, 2014).

$CD8^+$ cells recognize the antigen bound to MHCI and release granules containing perforin and granzymes, leading to lysis of the target cell. However, $CD8^+$ T cells also produce cytokines and chemokines, which can enhance immunity to pathogens. The most important of them is IFN-γ, which promotes a strong Th1 immune response. However, the effector function developed by $CD8^+$ T cells *Leishmania* specific and its consequence (protective, pathologic or irrelevant) seems to depend on the *Leishmania* species and/or model employed, as well as their location in the host (Novais and Scott, 2015). Few studies have demonstrated the involvement of $CD8^+$ lymphocytes in resistance to CanL. These lymphocytes were detected in the blood of asymptomatic dogs experimentally infected with *L. infantum* but not in the symptomatic ones, and they promote the direct lysis of *L. infantum*-infected MØ (Pinelli et al, 1995). Matralis et al (2016) showed that dogs with CanL have increased the number of peripheral blood $CD8^+$ T cells and a higher percentage of $CD8^+IL\text{-}4^+$ cells, particularly after *Leishmania* soluble antigen immunostimulation when compared with clinically healthy and subclinically infected

dogs. These data suggest a significant role of $CD8^+$ T cells in the regulation of humoral and cellular immunity and perhaps the progression of CanL (Papadogiannakis and Koutinas, 2015; Matralis et al, 2016).

2 EFFECTOR FUNCTIONS OF NEUTROPHILS

2.1 INTRODUCTION

2.1.1 General characteristics of neutrophils

The term polymorphonuclear leukocytes (PMN) is commonly used to identify granulocytes or granulocytic leukocytes and includes basophilic, eosinophilic and neutrophilic leukocytes, but is generally used with special reference to the neutrophilic leukocytes. Neutrophils are abundant white blood cells, comprising 10% to 70% of the total leukocyte count, depending on the animal species and are characterized by the presence of a multilobular nucleus (2-5 lobes) (Hurrell et al, 2016).

Neutrophils are an important component of innate immunity. These cells are essential to preserve homeostasis of the organism, constituting the primary defense against microbial infections (Zawrotniak and Rapala-Kozik, 2013). Neutrophils derived from the common haematopoietic stem cell (HSC) present in the bone marrow, which gives rise to the myeloid progenitor cell (termed granulocyte/macrophage colony-forming units), promyelocyte (committed to the neutrophil lineage) and subsequently matures into myelocyte. Cells in these three stages of development are able to proliferate and are known as the mitotic pool. After the myelocyte stage, the cells mature into metamyelocytes, band cells and finally into mature or segmented neutrophils (Faurschou and Borregaard, 2003; Kobayashi et al, 2005; Tak et al, 2013). Granulocyte colony-stimulating factor (G-CSF) is the principal factor regulating the neutrophil life cycle by increasing cell proliferation, survival, differentiation and trafficking/mobilization (Kruger et al, 2015).

Mature neutrophils are released into the bloodstream, with a previously described circulatory half-life of less than 7 h (Kobayashi et al, 2005; Borregaard, 2010; Tak et al, 2013). However, it has recently become clear that human neutrophils have a blood lifespan of 5.4 days (Pillay et al, 2010), which changes the paradigm of these cells as short living cells, produced to kill microbes (Bekkering and Torensma, 2013).

2.1.2 Neutrophil granules

Neutrophil precursors package and store proteins in cytoplasmic granules that are used to migrate and fight invading pathogens. Neutrophil granules are classified into three distinct subsets based on the presence of characteristic granule proteins: primary/azurophil granules containing myeloperoxidase (MPO), secondary (specific) granules enclosing lactoferrin, and tertiary or gelatinase granules characterized by the presence of gelatinase.

These granules are formed sequentially during granulocytic differentiation in the bone marrow (Borregaard, 2010). Primary granules are produced on the promyelocyte stage and contain proteolytic enzymes and antimicrobial peptides that are secreted into the phagocytic vacuole to kill phagocytized microorganisms. Secondary granules are formed during the myelocyte stage and enclose antimicrobial substances (*e.g.* proteases, antimicrobial peptides) and matrix metalloproteases (collagenase, gelatinase) that are secreted both into the phagocytic vacuole and into the extracellular space to fight pathogens and to degrade major structural components of the extracellular matrix during extravasation and migration, respectively. Tertiary granules are formed in the metamyelocyte and band stages and contain matrix metalloproteases and receptors necessary for diapedesis and migration. Secretory vesicles produced by endocytosis during the segmented neutrophil stage contain proteins and receptors that are rapidly integrated into the plasma membrane, allowing vascular endothelium attachment and tissue migration in response to several stimuli (Faurschou and Borregaard, 2003; Hostetter, 2012) (Table 3 and Fig. 5). The heterogeneity of granule content reflects the type of proteins being synthesized during the different stages of neutrophil maturation and differences in their mobilization (Borregaard, 2010).

Although neutrophils leave the bone marrow pre-equipped with cytoplasmic granules, they circulate in the blood stream as dormant cells. *In vitro*, stimulation with nanomolar concentrations of inflammatory mediators, such as N-formyl-methionyl-leucyl-phenylalanine (fMLP) leads to a rapid and almost complete discharge of secretory vesicles without significant release of granules. Stimulation with more powerful agonists like phorbol myristate acetate (PMA) induces exhaustive release of gelatinase granules, moderate release of specific granules and low-grade exocytosis of azurophil granules. A

similar hierarchy of exocytosis has been demonstrated *in vivo*. Upon neutrophil stimulation, secretory vesicles and tertiary granules, which are the last to be formed, are rapidly released, followed by secondary granules. Primary granules contain potentially damaging substances and are released later on, during neutrophil activation. The controlled exocytosis of granule content enables the delivery of potentially cytotoxic compounds in a targeted manner, thus preventing widespread damage of the host tissue (Faurschou and Borregaard, 2003; Hostetter, 2012).

Table 3: Content of secretory granules and of secretory vesicles. Adapted from Hostetter (2012).

	Primary granules	**Secondary granules**	**Tertiary granules**	**Secretory vesicles**
Adhesion		β2 Integrins	β2 Integrins	β2 Integrin
NADPH oxidase		$gp91^{phox}/p22^{phox}$	$gp91^{phox}/p22^{phox}$	$gp91^{phox}/p22^{phox}$
Antimicrobial	Myeloperoxidase Lysozyme Defensins Bactericidal permeability-inducing protein (BPI)	Lysozyme Lactoferrin Vitamin B12 binding protein	Lysozyme	
Enzymes	Elastase Cathepsin G Proteinase 3 Sialidase α-Mannosidase β-Glucoronidase Collagenase (nonspecific) Phospholipase A2	Alkaline phosphatase Gelatinase Collagenase (type IV) Sialidase Phospholipase A2	Gelatinase Acetyltransferase Acid phosphatase	Proteinase 3 Alkaline phosphatase
Receptors		u-PA, TNF, complement, fibronectin, thrombospondin, others	u-PA, TNF, complement, others	Complement, TNF, IFN, IL-1, IL-10, fMLP, TGF-β, others
Other Factors	Azurocidin α1-Antitrypsin	β2-Microglobulin Pentraxin-3 Plasminogen activator	β2-Microglobulin	Decay accelerating factor Azurocidin Plasma proteins

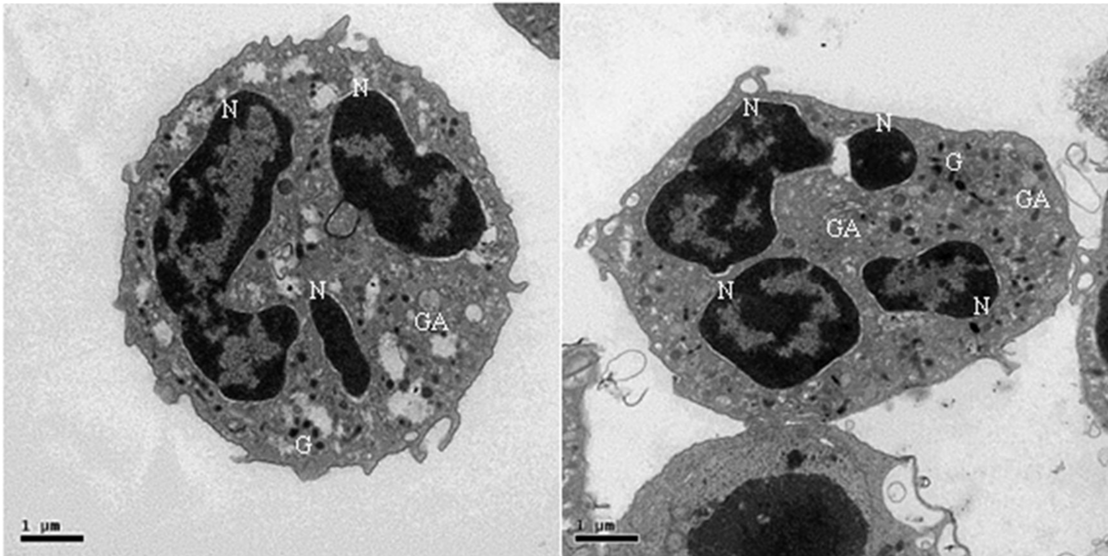

Figure 5: Intracellular morphology of quiescent dog neutrophil. Neutrophils were isolated from dog peripheral blood and incubated for 3 h at 37°C. Cells were fixed, contrasted and embedded in resin. Ultrathin sections were stained with 2% uranyl acetate and 0.5% lead citrate and observed under transmission electron microscopy. Mature neutrophils have about 5 µm of diameter, present a segmented nucleus showing highly condensed heterochromatin (dark) along the inner leaflet of the nuclear membrane. Close to nuclear pores heterochormatin is replaced by euchromatin (light spots). Euchromatin is mostly found in the center of nuclear lobules. The cytosol is filled with primary (bigger and more electro-dense), secondary and tertiary granules and vesicles. The Golgi complex is barely identifiable, and mitochondrias are rarely seen at this stage. N - lobules of the nucleus; G - granules; GA - Golgi apparatus.

2.1.3 Neutrophil recruitment, priming and activation

Upon stimulation with inflammatory mediators, vascular endothelial cells adjacent to the site of infection up-regulate the expression of several adhesion molecules on its surface, including E and P selectins and integrins, such as intercellular adhesion molecule (ICAM) and vascular cell adhesion molecule (VCAM). In neutrophils, the ligands and adhesion molecules that mediate the contact with endothelial cell receptors are L-selectin, E-selectin ligand-1 (ESL-1), and P-selectin glycoprotein ligand-1 (PSGL-1). The initial interactions between ligands and receptors signalize a cascade that leads to the incorporation of secretory vesicles on the cell membrane and cytoskeletal rearrangements to facilitate the firm contact with activated vascular endothelium and diapedesis. Several steps describe neutrophil diapedesis: capture, rolling, slow rolling, activation, spreading, arrest, intravascular crawling, and transcellular and paracellular migration (Hostetter, 2012). The exocytosis of gelatinase granules and partial exocytosis of specific and

azurophil granules release receptors of extracellular matrix components and matrix-degrading enzymes, including collagenase and serine proteases, which facilitate migration (Faurschou and Borregaard, 2003).

Neutrophils are actively guided from the bloodstream to their final destination in tissues by chemoattractants, a variety of diffusible molecules derived from other cells (*e.g.* platelets, MØ, neutrophils, endothelial cells), complement components, invading pathogens and damaged tissue. These chemoattractants are detected by membrane-associated G protein-coupled receptors. IL-8, activated complement components C5a and C3a, platelet activating factor, bacterial derived N-formyl peptides such as fMLP and L, particularly LB are potent chemoattractants for neutrophils (Hostetter, 2012).

In vivo studies showed that the inoculation of *Leishmania* parasites in mice or dogs through needle injection (Pompeu et al, 1991; Beil et al, 1992; Santos-Gomes et al, 2000; Matte and Olivier, 2002; Thalhofer et al, 2011; Ribeiro-Gomes and Sacks, 2012) induces a rapid dermal infiltration of neutrophils. Studies carried out in C57BL/6 mice-*L. major* infected through sand fly bite using two-photon intra-vital microscopy (2P-IVM) confirmed that neutrophils are the first cells to infiltrate the dermis within 30 min (Peters et al, 2008). Comparison of *L. major* injected and sham injected mice demonstrated that at 1 h the neutrophil infiltrate was similar, proving that in part it was induced by the tissue injury associated with the needle injection. However, in the subsequent time points, neutrophil recruitment seems to be dependent on the infectious status of the inoculum (Ribeiro-Gomes and Sacks, 2012). Importantly, neutrophil recruitment to the sand fly bite site was more massive, localized and sustained compared to the needle injection, but the response elicited by *L. major* infected or uninfected bites was proved to be similar (Peters et al, 2008; Peters and Sacks, 2009). These observations show that the contribution of wound repair and saliva components or microbiota inoculated into the skin by the sand fly might prevail over any parasite-derived signals on neutrophil recruitment (Ribeiro-Gomes and Sacks, 2012). Indeed, components of *L. intermedia* and *L. longipalpis* salivary glands were demonstrated to induce a rapid influx of neutrophils in BALB/c mice in response to *L. braziliensis* or *L. chagasi* (Teixeira et al, 2005; de Moura et al, 2010).

However, parasite-derived signals influence human neutrophils migration. The promastigote form of several *Leishmania* species releases a chemotactic factor

(*Leishmania* chemotactic factor, LCF) and induces the secretion of IL-8 by neutrophils, which in turn might amplify the recruitment of human neutrophils (van Zandbergen et al, 2002).

The early influx of neutrophils to the site of *L. major* or *L. infantum* infection in the skin is transient, with numbers returning to normal levels 3 days post-injection and also post-inoculation through the sand fly bite. However, in the last case a localized accumulation of cells was found in the bite site during 8 days after transmission. A second recruitment wave of neutrophils was observed following either needle or sand fly challenge, starting approximately one week after *L. major*-C57BL/6 mouse infection and coincident with the development of cutaneous lesions (Peters et al, 2008; Peters et al, 2009; Peters and Sacks, 2009). It is likely that the signals for recruitment of the first and subsequent waves of neutrophils will be quite distinct, with mediators arising from the adaptive immune response playing a major role (Ribeiro-Gomes and Sacks, 2012).

An important property of chemoattractants is that they can prime neutrophils for enhanced response. Neutrophil "priming" was described as the ability of a primary agonist, typically at a sub-stimulatory concentration, to influence/enhance further activation triggered by a second stimulus. Many priming agents are TLR agonists. Priming typically includes mobilization of secretory vesicles and secretion of cytokines, but fails to induce a complete degranulation or elicit production of superoxide (O_2^-) (Kobayashi et al, 2005; Hostetter, 2012). Interestingly, pre-incubation of neutrophils with extracted SAP from *L. donovani* blocks oxidative burst induced by fMLP (Remaley et al, 1985).

Activation of primed neutrophils occurs *via* exposure to numerous substances, including cytokines, chemokines and pathogen-derived products such as lipopolysaccharides (LPS). PAMP detection by neutrophil PRR (TLR, lectins) is critical for activation and full cell functionality. Neutrophils can also respond to DAMP, also referred to as alarmins, which are released from cells and tissues secondary to necrosis or other forms of tissue damage. Several classes of receptors recognize DAMP, including TLR and the intracellular NOD-like receptors. Detection of DAMP *via* these receptors also results in neutrophil recruitment and activation (Parker, 2005; Bourgeois et al, 2010; Hostetter, 2012).

2.1.4 Phagocytosis and intracellular killing

Phagocytosis is an active, receptor-mediated process by which pathogens are engulfed by the neutrophil and entrapped within a membrane bound vacuole, called phagosome. Ligation of neutrophil TLR, in particular TLR2 and TLR4, activates signal transduction pathways that ultimately prolong cell survival, facilitates adhesion and phagocytosis, enhances the release of cytokines, chemokines and ROS and promotes degranulation, thereby contributing to the microbicidal activity (Hayashi et al, 2003; Kobayashi et al, 2005). However, the efficiency of phagocytosis by neutrophils is markedly enhanced if microorganisms were opsonized with complement components and/or specific antibodies. Activation of the complement cascade promotes the deposition of complement components C3b, iC3b, and C1q on microbial surface. Complement-opsonized microbes are efficiently recognized by complement surface receptors (CR1, CR3 and CR4) on neutrophils. Microorganisms opsonized with specific antibodies are efficiently bound by antibody Fc receptors (FcR) on the surface of neutrophils, facilitating phagocytosis (Kobayashi et al, 2005).

Through the bite of a sand fly vector, *Leishmania* promastigotes are deposited in the dermis of the mammalian host and stay at the mercy of the hostile extracellular environment, such as the complement factors (Domínguez et al, 2002). However, neutrophils rapidly internalize the parasite and become the predominant parasitized cells over the first few hours following *L. donovani* and *L. infantum* infection (Wilson et al, 1987; Thalhofer et al, 2011). *In vitro* studies confirmed that neutrophils phagocytize *L. major* by using both opsonin-dependent and opsonin-independent uptake mechanisms, although with different kinetics. In the presence of fresh serum, the uptake of *L. major* was quicker and massive (47.2% of parasitized neutrophils in 10 min) when compared with heat inactivated serum or serum absence. *In vitro,* blocking of CR3 with anti-CD11b monoclonal antibody showed that the CR3 plays a major role in both serum-dependent and complement-independent uptake of the parasite. In contrast, opsonization through the heat-labile serum factor mannose-binding lectin (MBL) did not contribute to the serum-dependent uptake of *L. major* by neutrophils (Laufs et al, 2002).

Neutrophils use oxygen-dependent and oxygen-independent mechanisms to kill ingested microorganisms (Kobayashi et al, 2005). Phagocytosis of microorganisms triggers the

fusion of primary and secondary granules with the membrane of the phagosome and the rapid assembly of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Segal, 2005). NADPH oxidase (PHOX) is formed by the membrane-associated flavocytochrome b_{558} complex [gp91^{phox} (Nox2) and p22^{phox} (CYBA)] and cytosolic components (p40^{phox}, p47^{phox}, p67^{phox}), which are not associated in a resting or primed neutrophils. During phagocytosis, the cytosolic components translocate to the plasma and/or phagosome membrane and associate with the membrane-bound b_{558} complex to create a functional NADPH oxidase. The translocation of cytosolic components is mediated through the phosphorylation of multiple tyrosine and serine residues on p^{47phox} and p^{67phox} due to the phosphorylation activities of a variety of kinases. Various PKC isoforms, PKA and MAPK, are known to activate the respiratory burst. Then NADPH oxidase transfers electrons from the NADPH cytosolic to intraphagosomal molecular oxygen, thus producing O_2^-.

O_2^- is short-lived molecule that dismutates rapidly to hydrogen peroxide (H_2O_2), either spontaneously or through an enzyme catalyzed reaction involving superoxide dismutase. MPO, an abundant hemoprotein stored within the primary/azurophilic granules is released into the phagosome and catalyzes a reaction with chloride and hydrogen peroxide to produce hypochlorous acid (HOCl), a potent microbicidal agent (MPO-halide system) and other secondary ROS (Stafford et al, 2002; Kobayashi et al, 2005; Hostetter, 2012). However, some researchers suggest that the main function of ROS might be associated with the induction and increased activity of proteases and also of other granule-derived proteins (Reeves et al, 2002; Ahluwalia et al, 2004). Fusion of primary granules with phagosome enriches the vacuole lumen with numerous anti-microbial peptides, including α-defensins, cathepsin G (catG), proteinase-3, elastase (NE), azurocidin and lysozyme. Secondary granule compounds enrich the phagosome with flavocytochrome b_{558} and lactoferrin, augmenting the anti-microbial potential (Kobayashi et al, 2005). Defects in ROS production or granule content result in severe immunodeficiency, such as neutrophil specific granule deficiencies or chronic granulomatous disease (Perobelli et al, 2015).

L. donovani interferes with neutrophils ROS production (Remaley et al, 1984). *In vitro* assays revealed that human neutrophils kill *L. donovani* promastigotes through $H_2O_2^-$ peroxidade-halide pathway (Pearson and Steigbigel, 1981). Studies using neutrophils isolated from *L. infantum* naturally infected dogs showed contradictory results. Inhibition

of oxidative metabolism was observed by chemiluminescence assay (Brandonísio et al, 1996; Vuotto et al, 2000), whereas increased O_2^- production was assessed by the nitroblue tetrazolium reduction assay (Ciarlini et al, 2010; Gómez-Ochoa et al, 2010). A more recent work revealed that O_2^- production was dependent on CanL stage. Neutrophils isolated from dogs with moderate disease showed higher O_2^- production, while those with severe disease presented low amounts (Almeida et al, 2013).

2.1.5 Extracellular killing

Neutrophils kill microorganisms in the extracellular space by degranulation (exocytosis) of antimicrobial proteins (Kobayashi et al, 2005). Despite its microbicidal role, degranulation is also involved in immunomodulation (Kobayashi, 2015). Azurocidin, released from neutrophil secretory vesicles or primary granules, acts as a chemoattractant and activator of monocyte and MØ (Soehnlein and Lindbom, 2009). Pentraxin 3 (PTX3), which is stored in specific granules, functions as an opsonin and can also activate complement. However, PTX3 can limit neutrophil recruitment to sites of inflammation by binding P-selectin on endothelial cells, thereby reducing neutrophil rolling. PTX3 may have an additional role in tissue repair and remodeling (Hostetter, 2012).

Neutrophil Extracellular Traps (NET) are effector mechanisms elicited by activated neutrophils. NET are characterized by the extracellular release of decondensed DNA along with cytoplasmic, granular and nuclear proteins (histones), providing a localized high concentration of antimicrobial molecules able to trap microorganisms (Brinkmann et al, 2004; Brinkmann and Zychlinsky, 2007; Fuchs et al, 2007; Amulic and Hayes, 2011; Vorobjeva and Pinegin, 2014). NET include histones, defensins, NE, proteinase 3, heparin binding protein, catG, lactoferrin and MPO (Urban et al, 2009). The process of NET generation also called NETosis is considered a specific type of cell death different from necrosis and apoptosis (Fuchs et al, 2007; Borregaard, 2010; Zawrotniak and Rapala-Kozik, 2013). Several nuclear and cytoplasmic events must take place to complete NETosis. ROS are required to initiate NET formation (Fuchs et al, 2007). NE escapes from azurophilic granules and translocates to the nucleus, where it partially degrades specific histones. This enzymatic process is enhanced by MPO that promotes chromatin decondensation (Papayannopoulos et al, 2010; Metzler et al, 2011). During NET

generation, Histone H3 undergoes a modification (citrullination) that converts arginine residues to citrulline, resulting in chromatin decondensation. Histones citrullination is catalyzed by peptidylarginine deiminase 4 (PAD4), which is found in the neutrophil nucleus (Wang et al, 2009b). However, neutrophils primed with granulocyte/macrophage colony-stimulating factor (GM-CSF) and stimulated with LPS or C5a for a short period are able to generate NET, mainly comprised of mitochondrial DNA (Yousefi et al, 2009).

There are several microbial and chemical factors that stimulate NET formation (Guimarães-Costa et al, 2012). Human neutrophils activated by *L. amazonensis*, *L. donovani* and *L. infantum* (Guimarães-Costa et al, 2009, 2014; Gabriel et al, 2010) and mouse neutrophils activated by *L. mexicana* release NET (Hurrell et al, 2015). LPG was implicated in NET generation induced by *L. amazonensis*, *L. major* and *L. chagasi* promastigotes in human neutrophils and NET possess leishmanicidal activity conferred by Histone H2A (Guimarães-Costa et al, 2009). However, NET induced by *L. donovani* promastigotes in human neutrophils was LPG, gp63 and O_2^- independent. Furthermore, LPG protects the parasite from NET-mediated killing (Gabriel et al, 2010) and *L. infantum* avoided NET-mediated killing through their enzyme 3'-nucleotidase/nuclease (3'NT/NU) activity (Guimarães-Costa et al, 2014), whereas NET induced by *L. mexicana* did not kill the parasite (Hurrell et al, 2015). These differences might be due to the variation in LPG structure, parasite strains used and experimental design (Abi Abdallah and Denkers, 2012).

2.1.6 Neutrophil apoptosis

Normal turnover of aging neutrophils occurs in the absence of cell activation through a process known as constitutive apoptosis. Apoptotic neutrophils are then cleared by MØ in the liver, spleen and bone marrow (Saverymuttu et al, 1985; Kobayashi et al, 2005). Constitutive apoptosis is the final stage of the neutrophil life cycle and it is regulated by intracellular signaling and changes in gene expression ("apoptosis differentiation program"). The intrinsic ability of neutrophils to undergo apoptosis is essential to preserve the appropriate amount of cells circulating in the bloodstream (McCracken and Allen, 2014). Since apoptosis prevents the release of toxic intracellular components that can damage healthy tissue, precise regulation of neutrophil apoptosis is essential for the

resolution of inflammation (Borregaard et al, 2007; Geering and Simon, 2011). The loss of plasma membrane asymmetry in the apoptotic cells, characterized by the accumulation of phosphatidylserine (PS) in the extracellular leaflet, facilitates recognition of apoptotic cells by MØ and their internalization *via* a process called efferocytosis. Efferocytosis of dying neutrophils reduces proinflammatory cytokine production and reprograms MØ to a pro-resolution phenotype that favors the restoration of tissue homeostasis (McCracken and Allen, 2014).

Apoptosis is a conserved mechanism of programmed cell death. Three apoptotic pathways have been described: the extrinsic pathway, the intrinsic pathway and phagocytosis-induced cell death (PICD). The extrinsic pathway is activated by ligation and oligomerization of Fas, TNF receptor 1 or the TRAIL receptor that triggers the formation of a receptor-associated death-inducing complex for recruitment and activation of caspase-8. The intrinsic apoptotic pathway is initiated when the relative abundance of the proapoptotic Bcl-2 family members (Bax and Bak) exceeds that of their anti-apoptotic counterparts (Mcl-1 and A1), which allows Bax and Bak to oligomerize and disrupt the outer mitochondrial membrane, releasing cytochrome C into the cytosol that is an essential signal for the activation of caspase-9. The third apoptotic pathway, PICD, couples microbe killing to accelerated neutrophil death at sites of infection (McCracken and Allen, 2014).

Caspases are a family of cysteine proteases that reside in the cytosol as inactive pro-enzymes. This inactive state is preserved by direct association with molecules from the family of inhibitor of apoptosis protein (IAP). Caspase-8 and caspase-9 are the initiator caspases of the extrinsic and intrinsic apoptosis pathways, respectively, that in turn mediate activation of the executioner caspase-3 to induce many of the changes that occur during apoptosis (McCracken and Allen, 2014).

Regardless the specific pathway that is activated, apoptotic neutrophils undergo characteristic morphological changes, namely cell rounding and shrinking, loss of microvilli and membrane blebbing (zeiosis). At an early stage, condensed chromatin (pycnotic nuclei) tends to marginate in crescents around the nuclear envelope. The nuclear envelope disintegrates and lamin proteins undergo proteolytic degradation, followed by nuclear fragmentation (karyorrhexis). Many nuclear fragments of different sizes are

scattered throughout the cytoplasm. The nuclear fragments, together with constituents of the cytoplasm (including intact organelles) are packaged and enveloped by the remaining plasma membrane, originating the ''apoptotic bodies'', which are then shed from the dying cell (Darzynkiewicz et al, 1997; Silva et al, 2008). Activation of endonucleases generates nucleosomal and oligonucleosomal DNA sections that produce a characteristic ''ladder'' pattern during agarose gel electrophoresis. The accumulation of PS in the extracellular leaflet is another feature that can be used to quantify the apoptosis kinetics (Darzynkiewicz et al, 1997; McCracken and Allen, 2014).

The ability of microorganisms to manipulate neutrophil turnover has been largely demonstrated. Some pathogens delay neutrophil death to maintain viability of their replicative niche, others accelerate apoptosis or trigger neutrophil lysis to evade intracellular killing, while others still use the infected neutrophils as "Trojan horses" for subsequent infection of MØ (McCracken and Allen, 2014). Several species of *Leishmania* were seen to modify the neutrophil programmed cell death, prolonging life span or accelerating cell death. Although *L. amazonensis* accelerates the apoptosis of mouse neutrophils (Carlsen et al, 2013), *L. donovani* delays mouse neutrophil apoptosis (Gueirard et al, 2008) and *L. major* promastigotes inhibit human neutrophil apoptosis by decreasing caspase-3 activity (Aga et al, 2002). A recent work revealed that neutrophil apoptosis depended on the stage of CanL. The rate of neutrophil apoptosis was higher in dogs with very severe disease when compared with moderate stage disease (Almeida et al, 2013).

2.1.7 *Leishmania*-neutrophils interaction

The role of neutrophils in cutaneous leishmaniosis has been extensively studied. However, the impact of neutrophils in host resistance or susceptibility to the infection by *Leishmania* visceralizing species remains poorly characterized (Ribeiro-Gomes and Sacks, 2012).

The fate of ingested parasites is a question that needs to be clarified. Human and mouse neutrophils ability to efficiently kill *L. infantum* and *L. donovani* promastigotes *in vivo* and *in vitro* was confirmed by electron microscopy analysis (Pearson and Steigbigel,

1981; Rousseau et al, 2001). *In vitro* studies showed that *L. donovani* uptake by mouse and dog neutrophils *via* a lytic organelle-dependent pathway leads to the formation of large phagosomes and to parasite degradation, but the uptake *via* a lytic organelle-independent pathway, observed for around 20-25% of the parasites, was characterized by the formation of tight phagosomes that ensure parasite survival. Parasite survival was associated with their trafficking to an ER-like compartment (Gueirard et al, 2008). *L. donovani* promastigotes were also found to survive in human neutrophils due to the modulation of the granule fusion process. Tertiary and secondary granules, involved in vacuole acidification and O_2^- generation hardly fused with *L. donovani*-containing phagosomes (Mollinedo et al, 2010). Not surprisingly, intracellular parasite survival seems to be mediated by promastigote-derived molecules. Tartrate-resistant acid phosphatase isolated from the external surface of *L. donovani* promastigotes inhibits O_2^- production by human neutrophils (Remaley et al, 1985) and LPG prevents granule fusion (Gueirard et al, 2008).

Serum-dependent phagocytosis of *L. major* leads to human neutrophil activation, resulting in the rapid killing of intracellular parasites, whereas phagocytosis in the absence of fresh serum did not activate neutrophils and the majority of intracellular parasites survived the first day after being internalized. The activation of the oxidative burst was found to be associated with the elimination of intracellular parasites (Laufs et al, 2002).

Although neutrophils possess some direct leishmanicidal activity *in vitro*, parasite persistence at the site of infection indicates that promastigote killing by neutrophils is clearly insufficient in controlling the establishment of infection and the development of clinical disease (Carlsen et al, 2015). Indeed, several reports showed that a subset of parasites can survive inside neutrophils, but no multiplication has been observed (Müller et al, 2001; Laufs et al, 2002). *Leishmania* maintains their promastigote form, as concluded by electron microscopy and by time-lapse microscopy, which showed the flagella movement of *Leishmania* parasites inside neutrophils 42 h after infection (van Zandbergen et al, 2004). The parasite viability following *in vivo* phagocytosis by neutrophils was studied using a stain of *L. major* expressing RFP and mice expressing eGFP under the control of the endogenous lysozyme M promoter. Infected neutrophils isolated by sorting maintained the capacity to produce infection in naïve mice (Peters et

al, 2008). These findings suggest that although neutrophils do not kill *L. major* promastigotes, the intracellular environment is not suitable for replication. Therefore, neutrophils might serve as temporary host cells for the parasites within the first hours/days after infection (Aga et al, 2002; Laufs et al, 2002; van Zandbergen et al, 2004).

The role of neutrophils in promoting or suppressing the anti-leishmanial response *in vivo* was addressed primarily by treating mice with neutrophil depleting antibodies (functional studies) (Ribeiro-Gomes and Sacks, 2012). C57BL/6 mouse neutrophil-depleted and infected with *L. chagasi* showed a delay in the elimination of the parasite from the inoculation site. Furthermore, six days post-infection, depleted mice evidenced high IL-17 gene expression, a neutrophil regulatory cytokine and a reduction in IFN-γ gene expression which might favor disease development. On the contrary, infected mice evidenced high IFN-γ gene expression, indicating a Th1 polarization of the immune response, important for disease control (Marques et al, 2013). Neutrophil depleted BALB/c mouse infected by *L. donovani* showed a significant increase of parasite load in the spleen and bone marrow and, to a lesser extent, in the liver associated with a Th2 polarization of the immune response (McFarlane et al, 2008). Neutrophil depleted C57BL/6 mouse infected by *L. donovani* revealed a dramatic enhancement of parasite growth in both the liver and the spleen (Smelt et al, 2000). Studies performed in neutrophil depleted BALB/c mice-*L. infantum/chagasi* infected showed a significant slowdown in the clearance of the parasite from the inoculation site (Thalhofer et al, 2011) and an increase in the parasite load in the spleen, but not in the liver (Rousseau et al, 2001). Although these studies used different visceralizing *Leishmania* species and mouse strains, the effector mechanisms developed by neutrophils in the early phase of infection seem to confer resistance to *Leishmania* infection, influencing the parasite load in different organs and contributing to the polarization of the immune response.

2.2 OBJECTIVES

This study aims at characterizing *in vitro* the immune response developed by PMN isolated from dog peripheral blood when exposed to *L. infantum* by assessing (i) parasite-neutrophil interaction; (ii) phagocytic and (iii) chemotaxis capacity, (iv) O_2^- production, (v) granule content exocytosis, (vi) generation of NET and (vii) cell viability and

apoptosis. Additionally, (viii) parasite viability and replication after contact with neutrophils were also assessed.

2.3 MATERIAL AND METHODS

2.3.1 Animals

A group of 20 dogs with more than 20 kg of weight and aged between 1.5 and 5 years belonging to Grupo de Intervenção Cinotécnico da Guarda Nacional Republicana and to Faculdade de Medicina Veterinária, Universidade de Lisboa (FMV-UL) was selected. Animals were subject to a complete physical examination and blood was collected for hematology, biochemistry, ionogram and proteinogram to assess their health status. Detection of anti-*Leishmania* antibodies by indirect immunofluorescence assay (Kit *Leishmania* Spot IF®, BioMerieux, France) with a cutoff of 1:80 and blood and lymph node evaluation by q-PCR (Helhazar et al, 2013) (Applied Biosystems 7300 Real Time PCR System) were used to exclude *L. infantum* infection. The absence of *Dirofilaria immitis* infection was confirmed by Knott test. Other hemoparasites, such as *Babesia* spp., *Ehrlichia* spp., *Anaplasma* spp. and *Ricketsia* spp. were ruled out by microscopic observation of blood smears and by q-PCR, and *Mycoplasma haemocanis* by blood smear observation (Table 4). Only healthy animals with negative parasitological results were included in the experiments and their owners were fully informed about the objective and nature of interventions and official consents were obtained. The study was approved by the Ethics Committee of FMV-UL.

Table 4: Forward (FW) and reverse (RV) primers used to detect different microorganisms by q-PCR, the respective probes and base pair number (bp) of the amplified fragments.

Gene	Primers (FW, RV) and Probes	Fragment size (bp)
L. infantum	FW-5`GGAAGGTGTCGTAAATTCTGGAA3` RV-5`CGGGATTTCTGCACCCATT3` FAM AATTCCAAACTTTTCTGGTCCTCCGGGTAG TAMRA	124
Babesia spp.*	FW-5`ACCCATCAGCTTGACGGTAGGGT3` RV-5`AGCCGTCTCTCAGGCTCCCT3` JOE ACCGAGGCAGCAACGGGTAACGGGGA TAMRA	97
Ehrlichia spp. * *Anaplasma* spp. *	FW-5`ACCTATAGAAGAAGTCCCGGCAA3` RV-5`ACCTACGTGCCCTTTACGCCC3` FAM GCAGCCGCGGTAATACGGAGGGGGC TAMRA	100
Ricketsia spp. *	FW-5`AACCGCAGCGATAATGCTGAGTAGT3` RV-5`CCCTGCAGAAGTTATCTCATTCCAA3` JOE AGCGGGGCACTCGGTGTTGCTGCA TAMRA	130

* According to the protocol implemented in the Virology laboratory of FMV-UL.

2.3.2 Parasites

L. infantum zymodeme MON-1 (MHOM/PT/89/IMT151) virulent promastigotes collected in the stationary phase of growth of subcultures with less than five passages (Santos-Gomes and Abranches, 1996) and *L. infantum* expressing Green Fluorescent Protein (GFP) (Marques et al, 2015) were used for neutrophil infection. Parasites were maintained in Schneider medium (SCHN) (Sigma-Aldrich, Germany) supplemented with 10% (v/v) of heat-inactivated fetal bovine serum (FBS) (Sigma-Aldrich), penicillin-streptomycin (Biochrom, Germany) at 100 U.mL^{-1} and 100 µg.mL^{-1}, respectively (complete SCHN medium). In the case of GFP parasites, 100 µg.mL^{-1} of geneticin (Sigma-Aldrich) was also added to the culture medium. Parasite concentration was assessed in a Neubauer-counting chamber (Marienfeld, Germany).

2.3.3 Isolation and purification of dog PMN

The blood collection was made according to the veterinary norms with therapeutic shearing and antisepsis of venipuncture site with povidone-iodine and ethanol. Each time 20 mL of blood was collected using citrate phosphate dextrose adenine (CPDA)-1 solution as anticoagulant (Kawasumi, Germany). PMN isolation was achieved using a Histopaque double-density gradient, according to the technique described by Strasser et

al (1998). The opaque band between Histopaque-1077 (Sigma-Aldrich) and Histopaque-1119 (Sigma-Aldrich) containing PMN was collected and washed with 0.9% (w/v) NaCl. 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 resuspended in Hanks' Balanced Salt Solution (HBSS) (Sigma-Aldrich).

2.3.4 PMN cultures and controls

With some exceptions that will be mentioned in the respective assays, dog PMN (5×10^5 cells/well) were seeded in 96-well plates (Nunc, Denmark) with *L. infantum* or *L. infantum*-GFP promastigotes at a ratio parasite-PMN of 5:1 in 300 µL of HBSS supplemented with 5% heat inactivated FBS (v/v). Plates were incubated at 37ºC in a humidified atmosphere containing 5% of CO_2 for 1.5 h and 3 h. In parallel, PMN cultures were also established to serve as negative controls.

L. infantum total antigen (Ag) was used in the chemotaxis assay. Ag was produced as described by Diaz et al (2012). Briefly, promastigotes were centrifuged at 1830 ×*g* for 15 min at 4ºC and then washed three times with PBS/ETDA (Sigma-Aldrich) at 1830 ×*g* for 15 min at 4ºC. The pellet underwent six cycles of freezing and thawing (-20ºC, -70ºC and room temperature). The protein concentration was assessed using a spectrophotometer (NanoDrop 1000, Thermo Fisher Scientific, USA) and adjusted at 40 $\mu g.mL^{-1}$. The antigen was conserved at -20ºC until used.

LCF (van Zandbergen et al, 2002) was used in the chemotaxis assay as positive chemoattractant, promoting PMN migration. *L. infantum* supernatants obtained after culture centrifugation at 1800 ×*g* for 10 min were concentrated in an Amicon Ultra (Millipore Corporation, USA) device through centrifugation at 4000 ×*g* for 20 min. LCF was conserved at -20ºC.

Phorbol myristate acetate (PMA) (VWR, International) was used in the O_2^- and NET assays as a positive control. PMA is an activator of PKC and thus enhances the oxidative burst. PMA is also a recognized NET inducer (Brinkmann et al, 2010).

Escherichia coli lipopolysaccharide (LPS) (Sigma-Aldrich) was used as positive control in the exocytosis of NE and catG assays. LPS is a major constituent of the cell wall of most gram negative bacteria. It is a highly immunogenic antigen with the ability to enhance the immune response.

(S)-(+)-camptothecin (Sigma-Aldrich) (Campto) was used as apoptotic positive control. Campto binds irreversibly to the DNA-topoisomerase I complex, blocking the cell cycle in S-phase and inducing apoptosis in a large number of cells by cell cycle-dependent and cell cycle-independent processes.

2.3.5 PMN purity and viability

Cell purity was determined by flow cytometry analysis. PMN cultures were washed two times with cold 1× phosphate-buffered saline (PBS) (Lonza, Belgium) (300 ×*g*, 10 min, 4ºC), fixed with 2% paraformaldehyde (Sigma-Aldrich) (w/v) for 20 min at room temperature and resuspended in PBS. Flow cytometry acquisition was performed using a 4-color flow cytometer (BD FACSCalibur, BD Biosciences, USA) at Chronic Diseases Research Center (CEDOC), NOVA Medical School, Universidade Nova de Lisboa, Portugal with the collaboration of Professor Graça Alexandre-Pires. Forward scatter-height (FSC-H) *vs* side scatter-height (SSC-H) gate was used to remove debris and pyknotic cells in the lower left-hand portion of the plot as well as the very large (off-scale) debris found in the upper right-hand portion. Singlet gate was used to define the non-clumping cells based on pulse geometry FSC-H *vs* forward scatter-area (FSC-A), eliminating the doublets. PMN population was identified based on FSC-H *vs* SSC-H gate. Data was analyzed using Flowjo V10 (Tree Star Inc., USA). Neutrophil purity was determined by morphological analysis under an optical microscope (Olympus BX41). PMN suspension (100 µL) was deposited on glass slides, cytocentrifuged (citospin) at 55 ×*g* for 4 min (StatSpin 2 Cytofuge, USA), and fixed and stained with Hemacolor staining kit (Merck Millipore, Germany). Cell viability and concentration were assessed by trypan blue exclusion in Neubauer-counting chamber.

2.3.6 Chemotaxis assay

The migratory ability of *L. infantum*-exposed PMN in response to a chemoattractant stimulus, parasite antigen and viable promastigotes was evaluated in a 96-well modified Boyden chamber (Neuroprobe Inc., USA) containing a polycarbonate 3 µm pore membrane. Lower compartment was filled with 29 µL of *L. infantum* total Ag or 5×10^5 *L. infantum* promastigotes. LCF was used as positive control and HBSS as negative control. In the upper compartment of the chamber 25 µL of resting-PMN and PMN-*L. infantum* cultures were added. The chamber was incubated for 60 min at 37 ºC in a humidified atmosphere containing 5% of CO_2. Migration of PMN to the lower compartment was assessed in a Neubauer-counting chamber by optical microscopy (OM).

2.3.7 Interaction of *L. infantum* promastigotes with PMN

The interaction between *L. infantum*-GFP parasites and PMN was evaluated by flow cytometry. After 1.5 h, 3 h and 4.5 h of incubation, *L. infantum*-GFP, resting-PMN and PMN exposed to *L. infantum*-GFP were acquired by flow cytometry. The gating strategy referred above excluded the vast majority of free parasites, debris and pyknotic cells as well as the doublets. Resting-PMN and *L. infantum*-GFP promastigote cultures were used to calibrate the cytometer, compensating the overlap of emission spectra. Compensation was performed after signal detection, but before digitalization (hardware compensation). PMN population was identified in a pseudocolor plot based on FSC-H *vs* SSC-H gate. Percentage of PMN that internalize or bind the parasite was assessed on a histogram of only one parameter, plotting FL1-H/GFP *vs* the number of events. Resting-PMN were used as control. Additionally, the contact between parasites and neutrophils was morphologically evaluated in citospins, fixed, stained with Hemacolor staining kit or with the fluorescent nuclear dye 4’, 6-diamidino-2-phenylindole (DAPI) (Vectashield, Vector Laboratories, USA) and observed under optical or fluorescence microscope (Nikon Eclipse 50i), respectively.

