Leishmania spp. Infection Model Optimization for Drug Discovery Purposes

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RESUMO

A leishmaniose é uma doença negligenciada típica das áreas tropicais e subtropicais. Contudo, está a tornar-se comum nos países desenvolvidos, devido ao aumento da população imunossuprimida e à crescente frequência de viajantes pelo mundo.

São conhecidas mais de 20 espécies de *Leishmania* que infectam e causam doenças em humanos. Diferentes espécies podem causar distintas formas de doença, que podem variar desde manifestações cutâneas benignas até à morte.

Hoje em dia, a quimioterapia é quase a mesma de há 50 anos atrás, com poucas excepções como a introdução miltefosina e a anfotericina B (AmpB). Os antimoniais são fármacos de primeira linha. São altamente tóxicos e a sua resistência é já um problema. De todos os fármacos usados hoje em dia, só a miltefosina é de administração oral. A maior parte dos antileishmaniais disponíveis têm pelo menos uma das seguintes limitações: toxicidade, alto-custo, ineficácia, resistência ou necessidade de hospitalização.

Até agora, o desenvolvimento de fármacos contra a leishmaniose foi baseado em extensão de indicações terapêuticas de moléculas já existentes ou novas formulações de antileishmaniais já usados. Por isso, é urgente desenvolver novas moléculas que superem os problemas existentes. Têm sido usadas muitas estratégias para identificar movas moléculas. Um método promissor é o *high throughput screening* (HTS) de pequenas moléculas.

O HTS necessita de um modelo biológico robusto e bem estabelecido, capaz de responder a todas as solicitações e questões. Para tal, o objectivo deste projecto é desenvolver um modelo de infecção ideal para a descoberta de novos fármacos.

Foram optimizados modelos de infecção para *L. amazonensis*, *L. braziliensis*, *L. donovani* e *L. major* em linhas celulares de macrófagos humanos (THP-1) e de ratinho (Raw 264.7). Estes modelos foram optimizados, tendo em conta os diferentes tropismos e dinâmicas de infecção das diferentes espécies. As 4 espécies estudadas são responsáveis por formas de doença completamente diferentes, desde cutânea a visceral.

Portanto, este projecto fornece informação muito útil que pode melhorar o HTS de novos fármacos eficazes contra *Leishmania*. Estas condições ideais de infecção irão proporcionar uma selecção de compostos activos adequados a cada espécie que causa diferentes formas de doença.

PALAVRAS-CHAVE: *Leishmania*, leishmaniose, antileishmaniais, *high throughput screening*

ABSTRACT

Leishmaniasis is a neglected disease typically indigenous to tropical and subtropical areas. However, it is becoming more common in developed countries, because of the increasing immunosuppressed population and the higher frequency of travelers around the world.

More than 20 *Leishmania* species are known for infecting and causing disease in humans. Different species can cause distinct leishmanial disease forms, which can range from benign cutaneous manifestations to death.

Today, chemotherapy is almost the same as 50 years ago, with few exceptions such as miltefosine and amphotericin B (AmpB). Antimonials are first line drugs, but are highly toxic and resistance is already an issue. From all the drugs currently used, only miltefosine has oral administration. Most of the antileishmanials available are not adapted to the field, and have at least one of the following limitations: toxicity, high cost, non-effectiveness, resistance or hospitalization requirement.

Until now, drug development for leishmaniasis was based on label extension of already existing molecules or new formulations of old antileishmanials. Therefore, there is an urgent need to develop new antileishmanial molecules, in order to bypass or overcome the existing problems. To identify these molecules, many strategies have been used. One promising method to find new active chemicals is the high throughput screening (HTS) of small molecules, in order to select antileishmanial scaffolds.

HTS must have a strong and well established biological model able to answer all requests and questions. For that purpose, this project's aim is to develop an optimal infection model for antileishmanials' drug screening.

Infection models for *L. amazonensis*, *L. braziliensis*, *L. donovani* and *L. major* were optimized, and THP-1 (human) and Raw 264.7 (murine) macrophage cell lines were used as host cells. These infection models account for each specie's tropism and infection dynamics. These 4 species studied are responsible for completely different forms of disease, cutaneous and visceral.

Therefore, this project provides very useful information that can improve HTS for new antileishmanial drugs. With this valuable new data, optimal infection assays can be carried and optimal HTS results will be achieved. These optimal conditions will provide active compounds selection and testing against causative species of each disease form.

KEYWORDS: *Leishmania*, leishmaniasis, antileishmanials, high throughput screening

TABLE OF CONTENTS

TABLE OF FIGURES

TABLE LIST

LIST OF ABBREVIATIONS

AIDS	Acquired Immune Deficiency Syndrome
ACR2	Arsenate Reductase 2
ALX	Lipoxin A4 receptor
AmpB	Amphotericin B
APC	Antigen-Presenting Cell
AxA	Axenic Amastigote
BrdU	Bromodeoxyuridine
BSA	Bovine Serum Albumin
CanL	Canine Leishmaniasis
CL	Cutaneous Leishmaniasis
CLSM	Confocal LSM
CPB	Cysteine Protease B
CR	Complement Receptor
DALY	Disability Adjusted Life Year
DC	Dendritic Cell
DCL	Diffuse CL
DN	Double Negative
DNDi	Drugs for Neglected Diseases initiative
FACS	Fluorescence-Activated Cell Sorting
FBS	Fetal Bovine Serum
Fc-γ R	Fc-γ Receptor
FDA	US Food and Drug Administration
FLIM	Fluorescence Lifetime Imaging
FRET	Fluorescence Resonance Energy Transfer
GBD	Global Burden of Diseases
GFP	Green Fluorescent Protein
GIPL	Glycoinositolglycolipid
HAART	High Activity Antiretroviral Therapy
HCS	High-Content Screening
HIV	Human Immunodeficiency Virus
HTS	High Throughput Screening
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin

InA	Intracellular Amastigote
iNOS	Inducible NO Synthase
IPK	Institut Pasteur Korea
LACK	*Leishmania* homolog of receptor for Activated C Kinase
LPG	Lipophosphoglycan
LSM	Laser-Scanning Microscopes
MAPK	Mitogen-Activated Protein Kinase
MC	Mast Cell
MCL	Mucocutaneous Leishmaniasis
MHC	Major Histocompatibility Complex
MIP	Macrophage Inflammatory Protein
MRP1	Multiple drug Resistance Protein 1
MS	Mass Spectrometry
MTS	Medium Throughput Screening
NET	Neutrophil Extracellular Trap
NK	Natural Killer cell
NO	Nitric Oxide
NTD	Neglected Tropical Disease
O^{2-}	Superoxide anion
PBS	Phosphate Buffered Saline
PDP	Product Development Partnership
PE	Phorbol Ester
PFA	Paraformaldehyde
PGE2	Prostaglandin E2
P-Gp	Permeability Glycoprotein
PK	Pharmacokinetics
PKC	Protein Kinase C
PKDL	Post Kala-azar Dermal Leishmaniasis
PM	Paromomycin
PMA	4α-Phorbol 12-Myristate 13-Acetate
PMN	Polymorphonuclear
PMT	Photomultiplier Tube
PNA	Peanut Agglutinin
PRP1	Proline Rich Protein 1
PS	Phosphatidylserine
PV	Parasitophorous Vacuole

R&D	Research and Development
RNI	Reactive Nitrogen Intermediate
ROI	Reactive Oxygen Intermediate
ROS	Reactive Oxygen Species
SAR	Structure-Activity Relationship
SbIII	Trivalent Antimony
SbV	Pentavalent Antimony
SOD	Superoxide Dismutase
SSG	Sodium Stibogluconate
Tc	T cytotoxic cell
TCR	T Cell antigen Receptor
TGF	Transforming Growth Factor
Th	T helper cell
TipDC	iNOS-producing DC
TLR	Toll-Like Receptor
TNF	Tumor Necrosis Factor
TPLSM	Two Photons LSM
TR	Trypanothione Reductase
T_{reg}	T regulatory
VD_3	1,25-dihydroxyvitamin D_3
VL	Visceral Leishmaniasis
WHO	World Health Organization

I – Introduction

Leishmaniasis is a neglected disease that is becoming increasingly relevant in developed countries. Due to the great number of human infective *Leishmania* species, many disease forms can occur. Different species have different tropism, infection dynamics, immunology, and sensitivity to drug therapy. Current therapy often fails and has many limitations. Therefore, it is needed to develop new antileishmanial drugs. In order to do so, drug discovery processes must be adapted so that it incorporates earlier stages of the disease with different species. This way, future therapy will be more effective and drug resistance development can be avoided.

An effective method for such drug discovery is HTS. HTS relies on a biological model of an infection that is able to mimic the *in vivo* situation. This way, the screened molecules will be subjected to the closest to the real infection situation. We must consider that different species have different tropisms, infection strategies, and replication dynamics. For that, every infection model must be adapted to test these differences. At the time of writing of this thesis, as far as the authors are aware, there were no studies with this purpose.

In this thesis we want to contribute to *Leishmania* drug screening, providing optimized infection protocols for 4 *Leishmania* species: *L. amazonensis*, *L. braziliensis*, *L. donovani*, and *L. major*, for human (THP-1) and murine (Raw 264.7) macrophage cell lines.

Chapter II starts with a brief exposure about neglected tropical diseases (NTDs) and their impact on our world. After, there is a description about *Leishmania* parasite biology and the disease forms it may cause. It is also described *Leishmania*'s complex survival and infection strategies. On the last point, there is a summary about *Leishmania* induced immunological responses.

In Chapter III, current therapy used to treat all forms of leishmaniasis is extensively described. Also future therapy is discussed, focusing in new drugs that are currently being evaluated in clinical trials.

In Chapter IV, drug discovery process is exposed, and strategies to develop new drugs are described. Also, cell-based assays for HTS are explained, with special attention to *Leishmania* infection models. In the end, cellular imaging and confocal microscopy are high lightened.

In Chapter V, materials and methods are extensively described: cells culturing, infection and replication assays, and image analysis.

In Chapter VI, infection is phenotypically analyzed. Both infection images and statistical analysis are shown and properly discussed. Also, image-mining process is revealed. Finally, in Chapter VII all results are summarized.

II – *Leishmania*

1. Neglected Tropical Diseases (NTDs)

According to the World Health Organization (WHO), NTDs are chronic infectious diseases commonly associated with poor, warm and humid scenarios. Most are parasitic diseases, spread by insect vectors, contaminated water or soil. NTDs keep being perpetuated by poor standards of living and hygiene. They are pathologically different, but all of them can cause severe disability and life-long impairment. Many people are infected with 2 or more diseases at the same time (1).

NTDs are common among poor populations in developing countries, mainly in Africa and South America. These diseases put more than 1 billion poor people in poverty, and are an important economic burden in endemic countries. They also lead to social stigmatization and discrimination (1, 2).

According to WHO 2010 fact list, the top most prevalent NTDs are (1):

1. *Soil-transmitted helminthiasis*, more than 1 billion people infected;
2. *Schistosomiasis*, more than 200 million people infected, 120 million are asymptomatic and around 20 million are severely symptomatic;
3. *Lymphatic filariasis*, around 120 million people infected. It is the second leading cause of disability worldwide;
4. *Blinding trachoma*, around 80 million people infected and 6 million are blind. It is the first infectious cause of blindness;
5. *Onchocerciasis*, around 37 million people infected, mainly in Africa. Causes severe skin disease, visual impairment, blindness, and shortens life expectancy by 15 years;
6. *Chagas disease*, 13 million people infected. Mostly in Latin America, but with new endemic trends due to migration, blood transfusion, congenital via and organ donation;
7. *Leishmaniasis*, more than 12 million people infected, 350 million are at risk, and there is 1.5 to 2 million new infections per year.

NTDs do not cause massive deathly outbreaks, and tend not to affect richer countries. Therefore, they do not get media attention, are not included in health agendas and budgets setting (1). However, these diseases are becoming more common in the developed world, due to the increasingly larger immunocompromised population (3). In the end, there is no funding either for diagnostics and treatment, or research and development (R&D). NTDs' market is poor, and there are no real economic benefits in it (1). Only 1% of newly developed drugs are for NTDs (4).

NTDs disease burden assessment is difficult to measure. Disease burden is defined as the prevalence or incidence of disease morbidity and mortality. Currently, the disability adjusted life year (DALY) system is the most used method for disease burden measurement. Global Burden of Diseases (GBD) assessments, provided by WHO and World Bank, developed this system. The GBD program aims at cost-effective implementation of health programs (2, 5, 6).

DALY's goal is to quantify and compare regional aggregate and worldwide health burden. DALY is to measure disease impact for all health states, with an average disease impact per person, in a non-subjective manner. This system has been used to measure disease impact by health policy-makers and funding institutions, in order to prioritize investments. However, it might underestimate NTDs real priorities. According to World Bank and GBD, a serious investment in NTDs treatment can increase in 4 years life expectancy for the world's poorest 20% population (2).

Leishmaniasis DALY estimates is based on regional incidence and prevalence, case-fatality rates, and disability weights (5). Current leishmaniasis disease burden measurement do not account clinical and epidemiological diversity, medical, social and economic impact (6).

Current leishmaniasis statistics have not been updated since 1991. It is only notifiable in 33 of 88 endemic countries. Also, in many countries, most cases are treated by non-governmental organizations or by private sector, and they tend do not report the cases. One Sudanese study estimates that 91% of all deaths due to visceral leishmaniasis (VL) were undiagnosed. Data from a village-based study in India suggest that 20% of VL patients, poor and female, died before definitive diagnosis (5).

2. Leishmaniasis

Leishmaniasis is a vector-borne disease, and it can be caused by more than 20 *Leishmania* species (Table 1). It can cause varied clinical syndromes, from localized skin ulcers to lethal systemic disease. Among tropical diseases, it is the second in mortality, the fourth in morbidity, and, in terms of DALYs, the third most important vector-borne disease. Nevertheless, leishmaniasis is one of the most neglected diseases, because resources invested in diagnosis, treatment, and control are very rare. It is extremely associated with poverty conditions, and usually requires prolonged and expensive drug therapy. Other factors such as malnutrition or human immunodeficiency virus (HIV) co-infection can alter clinical course, complicating therapeutic strategies and outcome (5, 6).

Table 1. *Leishmania* species and disease forms.

Disease	**New World species**		**Old World species**	
Cutaneous	*L. (L.) mexicana* complex	*L. (L.) mexicana*	*L. (L.) major* complex	*L. (L.) major*
		L. (L.) amazonensis		*L. (L.) tropica*
		L. (L.) pifanoi		*L. (L.) aethiopica*
		L. (L.) venezuelensis		
	L. (V.) subgenus	*L. (V.) braziliensis*		
		L. (V.) peruviana		
		L. (V.) lansoni		
		L. (V.) naiff		
		L. (V.) panamensis		
		L. (V.) guyanensis		
Diffuse Cutaneous	*L. (L.) mexicana* complex	*L. (L.) amazonensis*	*L. (L.) major* complex	*L. (L.) aethiopica*
		L. (L.) pifanoi		
Mucocutaneous	*L. (V.)* subgenus	*L. (V.) braziliensis*		
Visceral	*L. (L.) donovani* complex	*L. (L.) chagasi**	*L. (L.) donovani* complex	*L. (L.) donovani*
				*L. (L.) infantum**

* CL cases caused by L. (L.) chagasi and L. (L.) infantum have been reported (9-11).

Leishmaniasis incidence is geographically heterogeneous (5). This disease is transmitted in urban, peri-urban, and rural areas (5), present in cities, deserts and rain forests on every continent, except Australia and Antarctica. More than 90% of reported VL cases are in Bangladesh, Brazil, Ethiopia, India, Nepal, and Sudan, and 90% of cutaneous leishmaniasis (CL) cases are reported in Afghanistan, Algeria, Brazil, Pakistan, Peru, Saudi Arabia, and Syria (5-7).