2.3.8 Superoxide production

O_2^- production was measured using a colorimetric nitroblue tetrazolium (NBT) assay (Marques et al, 2015). Yellow-colored nitroblue tetrazolium (Y-NBT) (Sigma-Aldrich) membrane permeable is absorbed by cells and reduced to water-insoluble blue formazan particles (NBT) by intracellular O_2^-. PMN cultured with *L. infantum* were incubated in FBS-free HBSS 0.4% (w/v) NBT (Sigma-Aldrich). Cells were washed with warm PBS to remove extracellular NBT and solubilized with 100 µL of 10% (w/v) sodium dodecyl sulfate (SDS) (Sigma-Aldrich) and 100 µL of 0.1N HCl. Absorbance was measured at 570 nm using a microplate reader (Anthos 2010, Austria). Resting-PMN were used as negative control and, PMA (0.2 $\mu g.mL^{-1}$)-stimulated PMN and PMA-stimulated PMN exposed to parasites were considered as positive controls. Cell free-wells were used as a blank.

2.3.9 Exocytosis of NE and catG

NE and catG exocytosis were measured in culture supernatants of resting-PMN (negative control), PMN stimulated with 1 $\mu g.mL^{-1}$ of LPS (positive control) and PMN exposed to *L. infantum* promastigotes, using specific colorimetric substrates (Marques et al, 2015). In a 96 well plate, 1mM of the NE substrate N-Methoxysuccinyl-Ala-Ala-Pro-Val-pNA (Sigma-Aldrich) and 2.5 mM of the catG substrate N-Succinyl-Ala-Ala-Pro-Phe-pNA (Sigma-Aldrich) diluted in 100 µL of 25 mM reaction buffer (Tris-HCl pH 7.5) was added to 100 µL of culture supernatants. Substrates were cleaved by the respective enzyme, producing a colorimetric reaction that was quantified at 405 nm in a plate reader (TRIADTM 1065, DYNEX Technologies, EUA) at ≈ 0 min (immediately after the addition of substrate to the supernatants) and after 15 min and 30 min of incubation at 37ºC in a humidified atmosphere containing 5% of CO_2. It was assumed that the intensity of the color is proportional to the activity of the enzyme under study.

2.3.10 Watching PMN-parasite relationship through different microscopy techniques

PMN-parasite interrelation was evaluated by scanning electron microscopy (SEM), immunofluorescence and transmission electron microscopy (TEM).

SEM uses a focused beam of high-energy electrons to interact with the surface of a solid sample, producing a variety of signals that contain information about the sample surface topography and composition. SEM generates 2-dimensional, high-resolution images (Vernon-Parry, 2000) and was used to study NET release and PMN-parasite interaction. Resting-PMN, PMN incubated with *L. infantum* promastigotes, PMN stimulated with 1 $\mu g.mL^{-1}$ of PMA and PMN stimulated with PMA and exposed to *L. infantum* promastigotes adhered to coverslips were incubated in HBSS 2% FBS. After incubation, slides were fixed with PBS 4% paraformaldehyde (VWR, International) (v/v) and prepared for SEM, according to Brinkmann et al (2010). Briefly, cells were postfixed with 2.5% of glutaraldehyde (Merck, Germany) (v/v), treated with 0.5% of osmium tetroxide (Sigma-Aldrich) (v/v) and 1% of tannic acid (Sigma-Aldrich) (w/v) and dehydrated with an ascending ethanol series. At the Unidade de Microscopia, Faculdade de Ciências da Universidade de Lisboa, Lisbon, Portugal, the samples were dried using the critical point drying method, coated with gold palladium and mounted on stubs. Cells were then observed under a scanning electronic microscope (JEOL 5200-LV) and images were acquired with the collaboration of Professor Graça Alexandre-Pires and Dr. Telmo Nunes. The number of PMN forming NET was counted in 50 cells for each condition and animal. The orientation of promastigotes bound to PMN (*via* flagellum, aflagellar pole, or by any other place of promastigote body) was evaluated in a total of 50 interactions PMN-parasite.

Immunolabeling assay was used to confirm the presence and composition of NET released by dog PMN stimulated as described above. Fixed samples were washed three times for 5 min with PBS and prepared for NET detection by immunolabeling, according to Brinkmann et al (2010). Coverslips were incubated in a drop of 0.5 % Triton X-100 (Sigma-Aldrich) (v/v) for 1 min at room temperature to permeabilize cells and watched again three more times in PBS for 1 min. In a humid chamber, coverslips were upside down on a drop of blocking buffer (PBS 5% FCS) and incubated for 30 min at 37°C. The

primary antibody Histone H1 (AE-4) FITC (Santa Cruz Biotechnology, Germany) was diluted in blocking buffer and coverslips were transferred directly onto a drop of diluted antibody. Coverslips were incubated for 1 h at 37°C and then washed three times with PBS for 5 min. Finally, coverslips were stained with DAPI, mounted on a glass slide with cells upside down and observed under a fluorescence microscope (Nikon Eclipse 90i).

TEM involves the passage of an electron beam through ultrathin sections of plastic-embedded samples. The differential transmission of the electrons creates a high resolution image (Graham and Orenstein, 2007). TEM was used to study PMN-parasite interrelation from an ultrastructural point of view. Resting-PMN and PMN exposed to *L. infantum* parasites for 3 h were centrifuged at 300 ×*g* for 10 min and fixed in PBS 2% glutaraldehyde and 0.1% tannic acid for 1 h at 4ºC. Then, pellets were washed two times in PBS, resuspended and maintained at -4ºC until used. At the Departamento de Patologia, Faculdade de Medicina da Universidade de São Paulo, Brazil, with the collaboration of Professor Felipe Passero, samples were post-fixed with a solution of 0.9% NaCl with 1% osmium tetroxide, contrasted with 0.9% NaCl 0.5% uranyl acetate (w/v) for 12 h and then embedded in araldite resin. Ultrathin sections (70 nm) obtained using the Reichert ultramicrotome were subject to double staining with 2% uranyl acetate and 0.5% lead citrate (w/v). Samples were observed under transmission electron microscope (Jeol 1010) and images were acquired.

2.3.11 PMN cell death

Cell viability, apoptosis and necrosis were assessed by flow cytometry analysis. Resting-PMN (negative control), PMN exposed to *L. infantum* promastigotes and PMN stimulated with 100 $\mu g.mL^{-1}$ of Campto (apoptotic positive control) were incubated in HBSS 10% FBS. After incubation, cultures were washed two times with 500 µL of cold PBS 1 × (300 ×*g*, 10 min, 4ºC) and incubated with the commercial kit TACS^{TM} Annexin V FITC (R&D Systems, USA), according to manufacturer's instructions. Prior to the flow cytometry acquisition, cells were treated with 10 µL of propidium iodine (PI) (R&D Systems). The gating strategy described in 2.3.5. was used to exclude the extracellular parasites, debris and the doublets. Untreated and annexin V FITC or PI treated cells were used to compensate for PMN autofluorescence and for the fluorochrome fluorescence

overlapping emission spectra. The 5% probability contour plots with outliers display were applied to gate PMN population based on SSC-H *vs* FCS-H strategy. FL1-H (Annexin V FITC) *vs* FL2-H (PI) gate on untreated-PMN was used to delimit annexin V $FITC^-/PI^-$ population (viable cells), annexin V $FITC^+/PI^-$ (apoptotic cells) and annexin V $FITC^+$ or $^-$ $/PI^+$ cells (total necrotic cells). Necrotic cells were also subdivided in annexin V $FITC^-$ $/PI^+$ (primary necrotic cells) and annexin V^+/PI^+ (secondary necrotic cells) and in three subsets: necrotic 1 (n1), n2 and n3, according to the level of PI expressed.

2.3.12 Viability of *L. infantum* promastigotes after PMN contact

The impact of dog PMN in the viability of *L. infantum* parasites was also evaluated. *L. infantum* promastigotes were added to PMN and cultures were incubated for 2 h. Then, 300 µL of culture were seeded on a 24-wells plate containing 300 µL of complete SCHN medium *per* well and incubated at 24ºC for 24 h. In parallel, cultures of *L. infantum* promastigotes were used as positive control. Viable promastigotes (moving parasites) were counted in a Neubauer-counting chamber.

2.3.13 Statistical analysis

Assays were realized in, at least, 10 different dogs, except SEM, TEM and immunofluorescent assays that were performed on three dogs. Each sample was analyzed in triplicate or quadruplicate. The non-parametric Wilcoxon Signed Ranks Test was used to compare paired groups in relation to each parameter studied. Differences were considered significant with a 5% significance level ($p<0.05$). The statistical analysis was performed using the SPSS 22 for Windows software (SPSS Inc., USA). Graphs were prepared by using GraphPad Prism version 6 for Windows (GraphPad Software, USA).

2.4 RESULTS

2.4.1 Double-density gradient protocol ensures higher cell purity

PMN were isolated from dog peripheral blood using the Histopaque double-density gradient protocol. Purity of cell suspension obtained from the lower opaque band was assessed by flow cytometry analysis after incubation for 1.5 h and 3 h, revealing PMN purity higher than 93% (Fig. 6A). Morphological analysis confirmed neutrophil purity higher than 95% and absence of contamination with red blood cells (Fig. 6B). Cell viability assessed by trypan blue exclusion was higher than 95%. This protocol ensures a cell yield between $1.5{\times}10^{6}.mL^{-1}$ and $2.5{\times}10^{6}.mL^{-1}$. Due to the higher recovery of purified and viable neutrophils, this protocol was used throughout the present study.

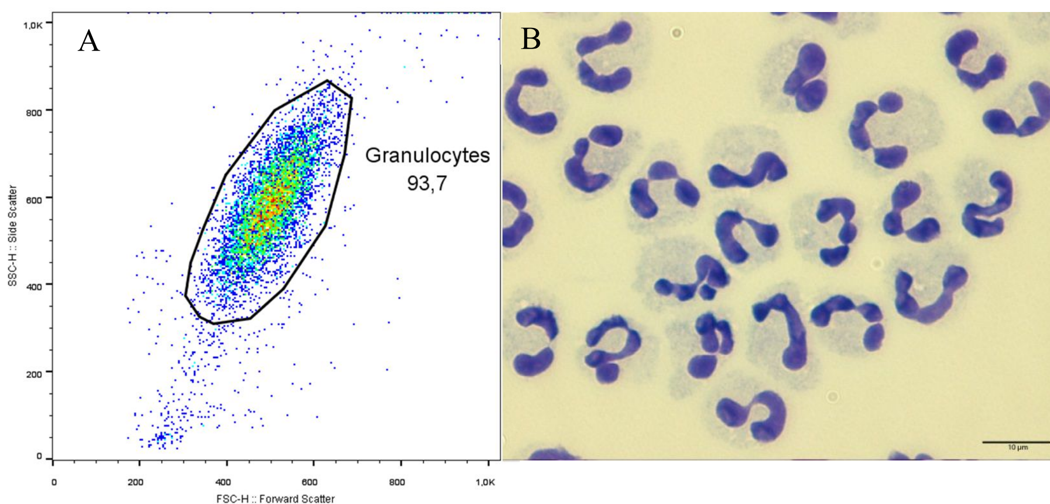

Figure 6: Purity of neutrophils isolated from dog peripheral blood. Isolated PMN were left to incubate for 1.5 h and 3 h and cell purity was evaluated by flow cytometry (A) and by morphological analysis through optical microscopy (B). Representative pseudocolor plot (A) showing the gating strategy based on FSC-H *vs* SSC-H analysis that was used to select PMN population. Citospin (B) of neutrophil culture incubated for 1.5 h (×1000 magnification).

2.4.2 *L. infantum* promastigotes bind to dog PMN in a orientated manner and are engulfed *via* funnel-like pseudopods

SEM analysis revealed that the binding of *L. infantum* promastigotes by PMN (Fig. 7) is not random. Parasites adhering to dog PMN through the flagellum tip (Fig. 7C, D, E and F) were observed in 63.9% of the interactions evaluated, indicating that this is the preferred way of contact. The binding through the aflagellar pole (Fig. 7A and B), or by another parasite body site was less frequent (Fig. 7G and H), reaching only 16.7% and 19.4% of the interactions observed, respectively. The engulfment of promastigotes takes place *via* funnel-like extensions of the phagocyte surface over the parasite (Fig. 7A, B and C). SEM images show for the first time the *in vitro* engulfment of *L. infantum* promastigotes by dog PMN, revealing the possible ways/types of *L. infantum* association with phagocytes and highlighting the PMN membrane extensions projected for parasite intake.

2.4.3 Dog PMN efficiently bind *L. infantum* promastigotes

In vitro, the binding of parasite to PMN was evaluated by flow cytometry analysis using *L. infantum* promastigotes expressing GFP. Resting-PMN cultures were used to identify the GFP^- cells (Fig. 8A). After 1.5 h of incubation the percentage of parasite-associated PMN (GFP^+ cells, Fig. 8B, G1-G3) was 33.7% (19.43%, 44.53%) [median (interquartile range, IQR, 25th percentile and 75th percentile)]. After 3 h (GFP^+ cells, Fig. 8C), the amount of PMN bounded to *L. infantum*-GFP slightly decreased to 28.75% (20.83%, 37.85%) and after 4.5 h (GFP^+ cells, Fig. 8D) about 29.0% (21.10%, 36.90%) of PMN still associated with parasites (Fig. 8E). However, these values did not evidence statistical differences. Besides that, the level of GFP fluorescence displayed by the subpopulation of GFP^+ PMN, expressed as the GFP median, remained unchanged throughout the incubation period (Fig. 8F). Taken together, these results suggest that the parasite rapidly bind PMN and the increase in the exposure time did not enhance the percentage of parasite-associated cells neither the number of *Leishmania* bound or internalized. Thus, the incubation period of 4.5 h was abandoned, because it did not contribute to an increase in the percentage of infected PMN and was associated with a boost of cell death.

The uptake of *L. infantum* promastigotes by neutrophils was evaluated by OM. After 1.5 h of incubation only 6% (5%, 8%) of PMN were parasitized, but at 3 h 23.5% (16.25%, 26.75%) of cells internalized the parasite, showing a significant increase ($p<0.001$) (Fig. 9). Taken together, flow cytometry and OM results indicate that parasite adhesion to PMN occurs rapidly, but parasite internalization requires an increased contact period.

8

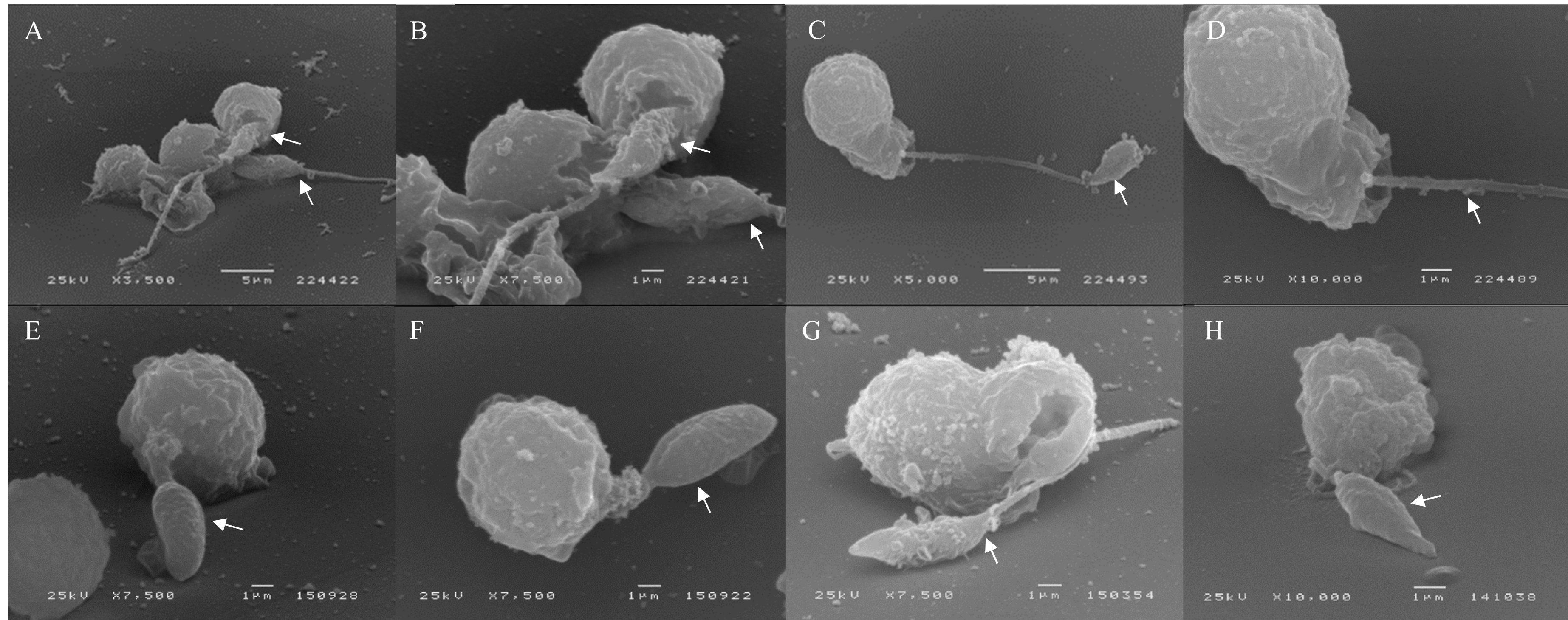

Figure 7: Attachment and phagocytosis of *L. infantum* promastigotes by dog PMN. Coverslip adhered-PMN exposed to promastigotes were incubated for 1.5 h (A, B, C, D and G) and 3 h (E, F and H) and evaluated by scanning electron microscopy (SEM). Attachment and engulfment of *L. infantum* promastigote *via* their posterior pole can be seen in A and in more detail in B. PMN orientated attachment *via* the tip of flagellum, presenting a well defined elongated tubular pseudopod advancing along the flagellum can be observed in C and also in more detail in D. Promastigote phagocytosis, revealing parasite internalization *via* the tip of the flagellum in different angles (E and F). Attachment through flagellum (G) and/or the base of the flagellum (H) can also be observed. White arrow - promastigote.

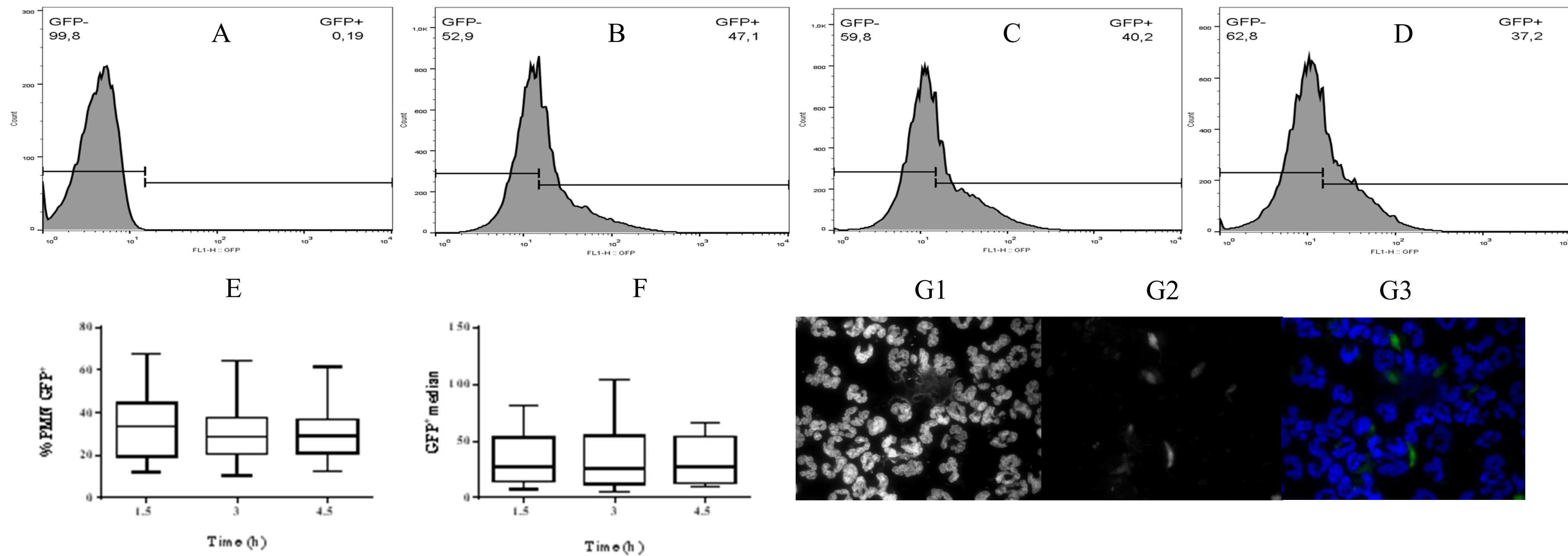

Figure 8: Levels of *L. infantum* associated (internalized or bound) to dog PMN. Resting-PMN and PMN-*L. infantum*-GFP were analyzed by flow cytometry. The histogram FL1-H/GFP *vs* number of events was used to determine the percentage of GFP^+ PMN in cultures incubated for 1.5 h (B), 3 h (C) and 4.5 h (D), using GFP^- PMN (A) as a negative control. Levels of PMN internalized or bound to the parasite at different incubation time points (E) and of GFP fluorescence in the subpopulation of GFP^+ PMN (F) of 10 dogs, using triplicate samples are expressed by medians (horizontal lines across the box), 75th percentile and 25th percentile (vertical ends of the box), and whiskers (lines extending from the box to the highest and lowest values). Representative images of *L. infantum*-GFP promastigotes (G2) incubated for 1.5 h with PMN and stained with DAPI (G1), and the respective merge image (G3) are shown (×1000 magnification).

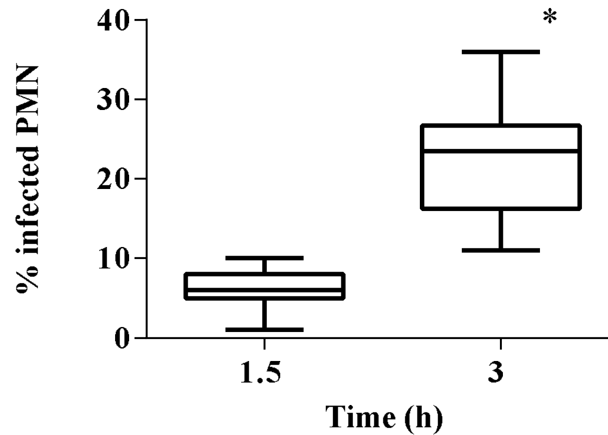

Figure 9: Levels of *L. infantum* internalization by dog neutrophils. PMN-promastigotes incubated for 1.5 h and 3 h were stained with Hemacolor staining kit and the intracellular parasitic forms were observed inside PMN under optical microscopy (×1000 magnification). Levels of parasitized PMN at both incubation time points are expressed by medians, 75th percentile and 25th percentile, and whiskers representing the highest and lowest values. The statistical analysis of 10 dogs and triplicate samples was performed using the non-parametric Wilcoxon test. Significant differences are indicated by * ($p<0.05$) when comparing 1.5 h *vs* 3 h.

2.4.4 Dog neutrophils efficiently internalize *L. infantum* promastigotes

Neutrophils exposed to *L. infantum* promastigotes observed under OM revealed some important features (Fig. 10). In some cells the parasite was found in the amastigote-like form (Fig. 10E), but the promastigote form was the most frequently morphological form observed inside neutrophils (Fig. 10A and D). A high proportion of neutrophils internalized two (Fig. 10E), three (Fig. 10B), four (Fig. 10C) or more parasites. Sometimes, images compatible with parasite division (Fig. 10B and C) were observed. These findings highlight the possibility that parasite replication might occur inside dog neutrophils. In a few dogs, the parasite was detected almost exclusively outside the cell, closely associated with the cell membrane, constituting concentric layers of parasites (Fig. 10F and G), suggesting different PMN-parasite responses in a host dependent manner.

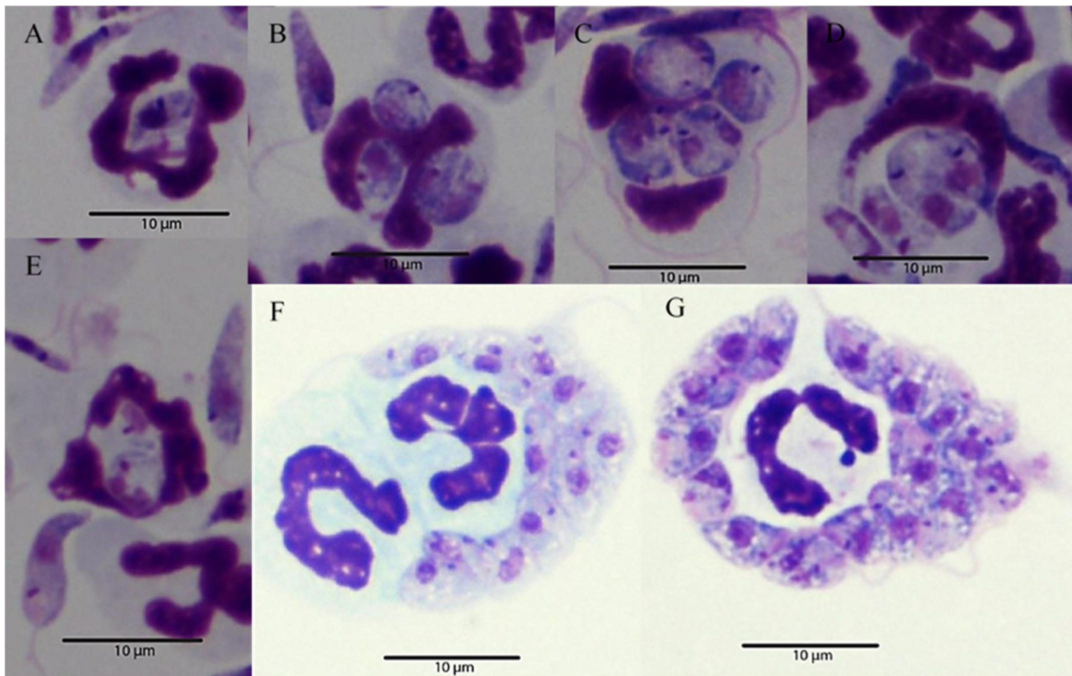

Figure 10: Promastigote uptake by neutrophils. Cultures PMN-*L. infantum* promastigotes were incubated for 1.5 h and 3 h, stained and observed under optical microscope. Intracellular promastigote (A and D) and amastigote-like (E) forms can be observed inside neutrophils. Images compatible with parasite division are also shown (B and C). Extracellular promastigote forms circumvent neutrophil membrane in concentric incomplete layers can also be observed (G and H) (×1000 magnification).

2.4.5 Previous contact with *L. infantum* decreases PMN migration

The ability of PMN to migrate from the upper to the lower compartment of a modified Boyden chamber was evaluated after incubation with *L. infantum* promastigotes. Migration of resting-PMN was significantly higher when the lower wells were filled with viable *Leishmania* promastigotes (p=0.001) and LCF (p=0.003) and significantly lower toward *Leishmania* Ag (p=0.001) when compared with lower wells filled with medium (negative control). PMN migration towards *Leishmania* Ag (p=0.002) was significantly lesser when compared with LCF (positive control). Interestingly, after 1.5 h of contact with the parasite PMN migration decreased significantly toward medium (p=0.008), *Leishmania* promastigotes (p=0.003) and LCF (p=0.002) when compared with the migration registered for resting-PMN in equivalent conditions, indicating that the contact with the parasite inhibits further chemotaxis (Fig. 11). These results showed that although the parasite functions as a quimiattractant for dog PMN driving the cell to the infection site, after parasite contact PMN chemotaxis was inhibited favoring PMN retention.

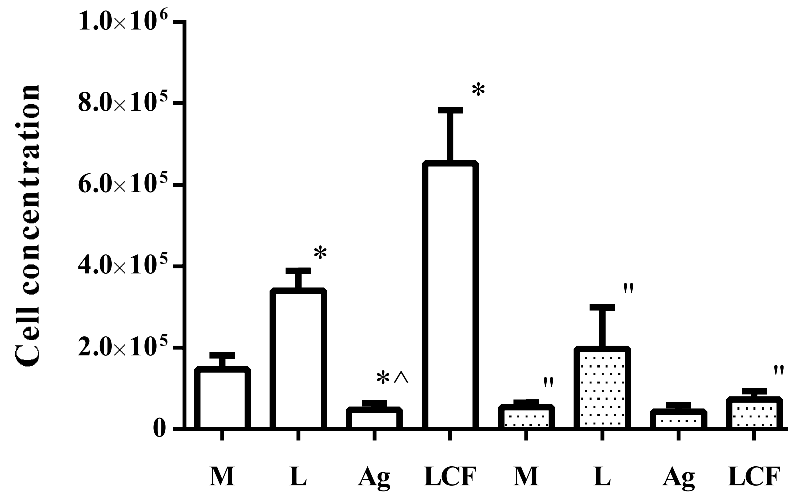

Figure 11: Ability of *L. infantum* to induce dog PMN chemotaxis. The amount of resting-PMN (white bars) and of PMN previously exposed to *L. infantum* promastigotes (dot bars) that migrate from the upper to the lower compartment of a modified Boyden chamber filled with culture medium (M), *Leishmania* promastigotes (L), *L. infantum* antigen (Ag), and *Leishmania* chemotactic factor (LCF) is indicated. Results of triplicate samples of 10 dogs are presented as mean ± standard error. Statistical analysis was performed using the Wilcoxon test ($p<0.05$). Significant differences are represented by * when comparing M (negative control) *vs* the other conditions, ^ when comparing LCF (positive control) *vs* the other conditions and by " ($p<0.05$) when comparing resting-PMN *vs* PMN+L in equivalent conditions.

2.4.6 *L. infantum* stimulates dog PMN oxidative burst

O_2^- production by PMN exposed to *L. infantum* promastigotes was assessed using the quantitative NBT assay. The intracellular production of O_2^- by PMA-stimulated PMN ($p<0.001$), *L. infantum*-exposed PMN ($p<0.001$) and, PMA-stimulated PMN exposed to *L. infantum* ($p<0.001$) was significantly higher than the production of O_2^- by resting-PMN at both 1.5 h (Fig. 12A) and 3 h (Fig. 12B) of incubation. The production of O_2^- by PMA-stimulated PMN exposed to *L. infantum* at 1.5 h was significantly higher when compared with PMA-stimulated PMN ($p<0.001$). Higher exposure (3 h) to PMA and *L. infantum* resulted in decrease of O_2^- production by PMN ($p=0.013$), suggesting that the early (1.5 h) oxidative burst observed in PMA-stimulated PMN exposed to *L. infantum* is modulate by the parasite in the later stage of infection.

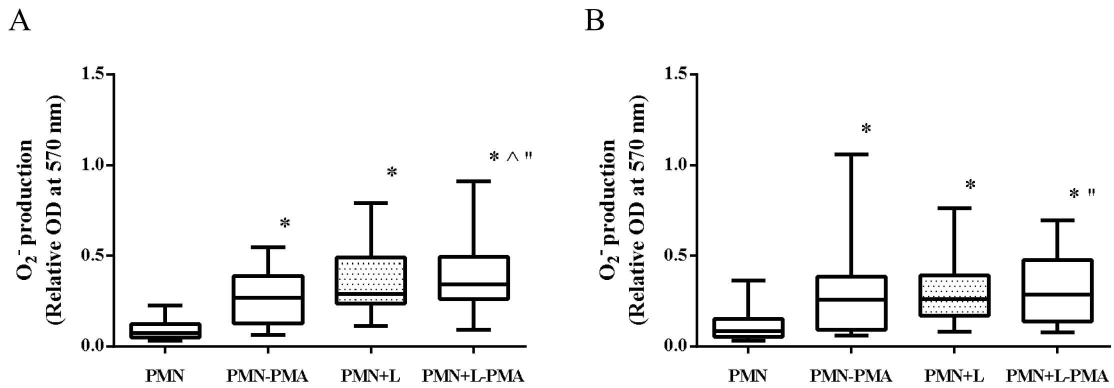

Figure 12: Superoxide (O_2^-) production by PMN exposed to *L. infantum* promastigotes. Cultures of resting-PMN (PMN), PMA-stimulated PMN (PMN-PMA), *L. infantum*-exposed PMN (PMN+L) and PMA-stimulated PMN exposed to *L. infantum* (PMN+L-PMA) incubated for 1.5 h (A) and 3 h (B) were used to measure O_2^-. Results of triplicate samples of 12 dogs are presented by medians, 75th percentile, 25th percentile and, whiskers indicating the highest and lowest absorbance values. The statistical analysis was performed using the non-parametric Wilcoxon test (p<0.05). Significant differences are indicated by * when comparing resting-PMN (negative control) *vs* the different conditions, ^ when comparing PMN+PMA (positive control) *vs* PMN+L-PMA, and by " when comparing PMN+L-PMA at 1.5 h *vs* 3 h of incubation.

2.4.7 *L. infantum* regulates NE and catG exocytosis

Exocytosis of NE and catG were measured in supernatants of PMN cultured with *L. infantum* promastigotes using specific colorimetric substrates. In supernatants of PMN-*L. infantum* cultures incubated for 1.5 h (p<0.001) (Fig. 13A) and 3 h ($p_{0\ min}$=0.001, $p_{15\ min,\ 30\ min}$ <0.001) (Fig. 13B), NE activity was significantly higher when compared with supernatants of resting-PMN (negative control). Also at 1.5 h, NE exocytosis by LPS-stimulated PMN (positive control) was significantly higher in comparison with resting-PMN ($p_{0\ min,\ 15\ min}$=0.012, $p_{30\ min}$=0.025). Furthermore, the release of NE by *L. infantum*-exposed PMN was higher than the positive control (p=0.012). The increase of parasite exposure (3 h) did not augment NE exocytosis when compared with 1.5 h of incubation (p=0.015). These results point towards NE exocytosis parasite induced in the early phase of the infection that is subsequently (late infection) modulated by the parasite.

On the other hand, the activity of catG remained without major variations. Of note a significant increase in catG exocytosis in resting-PMN after 3 h of incubation when

compared with 1.5 h ($p_{0\ \mathrm{min\ and}\ 15\ \mathrm{min}}<0.001$ and $p_{30\ \mathrm{min}}=0.001$) (Fig. 13C and D) pointing towards PMN activation.

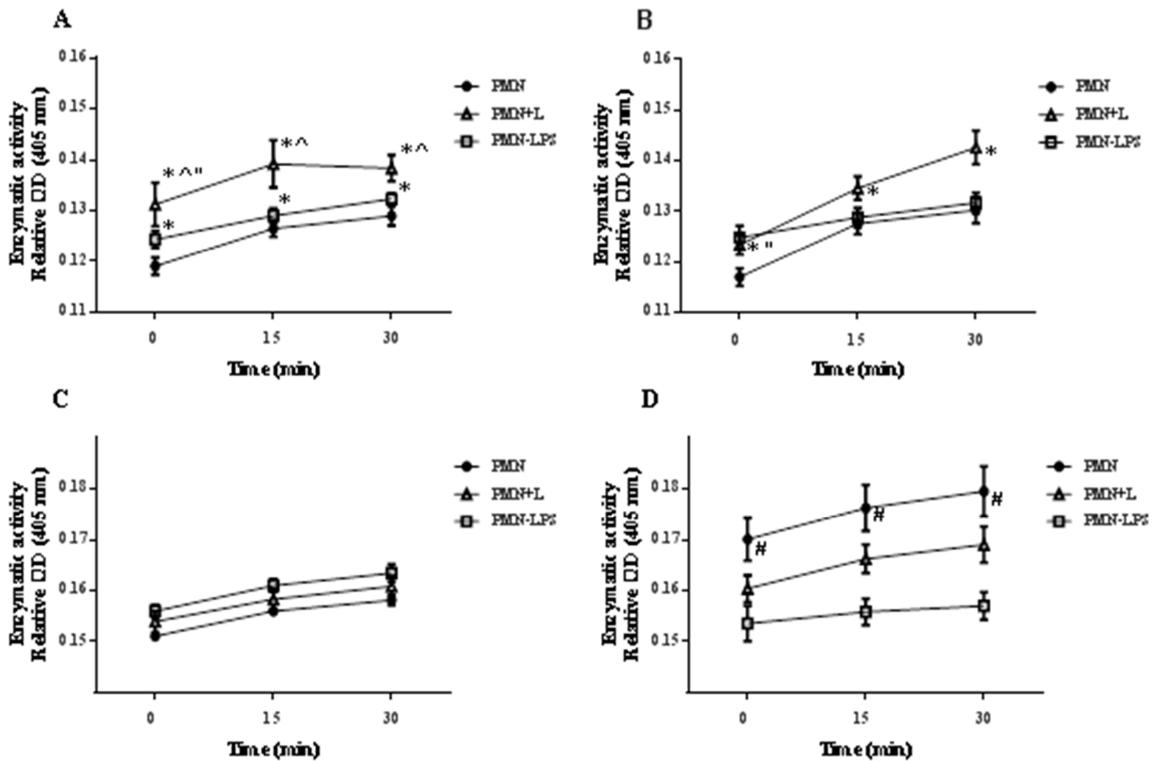

Figure 13: Enzymatic activity of dog PMN primary granules. Neutrophilic elastase (NE) (A, B) and catepsine G (catG) (C, D) exocytosis were quantified in supernatants of PMN, *L. infantum*-exposed PMN (PMN+L) and PMN stimulated by *E. coli* lipoplysaccharide (PMN-LPS) at 1.5 h (A, C) and 3 h (B, D) by a colorimetric enzyme-substrate reaction. Culture supernatants were incubated for 0 min, 15 min and 30 min with the specific colorimetric substrate and read at 405 nm. Results of triplicate samples of 10 dogs are presented as mean ± standard error. Statistical analysis was performed using the Wilcoxon test ($p<0.05$). Significant differences are indicated by * when comparing PMN (negative control) *vs* the different conditions, ^ when comparing PMN-LPS *vs* PMN+L, " when comparing PMN+L at 1.5 h *vs* 3 h and by # when comparing PMN at 1.5 h *vs* 3 h.

2.4.8 *L. infantum* precludes NET formation

NET generation induced by *L. infantum* was assessed by immunolabling and SEM in resting-PMN, parasite exposed to PMN, PMA-stimulated PMN and PMA-stimulated PMN exposed to *L. infantum* and incubated for 1.5 h and 3 h. PMA-stimulated cells were used as positive control and resting-PMN as negative control.

Immunolabeling assays showed extracellular structures stained with DAPI and anti-Histone H1 antibody, pointing towards NET emission by dog PMN. The parasite induced NET, but to a lesser extent than PMA-stimulated and PMA-stimulated PMN exposed to *L. infantum*. As expected resting-PMN did not released NET (Fig. 14).

The NET ultrastructure was observed as thin filamentous structures consisting of modified nucleosomes dotted with globular formations corresponding to proteins from granules and other cell compartments and as large cloud-like structures as described by Vorobjeva and Pinegin (2014) (Fig. 15C, D, E, F and G). The number of PMN generating NET was counted in 50 cells for each condition and experiment (Fig. 15H). Resting-PMN did not release NET (Fig. 15A). *L. infantum* almost abolished NET release in PMN cultures (Fig. 15B). However, NET formation was intense in PMA-stimulated PMN (Fig. 15C and D). PMA-stimulated cells exposed to promastigotes also showed an exuberant NET release (Fig. 15E, F and G).

A close contact between NET and the parasite was observed in PMA-stimulated PMN exposed to *L. infantum* promastigotes. NET structures were observed trapping the parasite, suggesting a limiting spreading effect (Fig. 16).

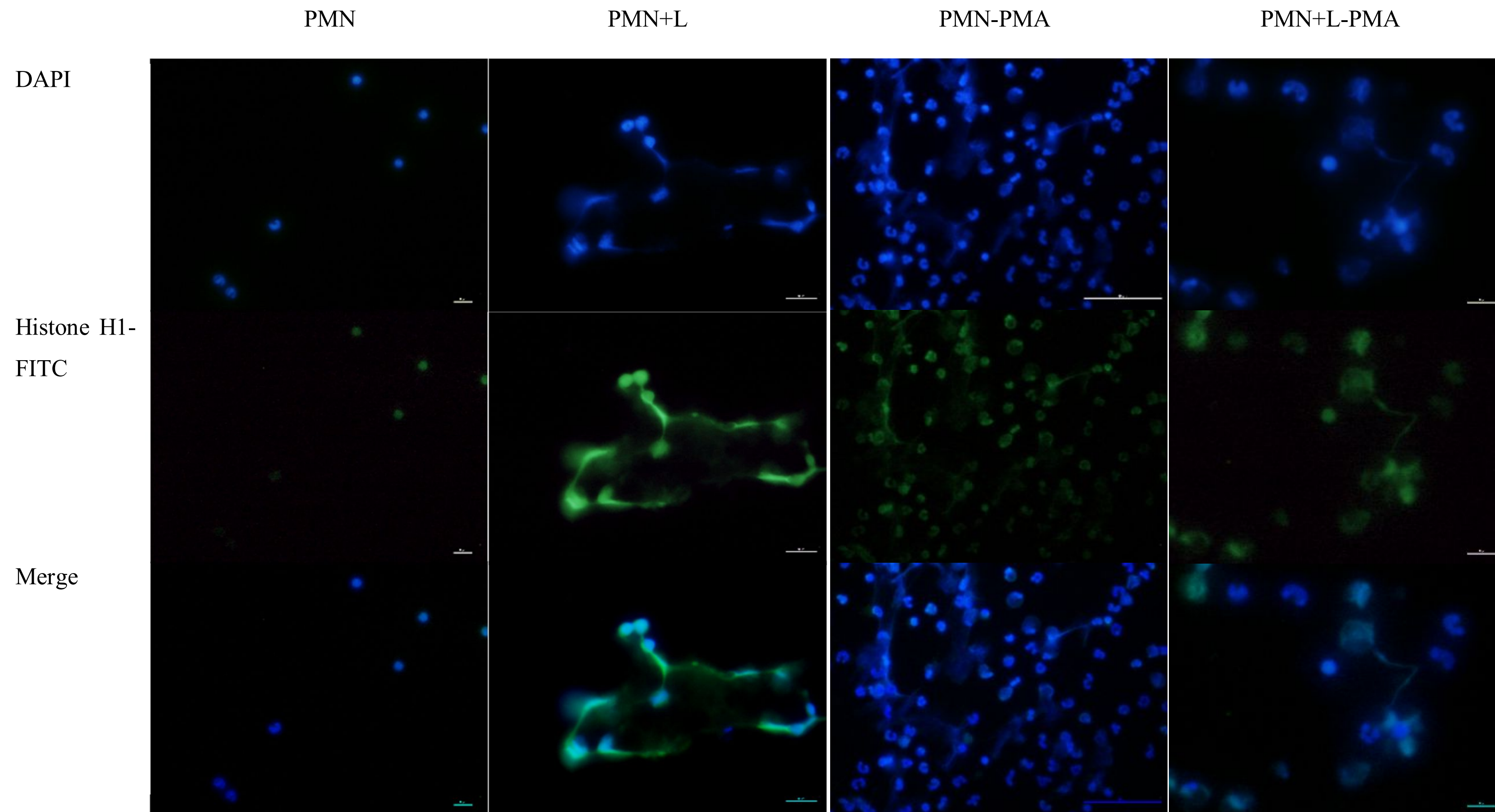

Figure 14: NET release evaluated by immunolabling. Coverslips adhered-PMN exposed to promastigotes (PMN+L) for 3 h were prepared for immunolabling. Additional resting-PMN (negative control), PMN stimulated with phorbol myristate acetate (PMN-PMA) (positive control) and PMN stimulated with PMA and exposed to *L. infantum* (PMN+L-PMA) were stained with anti-Histone H1 FITC and DAPI. Representative images stained with DAPI, anti-Histone H1 and merge (×600 or ×1000 magnification).

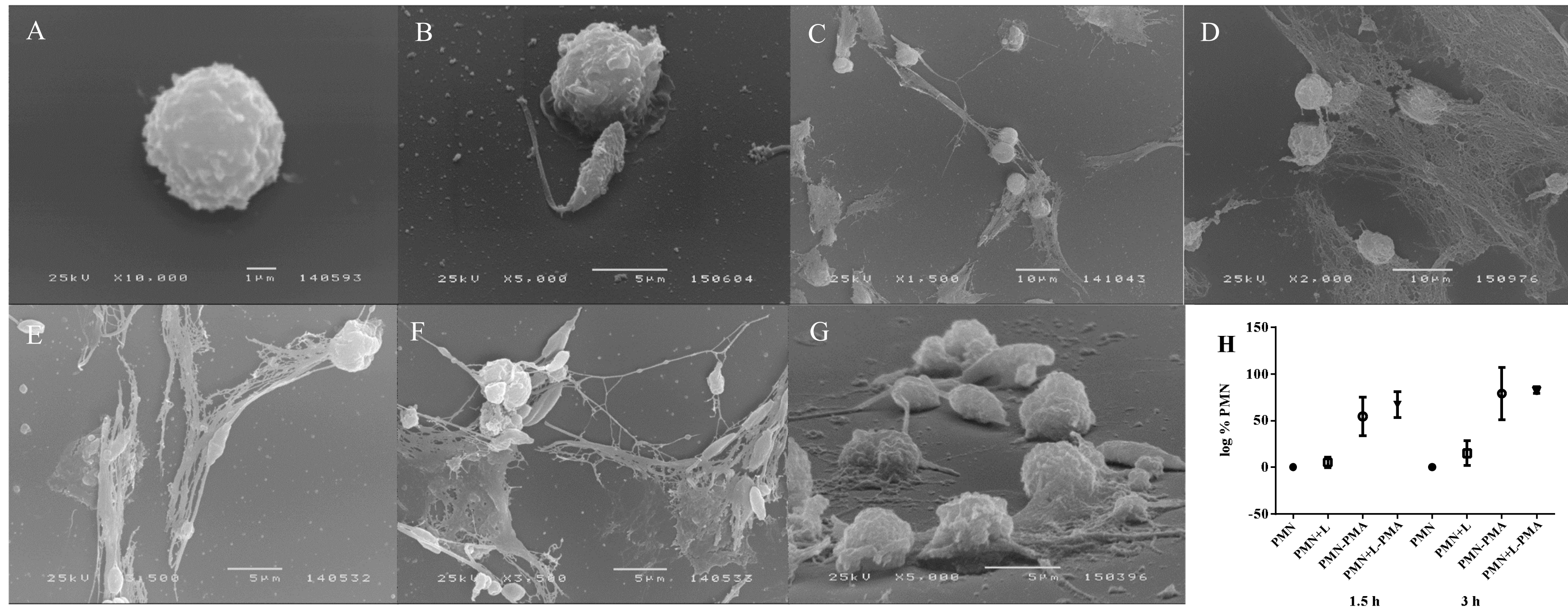

Figure 15: Extracellular interaction between dog PMN and *L. infantum* promastigotes. Coverslips adhered-PMN exposed to promastigotes (PMN+L) for 1.5 h and 3 h were prepared for scan electron microscopy (SEM). Additional resting-PMN and PMN stimulated with phorbol myristate acetate (PMN-PMA) coverslips were used as positive and negative controls, respectively. Representative SEM images of PMN (A), PMN+L (B), PMN-PMA (C, D) and PMN-PMA+L (E, F, G) incubated for 1.5 h (A, B, C, G) and 3 h (D, E, F) are shown at different magnifications. White arrow - filamentous NET; Black arrow - cloud-like structured NET. NET-generating cells were quantified *per* 50 PMN isolated from three dogs and the results are represented by the mean ± standard deviation of triplicates (H).

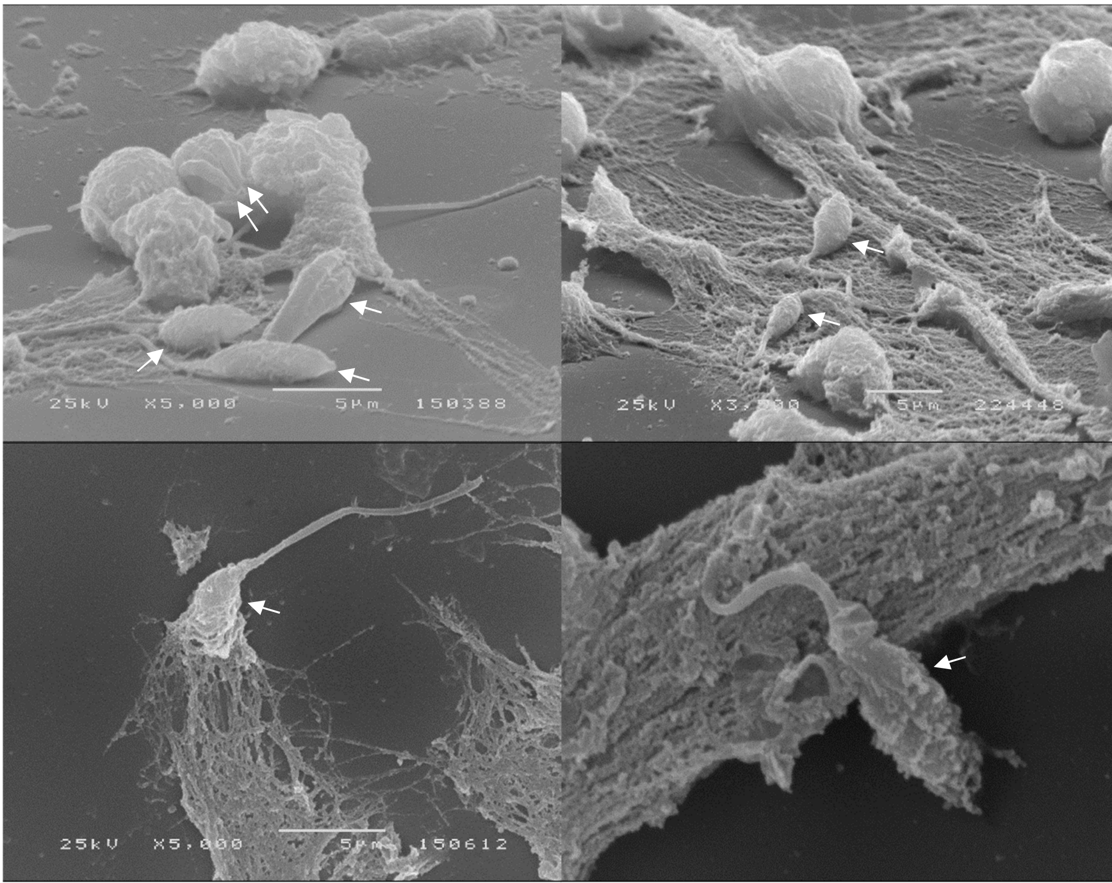

Figure 16: Neutrophil extracellular traps (NET) limit parasite spread. PMN cultures stimulated with phorbol myristate acetate (PMA) and exposed to *L. infantum* promastigotes (PMN+L-PMA) adhered on coverslips were treated for scanning electron microscopy (SEM). A close interaction between NET and the parasite (white arrow) evidence an entrapping effect.

2.4.9 *L. infantum* impacts PMN cell death

Cultures of resting-PMN (Fig. 17B and E), *L. infantum*-exposed PMN (Fig. 17C and F) and Campto-stimulated PMN (Fig. 17D and G) incubated for 1.5 h and 3 h were treated with annexin and PI and analyzed by flow cytometry. Untreated-PMN were used to gate annexin V FITC$^-$/PI$^-$ population (viable cells), annexin V FITC$^+$/PI$^-$ (apoptotic cells) and annexin V FITC$^+$ or $^-$/PI$^+$ (total necrotic cells) (Fig. 17A).

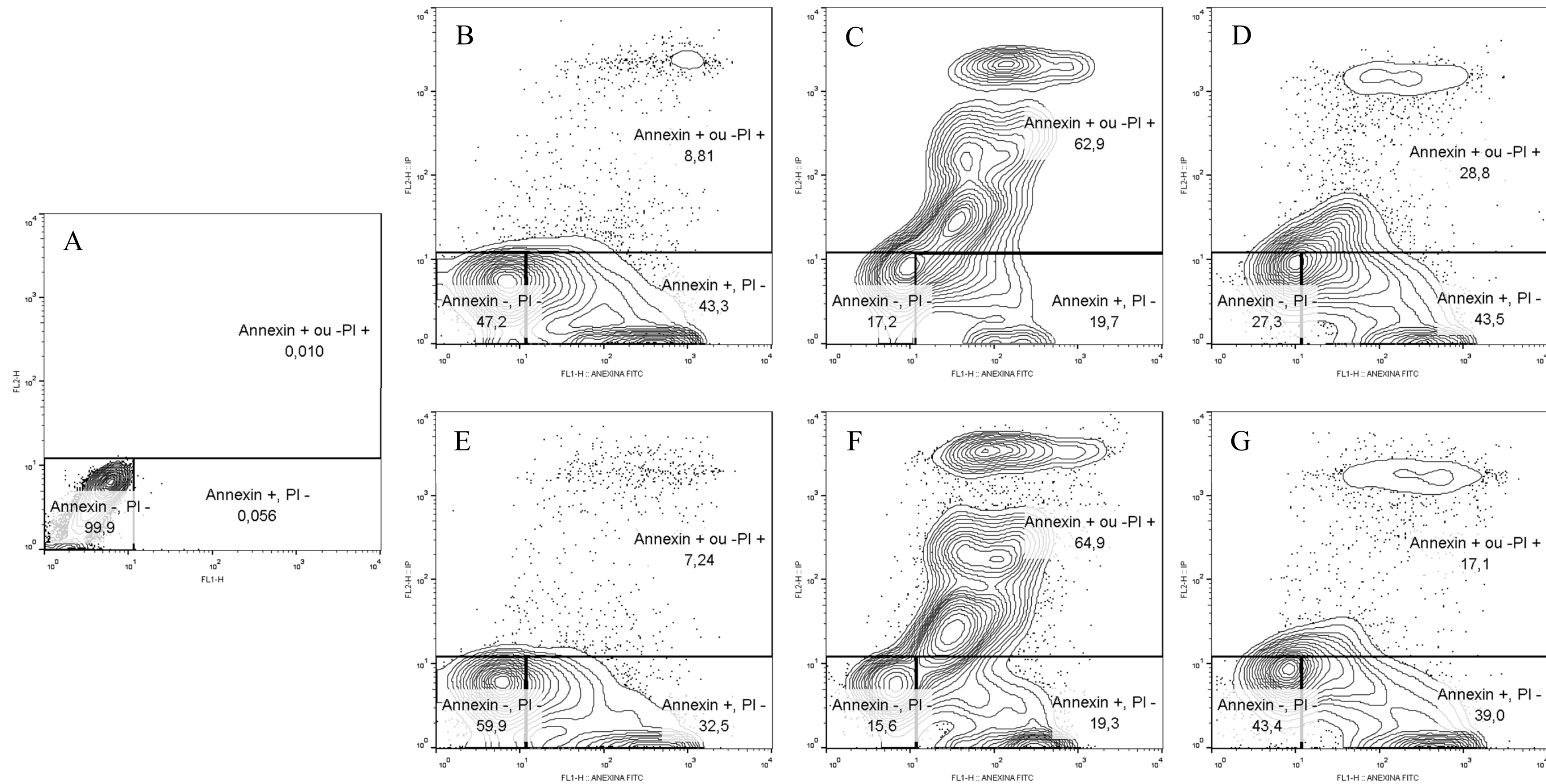

Figure 17: Gating strategy used to select viable, apoptotic and necrotic cells. Cultures of resting-PMN (B, E) and of PMN incubated with *L. infantum* (C, F) or stimulated with (S)-(+)-camptothecin (D, G) for 1.5 h (A, B, C, D) and 3 h (E, F, G) were treated with annexin V and PI and analyzed by flow cytometry. After gating on the PMN population based on forward scatter and side scatter characteristics, untreated-PMN (A) were used to gate annexin V FITC$^-$/PI$^-$ population (viable cells), annexin V FITC$^+$/PI$^-$ (apoptotic cells) and annexin V FITC$^+$ or $^-$/PI$^+$ cells (necrotic cells).

Campto stimulation ($p_{1.5\ h}$=0.011, $p_{3\ h}$=0.008) and *L. infantum* exposure ($p_{1.5\ h\ and\ 3\ h}$<0.001) had a negative impact on cell viability at both time points (1.5 h and 3 h) when compared with resting-PMN. The proportion of viable cells was significantly lower in Campto-stimulated PMN than in *Leishmania*-exposed PMN at 1.5 h ($p_{1.5\ h}$=0.008), but the scenario was reversed at 3 h ($p_{3\ h}$=0.038), indicating that with time the parasite exerts a more pronounced effect on cell viability (Fig. 18A). These results show that PMN viability is compromised by *L. infantum* parasites.

Significant differences in PMN apoptosis were only observed in late cultures (3 h). Campto stimulation caused a slight increase in apoptosis at 1.5 h, which became significant at 3 h ($p_{3\ h}$=0.008) when compared with resting-PMN. The proportion of apoptotic cells was also significantly superior in Campto-stimulated PMN when compared with parasite-exposed PMN at 3 h (*p*=0.008). Interestingly, the frequency of apoptotic cells decreased slightly in parasite-exposed PMN incubated for 1.5 h, but increased after 3 h of incubation when compared with unprimed-PMN (Fig. 18B). Although not statistically significant these results indicate that *L. infantum* seems to induce a late and slight increase in the level of cell apoptosis, probably in an attempt to promote its own transference to MØ, the definitive host cell.

However, the greatest impact of the parasite was on the dimension of necrosis subset. The proportion of total necrotic cells presented important increases in Campto-stimulated PMN ($p_{1.5\ h}$=0.005, $p_{3\ h}$=0.008) and parasite-exposed PMN (p=$_{1.5\ h\ and\ 3\ h}$<0.001) in comparison to resting-cells at both time points. Furthermore, after 3 h of incubation the proportion of necrotic parasite-exposed PMN increased significantly when compared with Campto-stimulated PMN (*p*=0.021) (Fig. 19A). The high proportion of cells experiencing *in vitro* necrosis may reflect the absence of scavenger MØ to remove the apoptosing PMN, leading to its disruption by secondary necrosis.

Interestingly, when total necrotic cells (annexin V FITC$^+$ or $^-$/PI$^+$) were decomposed in primary (annexin V FITC$^-$/PI$^+$, Q1) and secondary (annexin V FITC$^+$/PI$^+$, Q2) (Fig. 19B), the later cells prevailed in all the conditions and time points except in resting-PMN incubated for 3 h. Indeed, the proportion of secondary necrotic cells was higher in resting-PMN ($p_{1.5\ h}$<0.001), parasite-exposed PMN ($p_{1.5\ h\ and\ 3\ h}$<0.001) and Campto-stimulated PMN ($p_{1.5\ h\ and\ 3\ h}$=0.008) when compared with primary necrotic cells. These findings

reinforce the idea that in experimental conditions a large proportion of PMN that initiate the apoptosis pathway undergo secondary necrosis.

As observed with total necrotic cells, the proportion of primary and secondary necrotic cells in Campto-stimulated ($p_{1.5\ h}$=0.001, $p_{3\ h}$=0.028 and $p_{1.5\ h\ and\ 3\ h}$=0.008, respectively) and parasite-exposed cultures ($p_{1.5\ h}$<0.001, $p_{3\ h}$=0.005 and $p_{1.5\ h\ and\ 3\ h}$<0.001, respectively) was higher in comparison with resting-PMN at both time points. While the proportion of primary necrotic cells was superior in Campto-stimulated PMN when compared with parasite-exposed cultures ($p_{1.5\ h}$<0.001) at 1.5 h the amount of secondary necrotic cells was superior in parasite-exposed cultures when compared with Campto-stimulated PMN at both time points ($p_{1.5\ h\ and\ 3\ h}$=0.038) (Fig. 19C).

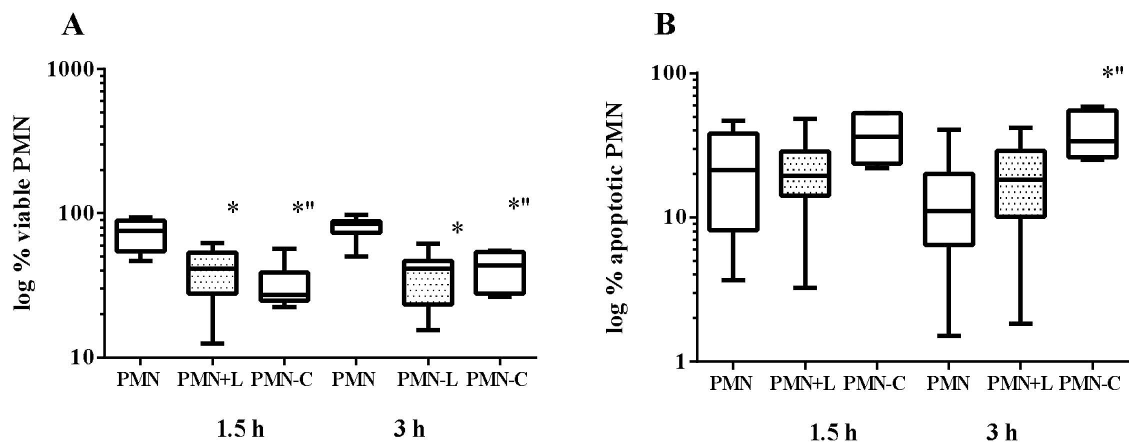

Figure 18: Levels of viable and apoptotic dog PMN after exposure to *L. infantum* promastigotes. PMN exposed to *L. infantum* (PMN+L) were treated with annexin V FITC and propidium iodine (PI) and analyzed by flow cytometry. Resting-PMN and (S)-(+)-camptothecin-stimulated PMN (PMN-C) were used as negative and apoptotic positive controls, respectively. The proportion of viable (A) and apoptotic (B) cells is presented by medians, 75th percentile and 25th percentile and whiskers representing the highest and lowest values. Assays were performed in 10 dogs using triplicate samples and statistical analysis was performed using the Wilcoxon test. Significant differences (p<0.05) are represented by * when comparing PMN (negative control) *vs* the other conditions, and " when comparing PMN+L *vs* PMN+C.

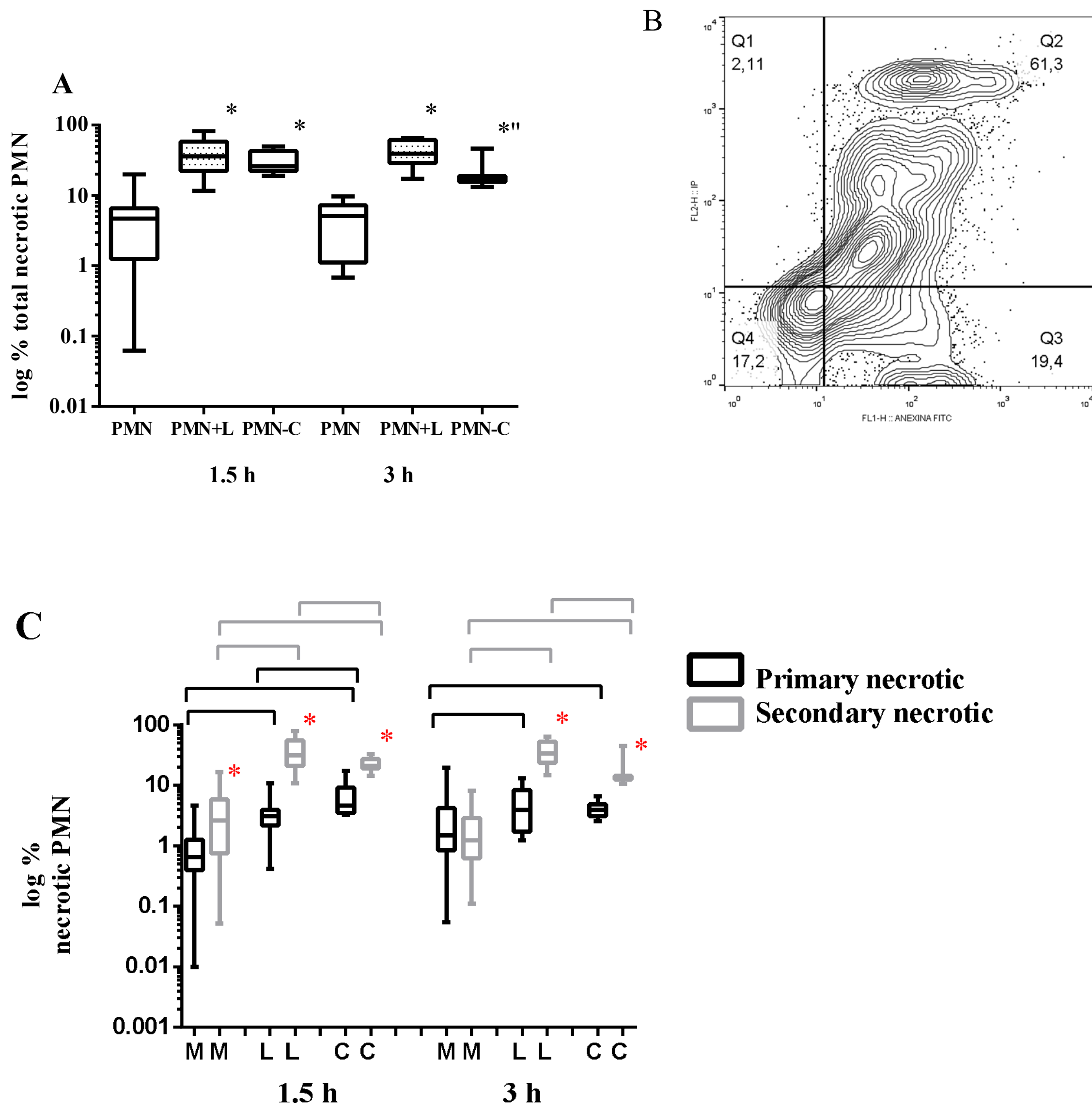

Figure 19: Levels of necrotic dog PMN. Parasite-exposed PMN (L) treated with annexin V FITC and PI were analyzed by flow cytometry. Resting-PMN (M) and (S)-(+)-camptothecin-stimulated PMN (C) were used as negative and positive controls, respectively. Total necrotic cells were identified as annexin V FITC^{+} or $^{-}$/PI^{+} and displayed in the quadrant 1 (Q1) and Q2 in the contour plot (B). Primary necrotic cell were identified as annexinV FITC^{-}/PI^{+} (Q1 in the contour plot and black boxes on graph C) and secondary necrotic cells as annexin V FITC^{+}/PI^{+} (Q2 in the contour plot and gray boxes on graph C). Results from triplicate samples of 10 dogs are presented by medians, 75th percentile and 25th percentile and whiskers representing the highest and lowest values. Statistical analysis was performed using the Wilcoxon test. Significant differences (p<0.05) are presented by * when comparing PMN (negative control) *vs* the other conditions, and " when compared PMN+L *vs* PMN+C (A). For primary and secondary necrotic cells significant differences (p<0.05) are presented by * when compared primary *vs* secondary necrotic cells in the same condition and black and gray lines connecting conditions in primary and secondary necrotic cells (C).

According to the PI level three necrotic cell subsets were identified: n1, n2 and n3, corresponding to cells with low, high and very high amounts of PI, respectively. The level of n1, n2 and n3 cells was evaluated in resting-PMN (Fig. 20A and D), *Leishmania*-exposed PMN (Fig. 20B and E) and Campto-stimulated PMN (Fig. 20C and F).

When compared with resting-PMN, n1, n2 and n3 cells prevailed in *Leishmania*-exposed ($p_{1.5\ h\ and\ 3\ h}$<0.001) and Campto-stimulated PMN (p=0.008) at both time points. After 3 h of incubation, n1 cells significantly increased in *Leishmania*-exposed cultures when compared with Campto-stimulated cells (p_{n1}=0.038). N2 cells also augmented at both time points in *Leishmania*-exposed cultures when compared with Campto-stimulated cells (p_{n2}=0.008). N1 cells decreased in Campto-stimulated PMN (p=0.038) and n3 cells increased in *Leishmania*-exposed PMN at 3 h when compared with 1.5 h (Fig. 20G, H and I).

Although the increase of apoptosis registered at 3 h of incubation was not statistically significant, the high levels of parasite-exposed PMN that reached necrosis *via* the apoptotic program (secondary necrosis) point towards the modulation of cell death program by the parasite. The well defined subsets of PMN, mainly annexin V^+, displaying different amounts of PI reinforce this possibility, indicating different stages of apotosis-necrosis transition.

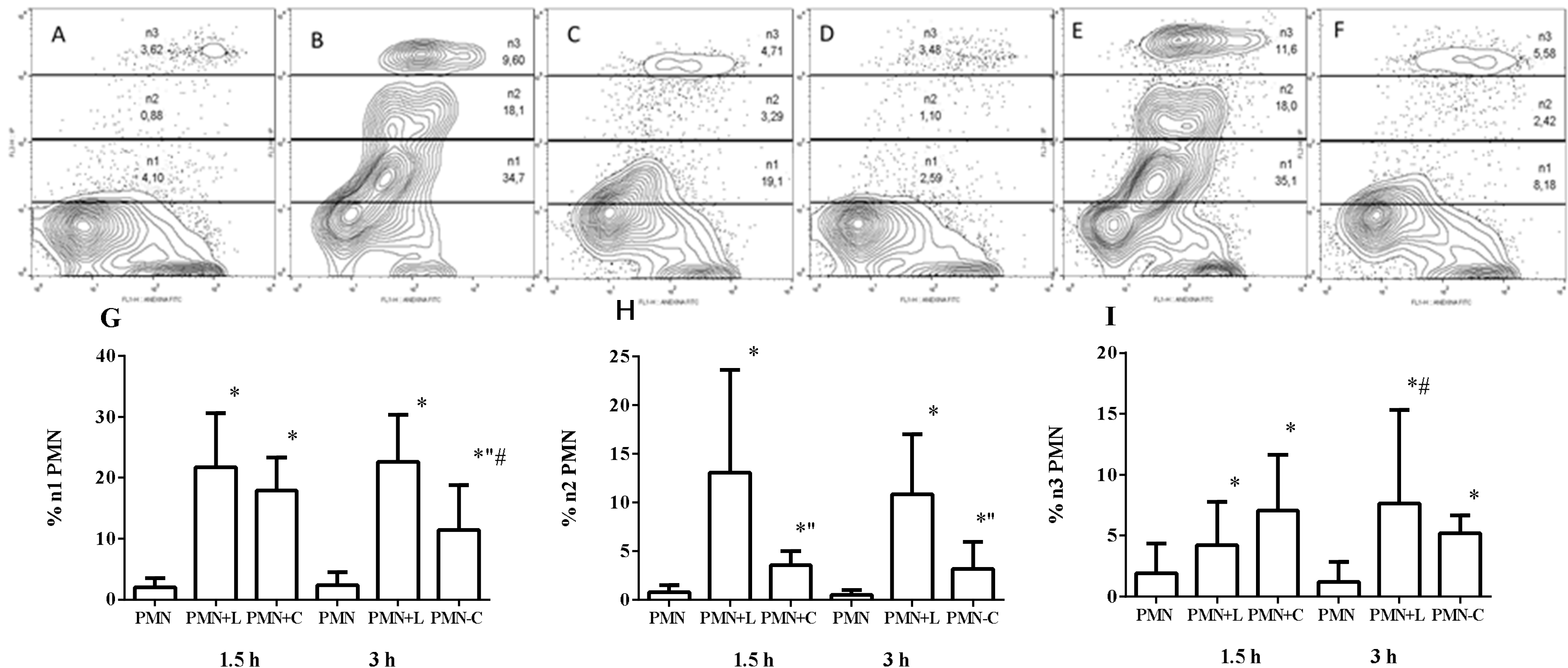

Figure 20: Levels of necrotic PMN expressing different amounts of propidium iodine (PI). Parasite-exposed PMN (PMN+L) treated with annexin V FITC and PI were analyzed by flow cytometry. Resting-PMN and (S)-(+)-camptothecin-stimulated PMN (PMN-C) were used as negative and positive controls, respectively. Necrotic cell subset identified as annexin V FITC^{+} or $^{-}$/PI^{+} was gated. Necrotic cells were classified as necrotic 1 (n1), n2 and n3 according to increasing PI levels in resting-PMN (A, D), PMN incubated with *L. infantum* (B, E) or stimulated with (S)-(+)-camptothecin (C, F) at 1.5 h (A, B, C) and 3 h (D, E, F). The proportion of n1 (G), n2 (H) and n3 (I) cells in PMN, PMN+L and PMN-C from triplicate samples of 10 dogs are presented as mean ± standard deviation. Statistically significant differences (p<0.05) are represented by * when comparing PMN *vs* others subsets, " when comparing PMN+L *vs* PMN-C, and by # when comparing equivalent conditions at 1.5 h *vs* 3 h.

2.4.10 *L. infantum* delays apoptosis of parasitized neutrophils

Microscopic observation of *L. infantum*-exposed neutrophils and of resting-neutrophils revealed the presence of apoptotic cells exhibiting the classical features, such as cell rounding and shrinking, and chromatin condensation (pycnotic nuclei), which tends to marginate, acquiring a crescent form. Late stages of neutrophil apoptosis were also observed as nuclear fragmentation (karyorrhexis)/apoptotic bodies. Although some infected neutrophils exhibited apoptotic nuclear morphology, after 3 h of parasite exposure most of the apoptotic neutrophils were not parasitized (Fig. 21), suggesting that *L. infantum* protects dog neutrophils from apoptosis. In spite of this fact the number of apoptotic cells in PMN-*L. infantum* cultures was higher when compared with resting-PMN cultures (Fig. 18B).

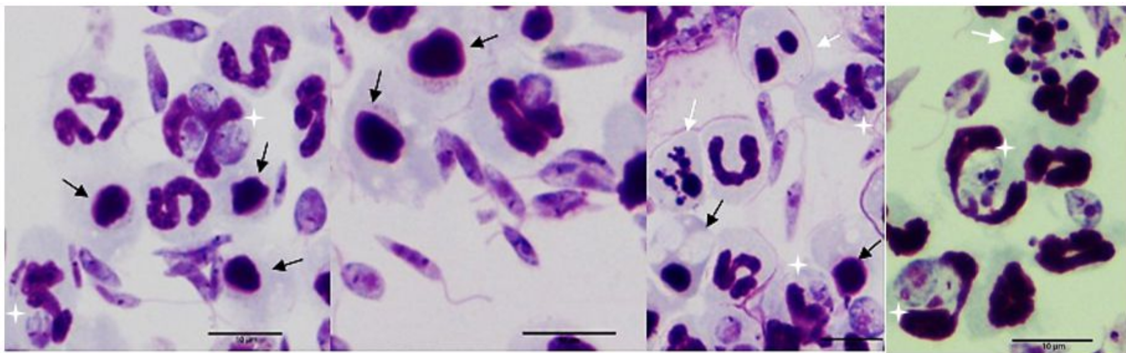

Figure 21: *L. infantum* delays apoptosis of parasitized neutrophils. PMN-*L. infantum* promastigotes incubated for 3 h were citocentrifuged, stained and observed under optical microscope. Not parasitized early (black arrow) and late (white arrow) apoptotic cells and parasitized neutrophils (white stars) can be observed (×1000 magnification).

2.4.11 PMN reduce L. infantum promastigote viability by extracellular and intracellular parasite killing

The viability of *L. infantum* parasites after contact with dog PMN was assessed by transferring PMN-*L. infantum* culture to complete SCHN medium for 24 h at 24ºC. *L. infantum* promastigote cultures were used as a positive control. Levels of moving promastigotes were estimated in a Neubauer-counting chamber. *L. infantum* parasites previously exposed to intracellular and extracellular PMN killing mechanisms showed

reduced viability [4.80×10^6 (1.92×10^6, 8.64×10^6)] when compared with *L. infantum* promastigote cultures [7.38×10^6 (4.80×10^6, 1.06×10^7)] ($p<0.001$). (Fig. 22).

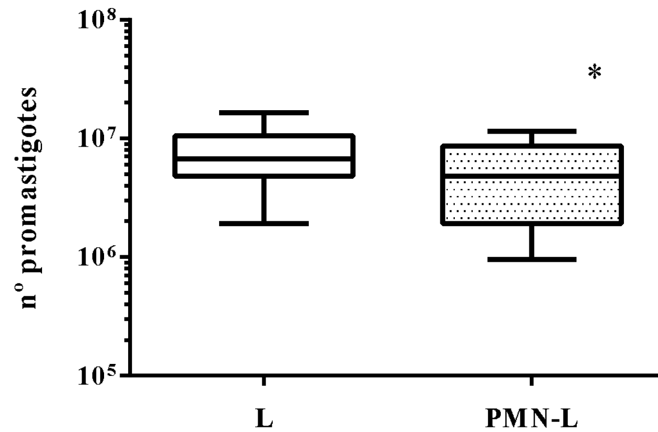

Figure 22: Viability of *L. infantum* promastigotes after exposure to dog PMN. Active *L. infantum* promastigotes incubated with PMN for 24 h (PMN+L) were counted in a Neubauer-counting chamber. Promastigotes not exposed to PMN (L) were used as positive control. The amount of viable parasites is presented by medians, 75th percentile and 25th percentile and, whiskers representing the highest and lowest values. Statistical analysis of triplicate samples of 10 dogs was performed using the Wilcoxon test. * ($p<0.05$) represents statistically significant differences when comparing L *vs* PMN+L.

To investigate the possible mechanisms involved in parasite killing, OM and TEM images were acquired. OM revealed that some intracellular parasites preserved the structural integrity, suggesting that parasites were intaken by nonlytic intracellular compartments. However, images of neutrophils contained large vacuoles surrounding cellular debris and distorted cell morphology, indicate that the parasite were enclosed by degradative vacuoles. Furthermore, evidences of degraded parasites were more frequent in some particular dogs (Fig. 23).

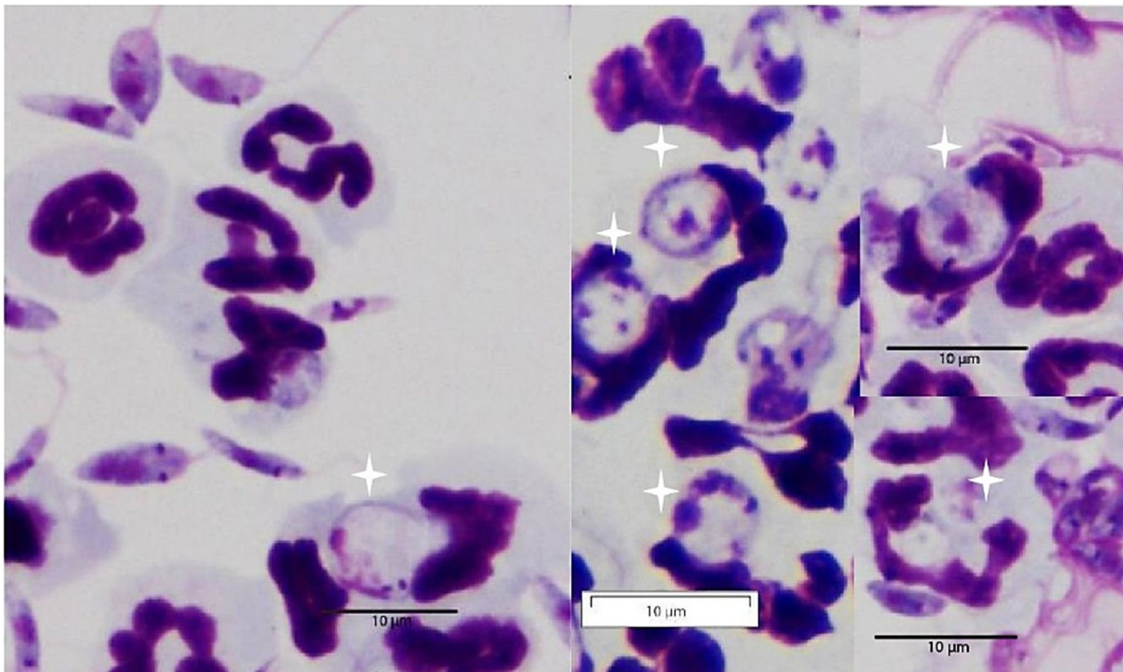

Figure 23: Large phagolysosomes ensure parasite killing. Cultures of PMN-*L. infantum* promastigotes incubated for 3 h were citocentrifuged, stained with Hemacolor staining kit and observed under optical microscope. Large phagosomes containing parasites that lose their structural integrity can be observed (white star) (×1000 magnification).

TEM images showed features of PMN activation after contact with the parasite. Filopodia protruding from the cell were visible (Fig. 24C and D). Different sizes and highly contrasted organelles, not delimited by a bilayer membrane and known as lipid bodies or lipid droplets (Fig. 24A and B) were observed in PMN cytoplasm. These dynamic and functionally active organelles are involved in a variety of functions, such as lipid metabolism, cell signaling and inflammation (Melo et al, 2013) and in this case indicate neutrophil activation.

PMN activation resulted in parasite killing. Images of TEM suggested parasite killing in the extracellular space (Fig. 24A, B, C and D), probably mediated by the exocytosis of the content of neutrophilic granules. The contact between the parasite and PMN in association with loss of parasite normal appearance was considered suggestive of extracellular killing. Parasite killing was assessed by the abnormal ultrastructural aspect of the main parasitic organelles, namely the nucleus that appeared more electro-dense and the loss of cellular membrane integrity revealed by cytoplasmic blebs (Fig. 24C and D).

The presence of the parasite within spacious phagosomes (Fig. 24E and F), indicates a possible fusion of PMN granules with the phagosome containing the parasite, culminating in the formation of the phagolysosome and of parasite degradation. Sometimes the advanced intracellular degradation process prevented the identification of any parasitic organelle.

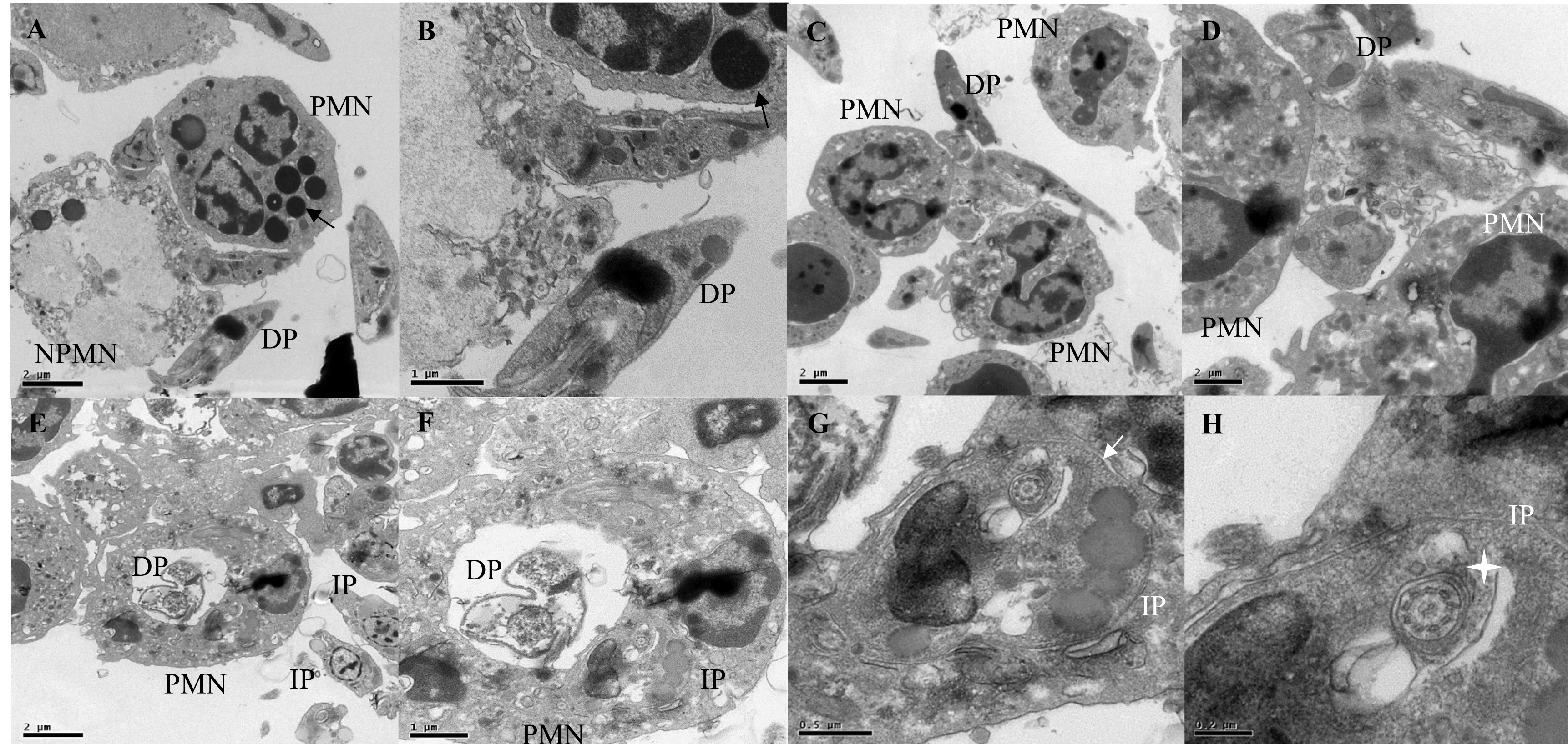

Figure 24: Parasite killing by dog neutrophils. PMN incubated for 3 h with *L. infantum* promastigotes were observed by transmission electron microscopy (TEM). Extracellular (A, B, C, D) and intracellular (E, F, G, H) parasite killing can be observed. A degraded parasite can be seen next to an activated and a necrotic PMN (A, B). Different amplifications of the contact between PMN and promastigotes reveal extracellular parasite killing (C, D). PMN containing a large phagolysosome with a degraded parasite and a tight parasitophorus vacuole containing an intact parasite (E, F) are shown. Amplification of the parasitophorous vacuole showing subpellicular microtubules and the flagellar cytoskeleton constituted by a canonical 9+2 microtubular axoneme (G, H). PMN - neutrophil; NPMN - necrotic neutrophil; IP - intact parasite; DP - degraded parasite; black arrow - lipid bodies; white arrow - subpellicular microtubules; white star - flagellum.