However, population migration, international traveling, lack of vaccines, vector control, international conflicts and resistance development have been increasing the number of infections throughout the world (8). Non-vectorial and human vertical transmission have been reported, but are rare (9). Other forms of human transmission have been reported,

such as iatrogenic route, especially in co-infection *Leishmania*-HIV cases and blood transfusion. Drug addicts are an important health problem issue, sharing contaminated needles (9, 10).

The factors determining the kind of clinical manifestation depend upon host genetics, general health and immune status, which can promote protective or susceptibility factors, host environment, previous immunological experience, infecting species, geographic localization and the vector species (11).

Leishmaniasis mortality is usually related to VL. Nevertheless, CL deaths occur, even though they are rare, usually due to co-infections or treatment complications. Death age varies according to the endemic setting. Younger groups are affected in established VL transmission conditions and older age groups are affected in new VL endemic areas. Because of the relatively benign nature of CL, inaccessibility of health services in rural, endemic areas, and the common non-availability of treatment, there is severe under-reporting. Also, VL has similar clinical symptoms to other diseases, which are more prevalent in endemic areas, such as malaria and schistosomiasis, which aggravates the under-reporting issue. Co-infection with other diseases may occur, such as malaria or, more recently, HIV. CL and VL have become an opportunistic infection of HIV/acquired immune deficiency syndrome (AIDS) patients. VL increases mortality risk by more than 3 folds (6).

VL, also known as kala-azar, is usually caused by *L. donovani* and *L. infantum*. VL is a chronic systemic disease characterized by fever, hepatosplenomegaly, lymphadenopathy, pancytopenia, weight loss, hypergammaglobulinemia, weakness, and, eventually, leads to death. Life threatening complications include immunosuppression, secondary bacterial infections, hemorrhage, anemia, and, during pregnancy, fetal wastage or congenital leishmaniasis (5, 6). During VL infection, there is a heavy parasite burden in spleen, liver, and bone marrow (12). VL disease can last up to 2.5 months, depending on infecting species, genetic host factors, and immunosuppression, malnutrition or HIV/AIDS co-infection. If not treated, VL patients do not tend to cure spontaneously, with 95% fatality rate. Depending on the drug and route of administration, the treatment itself can cause disability, due to severe toxicity, which can cause myalgia, gastroenteritis, pancreatitis, diabetes, hepato or cardiotoxicity (6). Even in treated patients, fatality rates can be 10% or higher. Jaundice, wasting, severe anemia, and HIV co-infection are commonly associated with increased risk of mortality (5).

A higher female incidence of VL has been reported. On average, women are ill longer than men, and are more likely to die from the disease. This might be explained by the social barriers women are confronted with when they seek healthcare, or because of their

poorer nutritional status, which may aggravate morbidity and mortality. Children have the highest risk for VL development, and fatality rate can approach 10% (5).

Post kala-azar dermal leishmaniasis (PKDL) is a chronic rash, seen in apparently cured VL patients, mainly in South Asia and the Horn of Africa. PKDL presents erythematous or hypopigmented macules that may progress to plaques or nodules. In Sudan, up to 60% VL patients develop PKDL; in South Asia, it is estimated to be from 10 to 20%. It is known that some years after VL incidence peaks, PKDL cases show up. PKDL patients remain infectious for years to decades, and require prolonged antileishmanial treatment. Some of them die during treatment, due to antimonial severe cardiotoxicity (5).

CL, also known as oriental sore, is the most common form of leishmaniasis, and *L. major*, *L. tropica*, *L. braziliensis*, and *L. mexicana* mostly cause it. It is generally non-fatal, is limited to the skin and may cure spontaneously. Though, spontaneous cure is slow and depends on the infective species (5, 6). However, CL can evolve into a more severe disease, such as leishmaniasis *recidivans* or mucocutaneous leishmaniasis (MCL) (6). MCL, also known as espundia, is mostly caused by New World species (12). It usually occurs months or years after healing primary CL infection, commonly due to *L. braziliensis*. MCL can lead to nasal septum, palate, throat, and associated tissues total or partial destruction, which originates facial mutilation and, rarely, death due to airway malfunction. Leishmaniasis *recidivans*, localizes slowly and progressive in non-healing lesions. Diffuse CL (DCL) is a diffuse nodular non-ulcerating form of disease that does not heal spontaneously and is difficult to treat (5). CL promotes a protective immune response that circumscribes the parasite to inoculation site. On the other hand, DCL has a poor immune response, which leads to uncontrolled parasite spread on the skin (13).

CL duration of disease is variable, and depends on the many infecting species. Clinical disease can spontaneously cure in 2 to 6 months, for *L. major*, but it can become chronic if not treated and become more severe, for *L. tropica* or *L. braziliensis*. For most species, if not treated, the disease lasts longer than 6 months. CL scars can be a social stigma, with great social impact, and may become a life-long burden. CL nodular lesions are similar to lepromatous leprosy, which deepens the stigma. Scars development depends on *Leishmania* spp., and type of clinical disease (5, 6).

CL is a major financial burden on the infected person, direct family and public health system (5). A recent Indian study showed that each VL episode could cost 71% of an annual household income, with medical expenses and loss of income, due to physical, psychological or social burden. VL's disability significance is comparable to disabling

leprosy, malaria episodes, dengue hemorrhagic fever, onchocerciasis and trachoma resulting in low vision. On the other hand, CL is in the range of malaria-induced anemia, hookworm-induced anemia, onchocerciasis-induced itching, and lymphatic filariasis characterized by hydroceles (6).

Disease burden estimation should include duration of active disease and scars, economic burden, physical and emotional disability. In order to bypass under-reporting issues, there should be a recommendation to uniform leishmaniasis definition, including clinical and non-clinical leishmaniasis diagnosis standardized algorithms (6).

As told before, leishmaniasis has been a disease typically endemic in poorer countries. However, these days, its epidemiology has evolved and it has become more present in more developed countries. Immunodepressed population and domestic dogs are the main reservoirs in these new endemic areas, and perpetuate *Leishmania* parasites' life cycle.

HIV infected individuals and people on immunodepressant therapy can develop leishmanial infection. Actually, HIV/AIDS patients are the most representative population of *Leishmania* infection in developed countries. In endemic areas, "HIV infection increases the risk of VL by a factor 100–1000" (14, 15).

East African countries have an increasing number of *Leishmania*-HIV co-infection cases, reaching up to 34% (14). In Brazil the number of co-infection reported cases is lower than expected, because of free distribution of antiretrovirals and possible under-reporting (5). Outside non-endemic areas, VL is an opportunistic infection in HIV patients (7), such as in Mediterranean countries. Portugal, Spain, France and Italy have more than 1,500 cases of co-infection reported (16). Up to 70% of leishmaniasis adult cases are related to HIV infection, and 90% are from the countries listed before (17).

Leishmania-HIV co-infected individuals are highly infectious to sandflies and can spread resistant parasites, which are an issue to control programs success (5). Immunocompromised patients can reactivate latent infections or be infected by zoonotic or anthroponotic transmission (9). The main reservoir of anthroponotic VL is the man, it is mainly caused by *L. donovani* and mostly occurs in Sudan, Ethiopia, India, Nepal and Bangladesh (16).

High activity antiretroviral therapy (HAART) reduces prevalence of co-infection and improves survival rate. The increase of $CD4^+$ T cell improves the control of leishmanial infection (18). VL promotes AIDS-defining illness conditions and its clinical progression, diminishing life expectancy. HIV-1 infection increases the risk of developing VL in endemic areas, enhances relapse occurrence and diminishes drug response. Without HAART, most co-infected patients die within 2 years, and relapse rate after treatment can

be up to 100% (5, 14, 15). HIV patients without severe immunosuppression, have manifestations similar to immunocompetent persons (5).

Leishmania infection maintenance in these new foci also relies on dogs' infection. Canine leishmaniasis (CanL) is more prevalent and widely distributed than VL, and it does not correlate with human disease prevalence. CanL is caused by *L. infantum*, and is endemic in China, Pakistan, Latin America (16), and Mediterranean countries. In southern Europe, it is considered a rural disease, but its prevalence in urban areas is increasing. In Mediterranean area, dog's seroprevalence ranges from 10 to 37%. More than half of seropositive dogs are asymptomatic, and they are an important reservoir for sandflies. Stray dogs usually spread and increase infection (9, 19).

Genetic predisposition, immunodepression, malnutrition, parasite load, species virulence, and phlebotomine saliva are important risk factors (16). CanL clinical manifestations are pleomorphic: nonpruritic skin lesions, such as exfoliative dermatitis and ulcerations, local or generalized lymphadenopathy, weight loss, poor appetite, ocular lesions, epistaxis, lameness, onycogryphosis, renal failure, diarrhea, musculoskeletal system abnormalities and eye lesions (10, 19). Standard treatment for dogs is meglumine antimonate for 28 days and allopurinol (16). Among dogs, the high prevalence of infection has suggested other forms of transmission, such as blood transfusion, venereal routes, and transplacental transmission (10).

3. *Leishmania* Biology

3.1 *Leishmania*

Leishmania is a protozoan parasite from the order Kinetoplastida, family Trypanosomatidae, and the causative agent of leishmaniasis. There are 30 species known to infect mammals, and 21 of them cause human infection (7, 20). Primitive *Leishmania* was divided into *Sauroleishmania* and *Leishmania*. *Sauroleishmania* species, *L.* (*S.*) *tarentolae* and *L.* (*S.*) *gymnodactyli*, infect reptiles, and current *Leishmania* species infect mammals. *Sauroleishmania* only infects lizards, so it is non-pathogenic for humans (21, 22). The genus *Leishmania* has 2 subgenera, *Leishmania* (*Leishmania*) and *Leishmania* (*Viannia*) (21).

Leishmania parasites are dimorphic, alternating between promastigote and amastigote forms during their life cycle (Figure 1). In the mammalian host, these protozoa are macrophage-dendritic cell (DC) lineages obligate intracellular parasites, and are called amastigotes. The promastigotes are the infective form and can be found in the insect vector, the sandfly (20). Promastigotes are phagocytized by macrophages, DCs, or neutrophils, where they transform into amastigotes. Amastigotes multiply by simple division and proceed to infect other mononuclear phagocytic cells. Sandflies ingest infected cells, and amastigotes differentiate back into promastigotes (7, 23).

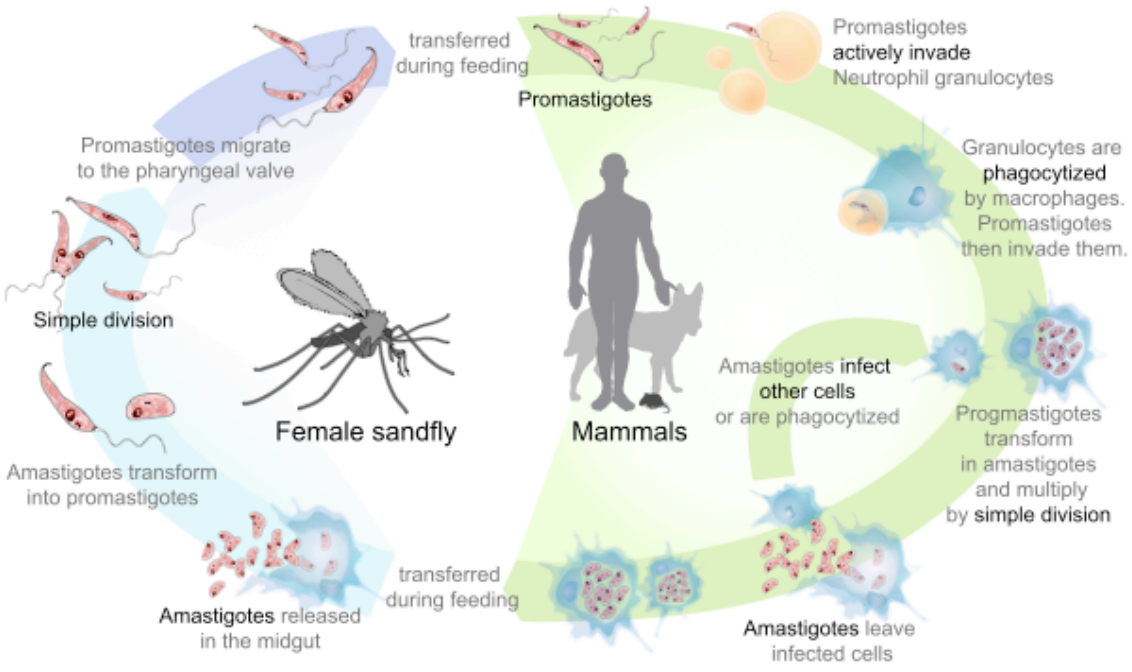

Figure 1. *Leishmania's* life cycle (24).

3.2 Species differences

The phylogenetic distance between *L. mexicana*/*L. amazonensis* and *L. major* (CL strains) is identical to the distance between *L. mexicana*/*L. amazonensis* or *L. major* and *L. donovani* (VL strains). Also, mammalian reservoir hosts are different and show some specificity for each *Leishmania* species, suggesting an evolutive adaptation. So, virulence factors and immunogenetics of host susceptibility and resistance vary greatly among species. Some genes are known to be commonly involved in *L. major*, *L. donovani*, and *L. mexicana* infection, such as *H-2* and *H-11*, but others, such as *NRAMP/Lsh*, only influence infection development for *L. mexicana*. There are specific genes related to disease visceralization. Infection control is very complex, and a multigenic combination may lead to healing or non-healing (25).

Different *Leishmania* species can cause a wide range of clinically distinct diseases. The evolution of visceralizing or disseminating phenotype of some species, such as *L. donovani* or *L. braziliensis*, and localized phenotype of others, such as *L. major*, is still unclear. The interaction host-parasite and immune response suggest an antigen-dependent pattern of disease, which depends on the species. VL disease clearly depends on immune response, receptors, co-receptor and parasite tropism (21). Nevertheless, there is a high gene conservation for *L. major*, *L. infantum* and *L. braziliensis* (26).

Berman et al. (1981) study showed that CL strains *temperature optima* for amastigote replication is 35 °C and for VL strains is 37 °C. Pereira et al. (1958) had already shown the relationship between skin and environmental temperature. *In vivo* CL strains multiply more rapidly at lower temperatures than VL strains. Cutaneous and mucocutaneous localization of CL might be related to this fact, so as primarily visceral and bone marrow localization for VL strains (27).

Parasitophorous vacuoles (PVs) are different between species. *L. mexicana* complex parasites, *L. mexicana*, *L. amazonensis*, and *L. pifanoi*, reside in communal PV, which get bigger during infection. On the other hand, *L. donovani* complex parasites, *L. donovani*, *L. chagasi*, and *L. infantum*, reside in tight individual PVs from where daughter cells segregate into their own PVs (28). *Leishmania* parasites reside inside PVs so they can evade the immune system, resisting to hydrolases and peptidases digestion (13).

3.3 Metacyclogenesis

Different *Leishmania* life cycle forms (Figure 2) are distinguished by their nutrients requirement, growth rate, division ability, expression of surface proteins, and morphology (29). During metacyclogenesis, there is a differential and enhanced expression of virulence factors (25). VL strains have faster metacyclogenesis than CL strains, which promote better ability to infect, and leads to stronger pathogenic effects (30). Metacyclogenesis can be triggered by low pH, high temperatures, such as human body temperature, and elevated CO_2 concentration (29, 31).