2.5 DISCUSSION

It is widely accepted that the adaptive immune response impacts on the final outcome of *L. infantum* infection. However, innate immune response constitutes the first barrier to the establishment of infection and the early intervention of neutrophils, the most abundant of the innate immune cells, might be decisive in the upshot of parasite-host interaction.

Neutrophils, which circulate in the blood stream as dormant cells are pre-programmed to find, phagocytosis and destroy pathogens. Secretory vesicles contain adhesion molecules, components of NADPH oxidase, enzymes and receptors that are rapidly incorporated into the surface membrane in response to several stimuli derived from other cells, complement components, invading pathogens and damaged tissues. These chemoattractants prime and guide neutrophils to the site of infection or inflammation (Borregaard, 2010, Hostetter, 2012). Indeed, neutrophils are rapidly recruited to *Leishmania* injection site (Pompeu et al, 1991; Beil et al, 1992; Santos-Gomes et al, 2000; Matte et al, 2002; Thalhofer et al, 2011; Ribeiro-Gomes and Sacks, 2012). Viable *L. major*, *L. donovani* and *L. aethiopica* promastigotes release LCF that induces the migration of human PMN (van Zandbergen et al, 2002). In the present study, live *L. infantum* promastigotes and especially culture supernatants induced a strong PMN chemotaxis, suggesting that some excretory/secretory substances released by the parasite are dog PMN chemoattractant. Thus, *Leishmania* modulates leukocyte recruitment at the early phase of infection, ensuring its internalization by neutrophils and consequently protection against soluble innate immune components, such as complement components that can destroy extracellular parasites. However, the previous contact with the parasite inhibited PMN migration, confirming the results obtained by Marques et al (2015) in C57BL/6 mice. These observations suggest that the parasite promotes PMN retention at the infection site, as observed for *Echinococcus granulosus* and *Borrelia burgdorferi* (Shepherd et al, 1991; Hartiala et al, 2008). In fact, some studies had already demonstrated that gp63 of *L. major* and LPG of *L. donovani* inhibit the migration of human and dog neutrophils, respectively (Sørensen et al, 1994; Panaro et al, 1996). Furthermore, in the present study *L. infantum* antigen suppressed PMN chemotaxis, probably blocking the chemotaxis receptors or inhibiting its expression. The *in vivo* interaction between fluorescent *L. major* parasites and neutrophils in mice using a two-photon microscopy reinforces this theory. While moving rapidly to the site of parasite

inoculation, neutrophils are about 80% slower after parasite phagocytosis (Peters et al, 2008). Retention of infected PMN in the site of parasite inoculation is a clever strategy that can be assumed as a standstill, ensuring *Leishmania* protection until MØ arrival and finally its transference to the definitive host cell.

Phagocytosis is the hallmark of neutrophils. It is an active, receptor-mediated process, facilitated by pathogen opsonization with specific antibodies and complement components. *L. infantum* uptake by PMN occurs rapidly at inoculation sites and at visceral organs (Rousseau et al, 2001; Thalhofer et al, 2011). *In vitro* studies confirm that PMN phagocytize the parasite using both opsonin-dependent and opsonin-independent uptake mechanisms, although with different kinetics. Opsonized parasites are more quickly phagocytized and to a higher extent (Laufs et al, 2002). However, neutrophils possess a variety of receptors, such as TLR, allowing them to recognize the parasite directly, without the need of opsonization (Hostetter, 2012). Our studies, using 5% heat inactivated FBS demonstrated that after a short parasite exposition, a relatively high number of PMN had strongly linked promastigotes, but only a few cells were in fact parasitized. However, the percentage of infected cells increases over time. These results indicate that parasite adhesion to PMN occurs rapidly, but its internalization requires an increased contact period. Interestingly, *in vitro* studies performed by Marques et al (2015) in C57BL/6 mice and using the same *L. infantum* strain showed a similar percentage (32.3%) of infected PMN after 3 h of incubation, although the percentage of infection at 1 h (15.7%) has been superior when compared with the present work. Furthermore, the described proportion of neutrophils harboring *L. infantum*/*chagasi* amastigotes in BALB/c mice at the inoculation site 24 h post infection was about 20% (Thalhofer et al, 2011). The spatial conditions achieved *in vitro* cultures and the high proportion parasite:neutrophil (5:1) used facilitates parasite attachment to neutrophils and its internalization. In addition, some *in vivo* microenvironmental factors that may render the interaction parasite-neutrophil more difficult and interfere with the internalization are overcome by *in vitro* studies.

As described for *L. donovani* and human PMN (Pearson and Steigbigel, 1981), the attachment between *L. infantum* promastigotes and dog PMN was non-random. Indeed, promastigotes preferentially adhered to PMN by the flagellum tip (anterior pole), which probably reflects the concentration of the main adhesion molecules (gp63 and LPG) in specific areas (adhesiotopes) of the parasite membrane (Rittig and Bogdan, 2000). The

attachment *via* the flagellum tip promotes the protrusion of symmetrical pseudopods that maintain the directional entry of the parasite into the PMN (symmetrical phagocytosis) and can influence the subsequent parasite fate, favoring parasite killing (Hsiao et al, 2011).

Our *in vitro* studies revealed that the majority of internalized parasites maintain an elongated appearance, suggesting that promastigotes did not differentiate into the amastigote form. Indeed, using TEM and by time-lapse microscopy van Zandbergen et al (2004) showed that *L. major* parasites preserve the promastigote form inside human PMN 42 h after co-culture establishment. Probably PMN intracellular medium does not provide the necessary conditions for the differentiation process and/or the period of time that the parasite remains inside the PMN is not sufficient to complete this process. This observation reinforces the role of PMN as ordinary transitional host cells.

Although some reports refer to the absence of parasite multiplication inside neutrophils (Müller et al, 2001; Laufs et al, 2002), in our study compatible images of intracellular promastigote division were observed. Although parasites of the stationary phase of growth were used we cannot exclude the possibility of neutrophils having phagocytized dividing parasites. Furthermore, there are no reports of promastigote division inside the mammalian host. The engulfment of dividing parasites denotes PMN ability to internalize large particles.

PMN are able to eliminate *Leishmania* parasites through phagocytosis-dependent and phagocytosis-independent mechanisms (Abi Abdallah and Denkers, 2012). Phagocytosis of microorganisms triggers the fusion of primary and secondary granules with the membrane of the phagosome and the rapid assembly of the NADPH oxidase (Segal, 2005). The high concentration of ROS and of antimicrobial peptides achieved into the phagosome is responsible for microorganism killing (Mayer-Scholl et al, 2004). ROS are critical components of the microbicidal armament of PMN and the induction of a strong oxidative burst results in the elimination of *L. donovani* and *L. major* promastigotes by human PMN (Pearson and Steigbigel, 1981; Laufs et al, 2002). The present study demonstrated that at the early stage of infection (1.5 h) and later on (3 h), *L. infantum* promastigotes activated PMN to release greater amounts of O_2^-. However, a more prolonged exposure to the parasite suppressed O_2^- production probably as a consequence of PMN death or as a result of parasite derivative ROS-deactivating molecules. Indeed, several *Leishmania* molecules

that detoxify the respiratory burst in phagocytes have been identified, as are the cases of tryparedoxin peroxidase and of iron superoxide dismutase secreted (Fe-SODe) by promastigotes (Longoni et al, 2013; Kima, 2014).

Phagocytosis independent killing occurs through NET formation with PMN granule content, DNA and histones being released into the extracellular milieu (Abi Abdallaah and Denkers, 2012). Due to the microbicidal effect, NET are an innate response that can contribute to diminish the parasite burden (Brinkmann et al, 2004), modulating the immune response towards a pro-inflammatory response (Luo et al, 2014). *L. amazonensis*, *L. donovani* and *L. major* induce human neutrophils to emit NET. Interestingly, our study demonstrated that *L. infantum* almost abolish NET formation by dog PMN, indicating that the parasite modulates negatively this effector mechanism, favoring parasite spreading and survival and preventing the detrimental pro-inflammatory response NET induced. The ability to suppress NET formation was described for *Bordetella pertussis* and *Streptococcus pyogenes* and attributed to *B. pertussis* adenylate cyclase toxin (Eby et al, 2014) and *S. pyogenes* streptolysin O (Uchiyama et al, 2015) exotoxins. Furthermore, bacteria that are small enough to be phagocytized, such as *E. coli* and *Klebsiella pneumonia* do not induce NETosis, but large pathogens such as *C. albicans* hyphae and extracellular *M. bovis* aggregates do, suggesting that neutrophils sense microbial size and selectively release NET in response to large pathogens that cannot be phagocytized (Branzk et al, 2014). As previously demonstrated, dog PMN readily phagocytize *L. infantum* promastigotes, which probably downregulate NET release, minimizing tissue damage.

Upon neutrophil activation, primary granule content is released in the extracellular medium where it may contribute to extracellular killing. Our study showed that *L. infantum* stimulates NE exocytosis as observed in C57BL/6 mouse neutrophils also exposed to *L. infantum* promastigotes (Marques et al, 2015) and as registered for both peritoneal and bone marrow derived neutrophils exposed to *L. braziliensis* (Falcão et al, 2015). Interestingly, NE was associated with the killing of *L. major* internalized by MØ and co-cultured with neutrophils of B6 mice (Ribeiro-Gomes et al, 2004). The activation of MØ for intracellular killing of *L. major* seems to be mediated by NE through recruitment *via* TLR4 (Ribeiro-Gomes et al, 2007). Furthermre, NE exocytosis probably prevents NE translocation to the nucleus where cleaves histones to decondense chromatin as part of NET formation (Papayannopoulos et al, 2010), thus avoiding the release of NET by dog PMN.

The role of PMN as invading microorganism destroyers is essential for organism survival. The capability of dog PMN to kill *L. infantum* promastigotes was investigated, *in vitro*. Just two hours of PMN-*L. infantum* contact impact negatively on the viability of promastigotes, pointing towards phagocytosis dependent or independent killing. OM and TEM images from cultures PMN-*L. infantum* promastigotes confirmed parasite death. Indeed, intracellular parasites showing no structural integrity located inside large vacuoles were observed, suggesting phagocytosis dependent killing. The presence of the parasite inside spacious phagosomes, indicates a possible fusion of neutrophil granules with the phagosome-containing parasite. Gueirard et al (2008) observed the uptake of *L. donovani* by mouse and dog neutrophils *via* a lytic organelle-dependent pathway, leading to the formation of large phagosomes and to parasite degradation. In the present study extracellular degraded parasites were also observed in close contact with necrotic PMN, pointing towards phagocytosis independent killing. Interestingly, images of parasite degradation were more frequent in some dogs, suggesting a different response depending on the cell donor.

Furthermore, in a few dogs the parasite was detected almost exclusively outside the cell, closely associated with the cell membrane even after a prolonged time of incubation (3 h). As the parasite undergoes phagocytosis as a passive partner, this finding suggests different PMN responses to the parasite in a host dependent manner. This situation is not surprising if we make the parallel with mouse models of *L. major* infection. Although resistance to the infection in C57BL/6, C3H and CBA were related to Th1 and BALB/c susceptibility to Th2 immune response, several studies showed that neutrophils may play a protective or a deleterious impact on the disease outcome (Charmoy et al, 2010; Hurrell et al, 2016). Indeed, the genetic background of the dog, their breed, age, sex and lifestyle may justify our results.

The minimal parasite phagocytosis evidenced by some dogs is a very surprising finding that lead us to speculate on the possible mechanisms involved. It is possible that the low expression of membrane receptors required for parasite internalization by dog neutrophils hampers phagocytosis. Another interesting question is the fate of these membrane associated parasites. It is probable that *in vivo* conditions, these extracellular promastigotes that do not hide inside neutrophils can be destroyed by soluble innate immune factors, namely by MAC. In this case, the dogs could have an evolutionary advantage being

resistant to *Leishmania* infection. However, it is also possible that these parasites might simply avoid the transitory neutrophil shelter, waiting for the arrival of definitive host cells.

Although the contact of promastigotes with dog PMN reduced parasite burden, a considerable proportion of parasites resist to PMN deleterious action. Indeed *L. infantum* promastigotes seem to be well equipped to evade killing by dog PMN as observed for other *Leishmania* species. For example, in the absence of serum supplementation *L. major* blocks the oxidative burst within human neutrophils and avoids elimination (Laufs et al, 2002) and *L. donovani* prevents the fusion between parasitophorous vacuole and mouse neutrophilic granules in a mechanism LPG dependent (Gueirard et al, 2008). LPG, the major surface phosphoglycan of *Leishmania*, has been involved in the parasite ability to inhibit phagolysosome formation (Desjardins and Descoteaux, 1997; Scianimanico et al, 1999). LPG was also proved to inhibit the association and retention of the NADPH oxidase cytosolic components $p47^{phox}$ and $p67^{phox}$ to the phagosome membrane, preventing O_2^- generation (Lodge and Descoteaux, 2005; Lodge et al, 2006). Interestingly, like Gueirard et al (2008), two distinct types of phagosomes were also found in the same dog cell: a large spacious phagosome containing a degraded parasite and a tight phagosome containing an intact parasite, suggesting that different parasites induce different PMN responses. Even thought that stationary growth phase parasites were used in the entire study, these cultures comprise a heterogeneous population of promastigotes with different abundance of virulence factors, namely gp63 and LPG (Ueno and Wilson, 2012). So, it is possible that the most virulent promastigotes survive dog neutrophil effector machinery while non-virulent or less virulent parasites are killed.

However, the parasite also impacts negatively on dog PMN viability. Actually, the presence of the parasite reduced the proportion of viable cells assessed by flow cytometry. In addition to NETosis that was already discussed, other types of neutrophil death can contribute to the observed reduction of viable cells, including apoptosis (programmed cell death) and necrosis.

The control of neutrophil cell death and the subsequent clearance of dying cells are crucial for the efficient resolution of inflammation. Neutrophils undergo spontaneous apoptosis at inflamed sites, which contributes to the safe disposal of engulfed microorganisms and avoids the release of potentially injurious granular enzymes (Geering and Simon, 2011; Iba

et al, 2013). Several microorganisms, including some species of *Leishmania* were able to modulate neutrophil apoptosis, prolonging its life span or accelerating its death (Aga et al, 2002; Gueirard et al, 2008; Carlsen et al, 2013; Falcão et al, 2015). *Leishmania* also uses apoptotic neutrophils as ‘‘Trojan horses’’ to gain access to their definitive host cells (van Zandbergen et al, 2004; Laskay et al, 2008; Peters et al, 2008). In our study, although not statistically significant, the proportion of apoptotic cells decreased early at the infection, but later on the proportion of apoptotic cells increased. Interestingly, an *ex vivo* study showed that dogs with severe CanL exhibited higher neutrophil apoptosis when compared with dogs with moderate disease (Almeida et al, 2013).

In vivo studies developed by our group showed that 3 to 4 h after experimental dermal injection, promastigotes were already phagocytized by dog neutrophils and by a few MØ, but infected cells progressively disappeared from the skin and at 24 h parasites were no longer observed at the inoculation site (Santos-Gomes et al, 2000). This scenario is quite different from the one observed for cutaneous *Leishmania* species that cause disease in the inoculation site. Indeed *L. major* delays the apoptotic death program of PMN by about 24 h, ensuring that the peak migration of MØ into the infected tissues coincides with time point when infected PMN become apoptotic, thus ensuring the transference of the parasite to MØ (Aga et al, 2002). In the dog-*L. infantum* model of infection, the kinetics of MØ recruitment seems to be faster, justifying the induction of apoptosis by the parasite to favour their rapid transference to the definitive host cell and dissemination throughout the body. Interestingly, infected neutrophils seem to secrete monocyte-attracting chemotactic factors such as MIP1-β, which participate in the recruitment of monocytes (van Zandbergen et al, 2004). Other molecules, such as neutrophil granule proteins and annexin A1 (or lipocortin I) are also chemoacttactants for monocytes although their involvement in the infection by *Leishmania* has not been proved. Curiously, annexin A1 that is present/released by apoptotic neutrophils, participates in the resolution of inflammation by promoting the elimination of death neutrophils and in the alternative activation of MØ, which downregulates the production of proinflammatory mediators (Sugimoto et al, 2016). *Leishmania* internalization by MØ *via* the uptake of infected apoptotic PMN prevents the direct interaction with surface receptors, avoiding the activation of MØ effector mechanisms and ensuring parasite survival and multiplication (van Zandbergen et al, 2004; Laskay et al, 2008).

In our study *L. infantum* induced a pronounced PMN necrosis. However, the secondary necrosis subset largely prevailed over the primary necrosis subset, suggesting that *L. infantum*-exposed PMN induce extensive cell apoptosis that undergo subsequent secondary necrosis. The well defined subsets of PMN displaying different amounts of PI reinforce this possibility, pointing towards different stages of apoptosis-necrosis transition. This kind of cell death combines the features of apoptosis, such as nuclear apoptotic changes (fragmentation and intense chromatin condensation) and of necrosis, including swelling of the cell and damage to the cytoplasmic membrane (revealed by PI positive staining) (Silva et al, 2008, Iba et al, 2013). Secondary necrosis occurs when cells are subjected to a strong stimulus (Iba et al, 2013) or when the removal of apoptotic cells by scavengers is not self-sufficient. Indeed, in monocultures apoptotic PMN are not removed, leading to its elimination by disruption (Silva, 2010).

Unlike apoptosis, cell death by necrosis is characterized by an uncontrolled release of toxic substances that can propagate the inflammatory response leading to tissue damage. The release of DAMP by necrotic cells that are recognized by PRR stimulates the synthesis of pro-inflammatory mediators, such as cytokines (Scaffidi et al, 2002), generating an environment unfavorable for parasite survival. However, *in vivo*, the majority of apoptotic PMN is probably removed by MØ and other phagocytic cells, avoiding secondary necrosis.

Despite the overall increase in apoptosis (annexin V $FITC^+$ cells) in prolonged parasite-exposed cells (3 h), the majority of apoptotic cells was not parasitized. Indeed, internalized *L. infantum* parasites or even their products seem to protect dog neutrophils from apoptosis at early stages of infection, promoting cell survival. The mechanism implicated in this interaction is unknown, but LPG might be involved (Lüder et al, 2001). On the contrary, non-infected cells might be directly affected by soluble factors released by extracellular parasites, undergoing apoptosis and discharging chemotactic factors for monocytes.

Although blood peripheral neutrophils may act differently from tissue neutrophils and the results obtained here must be interpreted with caution, these *in vitro* findings highlight the key importance of neutrophils in the early phase of *L. infantum* infection. The innate immune defense could be decisive, resolving the infection and preventing parasite access to the definitive host cell. However, as the first innate immune encounters, dog neutrophils retain the parasites in the inflammatory inoculation site, protecting them from the soluble

innate mediators. Furthermore, canine neutrophils seem to select the most infectious parasites that will be able to establish infection in the dog. Instead of killing the parasite, dog neutrophils act as temporary permissive host cell of the parasite. On the other hand, the parasite seems to use this phagocytic cell to assure CanL development. However, differences in terms of parasite killing capacity and the absence of parasite phagocytosis revealed by some dogs raise interesting questions in terms of individual resistance. This possibility is supported by the “in the field” observation of resistant dogs, which has been attributed to the ability of T lymphocytes to initiate a pro-inflammatory response. However, taking these findings, the contribution of innate mechanisms, namely neutrophilic activity requires further research.

3 EFFECTOR FUNCTIONS OF MACROPHAGES

3.1 INTRODUCTION

3.1.1 Monocytes and macrophages

Monocytes originate in the bone marrow from a common hematopoietic stem cell (HSC), which undergoes differentiation steps to give rise to the myeloid (granulocyte/macrophage colony-forming units) and then to the monocyte lineage (macrophage colony-forming units). In response to macrophage colony-stimulating factor (M-CSF), macrophage colony-forming unit divides and differentiates into monoblasts, pro-monocytes and ultimately into monocytes, which exit the bone marrow and enter the bloodstream (Mosser and Edwards, 2008).

Blood monocytes are a morphologically and phenotypically heterogeneous population (Chávez-Galán et al, 2015), which comprises 1-4% of the total white blood cells in the mice, 4-8% in humans and 1-10% in the dog. In mice, two populations of monocytes have been identified, based on the expression of cell-surface markers: a short-lived subset phenotypically described as $Ly6C^{+}$ CX_3CR1^{low} $CCR2^{+}$ $CD62L^{+}$ and found in inflamed tissue (inflammatory monocytes) and, a second subset with a longer half-life, phenotypically identified as $Ly6C^{low}$ CX_3CR1^{hi} $CCR2^{-}$ $CD62L^{-}$ and observed in non-inflamed tissues (resident monocytes) (Geissmann et al, 2003). These subsets of murine monocytes can be compared with the short and long-lived subpopulations of human monocytes. Their main phenotypic characteristics were related to different surface expression markers: the “classical” $CD14^{++}$ $CD16^{-}$ subset, which comprises approximately 90% of human monocytes, and the “non-classical” $CD14^{+}$ $CD16^{++}$ population, which are synonym of $CD62L^{+}$ and $CD62L^{-}$ murine subsets, respectively (Passlick et al, 1989; Mosser and Edwards, 2008; Chávez-Galán et al, 2015).

Monocytes migrate from the bloodstream into the tissues *via* chemokine receptor 2 (CCR2) and chemokine chemoattractant protein 3 (MCP-3) signaling (Tsou et al, 2007) and differentiate into MØ in a tissue-dependent manner, acquiring specific phenotypes and functional characteristics. According to their anatomical location, MØ are differently named, such as alveolar MØ in the lungs, microglia in the central nervous system, Kupffer

cells in the liver, osteoclasts in bone, histiocytes in the connective tissue (Mosser and Edwards, 2008; Chávez-Galán et al, 2015).

The colony-stimulating factor (CSF), macrophage colony-stimulating factor (M-CSF or CSF-1) and granulocyte-macrophage colony-stimulating factor (GM-CSF or CSF-2), were originally defined as hemopoietic growth factors. CSF-1 is ubiquitously produced by many tissues (Lacey et al, 2012) and CSF-1 receptor (CSF-1R), which is activated by the ligands CSF1 and IL-34, control the abundance of monocytes and of MØ in the tissues (Wei et al, 2010). CSF-2 is produced by specific tissues, retaining MØ locally (Lavin et al, 2015). Both CSF-1 and CSF-2 promote survival, proliferation, differentiation and activation of MØ (Lacey et al, 2012).

The concept that MØ are fully differentiated cells without proliferative potential that are replaced by circulating monocytes produced by the bone marrow has been questioned. Several studies have established the embryonic origin of some tissue-resident MØ, which self-maintain locally (Lavin et al, 2015; Sica et al, 2015).

Although it is accepted that monocytes produced in the bone marrow are recruited in large numbers to the injured tissues where they differentiate into MØ and DC, accumulating evidence suggests that the expansion of tissue-resident MØ also contributes to MØ replacement. However, the contribution of monocyte-derived MØ to the resident MØ population may depend both on the organ and the nature of the injury. In contrast, in most healthy tissues, MØ are maintained with a minimal contribution of circulating monocytes (Lavin et al, 2015; Sica et al, 2015).

3.1.2 *Leishmania* recognition and attachment by macrophages

Phagocytosis is a cellular uptake pathway for particles of greater than 0.5 µm in diameter (Mao and Finnemann, 2015). This process is essential for nutrient recycling, including iron recovery from dying erythrocytes, homeostasis and immune defense (Mosser and Edwards, 2008; Chávez-Galán et al, 2015; Arango Duque and Descoteaux, 2015).

Anti-pathogen phagocytosis, known as inflammatory phagocytosis is performed by professional phagocytes, such as neutrophils, DC and MØ (Mao and Finnemann, 2015). Anti-pathogen phagocytosis accomplishes two essential immune functions: the removal and degradation of microorganisms and direct antigens to both MHCI and II compartments for presentation, linking the innate and acquired immunity (Greenberg and Grinstein, 2002; Mao and Finnemann, 2015).

MØ infiltrate the canine dermis just a few hours after *L. infantum* intradermal inoculation (Santos-Gomes et al, 2000) and are considered the final host cells of this obligate intracellular parasite, ensuring its differentiation, multiplication and dissemination. *Leishmania* is successfully adapted to reside inside MØ, employing several evasion strategies (Liu and Uzonna, 2012). Quoting Arango Duque and Descoteaux (2015) "Living inside of the macrophage is an ideal way to escape the immune system, obtain nutrients, and proliferate". However, properly activated MØ are the major effector cells to eliminate the parasite (Liu and Uzonna, 2012).

The mechanism of parasite attachment and engulfment by MØ has been studied. Although early studies described the polarization of *Leishmania* parasites during attachment and phagocytosis by MØ as random, subsequent reports showed that *L. mexicana*, *L. tropica*, *L. braziliensis*, *L. major* and *L. aethiopica* promastigotes preferentially adhere with either the tip or the base of their flagellum, whereas *L. donovani* promastigotes attach either with the flagellar tip or the afagellar (posterior) pole and *L. enrietti* promastigotes bind MØ primarily by the posterior pole (Rittig and Bogdan, 2000).

Microscopic analysis revealed that the engulfment of adhering promastigotes takes place *via* funnel-like extensions of the phagocyte surface extending along the parasite. These funnel-like extensions were observed in human and murine MØ when internalizing *L. donovani*, *L. tropica*, *L. major* or *L. aethiopica* promastigotes. The formation of coiling rather than tubular (symmetrical) pseudopods around *L. major* or *L. aethiopica* promastigotes in human peripheral blood monocytes was also described (Rittig and Bogdan, 2000).

The phagocytosis is less efficient when promastigotes are positioned tangentially to the MØ membrane, compared with perpendicular position. "Coiling" phagocytosis occurs

when asymmetric pseudopod wrapped around an incoming particle, forming the characteristic ultrastructural multilayered coil and it could be related to promastigote reorientation after initial lateral attachment. Symmetrical MØ pseudopod extension characterizes the zipper-like phagocytosis mechanism (Hsiao et al, 2011).

Internalization of *Leishmania* promastigotes is a classical receptor-mediated process involving *Leishmania* and MØ surface molecules. MØ receptors reported to mediate *Leishmania* internalization include CR3, CR1, mannose receptor (MR), FcγR, in particular FcγRII-B2, fibronectin receptors (FnR) and MØ receptor with collagenous structure (MARCO) (Gomes et al, 2009; Ueno and Wilson, 2012). Two major *Leishmania* promastigote surface components have been shown to participate in the attachment process, the gp63 and LPG. However, some studies have revealed that internalization of LPG-defective *Leishmania* promastigotes is superior to that of wild-type parasites (McNeely and Turco, 1990; Descoteaux et al, 1992; Holm et al, 2003; Späth et al, 2003), suggesting that LPG does not play a major role in promastigote adhesion to MØ (Vinet et al, 2011).

MØ receptors used during phagocytosis impact the intracellular fate of the parasite (Ueno and Wilson, 2012; Liévin-Le Moal and Loiseau, 2016). Parasite uptake *via* CR seems to be a silent way of entry into the host cell, not eliciting the effector functions of MØ and consequently favoring parasite survival (Mosser and Brittingham, 1997; Bogdan and Röllinghoff, 1998; Ueno et al, 2009). However, different sources of MØ and different *Leishmania* species, culture growing phase or parasite stage vary in their surface molecular composition, leading to unique parasite-host receptor interactions (Hsiao et al, 2011; Ueno and Wilson, 2012). During a natural infection, it is likely that *Leishmania* simultaneously ligates more than one host cell receptor and that all receptors contribute to subsequent intracellular events (Mosser, 1994; Ueno and Wilson, 2012).

The interaction between receptors and complementary ligands guide local shape changes in the plasma membrane of the phagocytic cell to wrap around the microorganism. The structure beneath the bonded particle formed by a network of branched F-actin has been termed phagocytic cup (Swanson, 2008; Mao and Finnemann, 2015). F-actin polymerization is crucial for phagocytic cup formation. F-actin depolymerizing drugs cytochalasins and latrunculin block phagocytosis. The extensive F-actin re-organization required for phagocytosis is controlled by GTPase Rho family, whose members regulate

many aspects of intracellular actin dynamics. The closure of the phagocytic cup, which involves the fusion of protruded membranes and disassembly of F-actin, leads to particle internalization and phagosome formation (Mao and Finnemann, 2015). To replenish the extension of the plasma membrane used to form the phagosome of large particles such as *Leishmania* promastigotes, focalized exocytosis of endomembranes occurs at the phagocytic cup (Huynh et al, 2007; Vinet et al, 2011). Subsequently, the phagosome matures upon fusion with endosomes and lysosomes (Mao and Finnemann, 2015).

3.1.3 Phagosome maturation

The maturation of phagosome into phagolysosome is referred as phagolysosome biogenesis. This process is essential for the acquisition of microbicidal properties required for microorganism killing and processing of some of their proteins for antigen presentation (Desjardins et al, 2005; Jutras and Desjardins, 2005).

Phagosome maturation in MØ and neutrophils is strikingly different. While phagocytosis of microorganisms triggers the immediate fusion of primary and secondary granules with the membrane of the phagosome in neutrophils, maturation of MØ phagosome is achieved by continuous fusion and fission events (Harrison and Grinstein, 2002; Weiss and Schaible, 2015). As a result of highly regulated fusion and fission events with ER, early endosomes, late endosomes and lysosomes, the membrane of the phagosome sequentially acquires a set of different stage-specific markers and intravesicular components. Phagosome maturation involves the coordinated transfer of hydrolases, as well as the acquisition of the oxidative machinery, which comprises the following stages: early phagosome, late phagosome and phagolysosome, resulting ultimately in the microorganism killing (Liévin-Le Moal and Loiseau, 2016; Weiss and Schaible, 2015).

Fusion events require complex molecular machinery. The initial capture and docking of vesicles at the target membranes is controlled by several families of tethering factors, including the Rab family of small G proteins. Once docked, the vesicle and target membranes must be fused, a process that is mediated by SNARE [soluble-N-ethylmaleimide sensitive-factor accessory-protein (SNAP) receptor] family. On a donor membrane SNARE protein binds to cognate SNARE on the target membrane in a union (a

trans-SNARE complex) that transiently bridges the two membranes. Each cell type expresses different combinations of SNARE-family members that are selectively distributed on organelles and membrane domains (Stow et al, 2006).

The activity of SNARE complexes is controlled by synaptotagmins (Syt), a family of transmembrane proteins that regulates vesicle docking and fusion in a Ca_2^+-dependent manner (Arango Duque and Descoteaux, 2015). In MØ, Syt XI is a recycling endosome- and lysosome-associated protein that negatively regulates the secretion of TNF-α and IL-6 (Arango Duque et al, 2013) and Syt V, which is recruited to the forming phagosome and regulates the phagocytosis. Syt V contributes to phagolysosome biogenesis by regulating the acquisition of cathepsin D and vesicular ATPase (v-ATPase), which participates in phagolysomal acidification (Vinet et al, 2008, 2009). The important role of SNARE and Syt in vesicle trafficking make these proteins great targets for attack by intracellular pathogens (Arango Duque and Descoteaux, 2015).

The Rab family is part of the Ras superfamily of small GTPases. The different Rab GTPases localized at the cytoplasmic leaflet of phagosomal membrane are stage specific and regulate distinct steps in the membrane traffic pathways. In the GTP-bound form, the Rab GTPases recruit specific sets of effector proteins onto membranes. Through their effectors, Rab GTPases regulate vesicle formation, actin- and tubulin-dependent vesicle movement and membrane fusion (Stenmark and Olkkonen, 2001).

Early endosome antigen 1 (EEA1) localizes exclusively to early endosomes and has an important role in endosomal trafficking. EEA1, directly binds to the phospholipid phosphatidylinositol 3-phosphate (PI3P) and acts as a tethering molecule that couples vesicle docking with SNARE, bringing the endosomes physically closer and ultimately resulting in the fusion and delivery of endosomal cargo. Phospholipid PI is synthesized by phosphatidyl inositol kinases (PIK) at the cytoplasmic leaflet of phagosomal membranes and is stage specific, contributing to vesicle fusion and actin polymerization (Lawe et al, 2002; Weiss and Schaible, 2015).

When phagosome fuses with early endosome, it acquires markers such as the small GTPase Rab5 and the EEA1. Early fusion events involve the activity of PI3K, which generates PI3P on the phagosomal membrane. The early phagosome, which lacks the proton pumping v-

ATPase is therefore characterized by a mildly acidic pH of 6-6.5 (Vieira et al, 2001; Weiss and Schaible, 2015).

The late phagosomal stage loses early markers and acquires mannose-6-phosphate receptor (M6PR), Rab7 and the lysosome-associated membrane proteins (LAMP1 and 2) as late markers. The hallmark of the late phagosome is its luminal acidification (pH ≈5). A low pH is crucial for the release of newly synthesized lysosomal enzymes from M6PR into the phagosomal lumen. v-ATPase, which is assembled in the late phagosomal membrane generates and maintains the pH gradient using the energy of ATPase to pump protons into the lysosomal lumen (Lafourcade et al, 2008; Mindell, 2012; Weiss and Schaible, 2015).

The phagolysosome is the final stage and it is characterized by a pH of 4-4.5, high hydrolase activity and low amount of M6PR and PI3P. The hydrolases, including proteases, peptidases, phosphatases, nucleases, glycosidases, sulfatases and lipases have different target substrates and their activity leads to the degradation of all types of macromolecules. Among the lysosomal hydrolases, the best known are the cathepsin family of proteases. The acidic environment of the lysosomal lumen facilitates the degradation process and is optimal for the activity of lysosomal hydrolases. It was also proposed that the acidic pH and high temperature participate in promastigote-to-amastigote differentiation (Liévin-Le Moal and Loiseau, 2016; Weiss and Schaible, 2015), with the involvement of other signals, including stress signals (Späth et al, 2015), as described above.

Non-degradable material is removed from the cell by exocytosis. During the course of microorganism killing, some of their proteins are processed for antigen presentation by MHCII molecules. Furthermore, the interaction between phagosomes and a subset of the ER provides a novel pathway for the loading of exogenous peptides onto MHCI molecules, a process known as cross-presentation (Jutras and Desjardins, 2005; Campbell-Valois et al, 2012).

3.1.4 Modulation of phagosome fusion machinery by *Leishmania*

The predominant intracellular location of *Leishmania* is the phagolysosome or parasitophorous vacuole. Parasitophorous vacuoles harboring intracellular *Leishmania* are

not homogeneous, showing a species-specific heterogeneity. Members of the Old World *Leishmania* reside within ‘tight’ parasitophorous vacuoles usually bearing a single amastigote, whereas New World *Leishmania* including *L. mexicana* reside in ‘spacious’ vacuoles typically bearing many parasites (Rittig and Bogdan, 2000).

LPG contributes to the establishment of infection inside MØ, possibly by creating favorable conditions for the promastigote-to-amastigote differentiation (Lodge and Descoteaux, 2005). The importance of LPG in the establishment of infection inside MØ was evidenced by the demonstration that LPG-defective mutants are rapidly destroyed after phagocytosis and that passive transfer of purified LPG significantly prolonged their survival (Handman et al, 1986; McNeely and Turco 1990). Thus, without LPG promastigotes are unable to resist inside the maturing parasitophorous vacuole. However, *Leishmania* amastigotes synthesize little or no detectable LPG (McConville and Blackwell, 1991), but resist to lysosomal enzymes and to the acidic pH inside the phagolysosome (Antoine et al, 1990). Thus, the role of this molecule in parasite survival may be restricted to the establishment of infection, during promastigote-to-amastigote differentiation, which happens in 12 to 24 h (Van Assche et al, 2011; Naderer and McConville, 2011). Interestingly, LPG does not appear to be required for mice or MØ infection by *L. mexicana* (Ilg, 2000).

One mechanism used by promastigotes to evade the microbicidal consequences of phagocytosis is the inhibition of phagolysosome biogenesis, promoted by LPG repeating unit (Desjardins and Descoteaux, 1997; Lodge and Descoteaux, 2008). LPG disturbs the interaction between phagosome and late endosomes and lysosomes, delaying the recruitment of LAMP-1 and Rab7 to the phagosome (Scianimanico et al, 1999; Dermine et al, 2000; Späth et al, 2003). Preventing phagosome maturation gives the engulfed promastigotes time to evolve into amastigotes (Desjardins and Descoteaux, 1997).

Based on detergent-resistance, membrane rafts are defined as membrane microdomains enriched in glycosphingolipids and glycerolipids, containing mainly saturated fatty acid residues, cholesterol and lipid-modified proteins, including the GPI-anchored ones. These structures have been implicated in numerous cellular processes, including signal transduction, membrane trafficking and molecular sorting (Horejsi and Hrdinka, 2014). Lipid microdomains serve as platforms for actin organization and clustering of signaling

molecules involved in phagocytosis and also in exocytosis regulation, a process which is essential for the recruitment of endomembranes during phagocytosis (Hackam et al, 1998; Bajno et al, 2000; Salaün et al, 2004; Huynh et al, 2007). Furthermore, several processes take place within these domains, including the assembly of the NADPH oxidase and the recruitment of v-ATPase (Galli et al, 1996; Dermine et al, 2001; Vilhardt and van Deurs, 2004; Lafourcade et al, 2008).

When *Leishmania* promastigotes bind MØ surface, LPG is transferred from the parasite surface into the outer leaflet of the MØ plasma membrane and then to the inner leaflet of the nascent phagosomal membrane (Tolson et al, 1990), where it disrupts lipid microdomains (Dermine et al, 2005; Winberg et al, 2009). The insertion of LPG into the membrane of the phagosomes excludes Syt V from lipid microdomains, resulting in the reduction of the phagocytic capacity of MØ. Although LPG-mediated exclusion of Syt V reduces parasite internalization, in turn abrogates the recruitment of the v-ATPase and impedes phagosome acidification (Vinet et al, 2009, 2011).

Early phagosomes are surrounded by a distinct layer of F-actin, which progressively disappears as the phagosome matures (Greenberg et al, 1991). Actin turnover (disassembly) is mediated by PKC that phosphorylates myristoylated alanine-rich C kinase substrate (MARCKS) and MARCKS-related proteins (MRP) (Bhardwaj et al, 2010). This process is crucial for phagosomal maturation, whereas F-actin accumulation prevents phagosome-endosome fusion (Allen and Aderem, 1995). LPG induces a progressive accumulation of periphagosomal F-actin during phagocytosis of *L. donovani* promastigotes, which affects the recruitment of LAMP1, a marker of late endosomes, suggesting that F-actin form a physical barrier that prevents the interaction between the endosome containing the parasite and late endosomes and lysosomes (Holm et al, 2001; Lodge and Descoteaux, 2005). It was observed that F-actin accumulation is induced through phagosomal retention of the Rho-family GTPases Cdc42 and of proteins required for F-actin assembly (Arp2/3, WASP, Myosin, α-actinin) in a LPG-dependent manner (Lodge and Descoteaux, 2005). Furthermore, LPG also inhibits phagosomal maturation by inhibiting PKC-α dependent disassembly of periphagosomal F-actin (Bhardwaj et al, 2010).

The impact of other promastigote virulence factors on the phagosome maturation and function was also investigated. gp63 cleaves a subset of SNARE, including VAMP8, preventing the assembly of NADPH oxidase complex on the phagosome, which leads to decreased ROS production (Matheoud et al, 2013).

Processing exogenous antigens for cross-presentation within phagosomes is a highly complex procedure that requires limited proteolytic activity in part regulated by the intraphagosomal pH and the levels of hydrolytic enzymes (Delamarre et al, 2005). Phagosomal proteolysis and potentially pH is regulated by the activity of the NADPH oxidase (Savina et al, 2006; Rybicka et al, 2012). In the absence of VAMP8, the pH within the phagosome lumen decreased and the proteolytic activity increased. Matheoud et al (2013) showed that the inhibition of exogenous antigens presentation on MHCI molecules resulted in reduced T $CD8^{+}$ cell activation. These findings indicate that *Leishmania* subverts immune recognition by altering phagosome function.

Furthermore, using *L. major* strains that express or lack gp63 it was found that this protease degrades Syt XI and positively regulates the post-infection release of TNF and IL-6. RNA interference studies performed in RAW264.7 MØ showed that cytokine release was induced by gp63-mediated degradation of Syt XI. *In vivo* studies showed that early during infection, gp63 induces the release of TNF and IL-6 and the influx of neutrophils and inflammatory monocytes to the inoculation site. These phagocytes have been shown to be *Leishmania* target cells, proving that the parasite exploits the immune response to establish infection (Arango Duque et al, 2014).

Despite the modulatory effect exerted by the parasite, host genetic factors control the intraphagosomal conditions faced by the parasite. Slc11a1, formerly named N-RAMP1 is a proton efflux pump that translocates Fe_2^{+} and Mn_2^{+} ions from MØ lysosomes/phagolysosomes into the cytosol. Mutations in Nramp1 cause susceptibility to *Leishmania* infection, indicating that an available pool of intraphagosomal iron is critical to the intracellular survival and replication of the parasite (Huynh and Andrews, 2008). In the context of VL, genetically resistant mouse strains (*e.g.* CBA) have a functional Slc11a1 gene and are able to control early parasite growth, whereas genetically susceptible mouse strains (including C57BL/6 and BALB/c) have a mutant Slc11a1 gene, producing a nonfunctional Slc11a1 gene product that results in unrestrained parasite growth (Nieto et

al, 2011). A case-control study involving 97 dogs of different breeds associated three single nucleotide polymorphism (SNP) in the N-RAMP1 promoter region (T151C, A180G, G318A) with increased risk for CanL in different breeds and one haplotype (TAG-8-141) in the Boxer breed (Sanchez-Robert et al, 2005). Another case-control study including 152 Spanish dogs of different breeds and 12 Foxhounds from USA associated two more N-RAMP1 polymorphisms (A4549G, C4859T) with increased risk for CanL (Sanchez-Robert et al, 2008). These findings confirm the impact of host genetic background in the biology of MØ and its influence in the resistance/susceptibility to leishmaniosis.

3.1.5 Macrophage activation

The term classically activated MØ (or M1) is used to designate the effector MØ that are produced during cell-mediated immune responses. In the original characterization, the combination of two signals, IFN-γ and TNF, resulted in an MØ population that had enhanced microbicidal or tumoricidal capacity and secreted high levels of pro-inflammatory cytokines and mediators. Classically activated MØ are also induced by LPS and GM-CSF (Chávez-Galán et al, 2015; Sica et al, 2015).

NK respond to stress and infections with the production of IFN-γ, which is generally transient and therefore cannot sustain MØ activation. Consequently, an adaptive immune response is usually needed to maintain classically activated MØ and confer stable host defense against many intracellular microorganisms. This is typically provided by the sustained production of IFN-γ by Th1 cells and cytotoxic T $CD8^+$ cells (Mosser and Edwards, 2008). The original two-signals required for the classical activation can be achieved by TLR agonists, which induce both TNF and IFN-β production. TLR ligands that act in a MyD88-dependent manner will induce the transcription of TNF, whereas others acting in the TRIF-dependent pathway signal through IFN-regulatory factor 3 (IRF3) result in IFN-β production (Yamamoto et al, 2003; Mosser and Edwards, 2008). This endogenously produced IFN-β can replace the IFN-γ produced by NK and T cells and classically activate MØ (Mosser and Edwards, 2008).