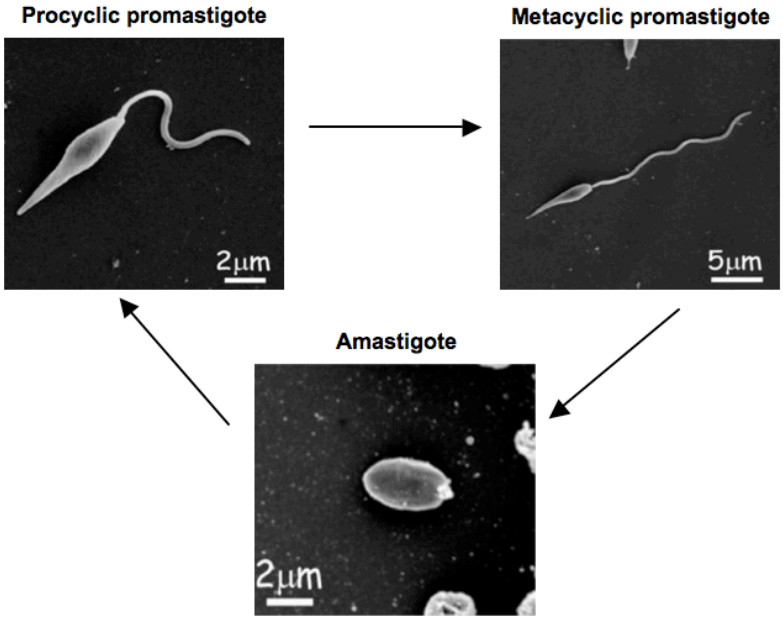

Figure 2. *Leishmania*'s metacyclogenesis morphological evolution (29).

Procyclic promastigotes (Figure 3) have rapid division ability (32), are non-infective, have an elongated spindle shape and are 20 μm long (29), are weakly motile (31), and do not present apoptotic features. *In vivo*, they are in the insect's midgut, and *in vitro*, they can be cultivated axenically (33).

Metacyclic promastigotes (Figure 3), are not able to multiply, have higher infectivity ability, have a rounder shape, have a longer flagellum, show a swollen kinetoplast, lack nuclear structures, and present apoptotic features. Metacyclic promastigotes have surface molecules that are complement-resistant, being more resistant to lysis (29, 30, 32, 33). They are at the insect's thoracic midgut and proboscis, and are more common during stationary growth (34). Metacyclic promastigotes do not adhere to phlebotomine sandfly digestive tract. During metacyclogenesis, surface carbohydrates change at D-galactose sites (30).

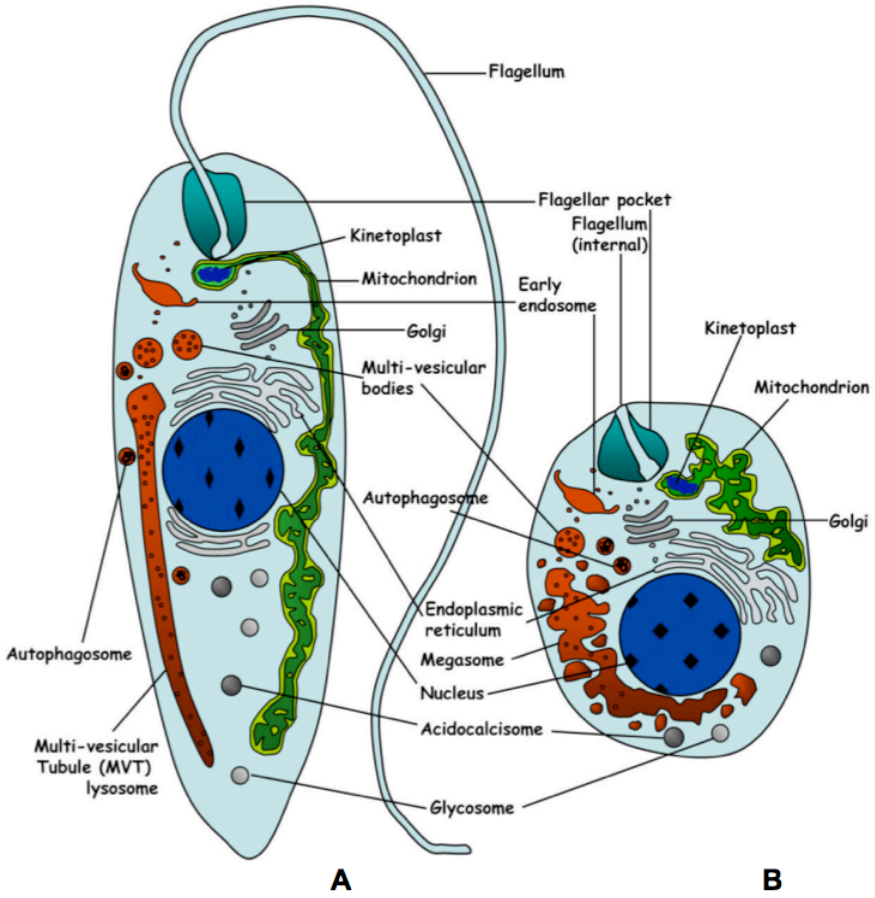

Figure 3. (A) *Leishmania* promastigote and (B) amastigote forms. The flagellar pocket marks the anterior cell end (29).

Lipophosphoglycan (LPG) is a complex glycophospphatidylinositol-anchored phosphoglycan molecule on promastigote's surface (25). LPG is over expressed in metacyclic promastigotes, and it is a complement resistance factor (35). Due to surface LPG differences during metacyclogenesis, peanut agglutinin (PNA), *Arachis hypogea*, is able to agglutinate procyclic promastigotes (PNA^+). This method is used to separate procyclic promastigotes from metacyclic ones (PNA^-) (30, 34).

After inoculation in mammalian host cells, metacyclic promastigotes differentiate into amastigotes, residing inside a vacuole with lysosomal features, the PV. Amastigotes are intracellular organisms, non-motile, with reduced size (around 4 μm), with a much-reduced flagellum located inside the flagellar pocket. They are acidophiles, and have an adapted energy metabolism (29). Amastigotes perpetuate infection, when they are taken during the insect vector's blood meal (31).

Intracellular macrophage environment promotes transformation into amastigotes (36). PVs import nutrients through the parasite surface and macrophage membranes. In order to survive to PV's hostile environment, for example, *L. amazonensis* changes its basic electrical properties. K^+ currents modifications explain sustained hyperpolarization elicited

by infection. Hyperpolarization appears to be related with macrophages activation and phagocytosis (37).

Lysosomes change shape and content. In procyclic promastigotes, lysosomes are a large single vesicle at the anterior end of the cell. Amastigotes show megasomes, which represent 15% of total cell volume. These megasomes can be related to nutritional requirements and species virulence. The number of autophagosomes increases, and autophagy can be enhanced, which is a survival strategy (29).

During metacyclogenesis, genetic expression changes, and there is a protein turnover (29, 31). However, because this process occurs in an environment poor in nutrients, *de novo* protein synthesis is limited (32). Studies made with *L. braziliensis* show differential mRNA transcripts expression throughout metacyclogenesis (38). *A2* gene, an amastigote stage protein, which contributes to tissue tropism, is important for *L. donovani* virulence, and promotes visceralization (25). Peptidases content also change, and metacyclic promastigotes show higher proteolytic activity. Peptidases release the heme group, important for Fe metabolism. Hemoglobin is internalized as a lysosomal compartment. Fe may be also obtained from transferrin or lactoferrin (29).

Post-translational protein modifications can be detected by mass spectrometry (MS) techniques. Many proteins exist throughout all *Leishmania* life cycle, but with different molecular weights and isoelectric points. Other proteins only exist in one of the life cycle stages. For instance, metacyclic promastigotes are more related to motility proteins. Overall, during metacyclogenesis, there is a down regulation of synthetic proteins and an up regulation of motility proteins. Also, there are specific modifications of mitochondrial enzymes (32).

3.4 Virulence and infection strategies

Since the first time of infection, strategies to enhance it and provoke a more virulent response are done, both by the parasite and by the insect vector. The sandfly saliva has exacerbating factors, such as anti-aggregation factors, vasodilators, anticoagulants and anti-haemostatics (31, 39). *Luzomia longipalpis* saliva and *Leishmania* parasites enhance macrophages recruitment and neutrophils in BALB/c mice (susceptible strain), but not in C57BL/6 (resistant strain) (39).

In the mammal host, myeloid cells, such as macrophages, DCs and neutrophils, are *Leishmania* hosts, final or intermediate (39). After ingestion, parasites are sequestered from the host cell cytosol by a membrane that turns into PV (28). If procyclic

promastigotes are phagocytized together with metacyclic promastigotes, they will survive inside the neutrophils (33). Survival and virulence strategies are summarized on Table 2.

Table 2. Long-term persistence mechanisms of intracellular pathogens *in vivo* (40).

Pathogen entry into safe target cells
Fibroblasts
Neurons
Pathogen resistance to host cell effector mechanisms
AO synthesis
↑ Proteasomal activity
Metabolic salvage pathways
Supression or avoidance of host cell effector mechanisms
↓ Expression of NADPH oxidase or iNOS
Blockade of NADPH or iNOS phagosomal recruitment
Inhibition of phagosome-lysosome function
Exit into cytosol
Pathogen-mediated immune deviation
Antigen presentation inhibition
↓ Co-stimulatory cell surface molecules
↑ IL-10
↑ Regulatory/Supressive T cells ($CD4^+CD25^+FoxP3^+$ T_{reg} cells, $CD4^+CD25^-FoxP3^-IL\text{-}10^+$ Th1 cells, $CD4^+FoxP3^-IL\text{-}10{+}INF\text{-}\gamma^-$ T cells)

Phagocyte functions are suppressed by phosphatidylserine (PS) exposure on apoptotic cells membrane. PS is an apoptotic feature, which appears when the plasma membrane phospholipids symmetry is altered (33), and its recognition leads to *Leishmania* intracellular survival. If there are no apoptotic parasites, *Leishmania* will not induce *in vivo* disease. A purified apoptotic population will not cause disease, because these parasites are dying or already dead. A purified non-apoptotic parasite culture will also not be able to induce disease. When non-apoptotic and apoptotic parasites are injected into BALB/c mice footpad, these develop large lesions (33, 41). *L. major* parasite apoptotic death activates cysteine proteinases, permeabilizes mitochondrion, and destroys DNA (34).

Amastigotes and metacyclic promastigotes can also have a silent phagocytic uptake, due to calreticulin exposure, which is also an apoptotic signal (42). Metacyclic promastigotes can be phagocytized by resident dermal mononuclear phagocytic leukocytes and/or by phagocytic leukocytes recruited from epidermis or blood. After an autophagic process, they differentiate into amastigotes. Mature mononuclear phagocytic leukocytes present colony-stimulating factor-1 receptor (CSF-1R), which regulates production, maintenance and function of phagocyte lineage derived macrophages (42).

PS exposure in promastigotes occurs by apoptotic death or apoptotic mimicry. The first is for infection and the second for disease progression. This leads to a permissive

host suitable for survival and proliferation, which is an adaptative survival strategy of Trypanosomatids (41). Apoptotic features will reduce inflammation signals and help the parasite to evade the immune system, facilitating its “silent entry” into neutrophils. Nutrient shortage in stationary phase and sandfly gut can be an explanation for apoptosis start with PS exposure (33, 34).

PS^+ amastigotes induce transforming growth factor (TGF) β and interleukin (IL) 10 production. They silence polymorphonuclear (PMN) cells effector functions, allowing intracellular survival of non-apoptotic parasites. Apoptotic *Leishmania* parasites ratio increases during *in vitro* culture. In stationary phase, more than 50% of the parasite’s population can be apoptotic (34).

PMN cells are the first line of organism defense against infectious agents or exogenous substances. These cells have a lobulated chromatin-dense nucleus and granules. The granules contain proteolytic and bactericidal substances and can be classified as primary or azurophil granules, secondary or specific granules, and gelatinase granules or secretory vesicles. Neutrophils are produced by bone marrow and are then released into blood circulation, representing more than 50% of leukocytes in circulation (33).

Neutrophils are the first phagocytes to leave circulation and to be recruited to infection sites. They can become primary host cells for intracellular pathogens, like *Leishmania* parasites. *Leishmania* chemotactic factor (LCF) recruits neutrophils to infection site and interacts with the chemokine receptor lipoxin A4 receptor (ALX) (12, 39). Neutrophils are primary antimicrobial effector cells and destroy invading pathogens by phagocytosis (33).

Neutrophils are an important link between innate and adaptive immunity during parasitic infections. They conduct inflammatory responses and tissue repair, which leads to infection control (43). Neutrophils can be recruited by IL-8, IL-17 and tumor necrosis factor (TNF). In skin lesions, TNF-α is crucial for inflammation resolution. So, in CL, macrophages and neutrophils seem to contribute to parasite clearance and protective immunity (12, 44).

There are 2 different microorganisms recognition mechanism by neutrophil granulocytes: opsonin-dependent and opsonin-independent. Opsonins are serum components that bind both to microorganisms’ surface and to specific receptors on phagocyte’s surface. They can be immunoglobulin (Igs), C3bi fragment or mannan-binding lectin. Phagocytosis can be mediated by direct recognition of pathogen-associated molecular patterns via pattern recognition receptors, which is a non-opsonic phagocytosis. After phagocytosis, the phagosome and cytosolic granules merge, generating the phagolysosome. Then, azurophil granules release hydrolytic enzymes and

bactericidal proteins, such as elastase, bactericidal permeability-increasing proteins and defensins (33).

PMN produce highly reactive oxygen species (ROS) (33). Inside phagolysosomes, parasites are phagocytized and exposed to enzymes, antimicrobial peptides or ROS. Neutrophils use nitric oxide (NO)-dependent and O-independent mechanisms for parasites elimination. To kill the pathogen, pre-formed proteinases or antibiotic proteins are released. Neutral proteases, such as neutrophil elastases (NEs) activate infected macrophages, in order to eliminate *Leishmania* parasites via toll-like receptor (TLR) 4 signaling. *L. major* block oxidative burst and evade elimination, and *L. donovani* is not found inside lytic compartments (39). PMN cells kill most ingested microorganisms. However, some can survive. This unexpected survival can be explained by escape from lytic compartments, blockade of phagosome-lysosome fusion or inhibition of oxidative burst induction (12, 33).

Leishmania is phagocytized by neutrophils in an opsonin-independent manner. This uptake does not activate oxidative burst, so parasites survive inside neutrophils. They are phagocytized silently (33).

Interferon (IFN) γ is a potent and critical modulator of PMN, regulating differential gene expression, ROS production, and surface markers expression, such as CD69 and Fc-γ-receptors (Fc-γ R). IFN-γ enhances bactericidal activity. *L. major* inhibits IFN-γ-signaling in PMN, which is a common evasion mechanism for intracellular pathogens. As a result, *Leishmania* compromises host cells IFN-γ-signaling, a potent inhibitory mechanism by which cellular activation is suppressed in macrophages and in neutrophils (33).

Comparing to macrophages, neutrophils are short-living cells. Their half-life ranges from 6 to 10 hours. Then, they undergo rapid spontaneous apoptosis, leading to their phagocytosis by macrophages (12). Apoptotic neutrophils show shrinkage, chromatin condensation, and loss of nucleus multilobed shape. Cell surface shows decreased expression of some receptors or new surface molecules. Neutrophils constitutively express the pro-apoptotic proteins Bax, Bid, Bak, and Bad. The anti-apoptotic Mcl-1 protein is expressed in bloodstream neutrophils and its levels decrease prior to apoptosis onset (33). Infection can increase their half-life in several days. For human neutrophils *in vitro* infection with *L. major*, their half-life is prolonged in 2 days, inhibiting procaspases processing (12).

Viable *Leishmania* parasites delay PMN cells apoptosis. Pro-inflammatory cytokines, such as TNF-α, IL-15, IFN-γ, granulocyte colony-stimulating factor (G-CSF), granulocyte

macrophage colony-stimulating factor (GM-CSF) and IL-8 delay neutrophils' apoptosis. IL-8 targets neutrophils, and creates an autocrine feedback loop that recruits more neutrophils to infection site, delaying apoptosis (33).

Senescent neutrophils are removed from blood circulation and replaced by mature ones. At local sites of infection or inflammation, it is very important how recruited neutrophils are removed from the inflamed spot. In *Leishmania* infection, the apoptotic neutrophils externalize PS, which facilitates recognition and silent clearance of apoptotic neutrophils by macrophages. *Leishmania* delays PMN apoptotic death up to 2 days. Infected PMN secrete high levels of macrophage inflammatory protein (MIP) 1b, which attracts macrophages. This secretion coincides with macrophages migration peak into the infected tissue. Macrophages phagocytize infected apoptotic PMN. Apoptotic cells clearance is a major macrophage function. Though ingestion of apoptotic cells, in general, does not result in the activation of antimicrobial effector functions. Phagocytosis of apoptotic neutrophils deactivates phagocyte functions, and no significant amounts of TNF are released. Intracellular parasites inside PMN do not have direct physical interaction with macrophages surface receptors. So, there is no macrophages activation (33). Neutrophils move rapidly to infection site, but become 80% slower after phagocytosis. There can be parasite release before apoptosis (39).

Apoptotic neutrophils are a temporary shelter for *Leishmania* parasites. For this, they are called "Trojan horses" (33, 39). This increases silent uptake of *Leishmania* and a higher level of survival inside the macrophage. Neutrophils conversion into "Trojan horses" occurs through the following steps (39) (Figure 4):

1. Neutrophils are recruited by ALX, and avoid activation of lethal functions. ALX activation by lipoxin A4 deactivates oxidative stress, and increases apoptotic cells phagocytosis;
2. Decrease phagocyte machinery for apoptotic cells uptake. PS^+ promastigotes induce TGF-β production, anti-inflammatory cytokine, and phagocyte functions will not be activated;
3. Apoptotic neutrophils die by apoptosis and recruit macrophages for their own phagocytosis. They regulate their silent clearance and, if there is an infection, promastigotes transfer will happen. *Leishmania* infection delays neutrophils apoptotic death up to 2 days. Apoptotic neutrophils release MIP-1b, providing a migration of macrophages into the infected tissue. There is almost no extracellular promastigotes.

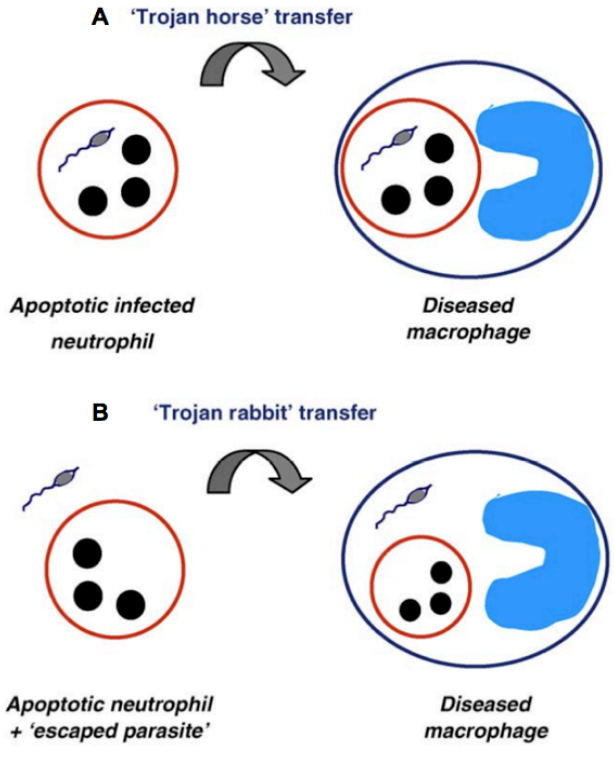

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Figure 4. "Trojan horse" infection model. Depending on *Leishmania* strain and model used, neutrophils can serve as a (A) "Trojan horse" to transfer parasites into macrophages. *Leishmania* parasites can also hide outside the apoptotic neutrophil (B) to get inside the macrophage, "Trojan rabbit". This is a free adaptation from the film "Monty Python and the Holy Grail" (39).

Recently, a new neutrophil-mediated antibacterial mechanism, the release of neutrophil extracellular traps (NETs), has been described. NETs are released by dying neutrophils, as well as antimicrobial factors. They are extracellular structures produced by stimulated neutrophils. These structures contain DNA, histones, granular proteins and antibacterial enzymes that bind and kill infective organisms. This cell death process is different from apoptosis and necrosis and depends on the generation of ROS by NADPH oxidase (33, 39, 45).

Macrophages clear apoptotic cells, and maintain tissue homeostasis. Resting macrophages phagocytize metacyclic promastigotes, allowing them to differentiate into amastigotes (42). When macrophages get infected, they start cell death process and start to spread parasites, see Figure 5. Amastigotes replicate extensively before spreading to other cells, until their number exceeds the lethal parasite density. Parasite transmission occurs when macrophages phagocyte dying primary host cells, such as neutrophils. Macrophages are recruited approximately 2 days post-infection (46).

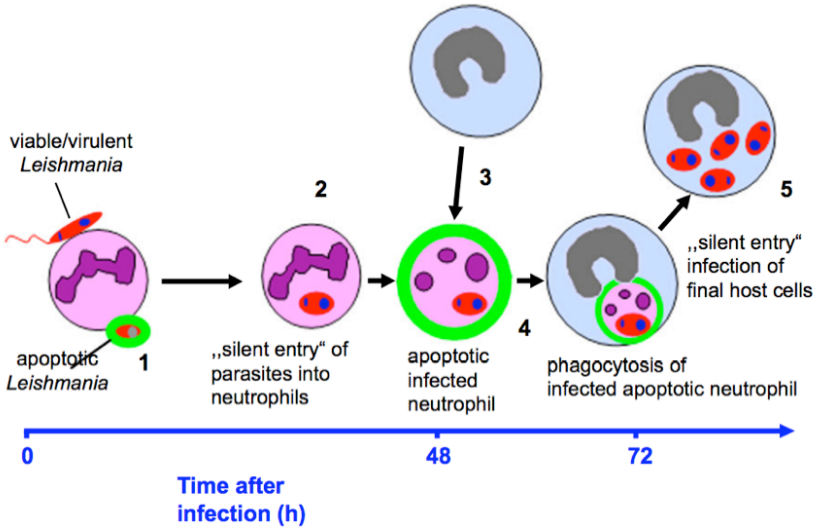


Figure 5. Early events in *Leishmania* infection. (1) Viable and apoptotic promastigotes in *Leishmania*'s virulent inoculum. Viable promastigotes survive inside PMN, due to a "silent entry". (2) *Leishmania* infection delays neutrophil apoptosis. (3) Monocytes/Macrophages migrate to infection site. (4) Apoptotic infected neutrophils are ingested by macrophages. (5) Apoptotic neutrophils' phagocytosis provides "silent entry" into macrophages (33).

There is a balance between macrophages activation and subpopulations, and *Leishmania* parasites survival mechanisms, which are highly related to their species and associated virulence. *Leishmania* parasites interfere with host cells signal transduction pathways, such as transient activation of mitogen-activated protein kinase (MAPK) and nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) signaling. *L. major* promastigotes were shown to downmodulate these pathways. Infection with amastigotes also modulates MAPK signaling, which can lead to pro-inflammatory signals suppression and IL-10 production promotion (28). This will further suppress TNF-α release by infected macrophages. Prolonged and repeated interactions between lipopolysaccharide (LPS) and macrophages can lead to its tolerance (47).

Phagocytosis is mediated by Fc and complement receptors, and suppressed by PS recognition on apoptotic cells membrane. Apoptotic cells uptake decreases pro-inflammatory cytokines, such as TNF-α, IL-1, and IL-12 secretion by macrophages. Anti-inflammatory cytokines production is induced, TGF-β and IL-10, and pro-inflammatory cytokines are downregulated, such as TNF-α. PS recognition prevents immune responses against internalized and processed apoptotic proteins remains. Cytokine production by macrophages is dependent on PS density. PMN cells secretion of TGF-β correlates positively with apoptotic parasites ratio. An inverse correlation is observed between apoptotic parasites ratio and TNF release. Disease is induced after PS recognition and TGF-β production (28, 33, 34). TGF-β prevents immune responses against internalized and processed apoptotic proteins, and its neutralization decreases

survival inside neutrophils (34). *Leishmania* parasites assure their survival and prevent tissue damaging due to inflammatory processes (42). This is a “silent entry”, because infection occurs without maturation induction (28).

DCs are important for parasite dissemination. DC entry depends on C-type lectin, ICAM-3-grabbing non-integrin, as the putative receptor on DC for *L. infantum* and *L. pifanoi* amastigotes. These molecules are parasite specific, and internalization by these receptors does not activate DCs (28).

Both amastigotes and promastigotes can start an infection. Promastigotes turn into amastigotes after 24 to 72 hours. Amastigotes are sustained and kept for days inside macrophages, which produce superoxide anion (O^{2-}) to infection with promastigotes and produce much less for amastigotes’ infection (28).

O^{2-} production suppression occurs due to NADPH oxidase enzyme complex inability to assemble inside PV, because p47 is not phosphorylated by protein kinase C (PKC), which is inhibited by amastigotes and promastigotes. Promastigotes do it through their LPG coating (28). Superoxide dismutase (SOD) detoxifies O^{2-}, converting it into H_2O_2 and H_2O. The genes *SODA* and *SODB* were identified in *L. chagasi*, and their overexpression protects the parasite from oxidative stress burden (48).

Leishmania infection can block NO production. NO is the product of inducible NO synthase (iNOS) gene, which is induced by IFN-γ. For *L. amazonensis* amastigotes, PS is displayed and parasites are internalized via PS receptors on macrophages. After, IL-10 and TGF-β secretion is induced, and these block iNOS. Therefore, there is no NO production (28).

Zn metalloproteases, and cysteine proteases are also virulence factors (26). Gp63 is a metalloprotease that cleaves C3b to C3bi on the parasite’s surface membrane. It is a virulence factor for *L. major*, *L. amazonensis*, *L. mexicana*, and *L. donovani*. So, complement mediated lysis is inhibited and parasite uptake by macrophage complement receptor (CR) 3 is promoted. Extracellular release of Gp63 facilitates parasite’s dissemination through tissue, degrading extracellular matrix components. It also subverts immune responses: cleaves major histocompatibility complex (MHC) class I molecules and CD4 to limit T cell responses, and downmodulates MARCKS-related (myristoylated alanine-rich C kinase substrate) protein. For amastigotes pathogenesis, it is significantly downregulated, though its role is still unclear (25).

LPG is a virulence factor for *L. major* and *L. donovani*, but not for *L. mexican*a (25, 34). LPG is important for NO production modulation, apoptosis inhibition, phagolysosome maturation delay, and macrophage signal transduction inhibition. LPG is the ligand for

C3bi deposition on promastigote surface, which is critical for CR3-mediated uptake and for inhibiting macrophage IL-12 and NO responses to infection (25). In amastigotes, it is minimally expressed, because they express high levels of glycoinositolglycolipids (GIPLs), and get other host cell lipids. This lipidic coating helps to avoid macrophages activation, and, possibly, to suppress IL-12 production in infected cells, which is responsible for immune response initiation (28).

Fe generates toxic reactive nitrogen intermediate (RNI) and reactive oxygen intermediate (ROI). Fe decreases iNOS expression by IFN-γ-activated macrophages, promoting pathogen survival. Fe^{2+} is translocated through Nramp2 into cytosol, and Fe^{3+} is produced. Nramp1 transporter shows up in later endosomes or lysosomes. *Leishmania* parasites compete with host Fe transporters. Fe uptake in *L. chagasi* occurs preferentially in Fe^{2+} form, which is essential for parasite growth and virulence. Fe^{3+} reduction is coupled to Fe^{2+} membrane transport by LIT1. LIT1 is a plasma membrane structure essential for parasite's intracellular replication and virulence (48).

3.5 Immunology

Leishmania infections induce strong humoral responses. Though, antibodies do not protect, but are associated with disease (49). Protective immunity is related to a classical cell-mediated immune response (25).

After a primary non-specific innate immune response, a specific T cell response is developed and determines disease development (49). This early innate response involves TLR2 receptors in macrophages, DCs and nature killer (NK) cells. Macrophages activate specific T cells by IFN-γ or TNF-α production (13). Macrophages and DCs are antigen-presenting cells (APCs) with phagocytosis characteristics (15). DCs promote mixed T cell immune response, are present in all lymphoid organs and are essential for immunity induction. Special inflammatory DCs, like TNF-α iNOS-producing DCs (TipDC), produce TNF-α, NO, IL-12 and stimulate T cells. iNOS production by TipDC is positively regulated by T helper (Th) 1 cell response and negatively by Th2 response, see Figure 6. DCs are essential in developing Th1 protection. During *L. major* infection, inflammatory DCs are the main iNOS producers. Their recruitment depends upon CCR2 expression, and iNOS expression induction requires a local Th1 environment (50).

Th1 cells mediate a protective immune response, but the identification of a precise role for Th2 responses remains yet to be clarified (25). Th1 cells secrete activators of cell-mediated immunity, such as IFN-γ, while Th2 cells secrete cytokines that promote antibody responses, such as IL-4. IL-4 induces Th2 response, and IL-12 induces Th1

cells differentiation (49). DCs are IL-12 producers during early infection. CD40–CD40L interactions enhance IL-12 production, and mice lacking this pathway are susceptible to CL. IL-12 is essential in Th1 responses.

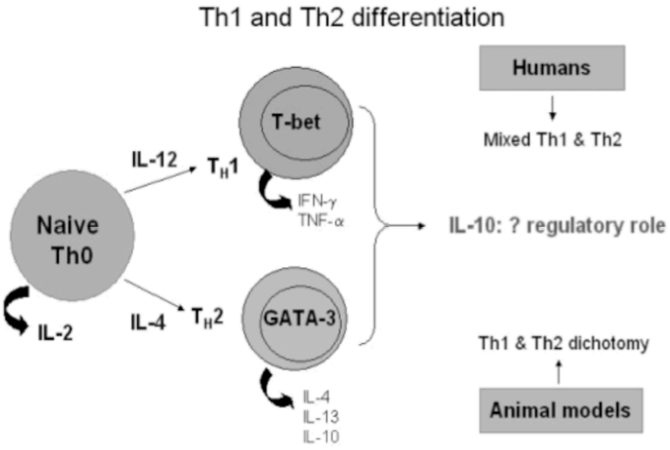


Figure 6. Th1 and Th2 dichotomy in *Leishmania* infection (49).

IL-12-driven Th1 response promotes protective immunity against all *Leishmania* species, although DCs are the primary source. IL-12 activates NK cells. NK cells produce IFN-γ, which controls innate resistance to infection, and influences Th1 response initiation. All resulting IFN-γ from NK cells, $CD4^+$ T and $CD8^+$ T cells, induce macrophages NO production (25, 50), providing parasite clearance and healing (12). The failure to regulate an effective Th1 immune response can lead to a bad immunological memory (11). IL-12 and IFN-γ are protective cytokines (49). IFN-γ-activated macrophages produce RNIs, but are inhibited by IL-4, IL-10, IL-13 and TGF-β. This downregulation might be crucial for acquired resistance development (51).

TNF-α is essential for *L. major* infection resolution. TNF-α enhances macrophage activation, NO production and parasite clearance. IFN-α/β is produced by APCs, and can activate macrophages, in order to produce NO. IL-12, TNF-α and IFN-α/β can develop a protective Th1 response to *L. major* infection (49). *L. major* infection control and resistance require IL-12 dependent differentiation of $CD4^+$ T cells. Amastigotes and promastigotes can subvert macrophages IL-12 production (28). Genetically resistant mice strains develop a strong Th1 response and restrict the spread of local parasite infection. Non-healing mouse strains have a Th2 response associated with high level of IL-4 and IL-13 production by $CD4^+$ T cells (50). DCs produce IL-12 during early infection. CD40–CD40L interactions enhance IL-12 production, and mice lacking this pathway are susceptible to CL (49).

Th1 responses (Figure 7) and macrophage activation are suppressed by IL-10 and TGF-β (49, 51). Genetic suppression of Th1, will lead to susceptibility. IL-12-IL-12R signaling is essential to develop a healing Th1 response. NK cells belong to innate immune response, participate in Th1 response, and release IFN-γ, which optimizes IL-12 production by DCs and IL-12R by T cells (6).