During the process of MØ activation several genes are triggered by a combination of transcription factors. These include signal transducer and activator of transcription (STAT)

molecules, which are activated following IFN-γ receptor ligation and, nuclear factor-κB (NF-κB) and MAPK, which are activated in response to TLR or TNF receptor ligation (Mosser and Edwards, 2008; O'Shea and Murray, 2008).

Classically activated MØ are characterized by its ability to secrete cytokines such as IL-1β, TNF, IL-12 and IL-18. Phenotypically, they express high levels of MHCII, CD68 marker and the co-stimulatory molecules CD80 and CD86, and initiate microbicidal mechanisms by the synthesis of NO, restriction of iron or nutrients for microorganisms and acidification of the phagosome (Chávez-Galán et al, 2015; Sica et al, 2015). The role of M1 in host defense against *Leishmania* has been well documented. The stimulation of MØ by IFN-γ and TNF before the infection generates a population of MØ that efficiently kills the parasite. Stimulation of MØ with exogenous TNF or by a TLR ligand, such as LPS, results in the complete clearance of the parasite, thereby confirming the importance of TLR activation or TNF production in the development of classically activated MØ. However, when MØ infected with *L. donovani* for 16 h were treated with IFN-γ the intracellular parasite killing decreased, because *Leishmania* prevented efficient MØ activation by interfering with IFN-γ signaling (Nandan and Reiner, 1995; Mosser and Edwards, 2008).

Although important in host defense, classically activated MØ can cause tissue damage and under specific conditions, exacerbate inflammatory processes that contribute to autoimmune pathologies (Mosser and Edwards, 2008; Chávez-Galán et al, 2015).

Alternatively activated MØ (or M2) can be elicited by a variety of stimuli, namely CSF-1, IL-4, IL-10, TGF-β, IL-13, TLR agonists, immune complexes, PG and glucocorticoids (Chávez-Galán et al, 2015; Hume, 2015), giving rise to biochemically and physiologically different types of M2 cells. M2 subpopulation produces IL-10 in high and IL-12 in low amounts and plays a central role in response to parasites, tissue remodeling, angiogenesis and allergic diseases (Chávez-Galán et al, 2015). According to its function, they can be subdivided into wound-healing and regulatory MØ (Mosser and Edwards, 2015).

The wound-healing MØ can be achieved by Th2-type cytokines IL-4 and IL-13 stimulation. One of the first innate signals to be released during tissue injury is IL-4 (Loke et al, 2007). Basophils and mast cells are important early sources of this cytokine, although neutrophils might also contribute to it (Brandt et al, 2000). This early IL-4 production

rapidly converts resident MØ into a population of cells that are programmed to promote wound healing. IL-4 stimulates arginase activity, allowing MØ to convert arginine into ornithine, a precursor of polyamines and collagen, thereby contributing to the production of the extracellular matrix (Kreider et al, 2007).

Wound healing MØ are maintained by IL-4 and IL-13 generated by the adaptive immune response (Mosser and Edwards, 2015). MØ treated *in vitro* with IL-4 and/or IL-13 fail to present antigen to T cells, produce minimal amounts of pro-inflammatory cytokines and are less efficient than classically activated MØ in producing ROS and RNS and, at killing intracellular pathogens (Edwards et al, 2006). These MØ can also exert indirect regulatory effects on the immune response, inhibiting T cell proliferation and the production of Th1 cytokines (Cordeiro-da-Silva et al, 2004).

IL-4-induced polyamine biosynthesis can contribute to the intramacrophagic growth of *Leishmania*. The activity of arginase promotes pathology and uncontrolled growth of *L. major*, *in vivo*, whereas its inhibition during the course of infection has a therapeutic effect, as evidenced by the reduction or absence of clinical signs and the efficient control of parasite replication. Thus, arginase regulates parasite growth directly by inducing the polyamine synthesis in MØ (Kropf et al, 2005).

Another functional type of M2 cells, the immune regulatory, can be elicited by several stimuli. Glucocorticoids released by adrenal cells in response to stress can suppress MØ-mediated host defense and inflammatory functions by inhibiting the transcription of pro-inflammatory cytokine genes and decreasing mRNA stability (Sternberg, 2006), giving rise to a population of regulatory MØ. Regulatory MØ can also develop during the later stages of adaptive immune response and its primary role seems to be dampening the immune response and limit inflammation (Mosser, 2003).

A combination of TLR agonists and immune complexes led to the development of a population of MØ that produce high levels of the immunosuppressive cytokine IL-10. Other factors can provide a signal for the differentiation of regulatory MØ, including PG (Strassmann et al, 1994), apoptotic cells (Erwig and Henson, 2007), IL-10 (Martinez et al, 2008) and some ligands for G-protein-coupled receptors (GPcR) (Hasko et al, 2007). Although there is a subtle difference between subpopulations of MØ generated by different

stimuli, some characteristics are common among them, namely the need for two stimuli to induce their anti-inflammatory activity. In addition to IL-10 production, a marker of regulatory MØ, these cells also down regulate IL-12 production. Therefore, the ratio of IL-10 to IL-12 could be used to define M2 cells (Edwards et al, 2006; Mosser and Edwards, 2015). Because IL-10 can inhibit the production and activity of various pro-inflammatory cytokines, regulatory MØ are potent inhibitors of inflammation, although they retain the ability to produce many pro-inflammatory cytokines. Regulatory cells express high levels of co-stimulatory molecules (CD80 and CD86) and therefore can present antigens to T cells (Edwards et al, 2006).

Regulatory MØ can be exploited by microorganisms. IgG-opsonized *Leishmania* amastigotes interact with FcγR on MØ, facilitating the internalization of the parasite and at the same time the activation of downstream signaling pathways, inducing the development of regulatory MØ and IL-10 production and therefore promoting intracellular parasite growth. In fact, the presence of IgG in *L. major*-infected mouse, in humans with VL (Miles et al, 2005; Bhardwaj et al, 2010) and in dogs infected with *L. infantum* correlates with the inability to resolve the infection.

At present, the pathways that regulate MØ polarization have not yet been fully understood. Different activation states require distinct transcription factors. The M1 phenotype is controlled by STAT1 and IRF5, whereas STAT6, IRF4 and peroxisome proliferator-activated receptor-γ (PPARγ) regulate M2 polarization (Lawrence and Natoli, 2011).

However, the M1/M2 concept, although useful, is an oversimplification of the complex biology of mononuclear phagocytes (Sica et al, 2015). Furthermore, this concept does not translate well across species (Hume, 2015).

Protozoans generally elicit an early M1 polarization that restrains parasite and controls the disease, followed by a partial M1 to M2 shift that limits inflammation-dependent tissue damages, but supports chronic infection (Raes et al, 2007). After recognition and phagocytosis of *Leishmania*, classically activated MØ ("effector cells") develop different cellular processes, including the production of lysosomal enzymes, ROS, RNS and/or nutrient deprivational mechanisms to kill the parasite. Hence, a delicate M1 *vs* M2 inflammatory balance tightly controls disease outcomes (Sica et al, 2015).

Numerous studies have examined the stability and longevity of activated MØ within the host. Several *in vivo* studies suggest that the phenotype of an MØ population can change over time. It is not clear whether this phenotype change is the result of exposure to new signals that induce the ''re-polarization'' of already differentiated MØ or the result of migration of a new MØ population into the tissue where they replace the original cells (Sica et al, 2015; Mosser and Edwards, 2015). The high functional plasticity, which allows the re-education of MØ is currently being studied for therapeutic purposes (Mantovani and Allavena, 2015).

3.1.6 *Leishmania* modulation of macrophage intracellular signaling pathways

The cell transmutes an extracellular stimulus into intracellular changes through signaling pathways. These stimuli are usually triggered by the ligation of an external ligand (*e.g.* cytokines, *Leishmania* molecules) to a receptor on the cell surface. This ligation causes activation of the receptor, commonly by phosphorylation and/or conformational changes, resulting in the activation of second messengers within the cytosol. These second messengers are often PK, which then phosphorylate other kinases to continue a cascade of events that ultimately results in the activation of effector molecules, such as transcription factors or actin filaments, promoting a cell response (Olivier et al, 2005).

Cell signaling is regulated by PK and phosphoprotein phosphatases, which transduce the signal through cycles of phosphorylation and dephosphorylation. *Leishmania* parasites are particularly effective at interfering with these signaling intermediates, such that the effector functions triggered by several cell surface receptors are either actively suppressed or altered, resulting in an immune response that promotes parasite survival (Bhardwaj et al, 2010; Olivier et al, 2012).

The MAP kinases play an important role as signal kinases and their activity is elicited upon phosphorylation of threonine and tyrosine residues in their regulatory domain. MAPK phosphorylate selected intracellular proteins, including transcription factors, which subsequently regulate gene expression by transcriptional and posttranscriptional mechanisms. There are three major groups of MAPK in mammalian cells: the extracellular signal-regulated protein kinases (ERK), the p38MAP kinases and the c-Jun NH2-terminal

kinases (JNK), which form three parallel signaling cascades activated by distinct or sometimes overlapping sets of stimuli. Activated by mitogens and growth factors, the ERK mediate signals promoting cell proliferation, differentiation and survival. JNK and p38 MAPK are predominantly activated by stress factors, such as osmotic changes and heat shock, but also by the inflammatory cytokines TNF-α and IL-1β and LPS (Bhardwaj et al, 2010).

Several studies have shown that MAPK are actively repressed and cannot be activated when *Leishmania*-infected MØ are stimulated by a variety of agonists. Inhibition of MAPK phosphorylation results in reduced expression of IL-12 and iNOS. *L. donovani* promastigotes failed to activate the phosphorylation of MAPK, affecting the activation of pro-inflammatory cytokines. The parasite surface molecule LPG has been implicated in this process, since phagocytosis of LPG-deficient *L. donovani* promastigotes caused MAPK activation, without the requirement of subsequent MØ stimulation (Bhardwaj et al, 2010).

IFN-γ plays a critical role in controlling *Leishmania* infection by inducing MØ leishmanicidal activities as well as by favoring Th1 response. The binding of IFN-γ to its receptor (IFN-γR) activates JAK1/JAK2 kinases and phosphorylates STAT-1, which in turn translocate to the nucleus and enhances the transcription of IFN-γ-induced genes to increase MØ microbicidal activity. Some of the important MØ functions IFN-γ-induced suppressed by *Leishmania* are NO production and MHCII expression. Inhibition of IFN-γ receptor responsiveness can be attributed to several mechanisms that are *Leishmania* modulated (Bhardwaj et al, 2010).

IL-10 suppresses the production of pro-inflammatory cytokines by activated monocytes/MØ and enhances B lymphocyte proliferation and antibody production. IL-10 decreases the expression of MHCI and MHCII, affecting antigen presentation, reducing the production of pro-inflammatory cytokines TNF-α, IL-12 and IL-18, and suppressing the induction of iNOS in MØ. It was demonstrated that MØ IL-10 is turned on by *Leishmania* amastigotes. IgG opsonized amastigotes recognized by MØ *via* FcγR induces ERK1/2 hyperactivation. The hyperactivation of ERK1/2 results in Histone H3 phosphorylation of IL-10 promoter making the promoter accessible to transcription factor, directing IL-10 secretion. The IL-10/IL-10R interaction engages the phosphorylation and activation of

receptor-associated janus tyrosine kinases, JAK1 and Tyk2, which in turn phosphorylate transcription factor STAT3. Then this factor homodimerizes and translocates to the nucleus where it binds with high affinity to STAT-binding elements (SBE) in the promoters of various IL-10-responsive genes (Bhardwaj et al, 2010).

PKC is involved in a wide range of immune cell functions. A number of studies has implicated PKC in the host defense against intracellular pathogens. *Leishmania* LPG and GIPL inhibit PKC activation. PKC is responsible for the phosphorylation of $p47^{phox}$ and $p67^{phox}$ components of NADPH oxidase, phosphorylation of myristoylated alanine-rich C kinase substrate (MARCKS) and MRP, which are involved in actin turnover, phagosomal maturation and lysosomal fusion. As PKC activation is inhibited by *Leishmania*, this results in subsequent inhibition of all the above mentioned processes, thereby favoring parasite survival (Bhardwaj et al, 2010).

Thus, *Leishmania* selectively manipulates the different signaling pathways to ensure its survival. The effector functions triggered by various MØ surface receptors are either actively suppressed or are altered by the parasite, preventing host cell activation (Bhardwaj et al, 2010).

3.1.7 Superoxide production

IFN-γ and TNF-α can prime MØ for ROS production. However, NADPH oxidase activation only occurs when MØ are appropriately triggered by innate stimuli, such as phagocytosis (Stafford et al, 2002; Van Assche et al, 2011). IFN-γ stimulates the affinity of NADPH oxidase for NADPH and induces the expression of $gp91^{phox}$, but not that of $p47^{phox}$, suggesting that the availability of $p47^{phox}$ may not be a factor that limits the overall enzyme activity. TNF-α acts as an autocrine stimulus, increasing MØ respiratory burst through the expression of both $p47^{phox}$ and $p67^{phox}$ transcripts. TNF-α may also mediate the exocytosis of granules containing the cytochrome component of NADPH oxidase (Stafford et al, 2002).

Phagocytosis acts as a critical factor, triggering the respiratory burst. Phagocytosis *via* antibody FcγR concentrates PKC in the phagosome membrane, mediating the

phosphorylation of the components of NADPH oxidase, $p47^{phox}$ and $p67^{phox}$ and its localized activation. Phagocytosis *via* CR also requires PKC activation. Synthetic activators of PKC, such as PMA are potent inductors of respiratory burst (Stafford et al, 2002).

However, the respiratory burst response depends on MØ subpopulation. Freshly isolated human monocytes triggered with PMA exhibit a vigorous response. In contrast, monocytes cultured *in vitro* (monocyte-derived MØ) have reduced respiratory burst activity, an effect that correlates with the length of *in vitro* cultivation (Cassatella et al, 1985).

In vitro studies showed a direct leishmanicidal effect of O_2^- on *L. chagasi* promastigotes (Wilson et al, 1994) and even a more toxic and dose-dependent effect of H_2O_2 on *L. donovani*, *L. tropica*, and *L. chagasi* promastigotes. Although both promastigotes and amastigotes induced oxidative burst when they enter the MØ, the response generated by promastigotes is higher compared to amastigotes. This finding can be attributed to parasite modulation of NADPH oxidase (Van Assche et al, 2011).

However, promastigotes can also interfere with O_2^- production. It has been demonstrated that although promastigotes do not affect the overall O_2^- production by MØ, they inhibit the assembly of NADPH oxidase in the phagosomal membrane (Lodge et al, 2006; Van Assche et al, 2011). *In vitro* experiments showed that LPG inhibits the recruitment of $p67^{phox}$ and $p47^{phox}$ to the phagosomal membrane, thus interfering with NADPH oxidase assembly (Lodge et al, 2006). Interestingly, amastigotes produced a general decrease in O_2^- levels, indicating that the parasite gradually exerts a greater influence on O_2^- production. In contrast to promastigotes, no amastigote linked molecule has until now been identified as the responsible for the decreased O_2^- levels (Van Assche et al, 2011).

3.1.8 Nitric oxide synthesis

Nitric oxide synthase (NOS) is the enzyme responsible for NO production. The isoform expressed by active immune cells is predominantly the inducible form (iNOS). NO synthesis is an antimicrobial mechanism primarily attributed to active MØ, although neutrophils can also produce NO in response to an immune stimulus (Stafford et al, 2002).

iNOS is a soluble enzyme that catalyzes the oxidation of L-arginine in a two-step process, leading to the generation of Nω-hydroxy-arginine (OH-arg) and the production of NO and L-citrulline. Secondary nitrogenous reactants, such as nitrite, nitrate and nitrosamines, produced as byproducts of the primary RNS are also toxic to pathogens. O_2^- and NO can synergize to form peroxynitrite ($ONOO^-$), a potent intermediate. iNOS activity requires the presence of Ca_2^+ and calmodulin (CaM), but also tetrahydrobiopterin (BH_4), flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) (Stafford et al, 2002; Van Assche et al, 2011; Kima, 2014).

In vitro studies evidenced the antileishmanial activity of exogenous donors of NO in *L. amazonensis* promastigotes and axenic amastigotes (Genestra et al, 2008) and also in *L. infantum* promastigotes (Salvati et al, 2001). Treatment of *L. major*-infected BALB/c mice with the NO donor trinitroglycerin induced a significant decrease in skin lesion size and in the number of amastigotes inside MØ (Nahrevanian et al, 2009). Indeed, several studies have defined the essential role of NO production by active MØ in the intracellular killing of *Leishmania* (Van Assche et al, 2011). MØ can control *Leishmania* infection when a Th1 response is mounted and pro-inflammatory cytokines like IFN-γ and TNF-α are released. This leads to the induction of iNOS and NO production, which is the major *Leishmania* killer molecule in the murine model of *Leishmania* infection (Stafford et al, 2002; Van Assche et al, 2011; Das et al, 2010).

Whereas MØ predominantly produces O_2^- in an attempt to kill *Leishmania* promastigote during phagocytosis, NO becomes increasingly important as a defense mechanism during the intracellular amastigote stage (Stafford et al, 2002; Van Assche et al, 2011). The induction of iNOS explains why NO does not play a role in the beginning of the infection (Van Assche et al, 2011). Phagocytosis is completed within 2 h, whereas the iNOS gene expression and protein generation take about 4 to 6 h to achieve detectable levels using reverse transcriptase-PCR and Western blotting, respectively (Wang et al, 2009). In contrast to ROS, NO is induced by Th1-type cytokines like IFN-γ, TNF-α, IL-1 and IL-2, but also by LPS and lipoteichoic acid (LTA) (Das et al, 2010; Kima, 2014).

IFN-γ and TNF-α are activating factors that work synergistically for the transcription of genes involved in iNOS synthesis. The regulation of iNOS transcription and translation is complex, involving several promoter and enhancer elements. Regulatory elements within

the iNOS gene complex include hypoxic-responsive elements, NF-κβ binding sites, STAT binding sites, interferon regulatory factor binding elements and an AP-1 binding transcription site (Stafford et al, 2002).

However, the production of NO varies depending on the type of cell. No significant increase in NO release was observed in dog monocytes 72 h after *L. infantum* infection (Panaro et al, 1998). However, NO production seems to be involved in the long-term protection of naturally infected dogs, particularly the asymptomatic ones (Panaro et al, 2008).

3.1.9 Modulation of the arginase pathway by *Leishmania*

Arginase is a manganese metalloenzyme that catalyzes the hydrolysis of L-arginine into urea and ornithine. There are two isoforms of the enzyme: arginase I is a cytosolic protein expressed almost exclusively in the liver, whereas arginase II is a mitochondrial protein with widespread tissue distribution, most prominently in kidney, lactating mammary gland, prostate, small intestine and brain, but also in monocytes and MØ (Munder et al, 1999; Das et al, 2010). Ornitine can be transformed in proline by ornithine aminotransferase (OAT) resulting in collagen synthesis, which is required for wound healing. Ornithine decarboxylase (ODC) converts the amino acid ornithine into the first polyamine, named putrescine, which is immediately metabolized into spermidine and finally into spermine (Fig. 25) (Das et al, 2010).

Some pathogens possess their own arginase (Munder et al, 1999; Das et al, 2010). *L. mexicana* have a unique gene coding for arginase, which is essential for parasite survival, since arginase-deficient parasites are unable to generate polyamines. However, these parasites could obtain polyamines from the extracellular environment (Roberts et al, 2004).

Arginase and NOS use arginine as a common substrate and compete with each other for this substrate (Wu and Morris, 1998). Interestingly, the balance between the two enzymes is competitively regulated by Th1 and Th2 cells *via* their secreted cytokines (Munder et al, 1999; Das et al, 2010). Th1 cytokines induce classical activation of MØ and iNOS generation, which oxidizes L-arginine into NO. Th2 cytokines result in the alternative

activation of MØ and the induction of arginase, with the production of ornithine, an amino acid that is the main intracellular source for the synthesis of polyamines (Kropf et al, 2005).

Murine monocyte/MØ lineage up-regulate arginase I upon activation by Th2 cytokines and GM-CSF, whereas Th1 cytokines increase NO production by NOS induction. Interestingly, MØ constitutively express low levels of arginase II, which is not significantly modulated by Th1 or Th2 stimulation (Munder et al, 1999; Kropf et al, 2005).

Using arginase inhibitors, Iniesta et al (2001) showed that arginase activity is necessary for the survival and growth of both *L. major* and *L. infantum* in murine MØ. *In vivo* studies, high splenic arginase I expression has been documented in the hamster-*L. donovani* model of HVL (Osorio et al, 2012). Arginase I was up-regulated in both the resistant C57BL/6 and the susceptible BALB/c mice strains when infected by *L. major*. In the first case, host arginase I was induced only during foot pad swelling, but in the susceptible strain the up-regulation was noticed throughout the infection period. Specific inhibition of host arginase I by nor-NOHA treatment decreased parasite load and delayed lesion development in susceptible mice, whereas ornithine supplementation increased the susceptibility of infection in the resistant strain, suggesting that the host arginase pathway is hijacked by the parasite for polyamine acquisition (Iniesta et al, 2005; Das et al, 2010). Inhibition of host arginase activity during the course of *L. major* infection has a clear therapeutic effect, as evidenced by markedly reduced pathology and efficient control of parasite replication. Arginase regulates infection by directly affecting the polyamine synthesis of MØ, which acts as a parasite growth factor (Kropf et al, 2005). Polyamines are not only beneficial for *Leishmania*, they also affect signaling transduction pathways and modulate immune functions. Spermidine and spermine inhibited the pro-inflammatory cytokine response of the host and spermine modulates immune function by inhibiting the TLR4 pathway (Zhang et al, 1997; Hasko et al, 2000; Perez-Cano et al, 2003).

However, since both the host MØ and the parasite express functional arginase enzyme, it is not clear whether one or both are needed for parasite survival. The generation of *Leishmania* mutants deficient in arginase enables an examination of the relative contribution of parasite and host cell arginases to parasite survival (Gaur et al, 2007). *L. major* lacking arginase retains infectivity in the susceptible BALB/c mouse strain, indicating that arginase-deficient parasite can survive in mice by salvaging the polyamines

synthesized by the host. However, the pathology in arginase-deficient *L. major* infection emerged less rapidly than in the wild-type infection (Reguera et al, 2009). The active role played by *Leishmania* arginase was demonstrated by the development of a Th1 immune response with NO production in mice infected with arginase-deficient *L. mexicana*, leading to a significant growth attenuation of the mutant parasite (Gaur et al, 2007).

Yet a question remains: how can parasite arginase, which is contained in the glycosome, an organelle unique of *Leishmania*, and is not secreted outside the parasite (Roberts et al, 2004) get access to the host arginine pool? It is hypothesized that *Leishmania* recruits host mCAT2B transporters to its parasitophorous vacuole to access the host cytoplasmic arginine pool (Wanasen and Soong, 2008). However, *L. donovani* promastigote uses its own arginine permease, LdAAP3, to transport arginine across its membrane (Shaked-Mishan et al, 2006).

Although would be tempting to target the polyamine biosynthetic enzymes as a therapeutic approach for leishmaniosis (Heby et al, 2007), it should be kept in mind that arginase induction benefits the host by reducing the detrimental effect of NO and supplying polyamines for cell proliferation and proline for collagen deposition. Furthermore, the urea cycle is an essential host biochemical pathway needed to clear the toxic waste product ammonia (Das et al, 2010).

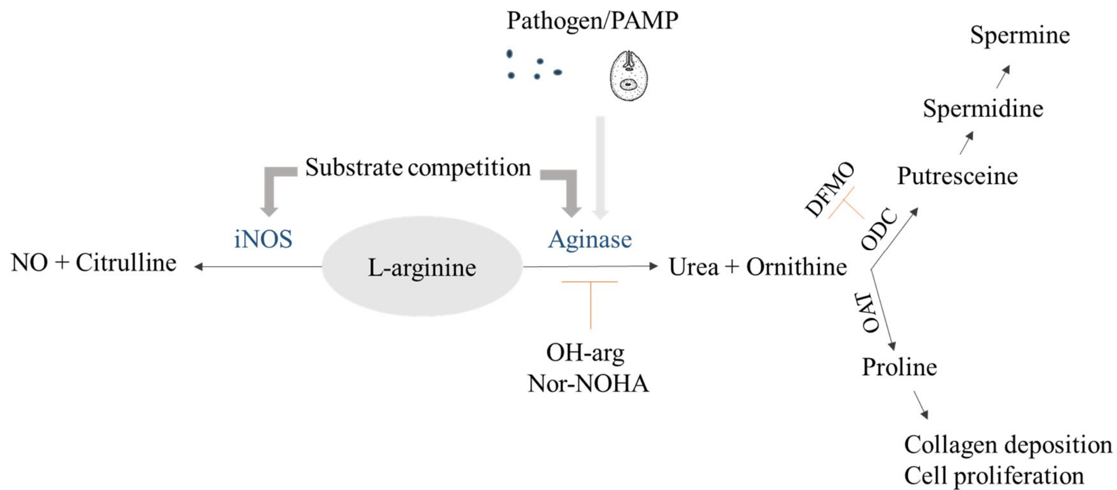

Figure 25: Simplified overview of the mammalian arginine pathway. Recognition of pathogen or pathogen-associated molecular patterns (PAMP) induces the production of inducible nitric oxide synthase (iNOS) by immune cells. Arginase and iNOS use L-arginine as a common substrate and compete with each other for the substrate. The intermediate product of iNOS reaction N^{ω}-hydroxy-arginine (OH-arg) and the synthetic amino acid N^{ω}-hydroxy-nor-L-arginine (nor-NOHA) inhibit arginase activity. Ornitine can be transformed into proline by ornithine aminotransferase (OAT) or into the polyamine putresceine by ornithine decarboxylase (ODC). Difluoromethyl ornithine (DFMO) inhibits putresceine synthesis. Adapted from Das et al (2010).

3.1.10 Macrophage extracellular traps

In addition to neutrophils, eosinophils (Yousefi et al, 2008, 2009), mast cells (von Köckritz-Blickwede et al, 2008) and MØ (Aulik et al, 2012) also have the ability to produce extracellular traps (ET). Indeed, several monocytes/MØ subsets and cell lines have shown to produce macrophage extracellular traps (MET) in response to several stimuli, such as bacteria (*C. albicans*, *E. coli*, *Histophilus somni*, *Mannheimia haemolytica*, *M. tuberculosis*, *Staphilococus aureus*), *Besnoitia besnoiti*, microbial products (*E. coli* hemolysin, *M. haemolytica* leukotoxin) and other agents (TNF-α, PMA, glucose oxidase (Muñoz-Caro et al, 2014; Boe et al, 2015).

Many of the components identified in the MET structure were already established as NET compounds. MET are composed of nuclear and mitochondrial DNA, as well as MPO and lysozyme. Elastase plays a critical role in METosis, similar to the cellular mechanisms that regulate NET formation. The differences in the structure and function (*e.g*. trapping *vs* killing activity, the reliance on ROS production, nuclear *vs* mitochondrial origin) of different ET types may reflect the diversity in cellular physiology and differentiation of

various leukocytes and their responses to microbial infection. However, in contrast to extracellular pathogens, the function of ETosis in intracellular microorganism infection is largely unexplored and many unanswered questions remain. Persistence of intact ET within tissue can lead to a number of deleterious effects, including tissue damage, local inflammation, thrombus formation or the development of an autoimmune response by the adaptive immune system. The further regulation and clearance of ET by MØ play a critical role in controlling inflammation (Boe et al, 2015).

3.1.11 Antigen presentation

In addition to their roles as phagocytic and killing cells, MØ also act as APC. The presentation of processed antigens to naïve T cells by APC can initiate its preferential activation and/or expansion as effector cells (Stafford et al, 2002; Saridomichelakis, 2009). The expansion of the Th1 subsets and production of IFN-γ can activate MØ and trigger their killing responses. The presentation of parasite antigens by MØ to T cells requires the involvement of MHCII molecules, which are found on APC. MHCII presentation of antigen alone is not sufficient to activate T cells that also require the costimulatory molecule colligation of B7-1 (CD80)/B7-2 (CD86) and CD40 (on MØ) with CD28 and CD40L (on T cells), respectively (Stafford et al, 2002).

Early studies showed that *L. donovani* suppresses MHCII expression in mouse MØ at the level of gene transcription (Reiner et al, 1987). Subsequently, other studies showed that antigen processing for MHCII presentation is defective in *L. amazonensis* and *L. major*-infected MØ, because the parasite interferes with the intracellular loading of MHCII molecules with antigenic peptides (Fruth et al, 1993; Prina et al, 1993; Stafford et al, 2002). Infected MØ also failed to up-regulate the expression of B7-1 after exposure to inflammatory mediators (Stafford et al, 2002). The expression of CD40 in *L. donovani*-infected MØ was also suppressed (Buates and Matlashewski, 2001). CD40-CD40L interactions result in Th cell subset skewing to a Th1 type. Apart from their role in Th1 immune response, CD40-CD40L interactions were also shown to stimulate MØ to produce a number of cytokines and inflammatory mediators, including NO. However, *Leishmania* infection down-regulates CD40-induced p38 MAPK phosphorylation, inhibiting the above mentioned processes (Bhardwaj et al, 2010). Collectively, these findings suggest that

Leishmania can prevent the activation of MØ by inhibiting the cognitive interaction with IFN-γ-producing Th1 cells through the down-regulation of MHCII and of co-stimulatory molecule expression (Pinelli et al, 1999; Stafford et al, 2002; Panaro et al, 2008).

After intradermal inoculation, *L. donovani* takes about 96 h to be found in the draining lymph nodes and in the spleen of dogs, where parasite antigens can be presented to T cells along with MHCII molecules (Saldarriaga et al, 2006; Saridomichelakis, 2009). An increase in the MHCII expression has been demonstrated in MØ infected with *L. infantum* promastigotes or exposed to *L. infantum* antigen in the presence of autologous canine lymphocytes (Diaz et al, 2012). *In vitro* studies showed that the expression of co-stimulatory molecules (CD80, CD86) by dog infected-MØ is compromised (Pinelli et al, 1999; Diaz et al, 2012). In fact, *Leishmania* is characterized by sophisticated mechanisms developed to evade or interfere with host antigen presentation processes, thus allowing the parasite to modulate T cell mediated immune responses (Pinelli et al, 1999; Panaro et al, 2008).

After antigen presentation, activated T cells migrate back to the site of parasite inoculation guided by specific chemokine gradients, *in situ* modulating the activation of MØ and LC (Papadogiannakis and Koutinas, 2015).

Furthermore, genetic factors involving MHC were identified. Significant correlation between certain MHCII alleles has been associated with susceptibility to CanL. In a cohort of Brazilian mongrel dogs, the MHCII allele DLA-DRB1*01502 was positively correlated with the risk of infection and with the titer of *Leishmania* specific IgG antibodies (Quinnell et al, 2003).

3.1.12 Efferocytosis

Efferocytosis is a specialized phagocytic process developed by MØ, aimed to remove apoptotic cells from the inflammatory milieu. It is an essential process of tissue homeostasis, embryologic development, resolution of inflammation and immunity (Korns et al, 2011).

Although efferocytosis resembles phagocytosis, it is a distinct process, mediated by specific receptors, bridging molecules, and downstream intracellular signaling pathways. During efferocytosis, RhoA activity is suppressed and Rac1 activity coordinates the engulfment of the apoptotic body and the formation of a spacious phagosome, termed efferosome (Martin et al, 2014).

The process of efferocytosis is highly regulated. Apoptotic cells exhibit surface changes, especially the exposure of PS in the extracellular leaflet of the phospholipid membrane that distinguish them from viable cells and allow them to be recognized by MØ efferocytic receptors. The release of chemokines by the dying cell recruits MØ to the site of cell death (Korns et al, 2011; Cullen et al, 2013; Martin et al, 2014). FasR (CD95)-induced apoptosis is associated with the production of MCP-1 and IL-8, promoting phagocyte chemotaxis ("find me" signals). In the "catch me" phase, recruited MØ increases the expression of bridge molecules and receptors that bind PS or other distinct ligands on the apoptotic cell (Martin et al, 2014). Lastly, the engagement of efferocytic receptors initiates a series of signaling events, resulting in the activation of Rac1, which promotes cytoskeletal rearrangement and the formation of lamellipodia and membrane ruffles that surround the apoptotic cell, ultimately leading to its engulfment in a large spacious efferosome ("eat me" step) (Korns et al, 2011; Martin et al, 2014). Rac1 and RhoA are small Rho GTPases with opposing roles in regulating efferocytosis: Rac1 enhances, while RhoA inhibits the process (Leverrier and Ridley, 2001; Nakaya et al, 2006). Successive lysosome fusion events deliver hydrolytic enzymes into the maturing efferosome which together with its gradual acidification establish harsh conditions that destroy the apoptotic cell (Kinchen and Ravichandran, 2008; Lu and Zhou, 2012; Martin et al, 2014).

Apoptotic cell efferocytosis is important for the resolution of inflammation, because dying cells are removed, preventing secondary necrosis, which is characterized by the disintegration of apoptotic bodies and release of cytotoxic compounds in the extracellular medium (Martin et al, 2014). Importantly, to prevent inflammation and autoimmunity efferocytosis typically evokes the secretion of anti-inflammatory cytokines, such as TGF-β, IL-10 and PGE2 by the engulfing cells (Fadok et al, 1998; Kim et al, 2004; Xiao et al, 2008).

Efferocytosis of infected cells can promote pathogen killing along with the engulfed apoptotic bodies (host antimicrobial activity), as is the case of *M. tuberculosis*. Several bioactive lipids cover the surface of this bacterium, interfering with phagosome maturation. It is believed that the 'double-wrapped' under the apoptotic cell membrane and the efferosome sequesters virulence factors of *M. tuberculosis*, leading to pathogen destruction through degradative enzymes delivered *via* the lysosomes (Martin et al, 2014).

However, some pathogens use the efferocytosis process to disperse into naïve host cells (Martin et al, 2014). *L. major* is a typical example of an intracellular obligate parasite that uses efferocytosis for its own benefit. Infection with *L. major* delays the apoptotic death program of human neutrophils by about 24 h (Aga et al, 2002). Infected human neutrophils secreted high levels of the chemokine MIP-1β, which attracts monocytes. When MØ arrive to the inoculation site, it is likely that they encounter the parasite inside neutrophils. *In vitro* studies showed that *L. major* infected apoptotic human neutrophils are readily phagocytized by MØ (van Zandbergen et al, 2004). However, other mechanisms of parasite transference from neutrophils to MØ have been described. Intra-vital microscopy studies showed viable *L. major* parasites being released from mouse apoptotic neutrophils in the vicinity of surrounding MØ (Peters et al, 2008).

In vitro studies using human cells showed that the engulfment of *L. major*-infected apoptotic neutrophils by MØ promotes TGF-β and suppresses TNF-α release, ensuring intramacrophagic parasite viability and multiplication. Efferocytosis of infected neutrophils prevents the direct contact between the parasite and MØ surface receptors, avoiding MØ activation. Furthermore, the uptake of apoptotic cells silences the MØ through the production of anti-inflammatory cytokines preventing the activation of its effector mechanisms (van Zandbergen et al, 2004). In this model of infection, intracellular parasites use neutrophils as ''Trojan horses'' to invade their definitive host cells (van Zandbergen et al, 2004; Laskay et al, 2008; Peters et al, 2008).

Later on parasitized MØ may interact with neutrophils and this contact can influence the course of the infection. Apoptotic and necrotic neutrophils had opposing effects on *L. amazonensis* promastigote survival and growth in human MØ. Neutrophils that had undergone apoptosis in response to ultraviolet irradiation strongly promoted parasite growth in MØ in a TGF-β1 and PGE2-dependent manner. In contrast, freeze/thawed

necrotic neutrophils facilitated parasite clearance in MØ *via* a TNF-α and NE-dependent mechanism. Surprisingly, living neutrophils had little effect on the percentage of infected MØ or total parasite burden (Afonso et al, 2008).

Specific differences in *L. major* susceptibility have been largely attributed to variations of T helper cell polarization. Susceptible BALB/c mice adopt a preferential Th2 response and resistant C57BL/6 mice a Th1-type response. However, it was recently demonstrated that the anti-parasite responses of these two strains of mice also differ in the innate immune level (Carlsen et al, 2015). Co-injection of dead neutrophils amplified *L. major* replication *in vivo* in susceptible BALB/c mice, but prevented parasite growth in resistant B6 mice (Ribeiro-Gomes et al, 2004). However, host background may be less important in the New World *Leishmania* infections (Carlsen et al, 2015), as neutrophils enhanced the ability of MØ to kill *L. amazonensis* (de Souza Carmo et al, 2010) and *L. braziliensis* (Novais et al, 2009). These findings indicate that MØ interactions with apoptotic or necrotic neutrophils play a role in host responses to *Leishmania* infection.

3.2 OBJECTIVES

The present study aims to evaluate neutrophil-MØ interplay during the initial phase of *L. infantum* infection in an attempt to mimetize the first steps of dog infection. The first goal of this study was to characterize the immune response developed by blood monocyte-differentiated MØ when exposed to *L. infantum* through the evaluation of (i) phagocytosis, (ii) M1 *vs* M2 activation by assessment of NO and urea production (iii) and MET release. The second goal was to investigate parasite transference from infected neutrophils to MØ and its impact on the immune response. To accomplish this objective co-cultures of neutrophil-MØ were established and analyzed by OM, SEM and TEM and, MØ activation was evaluated through NO and urea production.

3.3 MATERIAL AND METHODS

3.3.1 Animals and parasites

A group of 10 dogs housed in the Canil Municipal of Évora (Portugal) was selected as described in 2.3.1. *L. infantum* virulent promastigotes (MHOM/PT/89/IMT151) collected in the stationary growth phase and *L. infantum* promastigotes expressing GFP (Marques et al, 2015) were used for PMN and MØ infection. Parasites were maintained as described in 2.3.2.

3.3.2 Isolation of dog PMN and monocytes

Blood collection was made according to the veterinary norms with therapeutic shearing and antisepsis of venipuncture site with povidone-iodine and ethanol. About 30 mL of peripheral blood were collected twice from the same dog with four days of interval. PMN and mononuclear cells were isolated using a Histopaque double-density gradient, according to the technique described by Strasser et al (1998). The blood obtained from the first collection was used to isolate mononuclear cells that were left to differentiate into MØ. The second collection of blood was used to isolate PMN and mononuclear cells.

Isolated cells were washed with 0.9% NaCl and residual red blood cells were removed by osmotic lyse using H_2O and 1.8% NaCl (m/v) to adjust osmolality. PMN were resuspended in HBSS 5% of heat-inactivated FBS and used at once. Mononuclear cells were resuspended in 36 mL of complete RPMI supplemented with 15% (v/v) M-CSF, which induces monocyte maturation and MØ differentiation (Rodrigues et al, 2006).

Four days after the first blood collection, monocytes were already differentiated into MØ and these cells were used to establish a co-culture with PMN isolated from the second blood collection. The mononuclear suspension obtained from the second blood collection was differentiated into MØ and, on the 9th day of the experiment MØ were infected (Fig. 26). The differentiation process and infection are described below.

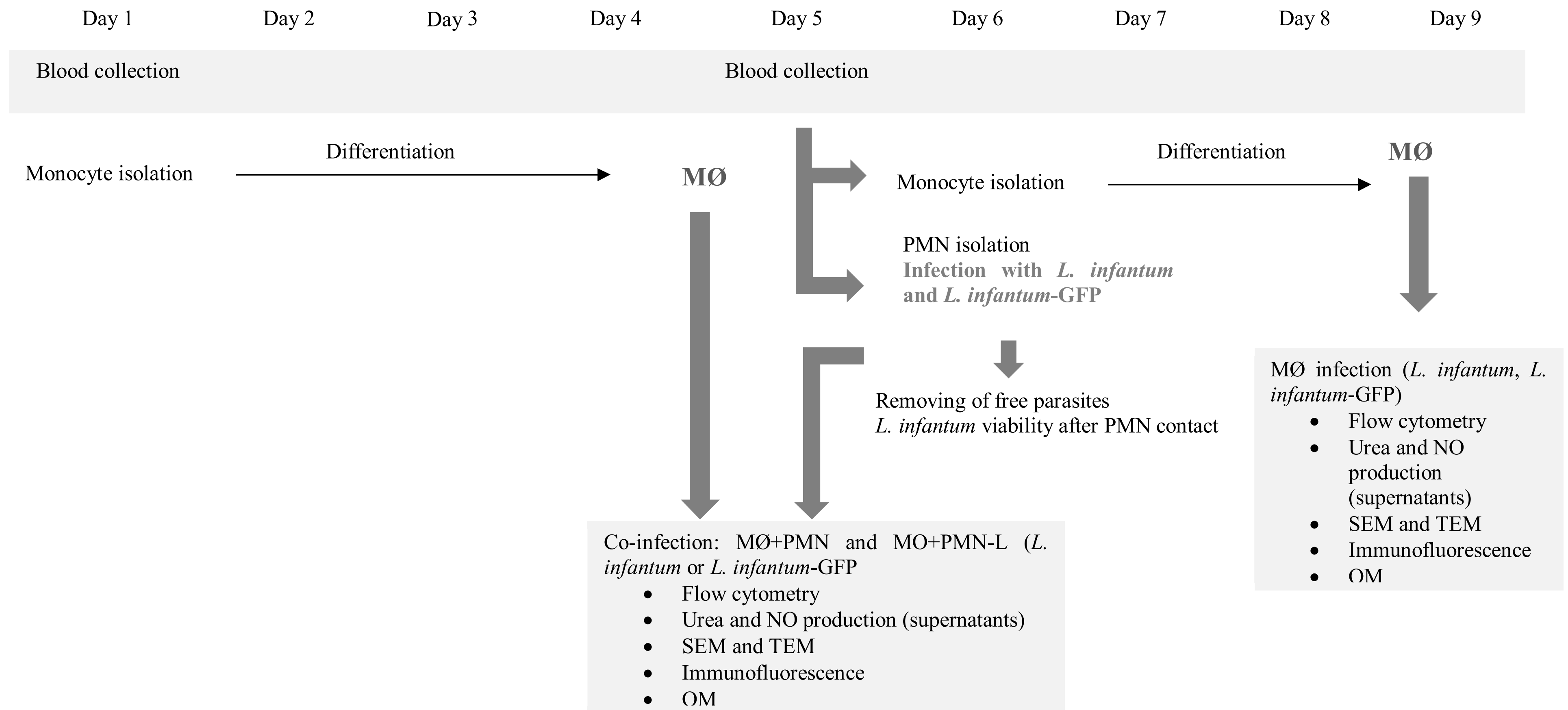

Figure 26: Timeline of blood collection, cell isolation and cell infection. MØ - macrophage; PMN - polymorphonuclear cells; Co-Infection - MØ+PMN or MØ+PMN-L; NO - nitric oxide; OM - optical microscopy; TEM - transmission electron microscopy; SEM - scan electron microscopy.