Th2 early response is significant for cytokines production and clinical development (51). Th2 response depends on *Leishmania* species. Th2 cytokines are immunoregulators for early infections (49) and secrete IL-4 and IL-13, which cause susceptibility (12). IL-4-driven Th2 immune response counter-regulates Th1 response and promotes disease development. Disease is due to no Th1 response, because of no IL-12 production. IL-4 cans downregulate IL-12 production, expression of IL-12Rb2, IFN-γ production and activity. After primary infection, IL-4 and IL-4Ra signaling are essential for parasite liver and spleen infection. IL-4 and IFN-γ activate macrophages against *L. major*. IL-13 has the same signaling pathway as IL-4, and also downregulates macrophage activation. It enhances monocyte IL-12 production, induced by IFN-γ, while IL-4 and IL-13 promote macrophages and DCs IL-12 production, induced by CD40L. IL-4 and IL-13 can be pro- or anti-inflammatory, depending on the extracellular environment (25). They are protective cytokines in *L. major* and *L. donovani* infections, but not in *L. mexicana* or *L. amazonensis* infections (49). Susceptibility to *L. major*, influenced by IL-4 and IL-13, is mediated by IL-10. IL-10 has a similar role in susceptibility to VL with *L. donovani*, and no disease exacerbatory role for IL-4 or IL-13 has been reported (25). IL-6 favors Th2 response (49).

IL-4 and IL-13 protect against *L. major* and *L. donovani* infections, but not for *L. mexicana* or *L. amazonensis* infections. In *L. major* early infection, resistant and susceptible hosts have mixed Th1 and Th2 responses of $CD4^+$ cell population. During early infection stages, IL-4-producing $CD4^+$ T cells population may be important for disease progression. IL-4 induction depends on other T cell factors, such as IL-2, which may be a susceptibility factor. IL-13 or IL-2 can substitute IL-4 (49).

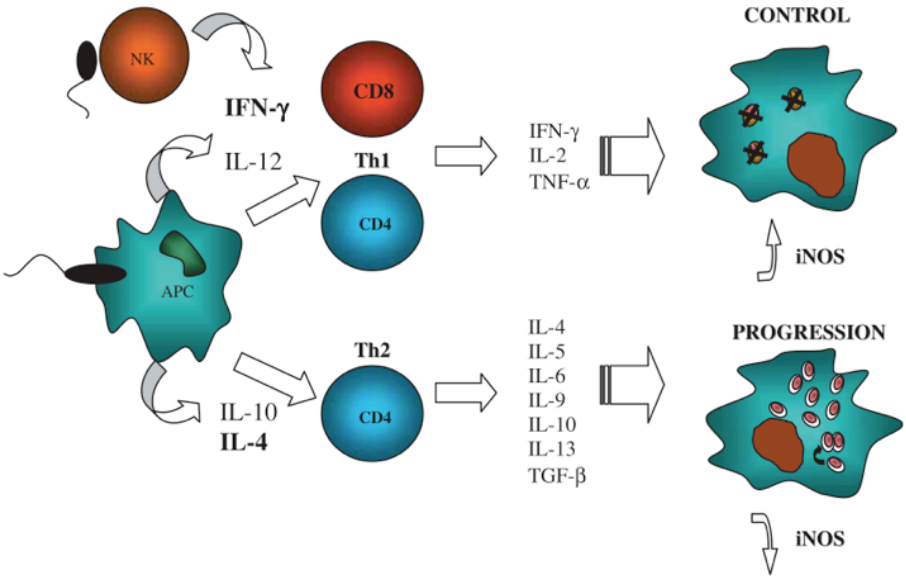


Figure 7. Immune response against *Leishmania* parasites. Susceptibility or resistance against the disease depends on the type of cytokines secreted (13).

Susceptibility is a multigenetic phenomenon (44). Susceptibility and resistance to *Leishmania* in mouse model are associated with emergence of T cells, T regulatory (T_{reg}) cells and cytokines, such as IL-10 (49). In localized human CL, Th1 cells predominate, and Th2 immune response markers are detected in DCL or MCL. Cure is associated with IFN-γ production and IL-10 with lasting lesions (44). Treatment of non-healing lesions with IFN-γ leads to cure. In human VL and DCL, IFN-γ absence allows parasite multiplication and disease progression (49).

The second IFN-γ significant producer is $CD4^-CD8^-$ T cells, double negative (DN) T cells, see Figure 8. DN T cell population contains T cells expressing γ/δ or α/β T cell antigen receptor (TCR) complex. α/β TCR^+ DN T cells are restricted to CD1 presented antigens, which express a restricted TCR and recognize lipid antigens presented by one of the CD1 family of molecules. These T cells are classified as invariant NK T cells and express a restricted TCR. 80% of peripheral blood DN T cells express γ/δ TCR. α/β DN T cells are highly activated T cells. α/β DN T cells express high IFN-γ or TNF-α to IL-10 ratios after soluble *Leishmania* antigen (SLA) induction (11).

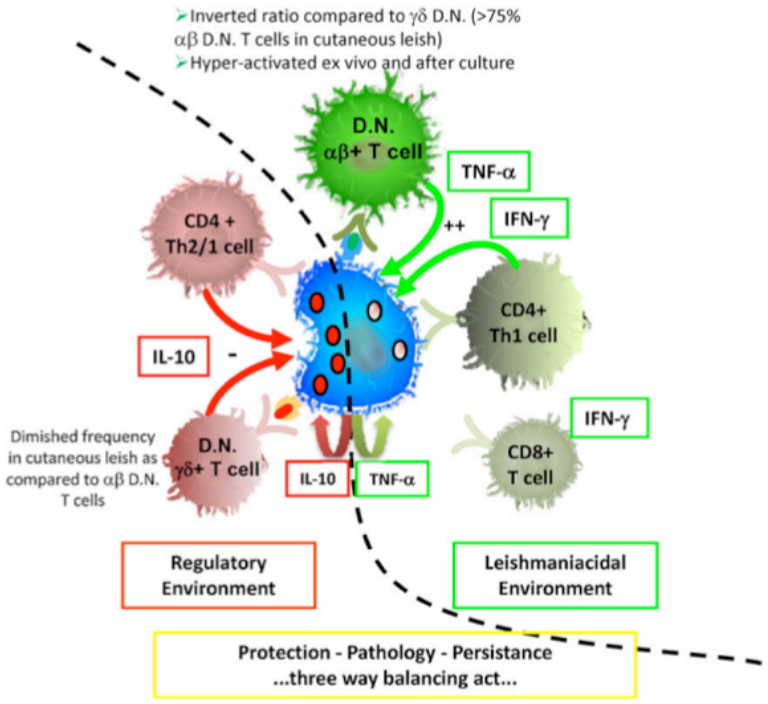


Figure 8. Cytotoxicity. $\alpha\beta^+$ DN T cells contribute to a leishmanicidal immune environment while, $\gamma\delta^+$ DN T cells appear to contribute for a downregulatory environment in human CL, caused by *L. braziliensis*. The overall balance will determine if an immune response is effective. Though, it allows some persistence, which is important for long-lasting immunological memory (11).

$CD25^+Foxp3^+$ T_{reg} cells downregulate specific immune responses against the parasite and can be linked to chronic progressive disease (13). T_{reg} cells produce TGF-β and IL-10. Localized immune responses show tissue tropism and localized growth patterns. T_{reg} cells suppress effector T cell activity through IL-10 production. T_{reg} cells produce most of the IL-10 responsible for chronic infection, and $CD25^-$ T cells produce most of the IFN-γ (49).

In VL patients, IL-12 enhances Th1 immune response and restores T cell proliferation, IFN-γ production and cytotoxicity. IL-12 decreases spontaneous or antigen-induced peripheral blood mononuclear cells apoptosis in VL patients. IL-12 and *Leishmania* antigen restore proliferation of these cells in VL patients (49). Infection control or exacerbation is regulated by IL-10 (Figure 9). $CD4^+$ T cells and monocytes are important sources of IL-10. A lower IFN-γ/IL-10 ratio is associated with better *L. braziliensis* infection prognosis (11). IL-10 is important for disease progression, it inhibits IFN-γ and promotes parasites persistence (49). It inhibits macrophage activation, and is the major cytokine involved in visceralization. IL-10 blocks Th1 activation and promotes a cytotoxic response by downregulating IL-12 and IFN-γ production. Active VL has increased production of IFN-γ, IL-2, IL-10 and IL-4. After cure, IFN-γ, from $CD4^+$ Th1 cells, IL-4 and IL-10 persist, suggesting mixed Th1 and Th2 responses in VL and cured individuals (11, 49). IL-12 and

IL-10 are critical for immune regulation during infection, pathogenesis and chemotherapy. Parasites may start modulating macrophages on early and later infection, on infected macrophages with T cells and induce IL-4 and disease-inducing factors from T cells, which help in disease and parasite survival. Infected macrophages produce IL-10. IL-10 is crucial in disease initiation independent of T cells and in disease progression later with IL-4 (49).

A Th1 immune response can lead to non-healing *L. amazonensis* infection, but not *L. major* infection. *L. mexicana* complex parasites downregulate Th1 responses by IL-4-dependent and independent mechanisms. In Th2 immune response, IL-4 and IL-13 production is associated with chronic infection. IL-10 and TGF-β are important to enhance VL development. IL-10 promotes non-healing *L. major* infections, but is less significant during *L. mexicana* and *L. amazonensis* infections. B cell mechanisms promote *L. donovani* and *L. mexicana* complex infections (25).

The *Leishmania* homolog of receptor for activated C kinase (LACK) antigen promotes IL-4 production through $V\beta4^{+}V\alpha8^{+}CD4^{+}$ T cells activation and is crucial for BALB/c mice susceptibility by *L. major*. It is required for parasite persistence within macrophages. In *L. mexicana* parasites complex, cathepsin L-like cysteine protease B (CPB) enzymes are considered virulence factors. CPB enzymes induce IL-4 production and Th2 immune response. *L. mexicana* can inhibit macrophage and DC IL-12 production, conditioning DCs to produce more IL-4. *L. donovani* and *L. chagasi* CPB enzymes activate latent TGF-β, which is a suppressive cytokine in *Leishmania* infection. Cathepsin L and B-like CPB may inhibit Th1 or promote Th2 response, respectively (25).

In CanL, gestation maintenance depends on the development of an immunoregulatory response. Th2 immune response is amplified and there is an increase in TGF-β, IL-6 and IL-10 release. Th1 response is depleted, with reduced production of abortive pro-inflammatory cytokines, such as INF-γ and TNF-α. TNF-α and INF-γ induce amastigotes' death in CanL. In pregnant dogs, polarization of Th2 response can increase parasite load (10).

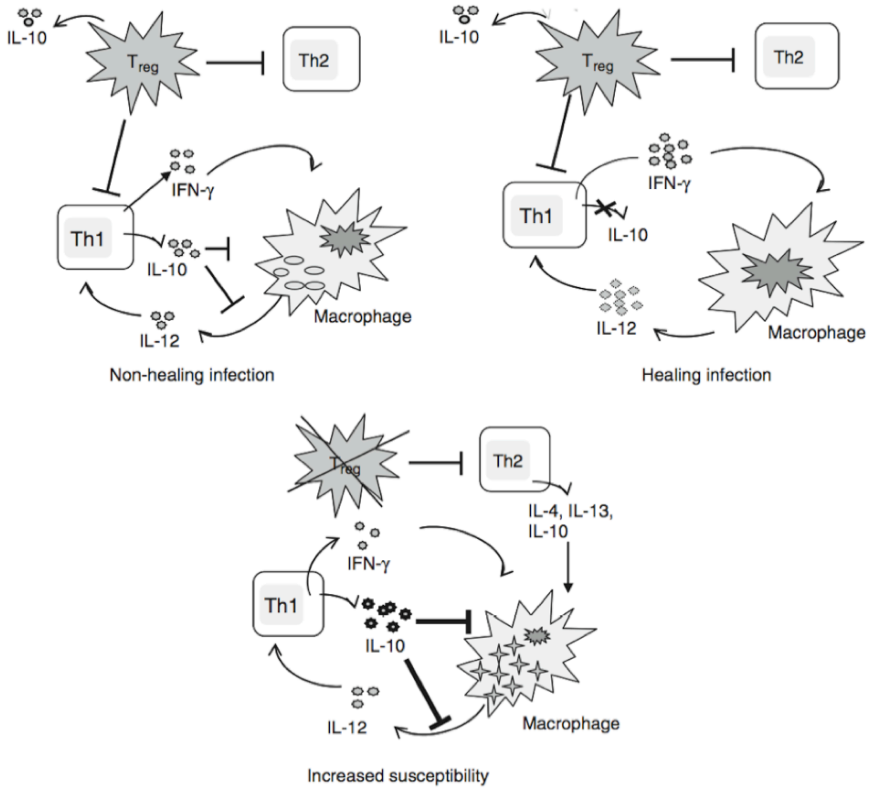


Figure 9. Immunological responses to *L. major* (49).

B cell-derived antibodies are important for adaptive immune responses. Antigen-specific IgG and natural IgG are responsible for opsonization (44). The role of B cells and antibodies in susceptibility depends on the *Leishmania* species and virulence factors. Downregulation of MHC class II presentation is normal, when parasites destroy it within PVs. Without antibodies, parasite uptake does not activate DCs and silent infection phase initiates. The role of antibody in pathogenesis mediates parasite uptake and regulates $CD4^+$ T cell activation and immune response at the local cutaneous site of infection. Nevertheless, systemic immune response to infection is not affected by antibody's absence. Though, a local immune response promotes parasite intracellular survival (25). Extracellular pathogens replicate outside the host cell, controlled by non-opsonic or opsonic phagocytosis. Th cells support neutrophils and macrophages by stimulating B cells, in order to produce antibodies and promote abscesses formation (40).

Leishmania species interactions with host cells are summarized on Table 3.

Table 3. *Leishmania* species-host interactions (50).

	L. major	***L. donovani***	***L. mexicana*** ***L. amazonensis***
Th1 relevance for cure	+	+	+
IL-12 resistance induction	+	+	-
B cells in pathogenesis	Dose dependent	Ab independent	Ab dependent
T cell deficient mice pathology	+	+	-
Virulence factors			
LACK	+	Unknown	-
LPG	+	+	-
A2	-	+	Unknown
Cysteine protease	-	Cathepsin B-like	Cathepsin L-like
Disease			
IL-4	Strain dependent	-	Host/Site dependent
IL-10	+	+	Somewhat

CD4 and CD8 T cells, NKs, and NK T cells mediate cytotoxic immune responses (13). $CD4^+$ and $CD8^+$ T cells are important for acquired resistance, sustained IL-12 production and parasite persistence (51). $CD8^+$ T cells develop immune memory and are involved in primary infection clearance (49). Cytotoxic mechanisms involve antigen-dependent or independent apoptosis of targeted cells (13), see Figure 10.

CD8 T cells produce IL-2, IL-4, IL-5, IL-10, IFN-γ, TNF-α and TGF-β. Two types of CD8 T cells have been proposed, T cytoxicity (Tc) 1 and Tc2 cells, depending on their cytokine production profile and migration ability (13). Tc cells release IFN-γ. IL-12 can induce Th1 and Tc1 cells responses (44). Cytotoxicity can then occur by lytic granules release, like perforin, and membrane expression of FasL/CD95L, which initiates apoptosis by caspase 8 activation. CD8 T cell response initiates when specific antigens presented by MHC class I molecules are recognized, in association with co-stimulatory signals on APCs. To become fully activated, they need CD4 T and DCs. DCs present antigens to CD4 T cells by MHC class II molecules, leading them to clonal expansion and differentiation, which upregulates CD40L expression. This interacts with CD40L, activating CD8 T cells that will further recognize MHC class I. If this does not happen, cells will not express CD40 and tolerance will occur. CD8 T cells have been associated to tissue damage. CD8 T cell activation through cross-presentation of apoptotic cells by DC can lead to immunity. CD4 T cells mainly regulate IFN-γ production. For *L. braziliensis* infection, the acute phase presents more CD4 T cells. During healing, CD8 T cells increase number until equilibrium. For *L. braziliensis*, CD8 T cells are also involved in chronicity and exacerbated tissue lesions (13).