3.3.3 Differentiation of blood monocyte-derived macrophages and purity assessment

The mononuclear cell suspension was cultured in 6-well plates at 37ºC in a humidified atmosphere containing 5% of CO_2. After 24 h and 72 h of incubation, culture medium was discharged to remove non-adherent cells and fresh medium (complete RPMI supplemented with M-CSF) was added. To assess MØ differentiation, culture triplicates were analyzed by flow cytometry at several time points (days 0, 4, 5 and 6). The results presented in figure 27 show that the optimal differentiation period is 96 h. Thus, after 96 h of culture, cells were detached by thermal shock on ice for 30 min and harvested by agitation. MØ were centrifuged at 300 ×g for 10 min and resuspended in RPMI complete medium. Cell viability and concentration were assessed by trypan blue staining using a Neubauer-counting chamber. In 24-well plates, 2.5×10^5 cells were left to incubate for 3 h and 24 h. The purity of cell suspension was evaluated by flow cytometry analysis after fixation with 2% paraformaldehyde for 20 min at room temperature. The FSC-H *vs* SSC-H gate was used to remove debris, pyknotic cells as well as the very large debris. Singlet gate was used to define the non-clumping cells based on pulse geometry FSC-H *vs* FSC-A as described in 2.3.5. MØ population was identified based on FSC-H *vs* SSC-H gate.

3.3.4 Macrophage *in vitro* infection

In 24-well plates, 2.5×10^5 MØ and MØ incubated with *L. infantum* or *L. infantum*-GFP promastigotes at a ratio parasite-MØ of 5:1 were cultured in 1 mL of RPMI complete medium for 3 h and 24 h, at 37ºC in a humidified atmosphere containing 5% of CO_2. Supernatants were collected and stored at -20ºC for further measurement of NO products and urea.

3.3.5 Binding and internalization of *L. infantu*m promastigotes by macrophages

In vitro binding of *L. infantum*-GFP parasites to MØ and internalization were evaluated by flow cytometry. MØ and *L. infantum*-GFP promastigotes were used to calibrate the cytometer, compensating for the overlap of autofluorescence spectra. After incubation, *L.*

infantum-GFP-MØ were detached by thermal shock on ice for 30 min, harvested by agitation and centrifuged at 300 ×*g* for 10 min. Cultures were washed with PBS, fixed in 2% paraformaldehyde and analyzed by flow cytometry. FSC-H *vs* SSC-H gate was used to remove small debris, free parasites, pyknotic cells as well as the very large debris. Singlet cells were gated as described in 2.3.5. MØ population was identified on pseudocolor graphs based on FSC-H *vs* SSC-H characteristics, applying the auto gate tool. The histogram FL1-H/GFP *vs* number of events was used to define the GFP^- population (negative GFP control) in MØ cultures incubated for 3 h and 24 h. The contact between parasites and MØ was morphologically evaluated in culture citospins stained with Hemacolor staining kit or Giemsa (1:9) and observed under OM.

3.3.6 Morphological features of macrophage activation

Morphological features of MØ activation induced by *L. infantum* promastigotes were assessed by SEM. Unprimed-MØ, MØ stimulated with 1 $\mu g.mL^{-1}$ of PMA, MØ incubated with supernatants of lymphocyte cultures stimulated with concanavalin-A (IFN-γ enriched medium)[1], MØ stimulated with 1 $\mu g.mL^{-1}$ of LPS and exposed to the parasite and, MØ treated with 10 $\mu g.mL^{-1}$ of cytochalasin D (Sigma-Aldrich) (Cyt D) for 30 min prior to *L. infantum* exposure (Abi Abdallah and Denkers, 2012; Muñoz-Caro et al, 2014) were adhered on coverslips and left to incubate for 3 h. Cyt D inhibits actin polymerization, thus preventing MØ phagocytosis (Elliott and Winn Jr, 1986). *L. infantum*-exposed MØ were left to incubate for 3 h and 24 h and also analyzed. After incubation, cells were fixed and treated as described in 2.3.10.

3.3.7 Evaluation of macrophage activation by immunostaining

Changes in DNA and Histone H1 induced by MØ activation were analyzed by immunofluorescence. Unprimed-MØ, MØ incubated with *L. infantum* promastigotes, MØ stimulated with Cyt D prior *L. infantum* exposure, PMA-stimulated MØ, MØ incubated with IFN-γ and, LPS-stimulated MØ were adhered on coverslips and left to

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[1] When referred the use of IFN-γ to stimulate MØ, consider IFN-γ enriched medium

incubate for 3 h. After incubation, cells were stained with anti-Histone H1 FITC and DAPI as described in 2.3.10.

3.3.8 PMN *in vitro* infection and removing of free parasites

In 96-well plates, 5×10^5 PMN were incubated with *L. infantum* and *L. infantum*-GFP promastigotes at a ratio parasite-PMN of 3:1 in 300 µL of HBSS 5% FBS. Cultures were incubated at 37ºC in a humidified atmosphere containing 5% of CO_2 for 3 h. After incubation extracellular promastigotes were removed by positive selection, using MicroBeads conjugated with monoclonal rat anti-mouse/human CD11b (Mac-1α) antibodies (Miltenyl Biotec, Germany), but employing the double of conjugated recommended by the manufacturer. CD11b antigen is strongly expressed in human myeloid cells and weakly expressed in NK and in some activated lymphocytes. In mice, the CD11b antigen is expressed in monocytes/MØ and to a lower extent in granulocytes, NK, $CD5^+$ B1 cells and in some DC subsets. A previous experience showed that this antibody also binds dog neutrophils, allowing the separation between PMN and extracellular parasites. After parasites removing, PMN were resuspended in complete RPMI medium and its viability and concentration were assessed by trypan blue staining using a Neubauer-counting chamber.

3.3.9 Viability of PMN-phagocytized parasites

To assess parasite viability after phagocytosis by dog PMN, free parasites from PMN cultures previously exposed to *L. infantum* promastigotes for 3 h were removed as described in 3.3.8. Then 5×10^5 PMN were resuspended in 300 µL of complete SCHN medium and incubated at 24ºC for 72 h to eventually promote amastigote-to-promastigote differentiation and parasite release from infected PMN. Viable parasites were estimated using a Neubauer-counting chamber after 24 h and 72 h of incubation.

3.3.10 Macrophage-PMN co-culture

In 24-well plates, 2.5×10^5 adherent MØ were co-cultured with PMN or with PMN-*L. infantum* or *L. infantum*-GFP (free parasites were removed) at a ratio MO-PMN of 1:2 in 1 mL of RPMI complete medium. Plates were incubated for 3 h and 24 h at 37ºC in a humidified atmosphere containing 5% of CO_2. Supernatants were collected and stored at -20ºC before being used for further NO and urea measurements.

3.3.11 Parasite transference from PMN to macrophages

MØ+PMN-*L. infantum*-GFP co-cultures were analyzed by flow cytometry to evaluate parasite transference from PMN to MØ. Cultures of PMN, MØ and *L. infantum*-GFP promastigotes were also established and used to calibrate the cytometer, compensating for the overlap of autofluorescence spectra. After incubation, MØ were detached by thermal shock on ice for 30 min, harvested by agitation and centrifuged at 300 ×*g* for 10 min. Then cultures were washed with PBS, fixed in 2% paraformaldehyde and analyzed by flow cytometry. FSC-H *vs* SSC-H gate was used to remove small debris, free parasites, pyknotic cells as well as the very large debris. Singlet cells were gated as described in 2.3.5. MØ and PMN populations were identified on contour graphs 5% probability based on FSC-H *vs* SSC-H characteristics. The histogram FL1-H/GFP *vs* number of events was used to define the MØ GFP^- population (negative GFP control) in MØ+PMN cultures incubated for 3 h.

3.3.12 Morphological characterization of parasite transference

Co-cultures of MØ+PMN and MØ+PMN-*L. infantum* were established and evaluated at 3 h and 24 h by TEM and SEM as described in 2.3.10 and also by OM in an attempt to decipher the mechanism of parasite transference. DAPI and Histone H1 FITC stained co-cultures (as described in 2.3.10) were also analyzed by immunofluorescence to investigate molecular features related to MØ activation.

3.3.13 Urea production

Urea is one of the products generated by arginase, an enzyme that catalyzes the hydrolysis of L-arginine. Urea production was quantified in supernatants of unprimed-MØ, *L. infantum*-infected MØ, and of MØ+PMN and MØ+PMN-*L. infantum* co-cultures at 3 h and 24 h, using the QuantiChrom^{TM} Urea Assay Kit (BioAssay Systems, USA). The procedure recommended for low urea samples was used and absorbance was measured at 450 nm on a microplate reader (Anthos 2010, Austria).

3.3.14 Nitrate plus nitrite measurement

NO is generated by NOS, an enzyme that catalyzes the oxidation of L-arginine in NO and L-citrulline. Secondary nitrogenous reactants, such as nitrite and nitrate are produced from NO. Total nitrate plus nitrite was measured in supernatants of unprimed-MØ, LPS-stimulated MØ, *L. infantum*-infected MØ, and of MØ+PMN and MØ+PMN-*L. infantum* co-cultures at 3 h and 24 h, using Nitrate/Nitrite Colorimetric Assay Kit (Abnova, Taiwan), according to the manufacturer's instructions and without sample dilution. Absorbance was measured at 550 nm using a microplate reader.

3.3.15 Statistical analysis

Assays were performed in 10 dogs, except SEM images that were obtained from five dogs, and TEM and immunofluorescence that were performed in three dogs. Each sample was analyzed in triplicate or quadruplicate. The non-parametric Wilcoxon Signed Ranks Test was used to compare paired groups in relation to each parameter studied. Differences were considered significant with a 5% significance level ($p<0.05$). The statistical analysis was performed using the SPSS 22.0 for Windows software (SPSS Inc., USA). Graphs were built up by using GraphPad Prism version 6.00 for Windows (GraphPad Software, USA).

3.4 RESULTS

3.4.1 Dog blood monocytes take 96 h to differentiate into macrophages

Flow cytometry analysis was used to monitor the process of *in vitro* differentiation of blood monocytes into MØ. Cultures were analyzed at several time points (days 0, 4, 5 and 6). On day 4 (Fig. 27B) a large and well defined cell population displaying high FCS characteristics, compatible with MØ was present and an amount of cells displaying low FCS, which characterizes the monocyte population was also also identified. On day 5 (Fig. 27C), the frequency of cells exhibiting low FCS characteristics increased. On day 6 (Fig. 27D), the number of acquired cells decreased, probably due to cellular death. However, a large population of monocyte cells was identified on the dotplots. Thus, the optimal differentiation period determined by flow cytometry was 96 h. The protocol used ensures a cell yield between $3.3\times10^{4}.mL^{-1}$ and $1\times10^{5}.mL^{-1}$. The viability of recovered cells assessed by trypan blue exclusion was higher than 95%.

3.4.2 The differentiation process ensures a high purity cell population

The purity of dog blood monocyte-differentiated MØ was assessed by flow cytometry analysis. MØ incubated for 3 h and 24 h were detached from the plate, fixed in paraformaldehyde and acquired. MØ population was identified in pseudocolor graphs based on FSC-H *vs* SSC-H characteristics using the auto gate tool (Fig. 28B and D). The differentiation protocol used ensures a population of MØ comprising more than 85% of recovered cells after 3 h and more than 70% after 24 h of incubation.

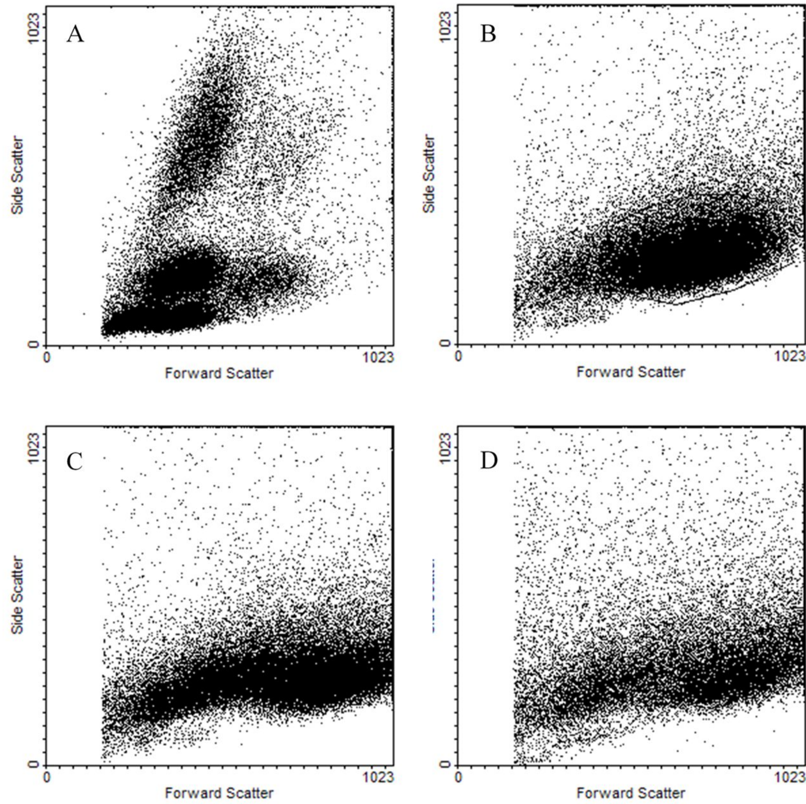

Figure 27: Flow cytometry analysis of macrophage differentiation. Mononuclear cells were isolated and cultured in RPMI complete medium supplemented with macrophage colony-stimulating factor (M-CSF). On day 0 (A), 4 (B), 5 (C) and 6 (D) cells were recovered, fixed with paraformaldehyde and analyzed by flow cytometry. Triplicates of each "in differentiation" culture were analyzed. Side scatter (SSC) *vs* Forward scatter (FSC) gate was used to analyze mononuclear cell suspension.

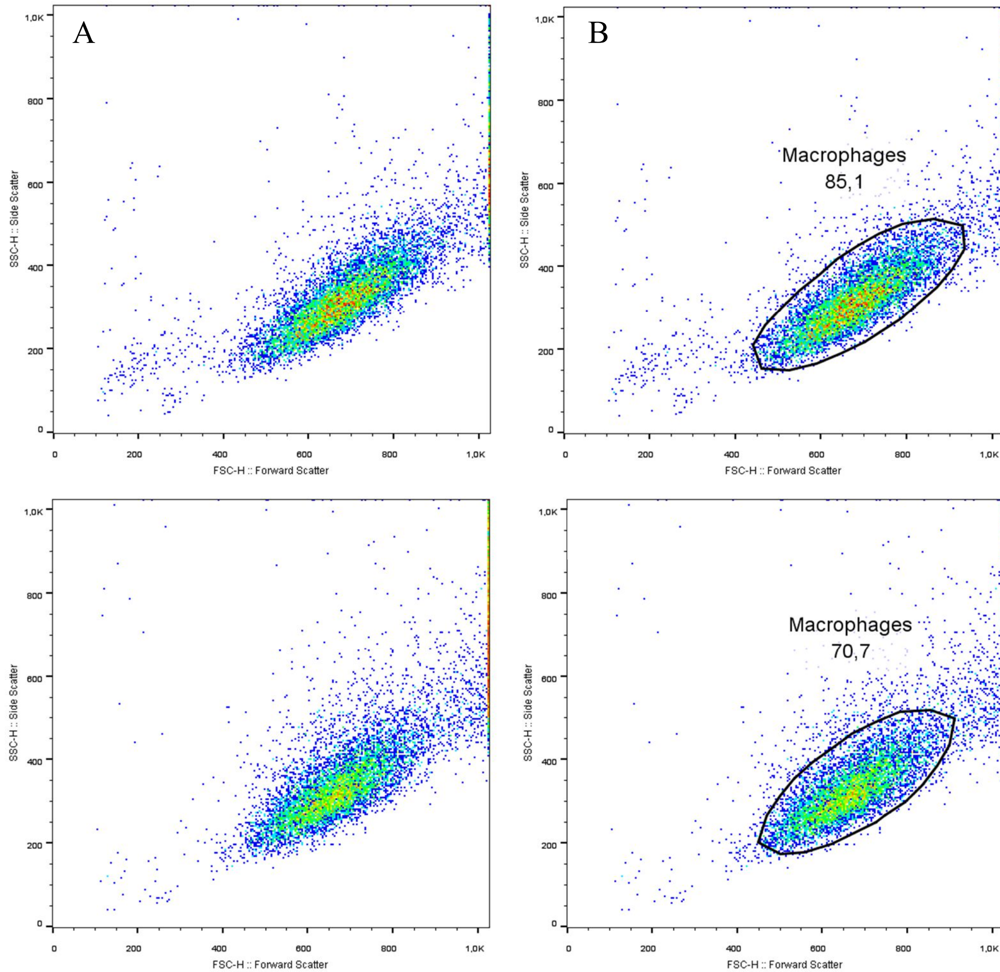

Figure 28: Macrophage purity assessed by flow cytometry. Cells were detached, fixed and acquired by flow cytometry. MØ population was gated on FSC-H *vs* SSC-H pseudocolor graphs using the auto gate tool. Representative images of 3 h (A and B) and 24 h (C and D) incubation cultures and of the respective gate strategy employed, indicating the percentage of MØ obtained (B, D).

3.4.3 Macrophages efficiently bind and internalize the parasite

In vitro parasite binding to MØ was evaluated by flow cytometry analysis using *L. infantum* promastigotes expressing GFP (Fig. 29G). After identification of MØ population and gating GFP^- MØ (Fig. 29A and C), the proportion of cells that internalized or bound the parasite was determined at 3 h and 24 h (Fig 29B and D). In cells incubated for 3 h, about 26.8% (18.25%, 31.55%) of MØ were *L. infantum*-GFP associated. At 24 h, a significant higher proportion of cells [46.9% (38.675%, 51.575%)] were parasite associated ($p<0.001$) (Fig. 29E). The level of fluorescence evaluated in GFP^+ cell sub-population, assessed as GFP median was also significantly higher at 24 h ($p=0.001$) when

compared with 3 h incubation incubated cells (Fig. 29F). Together, these results indicate that the proportion of MØ that are parasite-associated increases with time.

OM observation revealed that after 3 h of incubation the promastigote form was observed in the extracellular medium (Fig. 30A and C) and internalized (Fig. 30A, B, C, D, E and F) by MØ. Indeed, the promastigote had been the parasite morphological form most frequently observed inside the parasitoforous vacuole, even though some internalized parasites showed an amastigote-like form (Fig. 30D and E). After 3 h, *L. infantum*-exposed MØ showed morphological changes of the plasma membrane related to cell activation, namely the protrusion of numerous filodopodia (Fig. 30C, D and E) and cell extensions with a round (Fig. 30D) or elongated shape (Fig. 30E). Some MØ cultured with *L. infantum* for 24 h showed numerous intracellular amastigote-like forms (Fig. 30F), suggesting active multiplication. In some cells rupture of the plasma membrane was observed, leading to parasite release into the extracellular space (Fig. 30G and H). However, amastigotes were frequently observed in the cell periphery coming out from apparently intact MØ, suggesting parasite release by an exocytosis-like process without cell lysis (Fig. 30I).

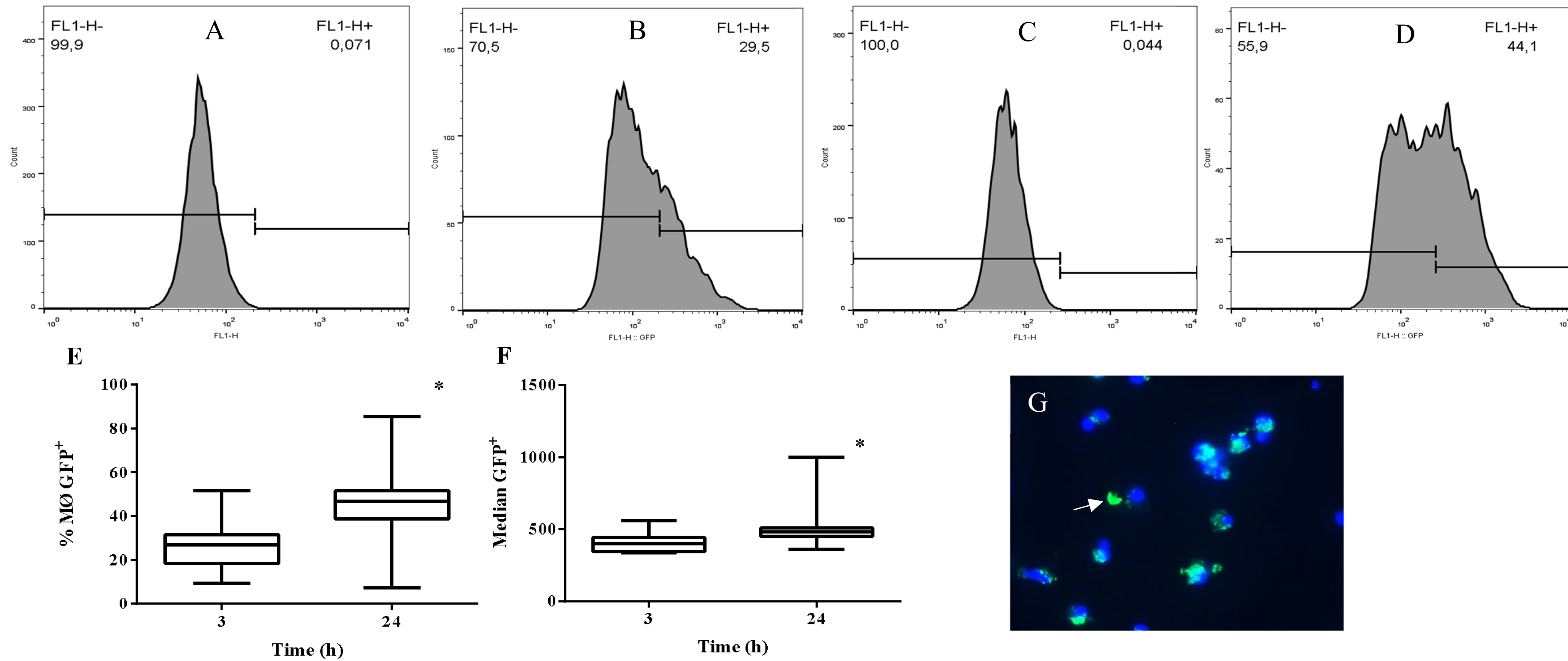

Figure 29: *L. infantum* bind or are internalized by dog macrophages. Unprimed-MØ and MØ-*L. infantum*-GFP were analyzed by flow cytometry. The histogram FL1-H/GFP *vs* number of events (count) was employed to determine the proportion of GFP^+ MØ in cultures incubated for 3 h (B) and 24 h (D). Unprimed-MØ were used as negative GFP control at both time points (3 h-A, 24 h-C). Level of parasite-MØ associated (E) and the intensity of GFP fluorescence in the subpopulation of GFP^+ MØ (F) at both time points are expressed by median (horizontal lines across the box), 75th percentile and 25th percentile (vertical ends of the box), and whiskers (lines extending from the box to the highest and lowest values). Statistical analysis of 10 dogs and triplicate samples was performed using the non-parametric Wilcoxon test. Statistically significant differences are indicated by * ($p<0.05$) when compared 3 h *vs* 24 h. MØ+*L. infantum*-GFP incubated for 3 h marked with DAPI (nucleus stained blue) show internalized parasites (green points around the nucleus) and extracellular promastigotes (arrow) (×400 magnification) (G).

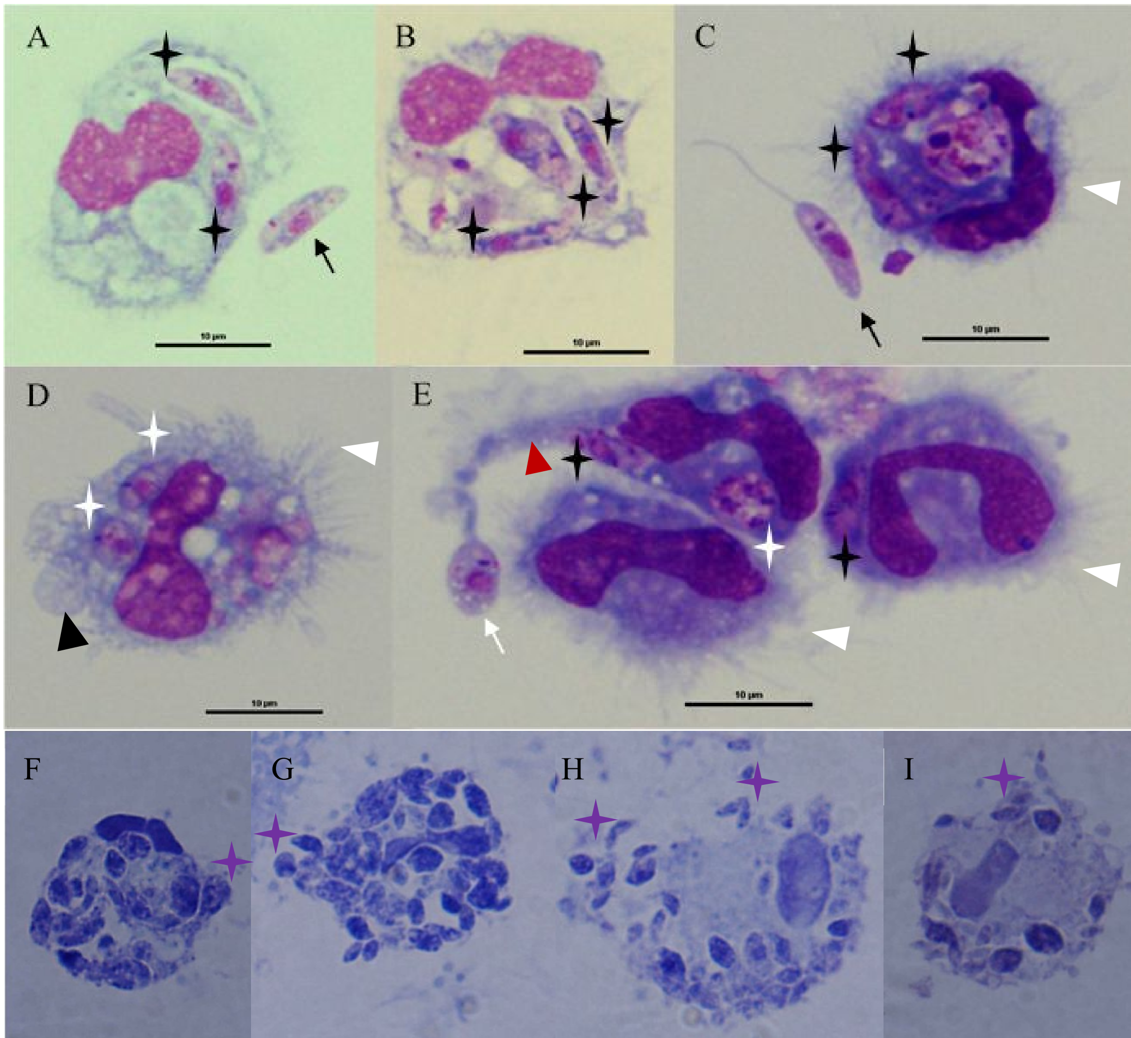

Figure 30: Dog macrophage internalizing the parasite and ensuring parasite differentiation into the amastigote-like form. MØ incubated with *L. infantum* promastigotes for 3 h (A, B, C, D and E) stained with Hemacolor staining kit and 24 h (F, G, H and I) or stained with Giemsa were observed under optical microscope and images were acquired. Extracellular promastigotes (black arrow), parasite being caught (white arrow) by a cellular extension (red triangle) filopodia-like, intracellular promastigote-like forms (black star) and, intracellular amastigote-like forms (white star) occupying part of the cytoplasm can be observed at 3 h. Parasites being released (purple star) from infected MØ can be observed at 24 h. Numerous pseudopodia (white triangle) and extensions of plasma membrane (cytoplasmic bleb) (black triangle) are observed in activated MØ (×1000 magnification).

3.4.4 *L. infantum* promastigotes adhere with the tip of the flagellum to dog macrophages

The analysis of the interaction between MØ and *L. infantum* showed that promastigotes used preferentially the flagellar tip to attach to dog MØ (Fig. 31A, B and D) and are engulfed *via* tubular extensions of the plasma membrane (Fig. 31F). Indeed, in 42.72%

of the interactions cell-parasite observed by SEM, *L. infantum* promastigotes adhere to dog MØ *via* the flagellar tip, 15.53% through the parasite posterior pole and 41.75% (Fig. 31C) *via* other regions of the parasite (Fig. 31B, C, D and E). In the last case, the contact with MØ was established by the promastigote body, the base of the flagellum or by the middle portion of the flagellum. MØ attachment of several promastigotes at the same time was frequently observed (Fig. 31B, C and D). Round parasites presenting a short or long external flagellum are often observed bound to MO in cultures incubated for 24 h. These parasites may represent transitional parasitic forms (parasites in differentiation from the promastigote to the amastigote form) or apoptotic parasites (Fig. 31F).

3.4.5 *L. infantum* induces morphological changes in dog macrophages

Dog monocyte-derived MØ stimulated for 3 h with PMA and IFN-γ were evaluated by SEM. LPS-stimulated PMN exposed to *L. infantum* and Cyt D-treated MØ exposed to the parasite were also assessed by SEM. Cell shape, surface appearance, presence of pseudopodia and of veil-like structures were used to characterize MØ activation.

Unprimed-MØ used as a negative control displayed a homogenous round shape and ranged in size from 6 to 10 μm or slightly more (Fig. 32A). PMA-stimulated MØ (Fig. 32B, C and D), IFN-γ-stimulated MØ (Fig. 32E, F, G and H) and LPS-stimulated MØ exposed to *L. infantum* promastigotes (Fig. 32M, N, O and P) showed a more variable cell shape, ranging from round to elongated (Fig. 32B, C, D, E, F, M, N, O and P), fusiform (Fig. 32G and H) or irregular and flat (Fig. 32E, M and O). Cell size was also heterogeneous and could reach three times the length of an unprimed-MØ. However, IFN-γ induced the most pleomorphic population of MØ. *L. infantum*-exposed MØ (Fig. 32I, J, K and L) and Cyt D-treated and parasite-exposed MØ (Fig. 32Q, R, S and T) showed a predominantly round shape.

Unprimed-MØ displayed a slightly irregular surface appearance (Fig. 32A), while differently stimulated cells exhibited a markedly irregular surface appearance, characterized by elongated and round raffles, small round bodies, protruding sucker-shaped formations (Fig. 32M), resembling phagocytic cups and large round bodies (Fig. 32M and P), which probably represent cytoplasmic blebs that characterize cell apoptosis.

Whereas unprimed-MØ showed scarce filodopodia formation, stimulated cells exhibited exuberant filodopodia. At least three types of thin filodopodia-like structures were observed: short and pointed that seem to promote firm contact with the substrate (Fig. 32B, C, D, G, H, I and S); long, pointed and sometimes branched that were also observed in contact with the substrate and/or with the parasite (Fig. 32E, F, J, P, R and T); and long structures that established contact with adjacent cells (Fig. 32D, M and Q), raising the possibility of tunneling nanotubes (TNT) (Rustom et al, 2004; Arrevillaga-Boni et al, 2014), which promote direct cell-to-cell communication through membrane continuity.

Furthermore, stimulated-MØ frequently exhibited thin, veil-like formations, racket-shaped with an irregular appearance, which is conferred by the presence of thick cords (Fig. 32C, D, F, I, K, N, O, Q, R, S and T). These formations emerge from the cell surface and adhere to the substrate and to neighboring MØ, contributing to the entrapment of the parasite (Fig. 32K and S). The veil-like formations may result from the release and spread of intracellular content in the extracellular space (ET-like), but may also represent extensions of the plasma membrane, named lamellipodia formations. Thick strands and hollow tubular structures, probably with similar function but different shape were observed connecting adjacent cells and entrapping the parasite (Fig. 32B, I and J). Interestingly, in the case of *L. infantum*-exposed MØ, the formation of long tubular extensions of the plasma membrane sucking the parasite were evident (Fig. 32L).

Thus, morphological markers of MØ activation (cell shape, surface appearance, pseudopodia and veil-like structures) were more exuberant in chemically-activated MØ and in LPS-stimulated and parasite exposed MØ than in *L. infantum*-exposed cells, suggesting that the parasite is a less potent inflammatory stimulus than chemical stimuli, which probably contribute for its own survival. Interestingly, MØ treated with the phagocytosis inhibitor Cyt D before *L. infantum* infection showed enhanced morphological changes, including exuberant filodopodia, veil-like formations and markedly irregular cell surface (Fig. 32G, H and I), probably in an MØ attempt to entrap and contain the parasites. However, the morphological changes parasite-induced are important to explore the surrounding milieu and to contact with the parasite.

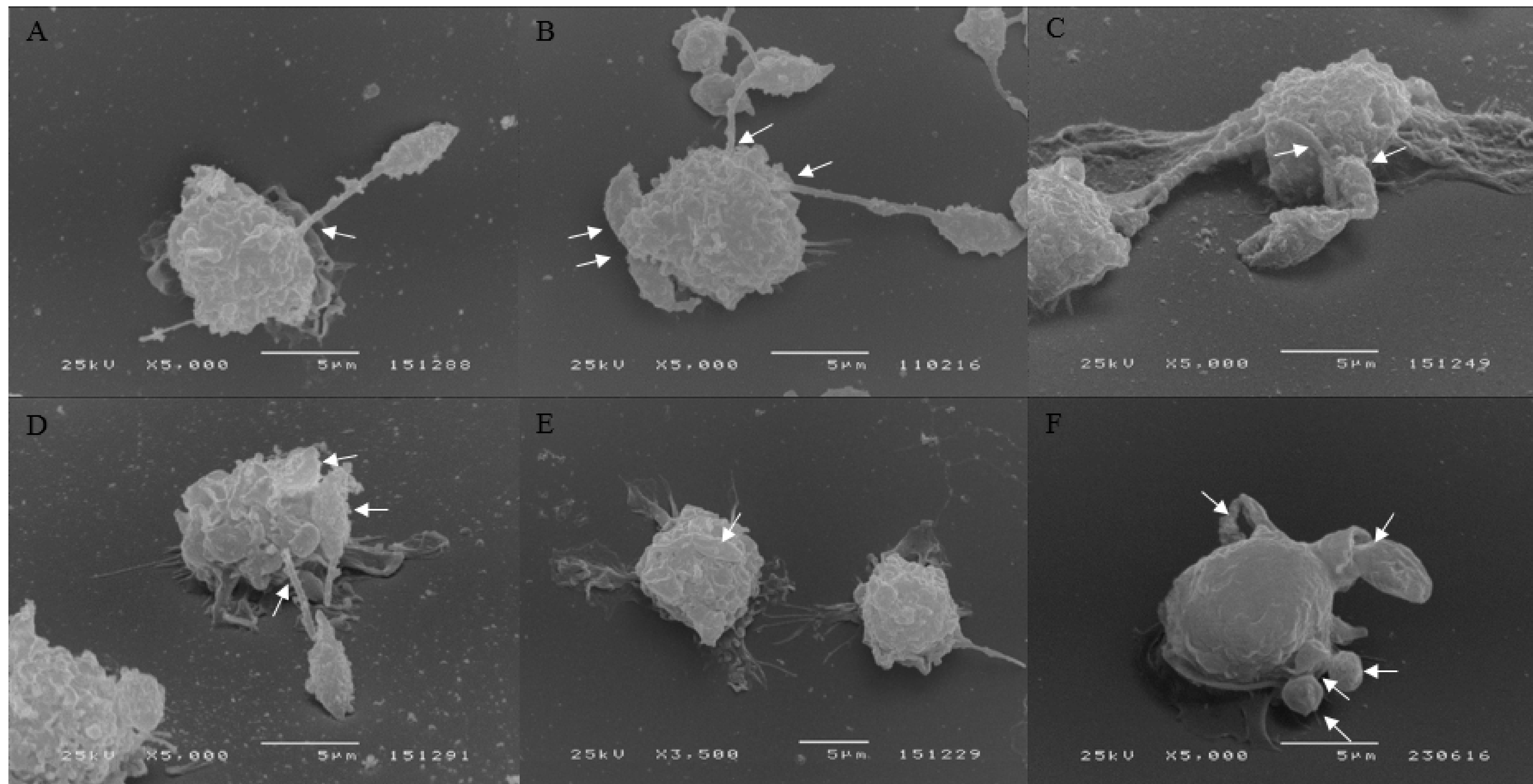

Figure 31: *L. infantum* promastigotes adhere by the tip of the flagellum to dog macrophages. Coverslips adhered-MØ exposed to *L. infantum* promastigotes were incubated for 3 h (A, B, C, D and E) and 24 h (F) and observed by SEM. Parasite attachment *via* the flagellar tip (A, B and D), the posterior pole (C) and *via* other regions of the parasite (C, D, E) is seen. The simultaneous attachment of two parasites was noted (B, C and D). A promastigote being engulfed through a tubular extension of the plasma membrane and the attachment of transitional parasitic forms (F) are shown.

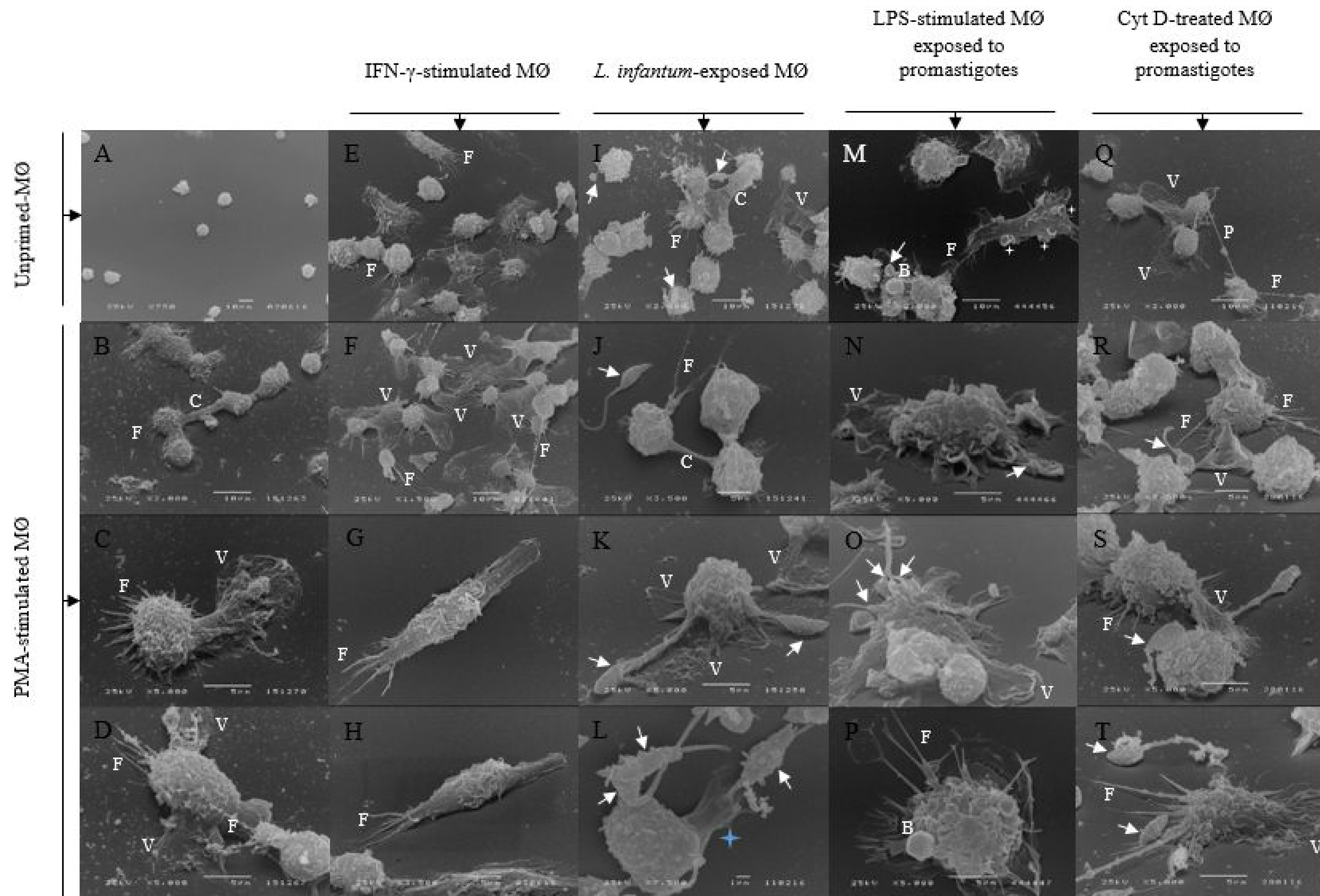
IFN-γ-stimulated MØ
L. infantum-exposed MØ
LPS-stimulated MØ exposed to promastigotes
Cyt D-treated MØ exposed to promastigotes
Unprimed-MØ
PMA-stimulated MØ
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
S
T

Figure 32: Morphological changes of dog macrophages. Coverslips adhered-MØ exposed to promastigotes (MØ+L), stimulated with LPS or treated with cytochalasin D before parasite exposure (MØ+L-LPS and MØ+L-Cyt D, respectively) for 3 h were observed by SEM and images were acquired. Additionally, unprimed-MØ were used as a negative control and, PMA (MØ-PMA) and IFN-γ (MØ-IFN-γ) stimulated-MØ were used as positive controls. Representative SEM images of unprimed-MØ (A), MØ-PMA (B, C, D); MØ-IFN-γ (E, F, G, H); MØ+L (I, J, K, L), MØ+L-LPS (M, N, O, P) and MØ+L-Cyt D (Q, R, S,T) are shown. Blue Star - phagocytic tube; Arrow - parasite; White Star - Phagocytic cup; B - Cytoplasmic bleb; C - Connecting formation; F - Filodopodia-like formation; V - Veil-like formation.

3.4.6 *L. infantum* induces the release of nuclear histone

With the objective of studying the nature of the veil-like formations observed in activated-MØ, cultures adhered coverslips were stained with DAPI and with the monoclonal antibody Histone H1 FITC. Unprimed-MØ showed a well-defined nucleus without loss of DNA and the cytoplasm was histone free (Fig. 33 MØ). *L. infantum*-infected MØ exhibited loss of nuclear definition, histone and DNA exclusion from the nucleus and their co-localization in the cytoplasm and in some cases in the extracellular space. Nuclear histone marked as large, round and intense green structures is distinct from parasite histone that exhibited a faint green signal and is close to the parasite kinetoplast, which showed a small but intense blue signal (Fig. 33 MØ+L). Nuclear histone presented in the cytoplasm and in the extracellular space maintained a granular aspect, suggesting an absence of denaturation. Compared with *L. infantum*-exposed MØ, Cyt D-treated MØ parasite infected evidenced more intense changes characterized by complete loss of nuclear structure, exclusion of nuclear DNA and extravasation of histone and DNA into the extracellular space (Fig. 33 MØ+L-Cyt D). PMA (Fig. 33 MØ-PMA), LPS (Fig. 33 MØ+LPS) and IFN-γ-stimulated MØ (Fig. 33 MØ+IFN-γ) used as positive controls displayed similar findings characterized by loss of nuclear definition, DNA and histone exclusion from the nucleus and its co-localization in the cytoplasm and in some cases in the extracellular space. Interestingly, these findings were generally more exuberant in chemically-stimulated MØ than in *L. infantum*-exposed MØ, suggesting that *L. infantum* is a weaker inflammatory stimulus. These results indicate that probably the parasite modulates or delays MET release, thus preserving the integrity of its host cell.