HIV infected patients have lower CD4 levels, so leishmaniasis exacerbation can be seen (44). In macrophages, *Leishmania* parasites increase HIV-1 gene transcription and release of progeny virus, enhancing the production of pro-inflammatory cytokines. Amastigotes increase IL-6 and TNF-α production, which leads to higher viral replication (15).

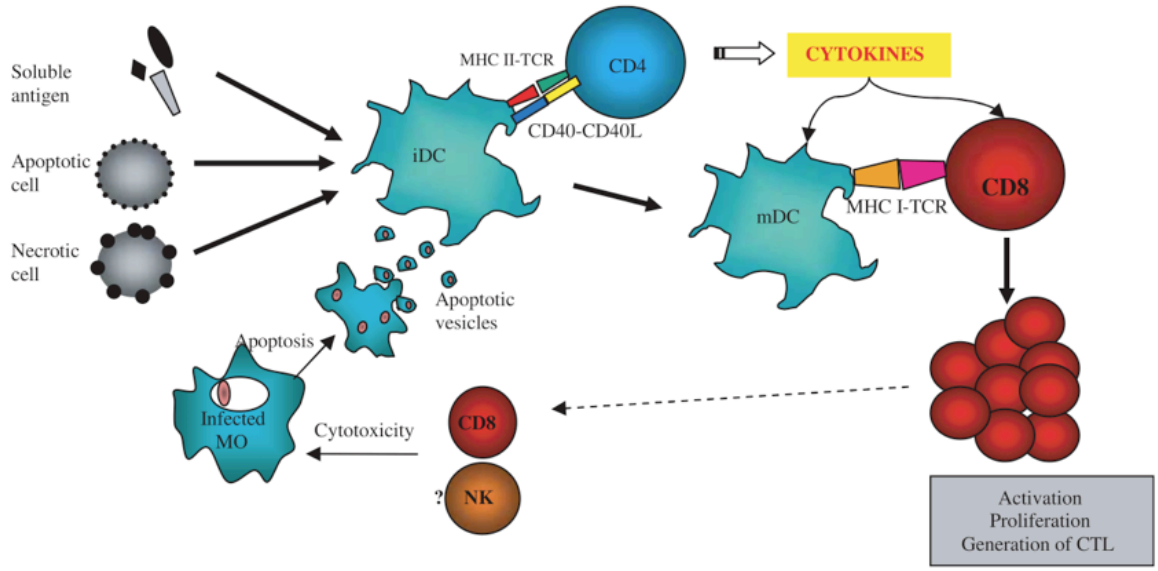


Figure 10. CD8 T cell activation by cross-presentation. iDC – Immature DC, mDC – Mature DC (13).

Leishmania species parasites are able to cause mice disease. This is a useful mean to study intracellular parasitism. However, its greatest value was the aid to understand the biochemical pathways responsible for Th1/Th2 immune responses dichotomy (25), see Table 4.

BALB/c mice do not control infection; they develop lesions and systemic disease. They are the animal model for non-healing human disease (51). BALB/c mice develop a typical Th2 immune response (49). Genetic susceptibility is related to IL-4 and IL-2 Th2 response, which causes disease. Th1 response releases IFN-γ, which leads to parasite clearance. In BALB/c mice infected by *L. major*, IL-12 drives to Th2 response and promotes resistance. Downregulation of IL-12 upregulates IL-4 production, which leads to disease. So, anti-IL-12 antibodies will exacerbate disease. Inoculation site might also influence T cell response, and DC subpopulations are able to induce Th1 or Th2 cells priming (51). Targeted disruption of IL-4 gene in BALB/c mice leads to high resistance, while IFN-γ and IL-4 gene disruption in C57BL/6 mice turns them into susceptible models (49). In BALB/c mice, LACK antigen induces IL-4 production by $V\beta4^{+}V\alpha8^{+}CD4^{+}$ T cells, which renders T cells resistance to IL-12 and leads to lesion development. Tolerance to LACK gives an enhanced Th1 response, a diminished Th2 response, and healing. IL-10

derived from $CD4^+CD25^+$ T_{reg} cells is responsible for maintaining latent infection in resistant mice, and ensuring long-lasting immunity (25). Neutrophil depletion leads to increased parasite elimination (43).

C57BL16 mice have localized dermal lesions that heal spontaneously. Healing is associated with immune activation of infected macrophages and parasites killing (52). Th1 response to infection does not eradicate all parasites, but provides cellular recruitment and parasite replication. Infection of T cell-deficient C57BL/6 mice with *L. major* or *L. donovani* is associated with parasite multiplication, dissemination, and disease exacerbation. *L. amazonensis* infection shows small lesions and low parasite levels. When *L. amazonensis*-infected mice are reconstituted with $CD4^+$ T cells, lesion development is restored. So, $CD4^+$ T cells contribute to susceptibility. For *L. major*, recruitment of macrophages occurs without T cells. *L. amazonensis* persistence in Th1 response is essential for macrophage activation, and macrophage recruitment is required for parasite survival and persistence (25).

L. mexicana and *L. amazonensis* produce chronic infections in most mice strains. IL-4 is crucial for initial *L. mexicana* lesion development, and chronic infection maintenance requires IL-4 and IL-13. For *L. amazonensis*, inhibition of IL-12Rb2 expression, independent of IL-4, is the main mechanism preventing Th1 response development and healing. There is a disease-promoting role for IL-13 during infection with both species. For *L. donovani*, protective immunity depends on an IL-12-driven Th1 response and IFN-γ production, which kills parasites by ROI and RNI production. Human VL shows a cytokine profile of mixed Th1/Th2 characteristics. IL-12 promotes Th2 and Th1 immune responses. IL-10 is the major immunosuppressive cytokine in VL. Neither IL-4 nor IL-13 exacerbates disease during VL. IL-10 has been suggested to be as important as IL-4 and IL-13 for *L. major* susceptibility (25).

Table 4. Genetic differences in phagocyte behavior in CL (44).

	BALB/c	C57BL/6
Neutrophils	+++	+
	Persisting infiltration	
	Depletion > ↑ Disease	Depletion > ↓ Disease
	Interaction of infected macrophages with apoptotic neutrophils	
	TGF-β + PGE_2 > Parasite persistance	TNF-α > Parasite killing
Macrophages	Less mature ($MRP14^+$)	More mature ($F4/80^+$)
	↓ Killing efficiency	↑ Killing efficiency
	↓ IL-12 release	↑↑ IL-12
DC	↓ IL-1α/β	↑↑ IL-1α/β
	IL-12p70	IL-12p70
	↑ IL-12p40	↓ IL-12p40
	↑↑ IL-12p80	↓ IL-12p80
	Unknown	IL-27

According to Stebut (2007), immune response against *Leishmania* parasites can be summarized into 4 distinct phases, see Figure 11:

1. *Leishmania* parasites activate complement system, and the parasite gets opsonized with C3bi and C3b. C3 breakdown attracts neutrophils. However, promastigotes are resistant to complement lysis. The CR3 provides *Leishmania* parasites entry into macrophages. This is a silent process and inhibits IL-12 synthesis pathway. This early phase of infection lasts for 4 to 5 weeks, without visible clinical skin affection. During this period, parasites differentiate into amastigotes that will induce macrophages rupture;
2. Innate immune system is activated. Skin lesions develop, and inflammatory cells are called to infection site, such as neutrophils, eosinophils, or macrophages. Macrophages induce proinflammatory cells recruitment, such as neutrophils, eosinophils, mast cells (MCs), and are involved in granuloma formation. TNF-α release from MCs promote neutrophil influx, which will further release MIP-1α/β and MIP-2, in order to recruit more macrophages. Neutrophils participate in granuloma formation and help to create protective immunity;
3. DCs $CD4^+$ and $CD8^+$ T cells migrate at the same time. DCs phagocytize amastigotes through Fc-γ R III and I. Induction of adaptive T cell responses by DCs in skin links innate to adaptive immunity. Infected macrophages and DCs, which are APCs, present *Leishmania* antigens to primed T cells, which can be primed by $CD4^+$ or $CD8^+$ T cells. Activated DCs are the only cells able to present both in MHC class II and I. Macrophages express low levels of MHC class II and co-stimulatory molecules and are unable to prime T cells. Phagocytosis mediated by Fc-γ R leads to *Leishmania* antigen presentation in MHC class I to CD8 T cells, and CR3-

mediated phagocytosis by macrophages leads to MHC class II presentation. DCs can induce Th1 or Th2 immune responses; it just depends on extracellular environment. In cutaneous infections, DCs preferentially induce Th1/Tc1 immunity. IL-12 family, such as IL-27 or IL-23, and IL-1 contribute to induce and maintain Th1 responses;

4. Infection control does not mean complete parasite clearance. Persisting parasites contribute to immunity perpetuation. Antigen persistence is important for T cell memory maintenance. Both effector memory T cells residing on skin and long life central memory T cells in lymph nodes require remaining parasites. Fc-γ R activation on infected macrophages induces IL-10 release, which prevents parasite elimination and promotes disease progression.

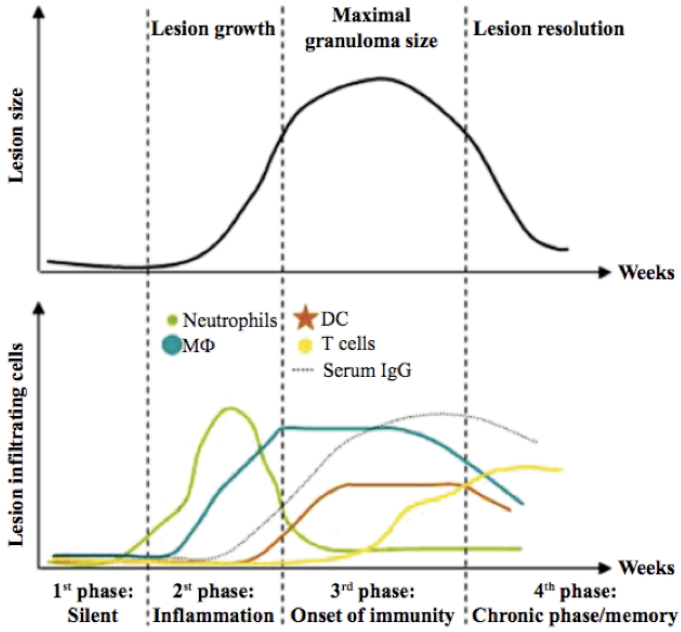


Figure 11. Immunological status in *Leishmania* infection. Lesion resolution coincides with DC infection and activation of T cells. Lesion development occurs when inflammatory cells are recruited to infection site. Later, lesion resolution is induced by DC-dependent recruitment of antigen-specifically primed T cells capable of producing IFN-γ (44).

Vaccination

Vaccination seems to be the better method for human disease eradication (21). If cured, a primary infection can provide protection against another infection. So, theoretically it is possible to produce a vaccine against *Leishmania* (53). Several vaccination strategies have been tested and a number of vaccine trials have been initiated without successful results (23). There are still no effective human vaccines yet.

Leishmania vaccination seems possible, because only a small percentage of individuals develop active disease in endemic areas and successfully cured patients rarely get re-infected. Many vaccination strategies have been tested for CL caused by *L. major*. Less effort has been done for VL, with attenuated or killed parasites, crude antigen fractions, purified *L. donovani* membrane proteins and DNA vaccines. Few vaccines have succeeded to reach phase I trials (49).

Th1 and Th2 immune response dichotomy for antigen selection in vaccine development has been used. *Leishmania* antigens that stimulate Th1 responses are regarded as potential protective antigens and promising future vaccines. Antigens that stimulate Th2 response are not interesting vaccine candidates, due to their possible relation with disease. Th1 response induction may not be protective, and should not be used alone for antigen selection (49), see Figure 12.

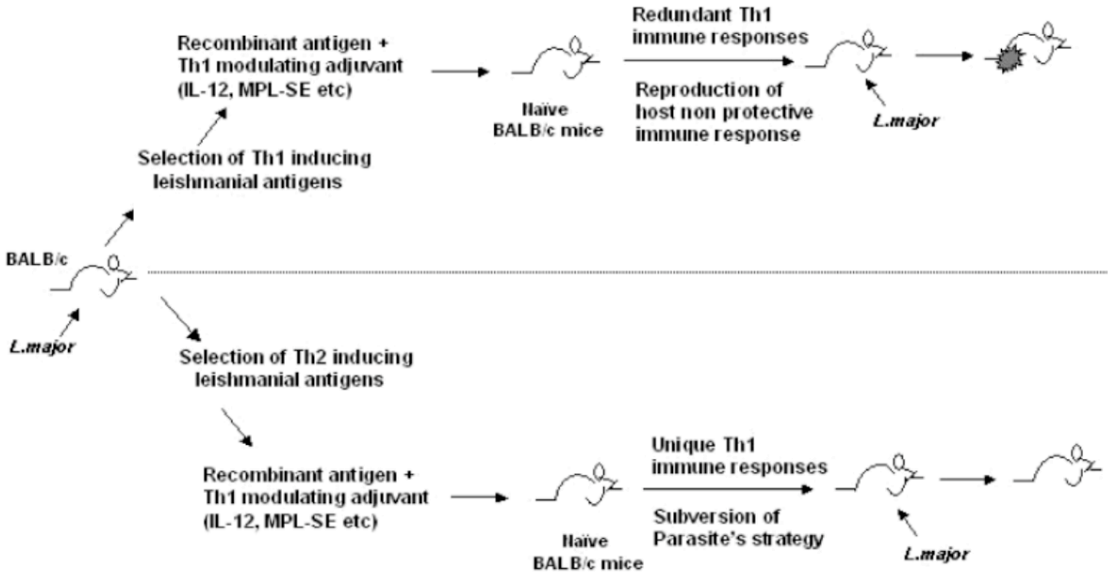


Figure 12. Th1 and Th2 paradigm in vaccine against leishmaniasis (49).

Live vaccination consists on virulent parasites inoculation in the arm and virtually provides complete and life-long immunization (51). Innate immune system modulation may be a valuable therapeutic approach, providing long-lasting immune protection. According to this approach, some vaccines use infected DCs or DCs loaded with antigen, and these are effective against leishmaniasis (44).

Non-living or protein-based vaccines promote poor $CD8^+$ T cell response and are less potent and lasting than live vaccines (51). Vaccination against *L. amazonensis* or *L. mexicana* depends on $CD4^+$, $CD8^+$ T cells and IFN-γ levels. Vaccines against New World species need to maintain a high-level immune response (25).

Currently, there are only 2 vaccines (1 live and 1 killed) licensed for human leishmaniasis, and 1 for canine prophylaxis. Anti-*Leishmania* vaccination can be based on live vaccines, vaccines with recombinant viruses and bacteria as delivery vehicles, vaccines based on purified *Leishmania* antigens, vaccines based on recombinant or on sandfly salivary antigens, or synthetic vaccines. The most promising vaccination strategy relies on vaccines composed of complex native antigens and well adjusted and developed adjuvants (54).

III – Therapy

1. Current Therapy

All leishmaniasis disease form has the same point in common. After infection by promastigotes, as explained on the previous chapter, these will be phagocytized and differentiate into amastigote form. Amastigotes are the ones who perpetuate infection and, eventually, lead to host cell death. This is why amastigotes must be considered the chemotherapy target.

Amastigotes and promastigotes have different morphology and biochemistry, which explains different drug sensitivity levels. The amastigote multiplies inside the macrophage phagolysosome, where pH is from 4.5 to 5.0. So, the molecular weight and pK_a of a molecule will affect its phagosomal accumulation. It also must be reminded that different species of *Leishmania* inhabit different phagosomes (55), reside in different macrophage types, and have different adaptation mechanisms (56). Therefore, it is expected that one drug or drug formulation could show different efficacy against the species and clinical manifestations leishmaniasis. VL and CL infection sites have different pharmacokinetics (PK), and infective species have different drug sensitivity (18).