However, the degree of histone and DNA extrusion was dissimilar among dogs. While in some dogs, parasite-exposed MØ exhibited cytoplasmic extensions and minimal release of DNA and Histone H1 into the cytoplasm without evidence of membrane rupture (Fig. 34A) in other animals the loss of nuclear DNA and Histone H1 was intense and the release of these nuclear components in the extracellular environment was evident, suggesting membrane breakdown (Fig. 34A and C). These findings suggest different degrees of MØ activation among dogs.

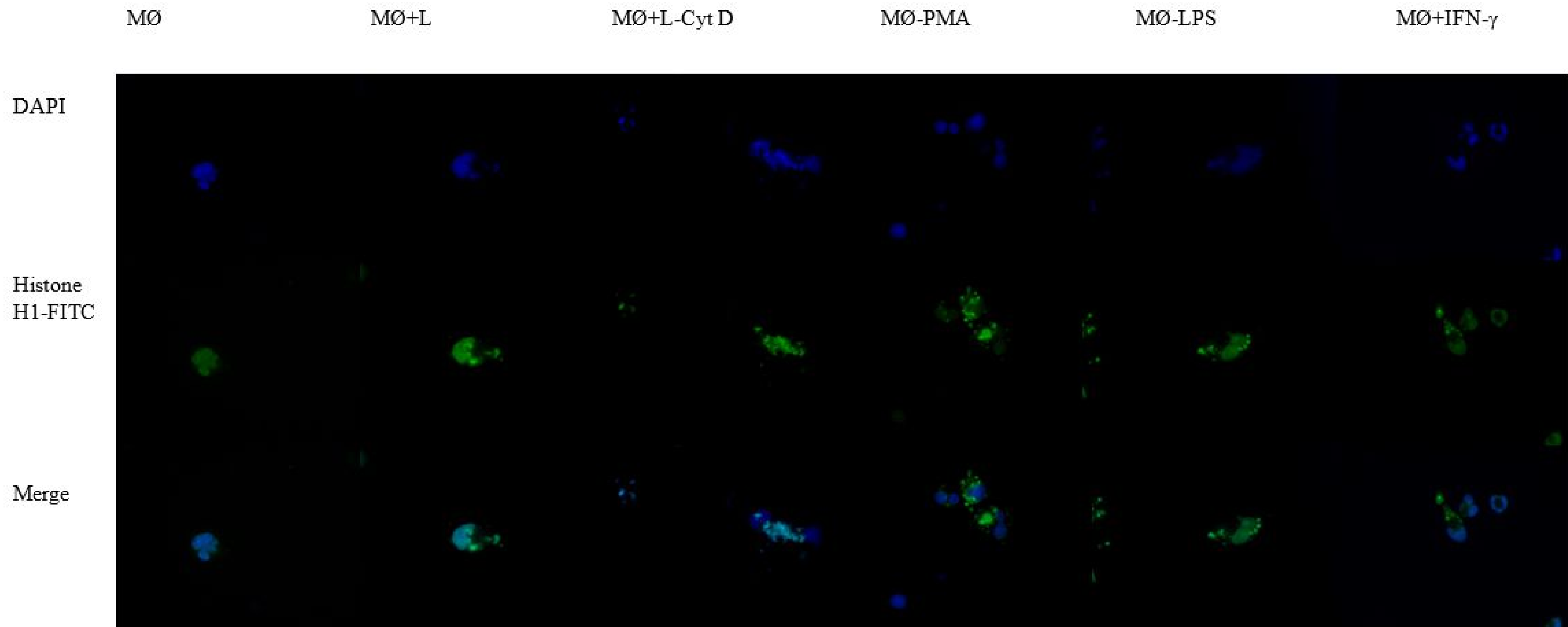

Figure 33: Stimulated dog macrophages release Histone 1. Coverslips adhered-MØ exposed to promastigotes (MØ+L) or treated with cytochalasin D before parasite exposure (MØ+L-Cyt D) were incubated for 3 h, and stained with Histone H1 FITC antibody and DAPI. Additionally, MØ were stimulated with PMA (MØ-PMA), IFN-γ (MØ-IFN-γ) and LPS (MØ-LPS) and used as positive controls and unprimed-MØ as a negative control. Representative images of MØ, MØ+L, MØ+L-Cyt D, MØ-PMA, MØ-LPS, MØ-IFN-γ stained with DAPI and Histone H1 FITC and the respective merge are shown (×1000 magnification).

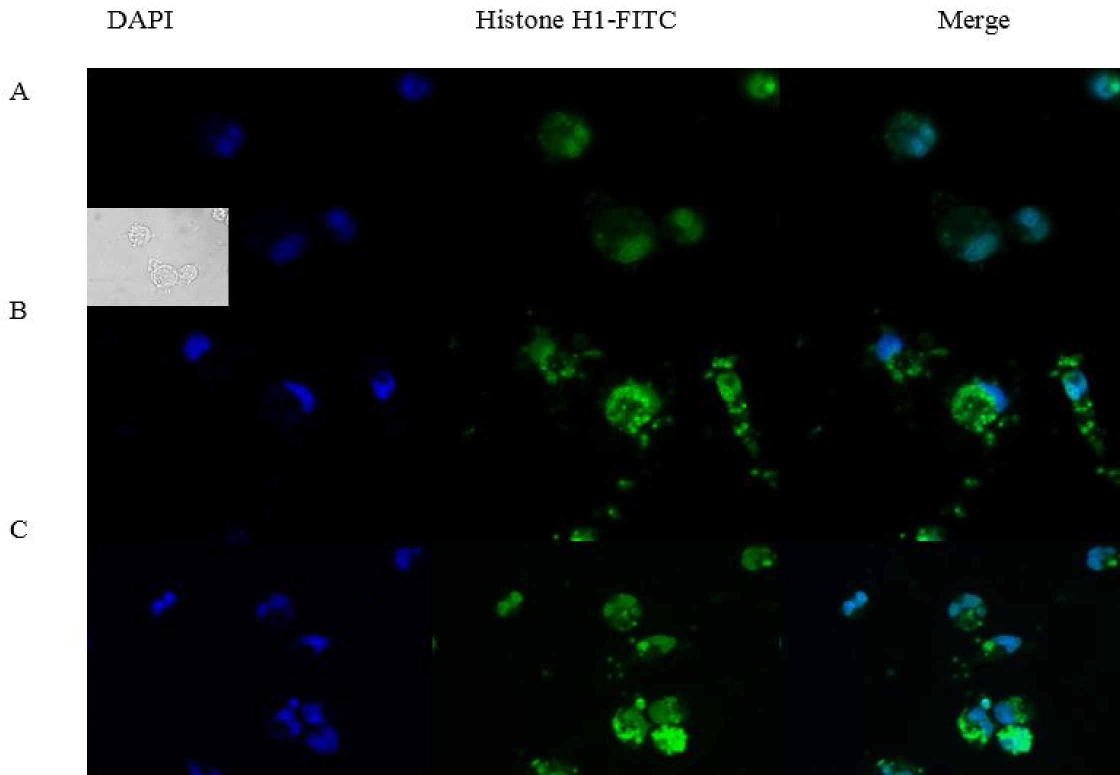

Figure 34: *Leishmania infantum* exposed-macrophages release Histone H1 into the extracellular medium. Coverslips adhered-MØ exposed to promastigotes for 3 h and stained with DAPI and Histone H1 FITC antibody were observed under fluorescent microscope. Cytoplasm expansion containing some parasites identified by the blue kinetoplast can be observed in A and some extracellular parasite membrane attached to the opposite pole of the cell can be identified in the visible light microphotography. Activated MØ presenting a polarized shape characterized by an elongated cytoplasm and the release of Histone H1 and DNA in the cytoplasm and in the extracellular environment can be observed in B and C (×1000 magnification).

Immunofluorescent images corroborate the features of SEM, confirming that veil-like formations observed in SEM images represent cell extensions and in some cases the membrane breakdown and the release of cellular content, namely Histone H1 and DNA in the extracellular space. These ET-like formations generated by MØ in response to *L. infantum* infection are different from those emitted by dog PMN that present a filamentous appearance.

3.4.7 Dog macrophages activate killing effector mechanisms in response to *L. infantum* infection

The interaction between MØ and the parasite was evaluated by OM and was ultrastructurally assessed by TEM. In some OM images, internalized parasites exhibited abnormal morphology (Fig. 35), suggesting phagolysosomal degradation.

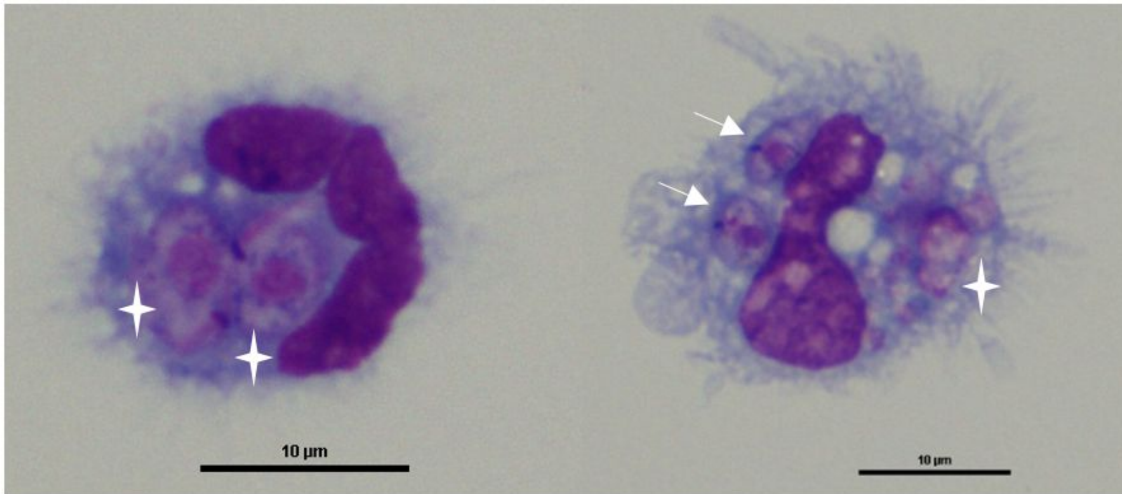

Figure 35: Dog macrophages promote intracellular parasite killing. MØ incubated with *L. infantum* promastigotes for 3 h and stained with Hemacolor staining kit were observed under an optical microscope (×1000 magnification) and images were acquired. MØ (left) with two intracellular parasites that have lost their normal morphology (white stars) and, another MØ (right) with two intracellular amastigote-like parasites showing the typical morphology (white arrow) and a parasite that has lost the normal internal organization (white star) are shown.

TEM images confirmed the presence of degraded parasites inside the phagolysosome (Fig. 36D) and in some cases the amorphous material was only identified based on the presence of intact subpellicular microtubules (Fig. 36E, F, G and H) characteristic of trypanosomatids. Interestingly, in some cells, both degraded and intact parasites were simultaneously observed (Fig. 36A, B and C), suggesting either different parasite uptakes through different surface receptors or/and the interference of some parasites with phagosome maturation.

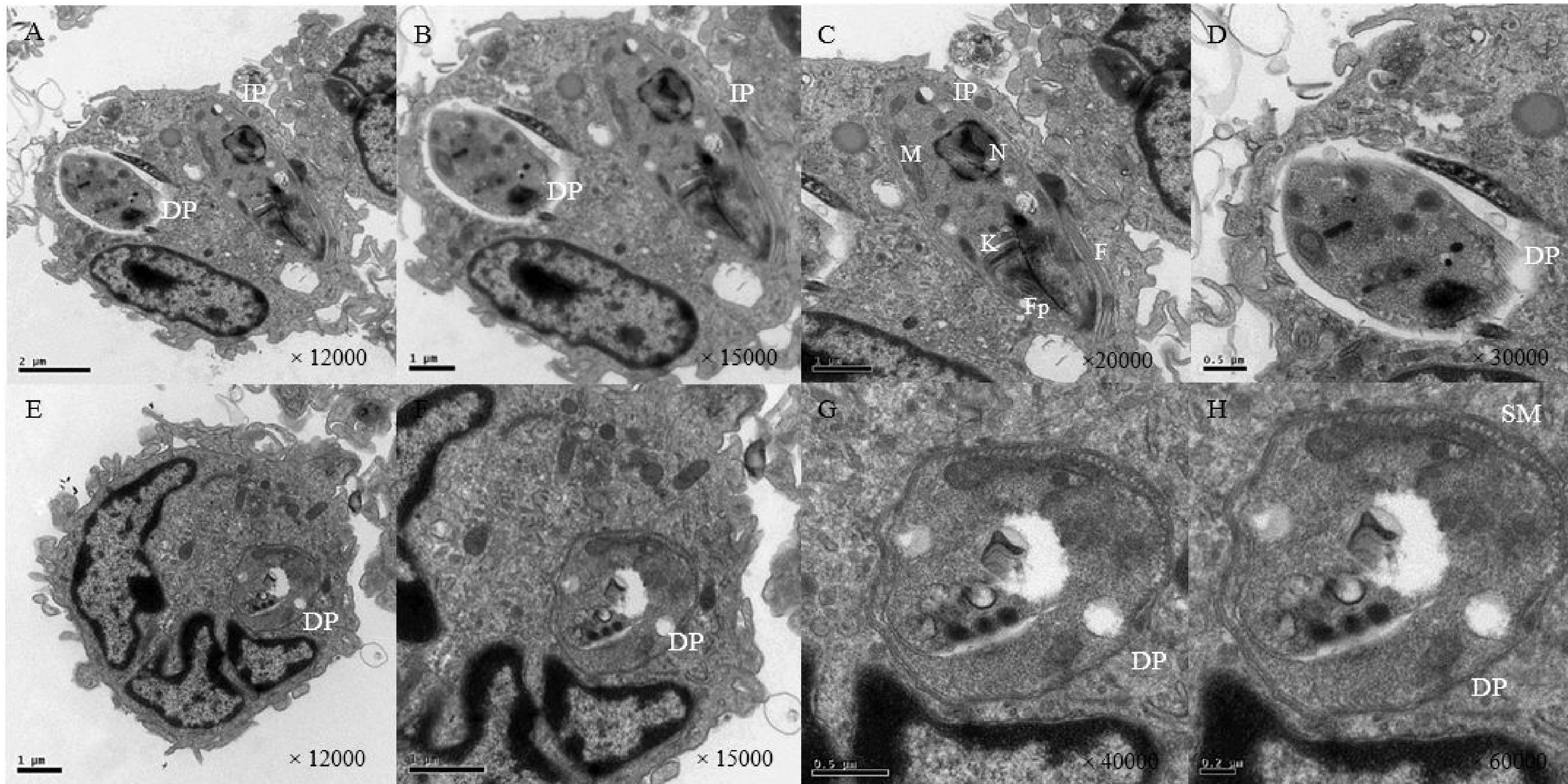

Figure 36: Intracellular killing of *L. infantum* parasites by dog macrophages. MØ incubated with *L. infantum* promastigotes for 3 h were observed by transmission electron microscopy (TEM) and images were acquired. A degraded (DP) and an intact parasite (IP) can be observed inside the same MØ (A). Further images with increase magnifications show details of the intra-structural organization of dying and alive parasites (B, C and D). IP show well differentiated nucleus (N), mitochondria (M) and the region corresponding to the kinetroplast (K), the flagellar pocket (Fp) and the extracellular flagellum (F). Another MØ containing a DP is shown at different magnifications (E, F, G and H). The subpellicular microtubules (SM) are indicative of a dying parasite (H).

3.4.8 The parasite maintains the viability and the capacity of multiplication after neutrophil phagocytosis

Cultures of PMN and *L. infantum* and of PMN and *L. infantum*-GFP were incubated for 3 h and the extracellular parasites were removed by positive selection using MicroBeads conjugated to monoclonal CD11b antibodies. Although this technique ensures the removal of extracellular parasites, extracellular promastigotes bound to cell membrane remain. In order to assess the viability of internalized parasites PMN were transferred to SCHN medium and incubated at 24ºC. After 24 h of incubation, 5×10^5 (3.3×10^5, 8.3×10^5) moving parasites were counted, suggesting that the internalized parasites actively escaped from PMN or were released after PMN death, maintaining the viability. Furthermore, after 72 h of incubation a significantly higher ($p<0.001$) number of viable parasites was counted [45.8×10^5 (21.7×10^5, 71.2×10^5)], indicating that the parasite also retained the capacity of multiplication (Fig. 37).

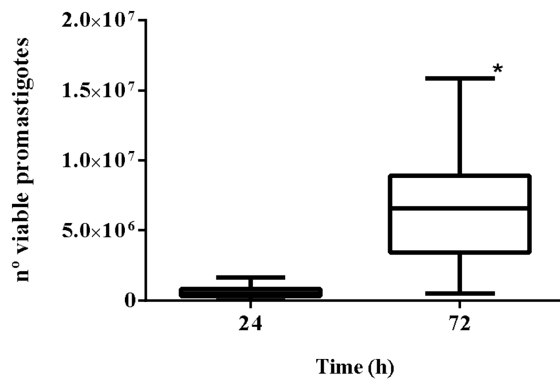

Figure 37: Neutrophil phagocytized parasites maintain the viability and the capacity of multiplication. After removing the extracellular parasites, PMN-*L. infantum* promastigotes incubated for 3 h were transferred to Schneider medium and incubated at 24ºC. Viable promastigotes estimated at 24 h and 72 h are represented by medians, 75th percentile and 25th percentile and whiskers indicating the highest and lowest values. A statistical analysis of triplicate samples of 10 dogs was performed using the Wilcoxon test. * ($p<0.05$) represents statistically significant differences when comparing 24 h *vs* 72 h.

3.4.9 *In vitro* efferocytosis of apoptotic neutrophils

Co-cultures PMN-MØ incubated for 3 h and 24 h were accompanied under OM to monitor efferocytosis of apoptotic neutrophils. The association between neutrophils and MØ was evident in co-cultures with 3 h of incubation. At this time point, most neutrophils showed apoptotic features, such as cell shrinkage, pycnotic nuclei and karyorrhexis (Fig. 38C and D), but some cells still maintained the characteristic multilobulated nuclei (Fig. 38A, B and C). In most microscopic fields the number of neutrophils doubled or equaled the number of MØ. However, in co-cultures with 24 h of incubation MØ were the predominant cellular component and frequently forming cellular aggregates. Pycnotic nuclei were observed inside MØ efferosome at several stages of disintegration (Fig. 38E, F and G). The reduced number of neutrophils observed in the microscopic images suggests possible disintegration or phagocytosis of neutrophils by MØ. DAPI and Histone H1 FITC stained cultures revealed the co-localization of DNA and histone in the MØ nucleus and in the pycnotic nucleus localized inside the MØ cytoplasm (Fig. 38H1, H2 and H3).

TEM was also used to ultrastructurally assess the process of neutrophils internalization by MO. Efferocytosis of necrotic (Fig. 39A and B) and apoptotic neutrophils were observed. In the case of necrotic neutrophils the efferocytosis process was characterized by the existence of amorphous material inside the efferosome, while the efferocytosis of apoptotic neutrophils was associated with the presence of several dense corpuscles, the apoptotic bodies (Fig. 39C, D and E).

Co-cultures incubated for 3 h were evaluated by SEM and the contact between MØ and PMN was recorded. Although MØ established connection with PMN throughout pseudopodia, PMN were also observed sending temporary cytoplasmic projections to contact with MØ (Fig. 40A). Clusters of PMN interacting with MØ were frequently observed and the interaction between several PMN and one MØ was also commonly seen (Fig 40B, C, D and F). Morphological features of MØ activation, such as pleomorphic shape, irregular surface appearance and pseudopodia (Fig. 40B, C, D, E and F) were detected. Different stages of PMN efferocytosis were documented: phagocytic cup formation (Fig. 40C and D), circumferential tubular extensions of MØ membrane progressively involveing PMN (Fig. 40C and D), and PMN engulfment.

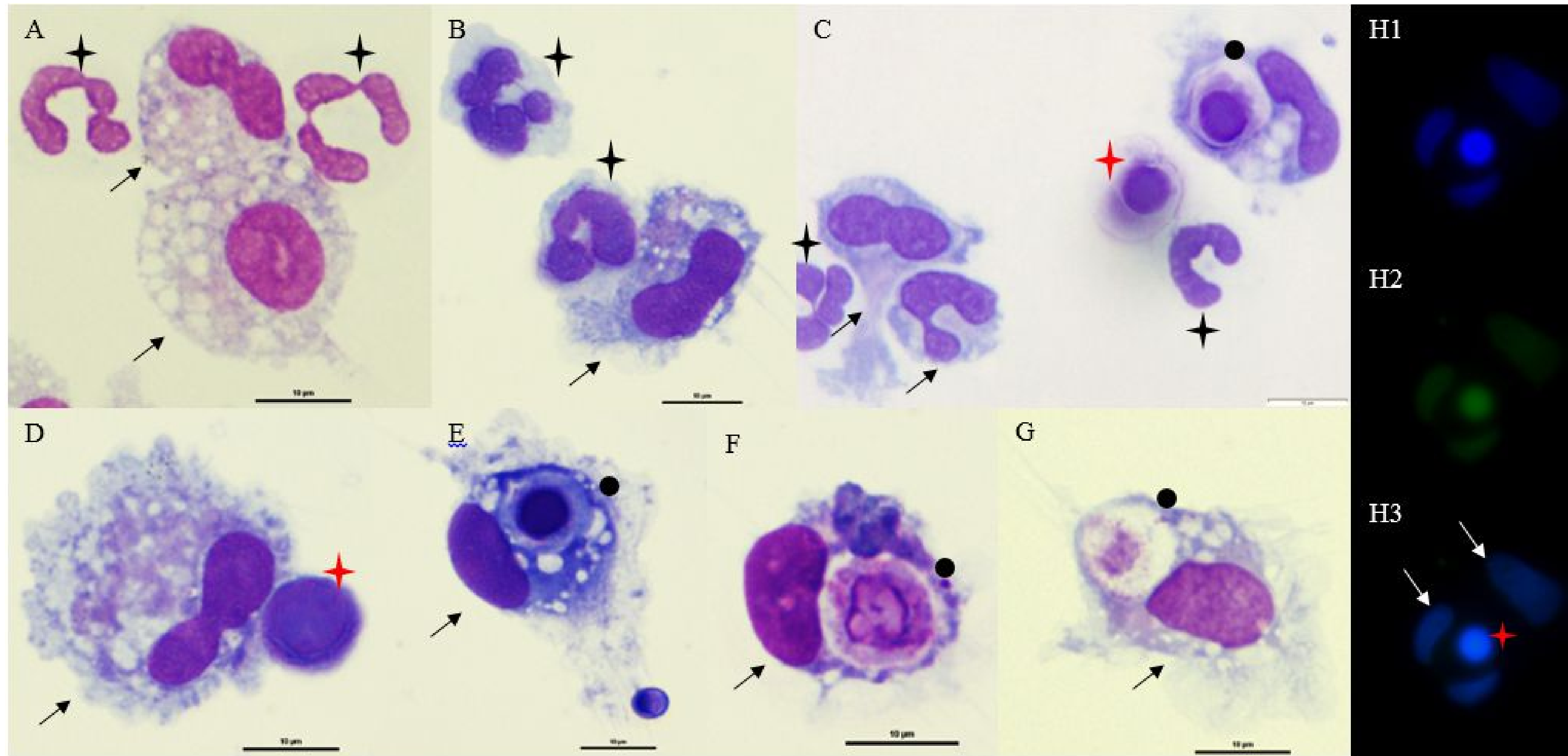

Figure 38: Apoptotic neutrophil efferocytosis. Citospins of co-cultures neutrophil-MØ (PMN+MØ) incubated for 3 h and 24 h were stained with Hemacolor staining kit or DAPI and Histone H1 FITC and observed under an optical or fluorescent microscope, respectively (×1000 magnification). Representative images of co-cultures with 3 h (A, B, C, D, H1, 2 and 3) and 24 h of incubation (E, F, G) are shown. MØ associated with PMN (A, B) and MØ associated with apoptotic cells (C, D) can be observed. Different stages of efferocytosis showing the appearance of the efferosome (C, E, F, G, H1, H2 and H3) can also be seen. Black arrow - macrophage; Black star - neutrophil; Red star - apoptotic cell; Black dot - eferosome containing apoptotic cell. H1 - DAPI staining; H2 - Histone H1 staining: H3 - merge.

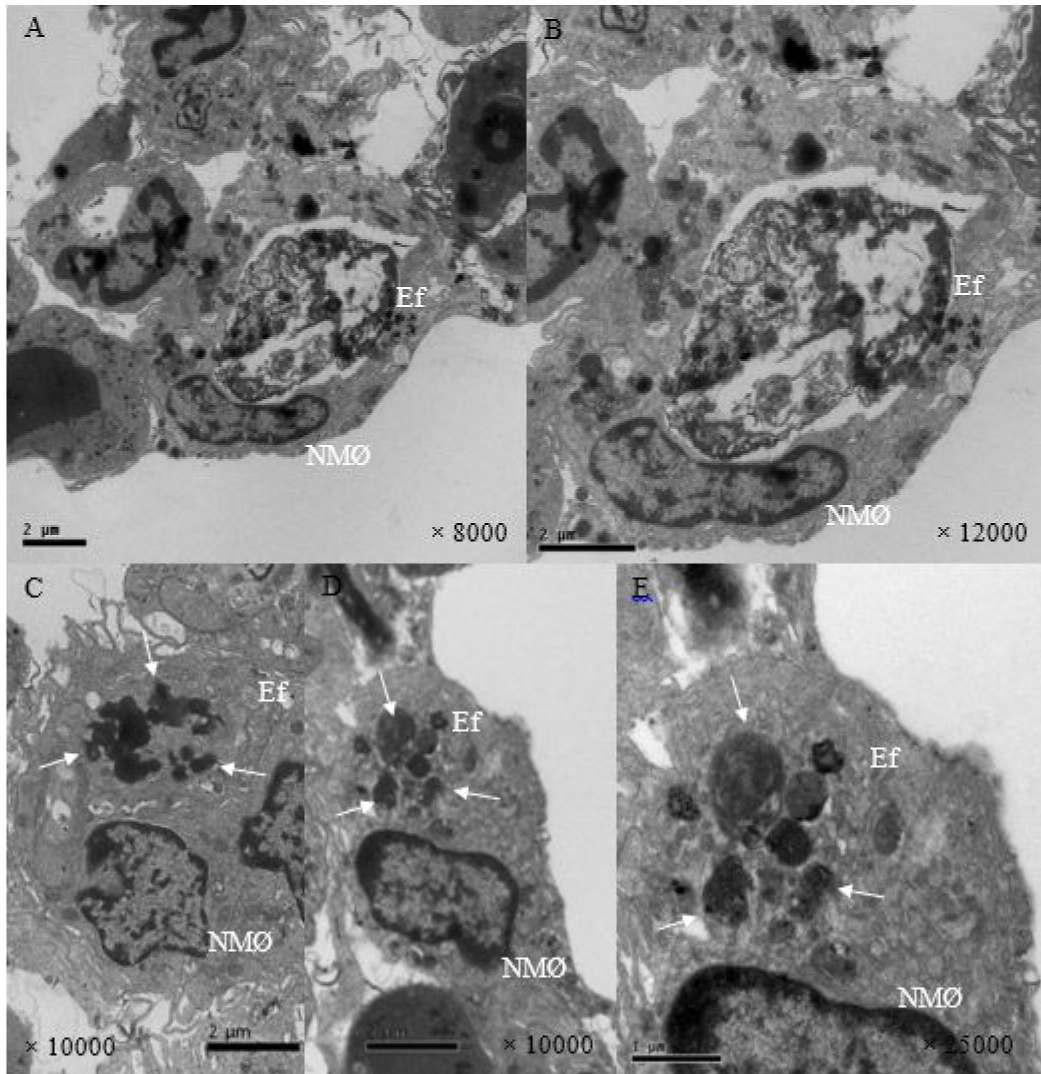

Figure 39: Neutrophil efferocytosis documented by transmission electron microscopy (TEM). Dog MØ and neutrophils previously exposed to *L. infantum* promastigotes were co-cultured for 3 h and 24 h. Ultrathin sections were observed by TEM and images were acquired. Magnifications of necrotic cell efferocytosis observed at 3 h (A, B) and of apoptotic cell efferocytosis observed at 3 h (C) and 24 h (D, E) are shown. The efferosome containing the necrotic cell (A, B) or several apoptotic bodies (arrows) (C, D, E) inside the MØ cytoplasm can be observed. D and E represent different magnification of the same cell. Ef - efferosome; NMØ - macrophage nucleus.

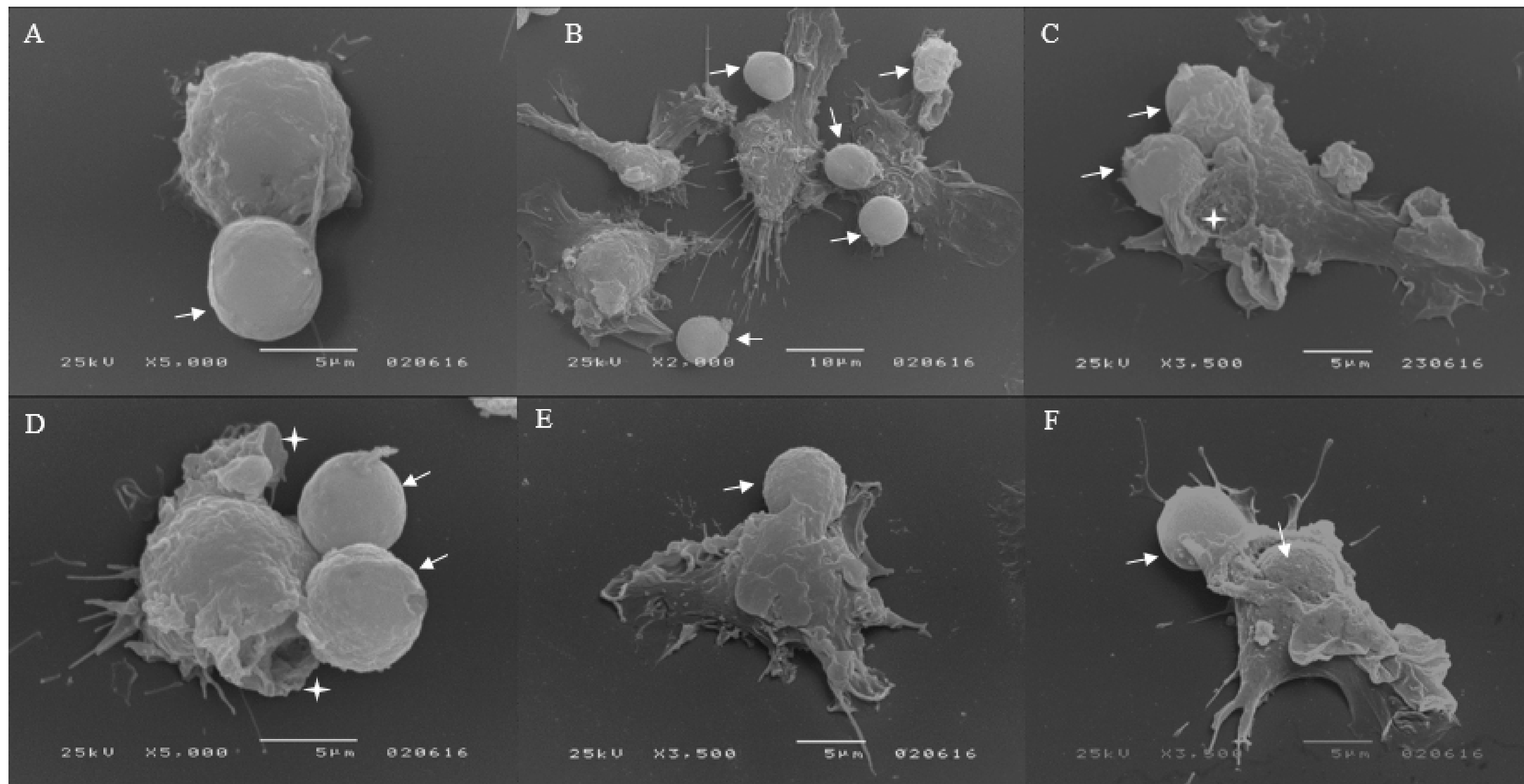

Figure 40: Efferocytosis documented by scanning electron microscopy (SEM). Dog MØ co-cultured with neutrophils for 3 h were observed by SEM and images were acquired. Contact between the two types of phagocytes is shown to be mediated by pseudopodia (A) or by veil-like structures (B). The progressive neutrophil engulfment by MØ can be observed (C, D, E, F). Neutrophils are indicated by the arrow and phagocytic cups by a white star.

3.4.10 The parasite is transferred from infected PMN to macrophages

PMN were exposed to *L. infantum*-GFP parasite for 3 h and then the extracellular parasites were removed. Co-cultures of MØ+PMN-*L. infantum*-GFP were established and incubated for 3 h and 24 h and then analyzed by flow cytometry to evaluate the level of transference of PMN internalized parasites to MØ. MØ+PMN co-cultures incubated for 3 h were used as GFP^- control (Fig. 41B and C). At 3 h [6.77% (3.78%, 14.3%)] and 24 h [11.25% (8.22%, 17.38%)] of incubation, the level of GFP^+ PMN (presented as median and interquartile range) was significantly higher than the level of MØ GFP^+ at 3h [4.41% (1.99%, 11.90%)] and at 24 h [6.16% (2.60%, 12.70%)] ($p_{3\ h}$=0.009, $p_{24\ h}$=0.008). Although, the level of GFP^+ PMN significantly increased at 24 h when compared with 3 h (p=0.007), the amount of GFP^+ MØ did not present important changes, suggesting either that parasite transference is limited, or that transferred parasites lose their replicative ability or are degraded by MØ effector mechanisms (Fig. 41H).

The amount of parasites internalized or bound to MØ was assessed by GFP^+ median. As expected, at 3 h of incubation the GFP^+ median (presented as mean ± standard deviation) was higher in infected MØ population (48.88±33.72) than in non-infected MØ population (38.09±21.96) although with no statistical significance. However, at 24 h of incubation the GFP^+ median was significantly higher in infected (59.53±35.01) when compared with non-infected co-cultures (36.90±17.46) ($p_{24\ h}$=0.028). These results reflect the uptake of infected PMN by MØ population and confirm the parasite transference to the definitive host cell (Fig. 41I).

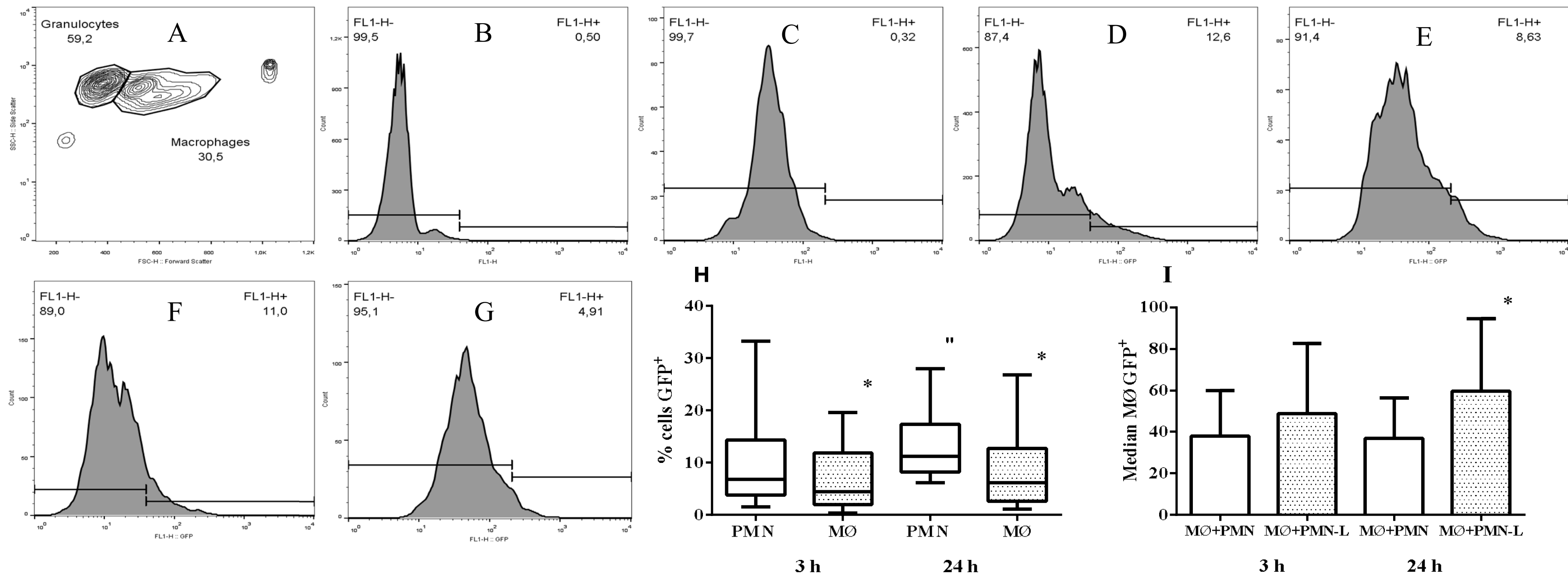

Figure 41: Dog neutrophil ensure parasite transference to macrophages. Co-cultures of MØ-neutrophils (MØ+PMN) and of MØ and *L. infantum*-GFP exposed neutrophils whose extracellular parasites were removed (MO+PMN-L) were analyzed by flow cytometry. MØ and PMN populations were gated on contour graphs 5% probability based on FSC-H *vs* SSC-H characteristics (A). PMN (B) and MØ (C) populations from MØ+PMN cultures incubated for 3 h were used to define PMN GFP$^-$ and MØ GFP$^-$ cells (negative GFP control) on the histogram FL1-H/GFP *vs* number of events. This gating strategy was used on PMN and MØ populations from MØ+PMN-L incubated for 3 h (D and F respectively) and 24 h (E and G respectively). Levels of PMN and MØ associated to the parasite (H) was expressed by medians, 75th percentile and 25th percentile and whiskers indicating the highest and lowest values and the level of GFP positivity in the entire PMN and MØ population (I) was expressed by mean ± standard deviation at different time points. The non-parametric statistical analysis of 10 dogs and triplicate samples was performed using the non-parametric Wilcoxon test. Statistically significant differences (p<0.05) are indicated by * when PMN were compared with MØ, " when time points were compared and by $^{\#}$ when MØ+PMN are compared with MØ+PMN-L.

3.4.11 Efferocytosis of apoptotic neutrophils ensures parasite transference to macrophages

L. infantum-exposed PMN co-cultured with MØ for 3 h revealed the presence of the parasite inside neutrophils in both promastigote (Fig. 42D and F) and amastigote-like forms (Fig. 42A, B, C, E and F). Parasitized neutrophils exhibited the characteristic multilobulated nuclei (Fig. 42A, B and C) or can be apoptotic (Fig. 42D, E and F). Rare parasites were observed in the extracellular space. Efferocytosis of infected neutrophils was observed (Fig. 42G, H and I), suggesting that at least in part this mechanism is responsible for parasite transference to the definitive host cell. After 24 h of incubation, MØ that had internalized apoptotic cells showed amastigote-like forms in the cytoplasm (Fig. 42J and K). MØ carrying promastigotes and amastigote-like forms (Fig. 42L) were also observed. These findings suggest that after being internalized by PMN, *L. infantum* parasites have two possible ways to be uptaken by MØ: they are either transferred by efferocytosis or internalized by MØ after escaping from apoptotic cells to the extracellular space.

Infected co-cultures were also analyzed by SEM in order to decipher the mechanism of parasite transference. Parasite associated to PMN in the vicinity of MØ (Fig. 43) and parasite MØ associated near degraded PMN were frequently noticed, giving evidence that the parasite can escape from infected neutrophils.

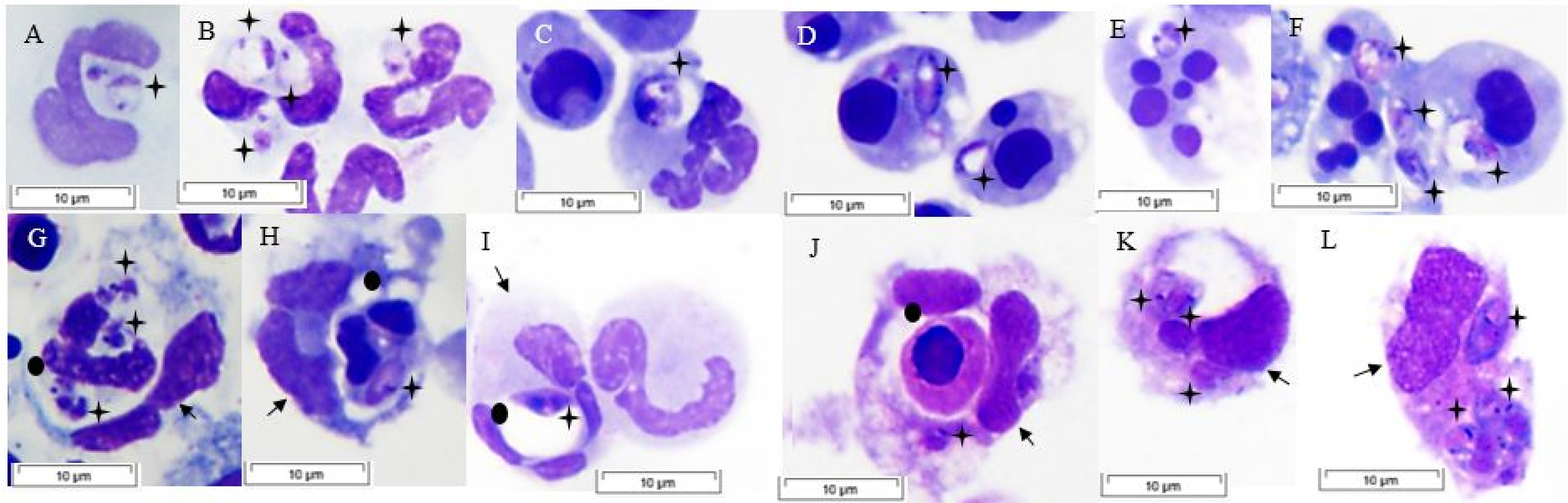

Figure 42: Transference of intracellular *L. infantum* parasites from neutrophils to macrophages. Citospins of *L. infantum*-exposed neutrophils co-cultured with autologous MØ (MØ+PMN-L) were stained and observed under an optical microscope (×1000 magnification). Representative images of 3 h (A, B, C, D, E, F, G, H and I) and 24 h co-cultures (J, K and L) show parasitized neutrophils evidencing normal nuclear shape (A, B and C), apoptotic neutrophils (D, E and F) with intracellular amastigote-like forms (A, B, C, E and F) and promastigote (D and F), efferocytosis of parasitized neutrophils (G, H and I), MØ evidencing simultaneously an intracellular apoptotic neutrophil and two parasites in the cytoplasm (J) and, parasitized MØ (K and L). Black arrow - macrophage; Black dot - efferosome containing an apoptotic cell; Black star - intracellular parasite.

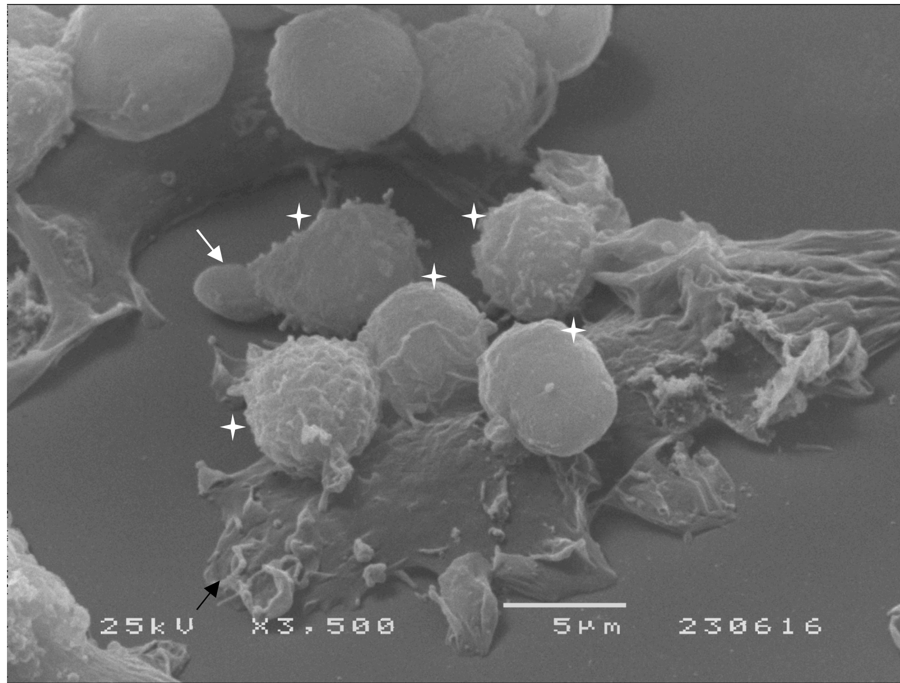

Figure 43: Parasite transference documented by scan electron microscopy (SEM). Dog MØ co-cultured for 3 h with infected neutrophil cultures subjected to previous extracellular parasite removal were prepared for SEM. A parasite escaping from a neutrophil is observed in the vicinity of a MØ. Macrophage - black arrow; Neutrophil - star; Parasite - white arrow.

3.4.12 Extracellular traps are released in co-cultures

Co-cultures stained with DAPI and Histone H1 FITC antibody revealed the exclusion of Histone H1 from the nucleus into the cytoplasm (Fig. 44A), as previously observed in activated MØ cultures. Filamentous NET that stained with DAPI and Histone H1 FITC were also documented (Fig. 44B, C, D, E and F). Interestingly, SEM images obtained from co-cultures clearly demonstrated the sequential morphological events that culminate with NET emission (Fig. 45A; B and C). MØ phagocytosis of parasites entrapped by NET may represent another type of cooperation between these two phagocytic cells in the defense against *Leishmania* parasites.

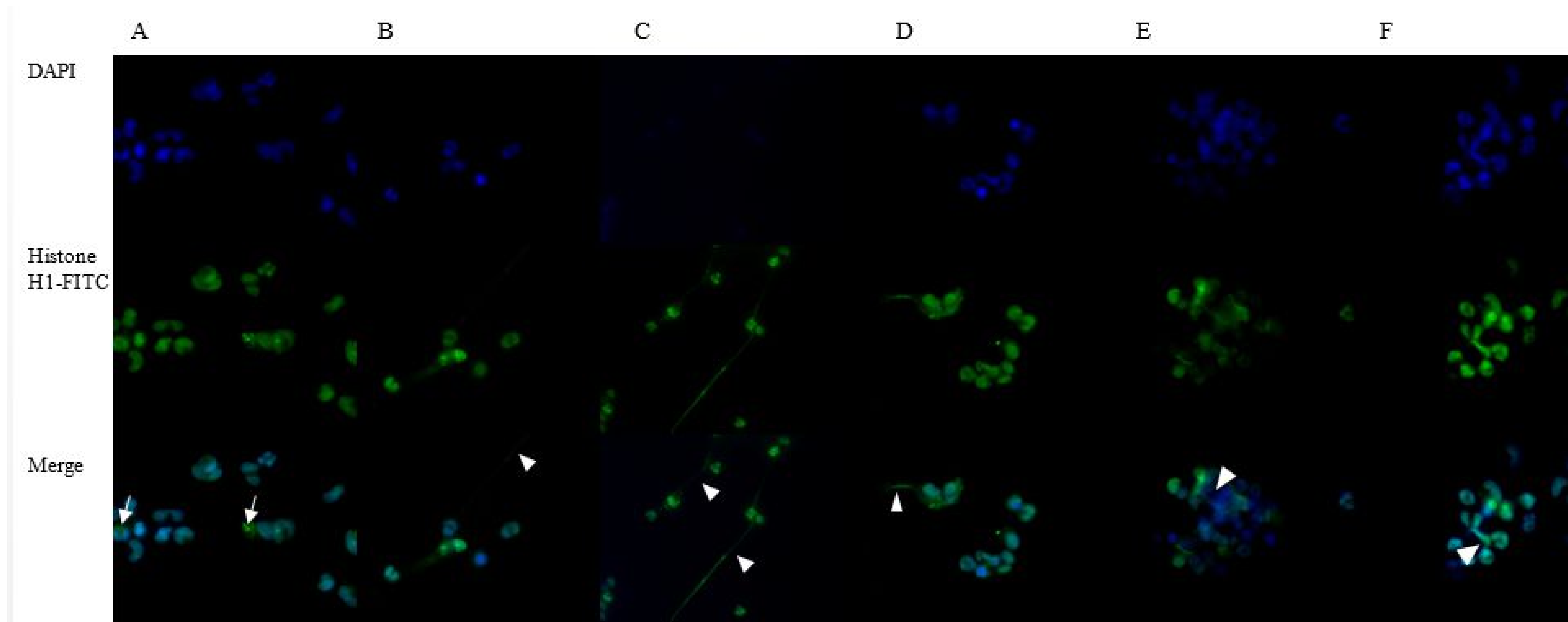

Figure 44: Neutrophils co-cultured with macrophages release neutrophil extracellular traps. Coverslips adhered-MØ co-cultured for 3 h with infected neutrophil cultures subjected to previous extracellular parasite removal were stained with DAPI and Histone H1 FITC antibody. Cells were observed under a fluorescent microscope and images were acquired. Exclusion of Histone H1 from the nucleus into the cytoplasm is observed in A and filamentous Histone H1 being released by small (B, C and D) or large agglomerates of neutrophils (E and F) stained with DAPI and Histone H1 FITC and merge are shown. Cytoplasmic histone is indicated by the white arrows and filamentous extracellular traps by the head arrows (×1000 magnification).

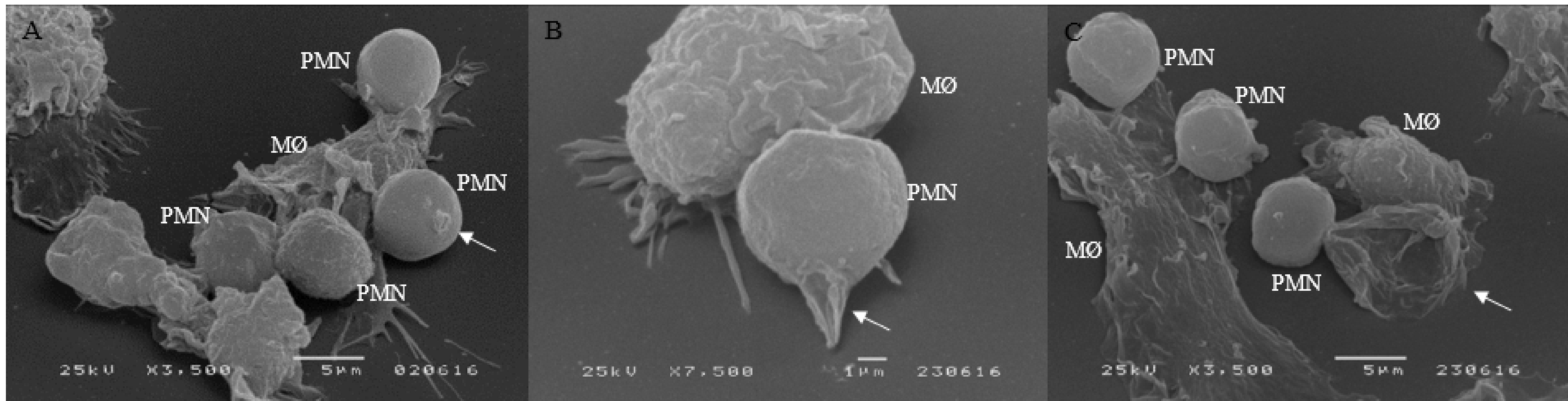

Figure 45: Neutrophil extracellular trap release by infected PMN in co-culture with macrophages. Dog MØ co-cultured for 3 h with infected neutrophil subjected to previous extracellular parasite removal were observed by scan electron microscopy and images were acquired. The sequential process of neutrophil extracellular trap release is indicated by arrows. Neutrophils - PMN; Macrophages - MØ.

3.4.13 Urea production is suppressed in infected co-cultures

Urea production was quantified in the supernatants of unprimed-MØ and MØ-*L. infantum* incubated for 3 h and 24 h. Supernatants of MØ+PMN and MØ+PMN-*L. infantum* co-cultures were also used. Urea levels did not show significant changes when analyzed under the different conditions. The levels registered in unprimed-MØ incubated for 3 h and 24 h were 4.54±0.44 mg.dL^{-1} and 5.08±0.28 mg.dL^{-1}, respectively. Although in some dogs *L. infantum*-exposed MØ produced low urea levels the mean value did not show significant changes at 3 h (4.24±0.70 mg.dL^{-1}) and 24 h (4.62±0.82 mg.dL^{-1}) when compared with unprimed-MØ. The production of urea observed in uninfected co-cultures at 3 h was 4.04±1.55 mg.dL^{-1} and at 24 h was 4.61±147 mg.dL^{-1}. However, the urea levels registered in infected co-cultures (4.12±1.48 mg.dL^{-1} at 3 h and 4.5107±1.13 mg.dL^{-1} at 24 h) had a significant decrease when compared with unprimed-MØ cultures ($p_{24\ h}$=0.005), indicating that infected co-cultures take around 24 h to suppress the arginase activity. As arginase and NOS compete with each other for the same substrate, the suppression of the first one suggests activation of NOS and proinflammatory activation of MØ induced by infected PMN.

3.4.14 Nitric oxide production is induced in co-cultures

NO production was quantified in the supernatants of unprimed-MØ, MØ-*L. infantum*, LPS-stimulated MØ, and of MØ+PMN and MØ+PMN-*L. infantum* co-cultures incubated for 3 h and 24 h. As expected, the production of NO at 3 h did not suffer significant changes when analyzed under the different conditions (Table 5).

The significant changes occurred at 24 h and were characterized by an increase in NO production in *L. infantum*-exposed MØ, LPS-stimulated MØ (positive control), PMN-stimulated MØ and PMN-L-stimulated MØ when compared with unprimed-MØ cultures (negative control) ($p_{24\ h}$=0.018). NO production by LPS-stimulated MØ ($p_{24\ h}$=0.028), PMN-stimulated MØ ($p_{24\ h}$=0.021), and PMN+L-stimulated MØ ($p_{24\ h}$=0.038) was higher when compared with *L. infantum*-exposed MØ (Fig. 46). These results point towards MØ classical activation induced by the parasite. Interestingly, the efferocytosis of uninfected and infected PMN also seems to be a potent NO inducer.

Table 5: Nitric oxide production by infected macrophages. Supernatants of unprimed-MØ, *L. infantum*-exposed MØ (MØ-L), LPS-stimulated MØ (MØ+LPS), MØ incubated with neutrophils (MØ+PMN) and, infected neutrophils incubated with MØ (MØ+PMN-L) for 3 h were used to measure NO levels. Results of triplicate samples of 10 dogs are presented.

	MØ	MØ+L	MØ-LPS	MØ+PMN	MØ+PMN-L
Median (µM)	35.24	35.76	41.09	35.68	38.23
25th percentile (µM)	29.84	33.06	33.84	30.07	24.44
75th percentile (µM)	36.73	38.53	45.18	40.47	44.52

Taking urea and NO production into account, significant changes were only registered at 24 h of incubation, suggesting that at the early stages of infection the promastigote form inhibited the proinflammatory activation of MØ which may contribute for the silent establishment of the parasite.

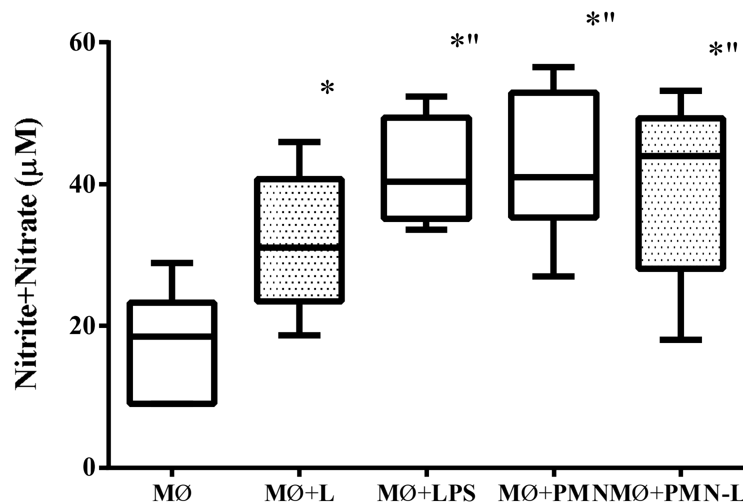

Figure 46: Nitric oxide production by infected macrophages. Supernatants of unprimed-MØ, *L. infantum*-exposed MØ (MØ-L), LPS-stimulated MØ (MØ+LPS), MØ incubated with neutrophils (MØ+PMN) and, infected neutrophils incubated with MØ (MØ+PMN-L) for 24 h were used to measure NO levels. Results of triplicate samples of 10 dogs are presented by medians, 75th percentile and 25th percentile and whiskers indicating the highest and lowest values are present. The Wilcoxon test was used for statistical analysis. * (p<0.05) represents statistically significant differences when unprimed-MØ were compared to the other conditions and " when MØ+L were compared to the other conditions.

3.5 DISCUSSION

Although several types of non-professional and professional phagocytic cells (neutrophils, monocytes and MØ) can uptake *Leishmania* parasites, a productive infection with intracellular parasite multiplication was rarely described in non-MØ cells (Rittig and Bogdan, 2000). Indeed, MØ are widely considered the primary host cells of *Leishmania* parasites, ensuring its replication, dissemination and long-term survival (Stafford et al, 2002; Liu and Uzonna, 2012).

Investigating dog MØ effector mechanisms in response to *L. infantum* is a very arduous task since it is difficult to obtain enough cells for *in vitro* studies. To overcome this obstacle, we used blood circulating monocytes that were *in vitro* differentiated into MØ through supplementation of the culture medium with M-CSF (Rodrigues et al, 2006). Circulating monocytes are most likely "quiescent" cells. The acquisition of a functional phenotype occurs after their translocation to the tissue and is depending on micro-environmental signals. M-CSF (CSF-1) and CSFR1 signaling are critical for MØ differentiation from monocytes in the tissues (Italiani and Boraschi, 2014).

MØ activation in response to external stimulus can be examined at several levels, namely phagocytic capability, antigen presentation and cytokine production (Gordon and Taylor, 2005). MØ are highly heterogeneous plastic cells, enabling a proper response to a diversity of stimuli, originated in other cells and in the microenvironment (Arrevillaga-Boni et al, 2014). Indeed, MØ surface is highly dynamic, experiencing rapid membrane turnover and morphological changes to optimize the interaction with the surrounding environment, during the search for debris, dead cells and microorganisms (Stow and Condon, 2016). Since the MØ surface is actively involved in *L. infantum* promastigote recognition and internalization, understanding the morphological changes that characterize this structure in response to infection is extremely important.

Dorsal ruffles, described as highly distinctive and non-adhesive veils of membrane, sometimes ring-shaped, extend vertically from the cell periphery or dorsal surface (Hoon et al, 2012; Stow and Condon, 2016). Dorsal ruffles act as signaling centers and can generate phagocytic cups for particles engulfment (Stow and Condon, 2016). Although seeming to occur constitutively in MØ, dorsal ruffles can be enhanced by LPS stimulation

(Bohdanowicz et al, 2013; Stow and Condon, 2016). Despite these structures were not easily identified in our SEM images, the parasite was frequently found lying on top of the cell and attached to the cell periphery. Dorsal ruffles act in a wave-like manner, rising up and then collapsing back onto the surface. Pathogens encircled by the collapsing ruffles are internalized in the phagosome (Stow and Condon, 2016). Its dynamic and transient character is probably what makes the visualization in our samples difficult.

Beyond ruffles, MØ produce other highly dynamic projections of their cell surface, such as filopodia and lamellipodia, allowing them to move and to survey the extracellular space (Stow and Condon, 2016). Indeed, *L. infantum* induced exuberant filopodia that were used to adhesion to the substrate and parasite. The extension of very long filopodia to wrap around and entrap the parasite in the coiling phagocytosis was also documented. In this case, filopodia were used as tentacles to draw the parasite towards the cell body and assist promastigote internalization and phagosome formation. Filopodia extension is a common characteristic of MØ and are used as ‘probes’ to explore the surrounding milieu. Their cholesterol-rich and highly ordered lipid-raft-enriched membranes contribute to receptor signaling and membrane trafficking (Stow and Condon, 2016).

In the present study, filopodia-like structures connecting adjacent cells were observed in IFN-γ- and PMA-stimulated MØ. Although these structures can represent long filopodia the possibility of TNT cannot be excluded. These channels F-actin-rich allow a highly dynamic process of intercellular communication apart from mediating the direct cargo transfer between cells (Gurke et al, 2008, Arrevillaga-Boni et al, 2014). In mammalian cells, TNT structures have been described in a variety of cell types, including kidney cells, fibroblasts, neuronal cells, T cells, NK, DC, myeloid cells, monocytes, and monocyte-derived macrophages (Onfelt et al, 2004; Watkins and Salter, 2005).

Filopodia and lamellipodia are essential for MØ migration and chemotaxis (Stow and Condon, 2016). However, in the present study, the veil-like formations looking like lamellipodia observed in stimulated dog MØ were encountered contacting and entrapping *L. infantum* promastigotes, which suggests the presence of extracellular traps. Lamellipodia are thin membrane sheets containing a branched actin substructure (Stow and Condon, 2016), but ultrastructurally they are hard to distinguish from ET formations, requiring the use of other discriminant imaging techniques.

Importantly, veil-like formations were more exuberant in chemically-activated MØ and in LPS-stimulated MØ parasite exposed than in *L. infantum*-exposed MØ, indicating that the parasite is not recognized as potent inflammatory stimuli. Interestingly, MØ treated with the phagocytosis inhibitor Cyt D before *L. infantum* infection showed enhanced morphological changes, including exuberant filopodia, veil-like formations and markedly irregular cell surface, probably in an MØ attempt to entrap and contain the parasites that cannot be phagocytized.

Actin supported protrusions (dorsal ruffles, filopodia and lamellipodia) are important transiently stable membrane platforms for juxtaposing receptors, integrins, signaling machinery and other molecules (Stow and Condon, 2016) that contact with *Leishmania* parasites and other immune cells.

In an attempt to confirm the nature of MØ veil-like formations, DNA and Histone H1 were stained with fluorescent dyes. Histone H1, also named the 5th histone or linker histone, interacts with the DNA entering and exiting the nucleosomal core particle, completing and stabilizing the nucleosome (Izzo et al, 2008). Among the histones, Histone H1 is the least conserved and genes encoding multiple variants and subtypes were identified (Schulze et al, 1993, 1994). H1-like or Histone H1 proteins have been identified in *Leishmania* species, but these H1 proteins are smaller than their counterparts from higher eukaryotes due to their lack of the central globular region (Fasel et al, 1993; Noll et al, 1997; Belli et al, 1999).

In this study, the impact of parasite exposition on MØ DNA integrity and Histone H1 location was compared with PMA and IFN-γ stimulation. MØ-exposed to *L. infantum* exhibited the characteristic changes of cell activation also evidenced by positive controls, namely the loss of the normal nuclear appearance, exclusion of DNA and Histone H1 from the nucleus and their co-localization in the cytoplasm and extracellular space. However, the intensity of these changes was lower when compared with chemically stimulated cells, suggesting once again, that the parasite does not act as a strong inflammatory stimulus. As observed in SEM images, features associated with MØ activation were exuberant in Cyt D-treated and infected MØ. Being prevented to phagocytize the parasite, MØ do not spare efforts to extracellularly interact and contain *L. infantum* promastigotes. The leishmanicidal effect of histones can in fact contribute for

parasite killing (Guimarães-Costa et al, 2009; Wang et al, 2011). However, once internalized the parasite appears to modulate MØ function, preventing excessive activation and histone release. From an evolutionary point of view, this can be considered a *Leishmania* survival strategy since the parasite tries to keep host cell (and also the surrounding) alive, long enough, to allow for their own replication and dissemination, and the toxic effects of extracellular histones mediated by direct interaction with several TLR receptors (Allam et al, 2014) can compromise parasite life cycle. On the other hand, histone release can induce immune mediated pathology (Koutinas and Koutinas, 2014) and kidney disease (Ginel et al, 2008) that negatively impact on dog survival.

Histones acting as antimicrobial agents are now considered critical components of the innate immune system (Parseghian and Luhrs, 2006). Histone H1 overexpression by *L. major* was related to decreased parasite infectivity *in vivo*, delayed parasite cell-cycle progression and parasite differentiation (Papageorgiou and Soteriadou, 2002; Smirlis et al, 2006). Furthermore, promastigotes treated with Histone H1 showed ultrastructural alterations, such as chromatin changes, cytoplasmic vacuolization, and a rounded appearance. Interestingly, parasites expressing mouse Histone H1 almost completely lost their capacity to survive in highly susceptible mice proving that the presence of a globular domain is detrimental for parasite survival in MØ (Masina et al, 2007). Another study proved the toxic effect of Histones H2A and H2B, but not H1 in *L. amazonensis*, *L. major*, *L. braziliensis* and *L. mexicana* promastigotes and the effect of these proteins in decreasing the infectivity of promastigotes to murine MØ (Wang et al, 2011). The effect of dog Histone H1 on *L. infantum* promastigote is unknown, but the exuberant release of the protein by some dogs fully justifies further investigation.

Although Histone H1 with a granular appearance was clearly observed in the cytoplasm and in some cases in the extracellular space associated with DNA, the characteristic appearance of filamentous ET was not observed. Indeed, NET observed in chronic obstructive pulmonary disease sputa revealed the cytoplasmic localization of granular citrullinated Histone H3 in early stages of NETosis (Obermayer et al, 2014). Thus, it is likely that the short period of incubation that the cells/MØ were subject to did not allow for the full development of these extracellular structures to take place, as observed in human PBMC-derived MØ infected with *Mycobacterium massiliense* (Je et al, 2016).

Although MØ can fight the parasite employing extracellular weapons, *Leishmania* have developed adaptive mechanisms to evade the host immune response by hiding intracellularly. Thus, binding experiments are critical to understand the relationship between the host cell and the parasite. In this study, *L. infantum* promastigotes binding to dog MØ were evaluated by flow cytometry. For this purpose parasites stained with fluorescent dye (Gonçalves et al, 2005) or transgenic parasites expressing fluorescent proteins, namely the GFP protein can be used (Marques et al, 2015; Tasew et al, 2016). We verify that GFP parasites efficiently bind to dog MØ even when using heat inactivated FBS to supplement culture medium. The relatively high levels of parasite association to MØ corroborate the idea that the direct interaction in the absence of opsonization, already well-established for several parasite species is also true for *L. infantum* promastigotes-dog MØ, although the identity of the host receptors remains unresolved. Furthermore, the amount of GFP^+ cells and the level of fluorescence in the subpopulation of MØ GFP^+ increased with time, indicating a time dependent association in terms of number of cells that bind the parasite and the number of internalized or linked parasites *per* cell. The direct binding between promastigote surface ligands and MØ receptors reflects the dog MØ capability to internalize *L. infantum* and may dictate the parasite ability to survive intracellularly.

Promastigote-to-amastigote differentiation is a critical step in parasite survival. While promastigotes are susceptible to the microenvironment inside the mature parasitophorus vacuole, amastigotes can resist lysosomal enzymes and the acidic pH inside the phagolysosome (Antoine et al, 1990). LPG has an important role in this process since it contributes to delay phagosome maturation, giving time to the engulfed promastigotes to evolve into amastigotes (Desjardins and Descoteaux, 1997). As expected, the promastigote form was the most frequently observed one inside the parasitoforous vacuole in early cultures, although some parasites presented an amastigote-like form, corresponding probably to “in differentiation” parasites, since promastigote-to-amastigote differentiation delays 12 to 24 h (Van Assche et al, 2011; Naderer and McConville, 2011). Indeed, in late stage cultures the amastigote form prevailed, demonstrating the capability of dog MØ to ensure parasite differentiation. Furthermore, compatible images with parasite multiplication and dispersion were frequently observed, confirming the role of dog MØ as *L. infantum* primary host cells. Amastigote release in a synchronized fashion, through an exocytosis-like process, mediated by parasitophorous

vacuole extrusions, was recently proposed by Real et al, (2014). Some of the images of late stage MØ infection obtained in the present work suggest that this type of amastigote release and further dispersion do not necessarily require host cell lysis. The release of amastigotes enclosed within host cell membranes allows for its transfer from cell to cell to occur without full exposure to the extracellular space, representing an important strategy developed by the parasite to evade the vertebrate host (Real et al, 2014).

Nevertheless, the interaction parasite-MØ resulted in different outcomes. While in some cases, the parasite was observed in active multiplication in others, suggestive OM images of parasite degradation were noticed. Indeed, TEM confirmed parasite killing in the early stage of infection. The presence of degraded parasites, probably the promastigote form, indicates phagosome maturation, which involves the fusion of the parasitophorous vacuole with lysosomes, as well as the acquisition of the oxidative machinery (Moal Liévin-Le and Loiseau, 2015; Weiss and Schaible, 2015) induced by phagocytosis (Stafford et al, 2002; Van Assche et al, 2011). Interestingly, in some cells, both degraded and intact parasites were simultaneously observed, suggesting either their different uptake and/or that some of them interfere with phagosome maturation avoiding its killing. Some promastigotes seem to enter through a route that offers protection against the MØ microbicidal machinery and it is possible that ligation through CR3 provides this survival advantage. Indeed, while metacyclic promastigotes of *L. infantum*/*chagasi* use CR3 but not MR to enter MØ, avirulent promastigotes use both receptors. It is already known that CR3 ligation does not trigger NADPH oxidase activation and subsequent respiratory burst at the phagosome membrane leading to enhanced intramacrophagic survival (Sehgal et al, 1993; Ueno and Wilson, 2012), while MR ligation has been shown to promote an inflammatory response (Linehan et al, 2000). *Leishmania* promastigotes ligate CR3 both directly and through opsonized iC3b. Stationary phase promastigote cultures used in the present work comprise a population of unpurified metacyclic parasites and it is known that promastigotes from different growth phases vary in its abundance of gp63 and LPG, and ligate different MØ receptors (Ueno and Wilson, 2012). Furthermore, the distinct content of virulence factors displayed by the different promastigotes can impact on phagosome maturation, which may explain the dissimilar fate of internalized parasites by the same MØ.

Some studies correlate the phagocytosis mechanism with the subsequent intracellular fate of internalized parasites. While U937 cells bound *L. infantum* promastigotes in diverse orientations and extended membrane lamellae to reorient and internalize parasites through coiling phagocytosis, human monocyte-derived MØ attached the parasite *via* their anterior pole and engulfed them through symmetrical pseudopods. In the first case, cell supported the intracellular replication and cell-to-cell spread of the parasite, but in the second case (when the parasite attacheed *via* the flagellum tip) that did not happen. Parasite engulfment by human monocyte-derived MØ occurs with the parasite positioned perpendicularly to the phagocyte, promoting a more efficient phagocytosis. In contrast, the attachment *via* parasite body sites can culminate in coiling phagocytosis and thus promote parasite survival (Hsiao et al, 2011). In this study, a large and similar proportions of interactions cell-parasite occurred *via* the anterior pole and *via* other parts of parasite body, which may explain the different fates of internalized parasites.

The metabolic activity of MØ can be used to study the type of activation (classical or alternative) induced by the parasite, which can dictate the infection outcome. In this study, *L. infantum* exposure did not induce changes in the production of urea by dog MØ but promoted NO synthesis in long term cultures. These findings point toward a classical or inflammatory activation of MØ, which is traditionally achieved by IFN-γ, TNF, LPS and GM-CSF stimulation (Chávez-Galán et al, 2015; Sica et al, 2015) but also by TLR agonists (Mosser and Edwards, 2008).

In contrast to O_2^-, which is predominantly produced in response to promastigote phagocytosis, NO synthesis becomes important as a defense mechanism during the intracellular amastigote stage (Stafford et al, 2002; Van Assche et al, 2011), justifying it increase in dog MØ exposed to *L. infantum* for 24 h. NO was also detected in supernatants of murine but not human MØ infected with *L. chagasi* and stimulated by pro-inflammatory cytokine (Gantt et al, 2001). However, Brandonisio et al (2002) observed NO production by peripheral blood-derived human MØ-*L. infantum* infected after treatment with the chemokines MCP-1 and MIP-1α. Interestingly, NO production by MØ seems to be involved in long-term protection of naturally infected dogs living in endemic areas, particularly in the asymptomatic ones (Panaro et al, 2008).

The interaction with other immune cells can also have an impact on the MØ activation status. Studies involving murine models of cutaneous *Leishmania* species documented the cellular interactions that take place in the early phase of infection. It is now widely accepted that PMN are the first cells to reach the inoculation site and that the parasite is subsequently transferred to the definitive host cells. In a previous study, our group showed that 3 to 4 h after dermal experimental inoculation promastigotes had already been internalized by neutrophils, proving the early involvement of these cells in dog infection (Santos-Gomes et al, 2000). Thus, it is probable that MØ encounter the parasite associated with PMN.

With the objective of investigating the interaction between these two types of phagocytic cells in the beginning of the infection, extracellular parasites from infected PMN cultures were removed and the viability of internalized promastigotes was assessed. As expected, some internalized parasites maintained the viability and thus, their capability of contributing to the establishment and progression of the disease. The survivors are probably the most virulent parasites that have managed to resist and overcome the oxidative and non-oxidative intracellular effector mechanisms. Some authors consider the possibility that these parasites might be transitional forms better adapted to intramacrophage survival (Ribeiro-Gomes and Sacks, 2012). Furthermore, these parasites maintained the proliferative capability and were transferred to the definitive host cells, albeit at a low level. In the presence of infected PMN, the lower level of MØ associated with parasites observed in our study could reflect a real low level of parasite transference. However, intramacrophagic parasite killing after transference should also be taken into consideration. Indeed, it is shown that while urea levels decreased in infected co-cultures when compared with unprimed-MØ cultures, the production of NO increased pointing towards classical activation of MØ, which can contribute for parasite killing.

Interestingly, the levels of NO also showed significant increases in uninfected MØ+PMN co-cultures, suggesting that the interaction with PMN and/or efferocytosis induce an inflammatory phenotype in MØ, contradicting the theory that efferocytosis typically evokes an anti-inflammatory response through the secretion of TGF-β, IL-10 and PGE2 by the engulfing cells to prevent inflammation and autoimmunity (Fadok et al, 1998; Kim et al, 2004; Xiao et al, 2008). In fact, efferocytosis of *L. major* infected apoptotic human neutrophils was related to the deactivation of MØ effector functions through secretion of

TGF-β and suppression of TNF-α release, ensuring intramacrophagic parasite viability and multiplication (van Zandbergen et al, 2004). We hypothesize that high NO production is related to the increase of PMN necrosis induced by *L. infantum* and by the subsequent efferocytosis of necrotic PMN. Actually, a previous study had already demonstrated that the interaction between necrotic neutrophils and *L. amazonensis*-infected human MØ induces parasite killing *via* TNF-α NE dependent (Afonso et al, 2008).

In an attempt to decipher the mechanism of parasite transference from infected PMN to MØ, infected neutrophil efferocytosis was detected in co-cultures incubated for a short time, revealing that this mechanism is responsible for part of the parasite transference to MØ and appears immediately after the contact between the two types of cells. Although efferocytosis has an antimicrobial effect due to the elimination of apoptotic infected cells (Martin et al, 2014), some intracellular microorganisms use this process for their own benefit. Entering the host cell surrounded by a seemingly harmless cell covered by “eat me” signals is a clever strategy. Indeed, this mechanism of entry, called “Trojan horse” prevents the direct contact between the parasite and MØ surface receptors and consequently MØ activation (van Zandbergen et al, 2004).

Despite this fact, the parasite was more frequently observed inside MØ in combination with an efferosome containing apoptotic cell. This observation suggests two possibilities: *L. infantum* transference from the eferosome to MØ cytoplasm or parasitoforous vacuole after infected neutrophil efferocytosis; or the simultaneous efferocytosis of an apoptotic cells and phagocytosis of free parasite. Indeed, we hypothesize that *L. infantum* can escape from infected apoptotic PMN to the extracellular space to be subsequently internalized by the dog MØ, suggesting another mechanism of parasite transference. Actually, *in vivo* studies showed viable *L. major* parasites being released from mouse apoptotic neutrophils in the vicinity of surrounding MØ (Peters et al, 2008). This mechanism was called “Trojan rabbit” (Ritter et al, 2009).

However, the interaction and cooperation between dog PMN and MØ could be much more complex. Indeed, NET were emitted in PMN+MØ co-cultures. The stimulus that triggers NET release is probably the MØ or parasite derived molecules. Persistence of intact ET structures in the tissue can induce inflammation, tissue destruction and autoimmune responses. Thus NET clearance promoted by DNAse I-dependent digestion,

complement opsonization and phagocytosis by MØ is essential. ET clearance by MØ can be an immune silent process or, by contrast, induce an inflammatory response. In the absence of microorganisms or DAMP, silent clearance avoids inflammation and maintains tissue homeostasis. However, in the context of infection, NET removal can influence MØ phenotype in order to control invading microorganisms (Boe et al, 2015). Indeed, some studies have shown that the ability of MØ to kill intracellular microorganisms is supported by the uptake of neutrophil-derived exogenous proteins. For instance, NE, a NET component, stimulates *Leishmania*-infected MØ *via* TLR4 and assists parasite elimination (Ribeiro-Gomes et al, 2007) and human MØ internalize NET-bound cathelicidin, which is transported to lysosomal compartments and attacks mycobacteria in MØ phagolysosomes, resulting in antimicrobial activity (Stephan et al, 2016).

Taking these findings into account, the interaction between the dog PMN and the autologue MØ at the early stage of the *L. infantum* infection is extremely complex and can definitely have an impact on the final outcome.

4 CONCLUDING REMARKS

CanL is a serious illness not only from a veterinary point of view, but also because it is a zoonosis. The disease causes suffering for the affected animals and also for their owners and families. Infected symptomatic and asymptomatic dogs represent the main parasite reservoir for human population. Furthermore, the infection is globalizing due not only to the fact that when travelling, many pet owners take their animals with them to endemic areas, but also to the growing importation and adoption of animals from endemic areas.

Although the therapeutic options currently available induce clinical cure, they do not promote parasitological cure, thus animals can suffer relapses and eventually continue to transmit the parasite. Environmental vector control is not only difficult to achieve, but also expensive, and it can promote vector resistance to insecticides and environmental contamination. Besides, the use of topical and impregnate collars with insecticides is expensive and requires the constant commitment on the part of the owners, mainly in endemic areas where the transmission is continuous. In Europe, the available vaccine is expensive, it requires the administration of three doses in prime-boost vaccination and annual boosters and several cases of vaccine failure have been referred by veterinarians. In China and Brazil seropositive dogs continue to be euthanized. However, in Brazil there are two commercial vaccines approved for use. Thus the problematic of CanL is still far from being solved.

Given this reality, we must continue to invest in the research of immunological mechanisms that determine the resistance and susceptibility to the disease. The existence of a large proportion of infected animals that develop a cellular immune response, preventing disease development suggests that individual factors, in particular the genetic background and the type of immune response are decisive for parasite control. In fact, the adaptive immune response is important in controlling parasite replication and in promoting parasite killing. However, immediately after parasite inoculation the innate immune mechanisms are crucial because they can prevent the establishment of the infection or diminish the proportion of viable parasites that can reach the final host cell.

The above described *in vitro* studies investigated the role of dog neutrophils in the early phase of *L. infantum* infection. This extensive analysis, performed for the first time allows us to propose a model that reflects the effector functions parasite induced and the

interaction between these innate immune cells and MØ, the final parasite cell host that ensures *Leishmania* multiplication and dissemination.

Neutrophils represent the most abundant dog blood leukocytes. *Leishmania* releases chemotactic factors that attract neutrophils, which rapidly reach the inoculation site. Neutrophils readily internalize the parasites and ensure their intracellular survival, probably in the promastigote form. Parasite phagocytosis induces intracellular O_2^- production and secreted/excreted *Leishmania* molecules activate the effector functions of neutrophils, namely NE exocytosis and NET release, which induce extracellular and intracellular parasite killing. Despite this fact, some parasites resist the effector functions of neutrophils and maintain the proliferative capability. The parasite negatively impacts on neutrophil viability, inducing neutrophil apoptosis and secondary necrosis. Dog MØ rapidly reach the inoculation site such that 24 h after inoculation the parasite is already cleared from the dermis and is probably in active dissemination throughout the body. The parasite maintains its viability and multiplication capability after internalization by neutrophils, which ensure its transference to MØ by two possible ways: infected neutrophil efferocytosis and phagocytosis of free parasites that escape from dying neutrophils, assuring dog infection. Although the parasite may survive the neutrophil effector machinery and move into the host cell the mechanism used for parasite transference can activate MØ oxidative burst, probably causing parasite reduction. Furthermore NET clearance promoted by MØ could improve its ability to kill the parasite (Fig. 47).

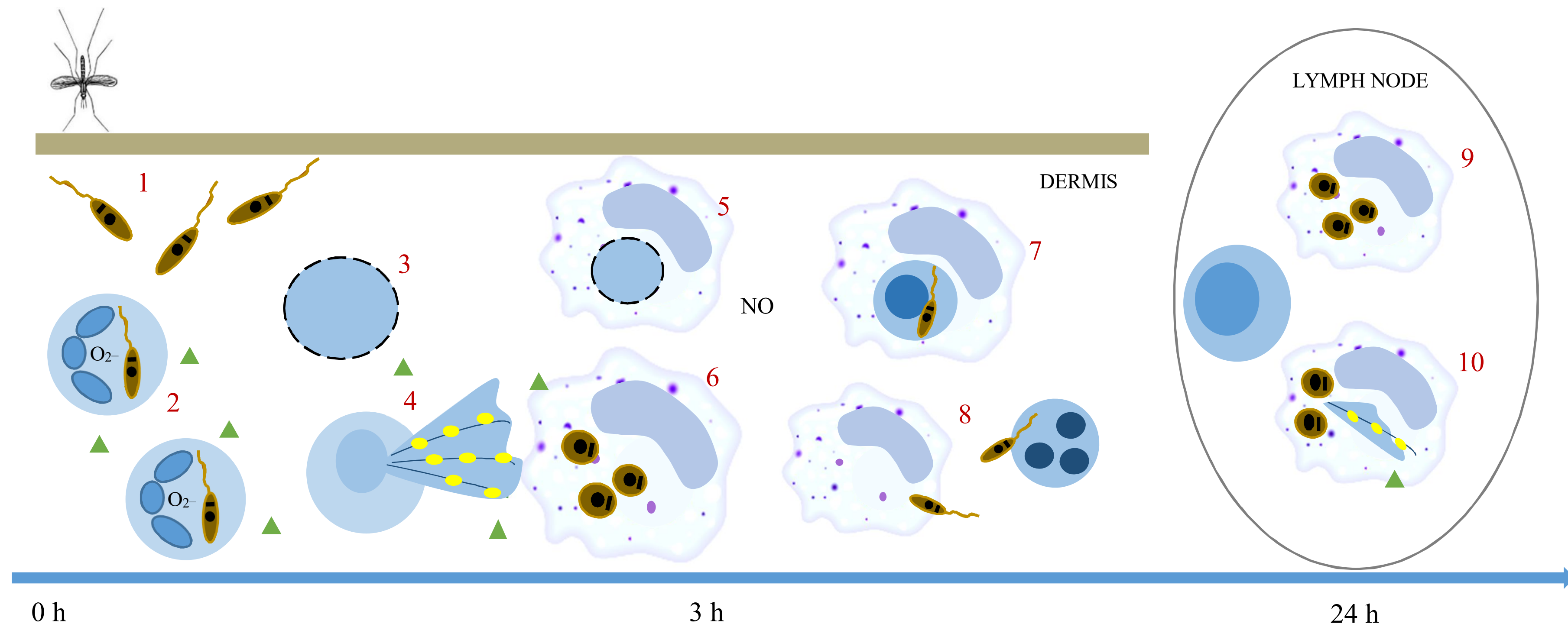

Figure 47: Interaction between dog neutrophils and macrophages at the early phase of *L. infantum* infection. 1- Neutrophils are the first cells to reach the inoculation site and rapidly phagocytize the parasite; 2- The parasite induces the superoxide (O_2^-) production and the exocytosis of neutrophil elastase (big green triangle); 3- The parasite induces neutrophil apoptosis and secondary necrosis; 4- Neutrophils release neutrophil extracellular traps (NET), containing DNA (blue lines), histones (yellow circles) and NE (small green triangles) ; 5- MØ internalize necrotic neutrophils and produce nitric oxide (NO); 6- MØ and eventually infected MØ contact with NE that was released by PMN; 7- Efferocytosis of infected neutrophils ensure parasite transference; 8- MØ internalize parasites that escape from dying neutrophils; 9- 24 h after inoculation, parasite dissemination takes place. 10- Eventually in the regional lymph node, parasitized MØ that had removed NET compounds and contacted with NE released by neutrophils kill the parasite and present parasitic antigens to lymphocytes.

These studies highlight the key importance of neutrophil-MØ interaction in controlling the infection immediately after parasite inoculation and draw attention to some unexplored aspects that can be used to find new targets for vaccine and drugs, namely cell surface morphological changes, the leishmanicidal effect of histone and the pro-inflammatory effect of NET clearance, and design more efficient and precocious diagnostic tools.

Although CanL and HVL share some common characteristics, such as the severity and systemic nature of the disease, there are some important differences at the clinical level that can be explored, namely the possibility of parasitological cure in the case of human disease. These clinical differences are certainly the reflex of cellular and subcellular mechanisms differently modulated. Thus, comparing the interaction parasite-host in these different host species can help understand the mechanisms that govern resistance and susceptibility and the possibility of parasite clearance, improving our knowledge and shedding a light on the design of new tools for disease control.

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