Pentavalent antimonials are the recommended drug for VL and CL, and were introduced 60 years ago. Over the past two decades few alternative drugs or new formulations of old ones became available (57). The few drugs available are expensive, toxic, and most of them require IV administration. Because of these adversities, patients often do not complete the treatment, enhancing the chances of drug resistance development (18). Drug combinations can help to delay or prevent the emergence of resistance, increasing efficacy, or shortening the course of treatment (57).

CL can be treated intralesionally with antimonials, and antiseptics should be applied only if the ulcer is infected. Under other circumstances, all lesions should be treated systemically. VL is usually treated with injectable drugs, or with oral drugs, such as miltefosine. Generally, the injectable drugs are toxic and it is necessary to monitor the patient. Cure happens if, 6 months after chemotherapy, there is no fever nor splenomegaly (18). Table 5 summarizes current leishmaniasis therapy.

Table 5. *Leishmania*'s current therapy schemes (7).

Visceral			
Drugs of choice		Liposomal AmpB	3 mg/kg/d IV 1-5, 14 and 21
	OR	SSG	20 mg/kg/d IV or IM x 28 d
	OR	Miltefosine	2.5 mg/kg/d PO (max 150 mg/d) x 28 d
Alternatives		Meglumine antimonate	20 mg/kg/d IV or IM x 28 d
	OR	AmpB	1 mg/kg IV daily x 15-20 d or every second day for up to 8 wks
	OR	PM	15 mg/kg/d IM x 21 d
Cutaneous			
Drugs of choice		SSG	20 mg/kg/d IV or IM x 20 d
	OR	Meglumine antimonate	20 mg/kg/d IV or IM x 20 d
	OR	Miltefosine	2.5 mg/kg/d PO (max 150 mg/d) x 28 d
Alternatives		PM	Topically 2x/d x 10-20 d
	OR	Pentamidine	2-3 mg/kg IV or IM daily or every second day x 4-7 doses
Mucosal			
Drugs of choice		SSG	20 mg/kg/d IV or IM x 28 d
	OR	Meglumine antimonate	20 mg/kg/d IV or IM x 28 d
	OR	AmpB	0.5-1 mg/kg IV daily or every second day for up to 8 wks
	OR	Miltefosine	2.5 mg/kg/d PO (max 150 mg/d) x 28 d

1.1 Pentavalent antimonials

The pentavalent antimonials, see Figure 13, (meglumine antimoniate (Glucantime) and sodium stibogluconate (Pentostam, SSG) have variable efficacy against VL and CL, require parenteral administration, and are highly toxic. Antimonials should not be used in elder patients, in individuals with cardiac and renal disease and during pregnancy (58). The emergence of antimony resistance has jeopardized the treatment of VL in many countries, such as India (18). Variation in the clinical response is common (59). Antimonials are rapidly absorbed and excreted, and their half-life is around 2 hours (55).

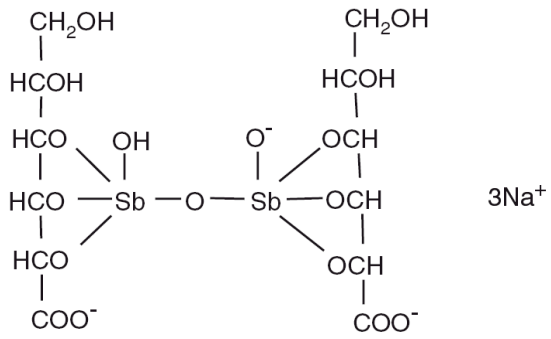


Figure 13. Sodium Stibogluconate (SSG) (18).

The mechanism of action of antimonials is still uncertain. However, they compromise the thiol redox potential of the cell by inducing the efflux of intracellular thiols and inhibiting trypanothione reductase (TR). Pentavalent antimony (SbV) enters the host cell, crosses the phagolysosomal membrane and acts against amastigotes. It is also likely that SbV is also converted to trivalent antimony (SbIII). Glutathione (GSH), glycylcysteine and trypanothione reduce SbV non-enzymatically under acidic conditions. Thiol dependent reductase 1 (TDR1) and arsenate reductase 2 (ACR2) catalyze the reduction, and ACR2 increases the sensitivity of *Leishmania* to SbV. SbV impairs energy metabolism, by inhibiting glycolysis and fatty acid oxidation (53, 59). SSG is a potent inhibitor of protein tyrosine phosphatases, which leads to increased levels of cytokines. So, SbV may kill the parasites by direct and indirect mechanisms, with the host response essential for SbV activity. SbIII and SbV have also shown to mediate DNA fragmentation in *Leishmania*, suggesting an induced apoptosis (59).

Now, antimonials are almost obsolete in India because of drug resistance. Nevertheless, they are still useful in the rest of the world, where generic brands lowered the prices (60), though resistance has started to be reported (58). The failure of clinical response results from acquired resistance and strains with low antimonials sensitivity. The inadequate treatment by unqualified personnel, the inability to follow WHO guidelines, or the use of poor quality drugs are some of the reasons for the increasing treatment failure (18).

SbIII and SbV routes of entry of in *Leishmania* are different. SbIII resistance can result from reduced uptake or increased efflux (53, 61, 62). Infection with Sb-resistant *L. donovani* induces the upregulation of multidrug resistance-associated protein 1 (MRP1) and permeability glycoprotein (P-Gp), avoiding antimonials accumulation. The inhibition of MRP1 and P-Gp leads to antimony accumulation and parasite killing within macrophages. Unresponsive VL patients overexpress P-Gp and MRP1 (63). At least 2 transporters of the ABCC family appear to be involved in antimony resistance: PGPA (MRPA) by gene transfection and the proline rich protein 1 (PRP1). Also, total thiols increase happens in metal-resistant *Leishmania*, due to overexpression or amplification of genes involved in trypanothione synthesis (53, 61, 62). Antimonials also inhibit DNA topoisomerase I (64).

Antimonials are cardio and embriotoxic (18). They can induce abdominal pain, anorexia, vomiting, nausea, myalgia, arthralgia, headache, malaise, T-wave inversion and prolonged QT interval (58). Drug hypersensitivity syndrome, drug reaction with eosinophilia and systemic symptoms, and thrombocytopenia induced by parenteral meglumine antimoniate were reported (65, 66). As was told before, SSG influences the

immune response which can explain the occurrence of PKDL after antimonial treatment (67).

1.2 Amphotericin B (AmpB)

AmpB deoxycholate, see Figure 14, (Fungizones) is an alternative drug for VL and it is produced by *Streptomyces nodosus*. It has been used as first line drug, due to the increasing resistance to antimonials. AmpB is as a systemic anti-fungal and a highly active antileishmanial. It is a highly toxic drug, so it requires careful and slow intravenous administration. Lipid AmpB formulations have been developed in order to improve the toxicokinetics and PK drug properties (18).

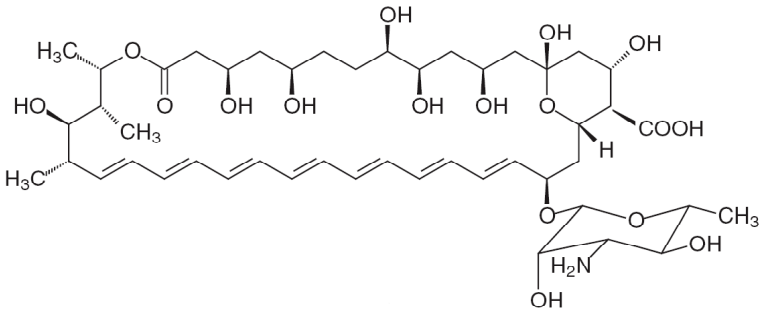


Figure 14. Amphotericin B (AmpB) (18).

In biological membranes, AmpB complexes with 24-substituted sterols, like ergosterol. These complexes open pores which alter ion balance and lead to cell death (56). Relapses after treatment can happen, mainly in HIV-positive patients, and resistance has already been induced *in vitro* by gene amplification. Also, membrane fluidity can be altered, which decreases binding affinity of AmpB (53).

Liposomal AmpB formulations, AmBisomes, are an approved treatment of VL. AmBisomes have reduced toxicity, better half-life, and high level of efficacy in VL treatment, with 90% cure rate. The main limitation is its high cost. Lesser expensive AmpB lipid formulations have also been used, but are less efficient and have higher toxicity. These novel AmpB formulations have been successfully used to treat CL in immunocompromised patients and children (68). In experimental VL models, AmBisome has hepatic accumulation, and reaches therapeutic levels faster than antimonials. It also has a longer half-life (69). These new formulations can lead to the emergence of resistance because of their longer half-lives, as reviewed by Croft and collaborators (59).

1.3 Miltefosine

Miltefosine (Figure 15) was first developed as an anticancer drug. Today, it is the first effective oral treatment for VL and the most recent antileishmanial drug to enter the market, being also an alternative treatment for HIV patients. Its oral administration provides a good PK profile (70, 71), facilitating treatment access, with low costs to health sector without the need of hospitalization. Nevertheless, the uncontrolled use of this drug can lead to toxicity and resistance events (72).

The major limitation in miltefosine use is its teratogenicity. Women of child-bearing age must take anticonceptionals during the treatment and for more 2 months (18). Also, there is a case report of PKDL developed after successful treatment of VL with miltefosine (73, 74). It has also shown to induce severe thrombocytopenia (66).

Miltefosine is effective and well tolerated in VL therapy. It can be recommended as the first line drug for childhood VL (75). The combination of miltefosine and AmpB or paromomycin (PM) is better than miltefosine and SSG (76). This could be helpful to treat antimony-resistant VL infections in India. Considering toxicity and cost, PM is the best option (57).

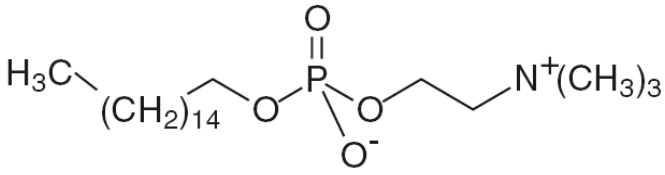


Figure 15. Miltefosine (18).

Miltefosine impairs the alkyl-lipid metabolism, and phospholipid biosynthesis (53). In promastigotes it induces an apoptosis-like cell death. Miltefosine stimulates the hematopoietic and immune system, leading to T cells, macrophage and IFN-γ activation (70). Miltefosine also promotes p38MAP kinase-dependent antileishmanial functions and IL-12-dependent Th1 response (77).

Due to its long half-life (120 hours) and uncontrolled accessibility, the emergence of resistance might become an issue. The combination with other drugs will help to avoid this. *In vitro* studies show that resistance is related to 2 point mutations on an aminophospholipid translocase, LdMT, and that miltefosine-resistant clones can be readily selected (18, 53, 78). Possible resistance mechanisms already described are: reduced drug uptake, impaired membrane permeability, faster metabolism and increased drug efflux. Multidrug-resistant *L. tropica* lines overexpressing P-Gp are less sensitive to miltefosine (59). Multidrug resistance 1(*MDR1*) gene may also be implicated (53).

1.4 Paromomycin (PM)

PM (Figure 16) is an aminoglycoside antibiotic with antileishmanial activity, and is produced by *Streptomyces rimosus* (79). This molecule is highly hydrophilic, has high molecular weight, and is relatively lipid insoluble (80). PM is used as a topical treatment for CL and as a parenteral drug for VL (60). For CL treatment, liposomal formulations have been developed (80). This molecule is off-patent and has received Orphan Drug status by US Food and Drug Administration (FDA) and the European Medicines Agency (EMEA) (18).

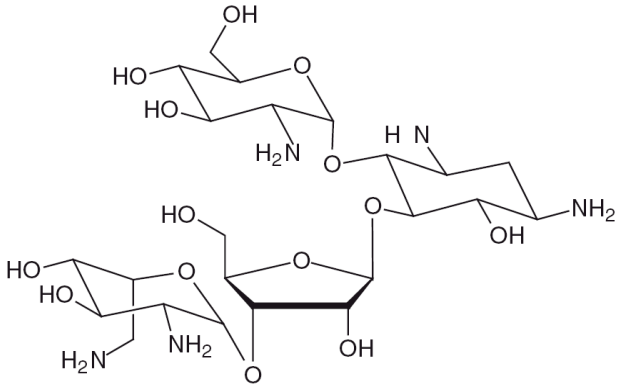


Figure 16. Paromomycin (PM) (18).

PM impairs the mitochondrial membrane potential, interacts with ribosomes, inhibiting protein synthesis, and leads to respiratory dysfunction. PM also alters membrane fluidity, lipid metabolism, and mitochondrial function (81).

PM has not been used extensively, so resistance is not a problem yet (53, 56). But at least 3 possible mechanisms of resistance are already known: reduced uptake or decreased cell permeability changes at the ribosomal binding sites, or production of PM modifying enzymes. PM does not induce cross-resistance (81).

1.5 Pentamidine

Pentamidine (Figure 17) has been used as second-line drug in the treatment of antimony-resistant VL. Pentamidine causes some toxicity, such as hypotension, hypoglycaemia, diabetes, and nephrotoxicity. In fact, its toxicity has led to its complete abandonment in India. However, this compound can still be valuable for combined therapies (53).

The cellular target of pentamidine is unknown, but it seems to bind to kinetoplast DNA. Resistance to pentamidine has been induced *in vitro* in several species, and it has been related to changes in intracellular concentrations of arginine and polyamines (56). *In vitro* pentamidine-resistant mutants are not able to accumulate pentamidine in the mitochondria and the drug is effluxed, by, possibly, PRP1 (53).

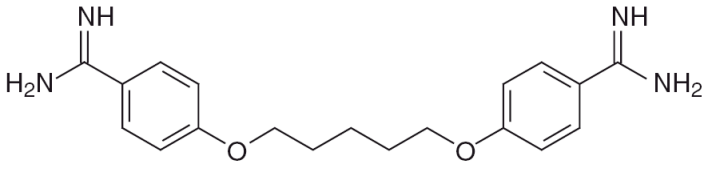


Figure 17. Pentamidine (18).

2. Future Therapy

In the last decades, the drug development of antileishmanials has not been a real purpose. The few advances done were limited to reformulation of already existing drugs and molecular screening, instead of rational design and immunology comprehension. New approaches led to novel compounds and molecular targets (60).

New ideal antileishmanial drugs must be active against CL (topical formulations) and VL (oral treatment), have a short period of treatment (less than 14 days), and have a single daily dose, have reduced IV treatment time, have lower toxicity, be safe for children and pregnant women, have lower cost, have robust formulations, and be suitable for combination with existing agents (82). Below, there are some examples of the most promising drugs being currently evaluated in clinical trials.

2.1 In clinical trials

Sitamaquine

Sitamaquine (Figure 18) is an oral 8-aminoquinoline, and little is known about its mechanism of action or resistance (18). Sitamaquine is a lipophilic weak base, which is rapidly metabolized, forming desethyl- and 4-CH_2OH derivatives that might retain its activity. Toxicity appears to be low, but it can cause mild methaemoglobinaemia and hemolysis. Sitamaquine seems to affect the electron transport chain (53, 56, 83). Another purposed mechanism of action is related to sitamaquine's fast accumulation in acidic compartments, such as acidocalcisomes. These vacuoles are involved in polyP and Ca storage, pH homeostasis and osmoregulation. Acidocalcisomes accumulation is different between species (84).

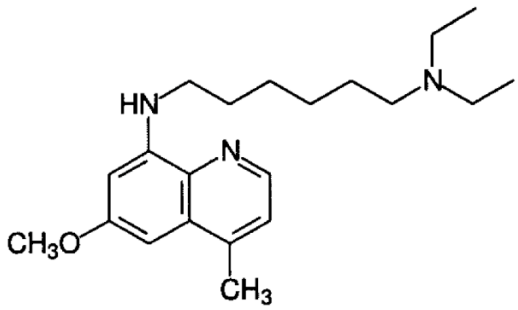


Figure 18. Sitamaquine (59).

Imiquimod

Imiquimod is an immunomodulatory imidazoquinoline used for the topical treatment of human papillomavirus. It is a potent inducer of IFN-α, TNF-α, IL-1β, IL-6, IL-8, cytoquines, and NO. Its target cells are monocytes and macrophages (85, 86). Imiquimod has been combined with antimonials to treat patients with CL, which were antimony-unresponsive. Alone, imiquimod is not effective as a topical agent (18, 78). The topical treatment activates localized macrophages, while the antimonial eliminates systemic amastigotes, which are responsible for infection persistence (78).

Allopurinol

Allopurinol is a purine analogue and is used as substrate by enzymes of trypanosomatids purine salvage pathway. It is selectively incorporated into nucleotide intermediates and nucleic acids in parasites. Because of its oral bioavailability and wide use for other clinical indications, clinical trials for VL and CL were already started. However, the results were not good. Right now, allopurinol is used as maintenance therapy in CanL (56).

Azoles

Azoles are antifungic drugs. *Leishmania* produces 24-substituted sterols, such as ergosterol. Azoles inhibit 14α-demethylase, a main enzyme in this biosynthesis pathway. Ketoconazole, itraconazole and fluconazole (*N*-substituted) have been submitted to several trials for CL and VL, but the results were equivocal (56, 87). Metronidazole and *N*-substituted azoles are well tolerated, and are useful in combination therapies (87).

Bisphosphonates

Bisphosphonates, such as risedronate and pamidronate, are used in bone disorders treatment. They have also shown activity against VL and CL in experimental models. Bisphosphonates accumulate in tissues susceptible to infection, are immunomodulatory, and are not toxic. They inhibit *L. donovani* promastigotes and amastigotes' proliferation (88). Bisphosphonates interfere with pyrophosphate metabolism, and the prime target might be farnesyl pyrophosphate synthase (FPPS) (56). In BALB/c mice, risedronate is effective for VL and pamidronate for CL. Overexpression of farnesyl diphosphate synthase was already identified as mechanism of resistance (89).

IV – Drug Discovery

1. Drug Discovery

Product development partnerships (PDPs) are non-profit companies focused on drug development for neglected diseases. PDPs manage new-product portfolios, preclinical and clinical development, decisions and clinical trials in developing countries. They manage R&D activities through partnerships with industry, and contract research organizations and academic groups. Some PDPs have R&D in their laboratories and manage major programs. Others mix these models. New drugs pipeline is not enough for most neglected diseases. Small-molecule discovery platforms should be available to create new hits and leads (3). The development of non-patentable drugs can make drug candidates available to anyone who wants to develop them. So, prices can be reduced. There are successful historical precedents for private companies developing drugs off patent, for instance, polio vaccines (4). Big pharmaceutical companies are placing early drug discovery in academic institutions, mainly for non-profitable diseases, such as rare genetic disorders and neglected diseases (90).

Private donors are providing a new generation of drugs for neglected diseases, such as the Institute for One World Health, from Bill & Melinda Gates Foundation, and the Drugs for Neglected Diseases initiative (DNDi). These donors are non-profit organizations, which gather resources for R&D, and act as virtual drug development industries. They manage the project and coordinate R&D funds. The aim is to create a robust pharmaceutical pipeline with new products registered every few years. Initially, known molecules with promising data in target diseases are acquired or new formulations or fixed-dose combinations are created. This synergistic approach has already produced new drugs for VL. Because many screening projects must be done simultaneously, there can be target overlap (3).

The DNDi was created in 2003. It is a non-profit Swiss foundation. Its partners are institutions from developed and developing countries, but the main is *Médecins sans Frontières*. DNDi goals are to develop new drugs for neglected diseases and transfer new technology (91). To identify opportunities in R&D, DNDi sends out calls for letters of interest to the scientific community. DNDi's portfolio currently has 9 projects at different stages of development for VL, sleeping sickness, Chagas disease and malaria (4). At discovery stage, DNDi is working on validating the kinetoplastid enzyme dihydrofolate reductase (DHFR) as a potential target for trypanosomatids, and on identifying inhibitors of the kinetoplastid enzymes TR and protein farnesyl transferase. At Institut Pasteur Korea (IPK), HTS on whole trypanosome cells is being made, in order to discover new lead compounds (92).

Several developments improved the ability to discover drugs for neglected diseases, such as genome sequencing, protein structures determination, compound libraries, and bioinformatics analysis (93).

Globally, there has been a decline in number of drugs against new therapeutical targets. Mainly, because of their low clinical success (94). Also, the regulatory authorities have been hampering the preclinical development. More sensitive biomarkers are needed to facilitate PK and toxicokinetics determination of new molecules (95). The FDA has been adopting higher drug approval standards, and is currently requesting extremely detailed safety data. This, will prolong clinical trials, increase costs and lower the chances of approving a new drug. So, pharmaceutical industry is maximizing sales from the most popular drugs by approving new formulations, adding indications and, when patent loss is close, follow-on compounds (96). These limitations to drug development led to a lack of new molecules which coincides with the development of combinatorial chemistry and HTS (95).

Little development has been made in antileishmania drug discovery. Many of the drugs currently used in therapy were developed for other purposes, and their antileishmanial use is just an extension of label indications (97). Drug development is very expensive, and resources available for antileishmanial research are sparse (56). So, rational development is useful, since it lowers the cost of drug discovery and/or improves those that already exist. It is essential to determine the leading compound structure-activity relationship (SAR), so the newly synthesized derivatives retain its activity (70).

1.1 Development of a new drug

Drug development can be divided into 3 main phases (Figure 19): *(i)* drug discovery, identification of biochemical, cellular or pathophysiological mechanism, identification and validation of a molecular target; *(ii)* drug development, identification of lead structure, followed by design, testing and fine-tuning; *(iii)* and commercialization (94).

Nowadays, HTS is becoming more useful to identify hits with significant activity. Biological screening has been the main technology used in hit discovery, but biophysical or *in silico* screening is also becoming popular. Hits validation is needed in order to eliminate artifacts and determine SAR. Then, these hits will be clustered and prioritized. If the hit belongs to a class of compounds, a hit series, it could be the starting point for derivative synthesis. Validated hits follow to hit-to-lead optimization, and chosen leads will have its PK and toxicity evaluated. In lead optimization step, new lead series derivatives

are synthesized, and have their toxicity, PK and pharmacology evaluated. If the project is successful, these leads can be tuned and follow to preclinical development (94, 98). It is common to centralize early phases of drug discovery until identification of lead series. Meanwhile, parallel screens with large compound libraries, aiming at drug targets, are done (94).

The drug discovery process used to last 7 to 12 years, but now it can be completed in 3 years. This time reduction in mainly due to HTS help in the identification of lead compounds from large libraries, which accelerates the process into lead optimization. Now huge amounts of derivatives can be generated and characterized (94, 98).

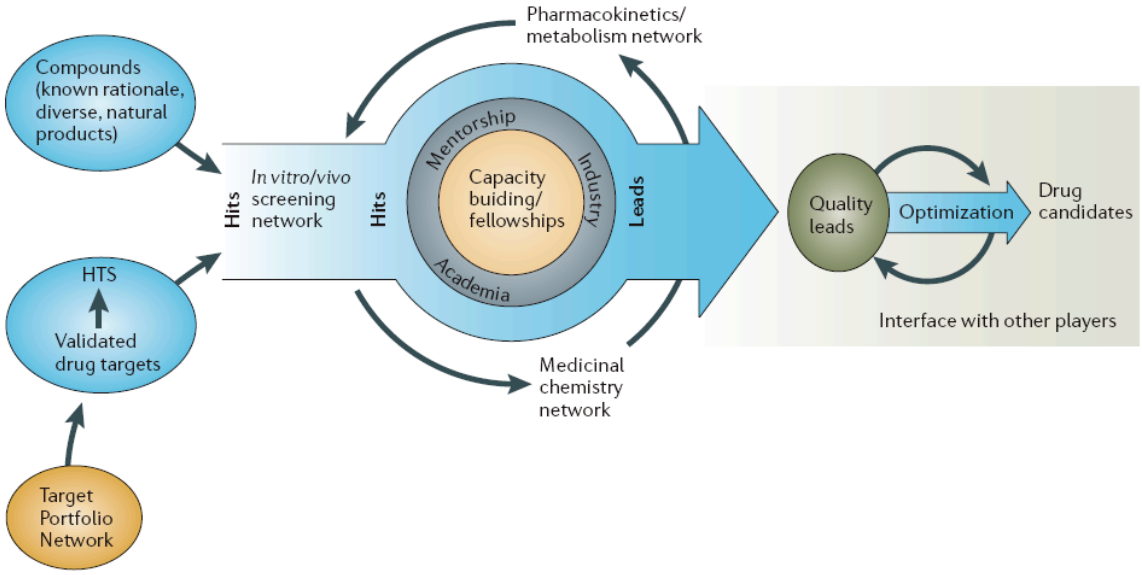


Figure 19. An innovative lead discovery strategy for NTDs (59).

1.2 Screening methods

The discovery of new drugs for NTDs has been carried by 3 different strategies (82):

• *Label extension* of existing treatment indications to NTDs. This approach reduces cost and time to market for new drugs. However, many companies do not allow their products to be developed for NTDs, because unexpected toxicities could deteriorate the drug image.

• *The 'piggy-back' strategy* is used when a parasite molecular target is being evaluated for other indications. SAR from parasite assays is not the same as the original indication. So, clinical candidates from will be mainly disease-specific.

• *De novo* drug discovery focuses on the identification of new chemical molecules. It relies on HTS and medium throughput screening (MTS) in whole-parasite assays against

specific proteins and whole parasites. HTS techniques for whole-cell screening have been preferred, because of its simpler use. Studies using compound libraries with registered drugs have led to great results. Also, chemo-informatics methods plus genomics, *in silico* screening, structural determination of proteins and their co-crystallization with small molecules, are being applied in drug discovery. The main disadvantage of this approach is the low throughput of the available assays, and the limited investment in development of new robust assays. So far, the target-based HTS has yielded few success stories, because many compounds active in target-based assays are inactive in whole cells. This can be due to the inability of the compound to permeate through cells and because the chosen targets are not in fact the real target. So, whole-cell-based HTS is becoming more useful and target-based HTS should be seen as complementary.

HTS has become one of the most used techniques for drug discovery in pharmaceutical industry (99). The development of a HTS assay has high maintenance and support costs. Besides having possible limited accuracy and low quality of the screening, it is highly compensated by the large number of compounds evaluated. Increasing the size of screening libraries did not led to new hit classes, and produced a high false positive rate (94). Computational filtering of the libraries optimizes the physical properties of molecules for drug likeness and rejects the unfavorable ones. Screening with compound libraries based on quality, instead of quantity, should increase the quality of data obtained from HTS (99). HTS has an automated process for plate preparation, liquid handling and detection. It also has data management for tracking, analysis, storage and visualization (93). Comparing HTS leads to their corresponding hits, it can be seen that leads are more complex, more lipophilic and more flexible than the original hits. HTS leads are more drug-like than lead-like. The molecular structure of HTS hits is kept during hit-to-lead optimization (94).

HTS primary screens have a single read-out to facilitate active compounds selection. For this, data from multi-parameter assays are reduced into crucial parameters for hit selection. Actually, most multi-parameter assays are seen as a single parameter read-out. Other parameters are only used to indicate toxicity or to divide sub-populations of cells. Secondary HTS screens are smaller and are more flexible. The goal is to understand the mechanism of action or toxicity. Selected parameters are scrutinized in a dose-response manner. Multi-parameter profiling provides more information about the mechanism of action of a compound than a biochemical screen. Compound activities are concentration dependent and non-specific toxicity occurs at high concentrations. The compounds must be compared at effective concentrations (100).

Alternative techniques have been used, but the pipeline always begins with HTS, unless a suitable assay cannot be established. Most of the non-HTS based hit discovery is fragment-based approaches, followed by virtual screening and natural-product-based approaches. The non-HTS hits are weak binders, and HTS-based leads are more lipophilic and have higher molecular weight. Fragment hits have low molecular weight and complexity, but leads are larger, and have similar physicochemical profiles to other leads. Natural products are more complex and more difficult to optimize. HTS achieve this optimization by lipophilicity, while fragments and natural products attain by complementarity and balanced properties. Increased potency is one of the main goals of the hit-to-lead phase, and it is lipophilicity dependent (94). RNA interference (RNAi) is also a screening method and it is very useful. A knock-out key gene and its related protein lead to impaired parasite function, and it may indicate a potential drug target (97).

Quantitative HTS (qHTS) increases drug discovery efficiency and provides a “chemical genomics” database. Complex biological responses can be read from curve shape. This technique bypasses chemical activity identification from post-HTS confirmatory assays to automated primary HTS (90).

High-content screening (HCS) method enables scientists to extract and better understand multiparametric data, produced by high-throughput cellular imaging. HCS is a valuable tool for biological pathways comprehension, efficacy and compounds safety characterization, screening identification and liable evaluation. This allows drugs acting on new targets identification with almost no knowledge about its pharmacology. It is a “target agnostic-nature” strategy (101).

1.3 Drug discovery strategy at Institut Pasteur Korea (IPK)

Usually, drug discovery process is developed for one specific drug target, and the drug is designed according to it. Instead of, for example, targeting an enzyme, the whole parasite cell is targeted. Then, by viability assays, the activity of the drug can be measured. The efficient drug is the first to be identified, and only after the target will be researched. The target-to-drug approach is not very efficient when compared to drug-to-target. The lack of promising molecular targets is clear and can be seen in this review. Many promising drug candidates are being evaluated as antileishmanials and their mechanism of action is not completely known yet. Many of these compounds were identified in natural products extracts screenings. It is also known that data obtained from compounds screening is less equivocal and in higher volume.

At IPK, the screening is made directly into the macrophage-amastigote model. Therefore, several steps are bypassed. Only the molecules able to penetrate through the macrophage and parasite will be efficient. The others will be ruled out, and the discovery process will become faster and simpler. Specific software was developed for these screenings and it enables automation of the process, turning it into a faster and more accurate method. Software provides better predictivity. HCS images with high quality can provide important information (101).

Large library of small compounds can be screened by HTS. These compounds are the molecules active backbones. Cellular imaging is used for the identification and provides accurate large-scale assays. After the identification of the hit compounds, SAR studies are made, derivatives are synthesized and the active scaffold is identified. After hit-to-lead optimization, the lead optimization will provide a molecule that will be submitted to *in vivo* PK assays. This molecule will have, approximately, a 100 times higher potency. The lead optimization process can be more difficult because of the ignorance of its structure.

2. Cell-Based Assays

A cell-based assay may be sufficient for preclinical development, if the phenotype of interest is well defined. Quantitative phenotypic profiles can help to predict SAR. The cell-based SAR may not be as precise as biochemical assays SAR, but it reflects the therapeutic value. Knowing a biological target can accelerate the hit-to-lead phase but is not a limiting factor (100).

Cell-based assays are increasingly being used in drug discovery because they reflect more accurately the complexity of the entire living organism. Cell-based screens are called black-box screens, and have higher hit rates than biochemical screens. It is mandatory to filter these hits for further development and evaluate their efficacy and toxicity. The cellular phenotype is complex and multiplexed measurements must be done. Filtering HTS hits requires high throughput, while studies to determine the mechanism of action and toxicity requires a greater range of read-outs. Multi-parameter profiling technologies and multi-parameter phenotypic profiling include transcriptional, proteomic and cell imaging measurements. Transcription profiling using a complementary DNA (cDNA) micro-array is a standard technique. Its application in MTS has limitations, such as high cost and poor results comparability. Quantitative polymerase chain reaction (qPCR), MS and bead-based immunofluorescence technology are higher throughput gene signature-based methods. So, they are better for large-scale profiling, but very expensive. Data analysis in phenotypic profiling goals is the stratification of hits, identification of mechanism of action and characterization of toxic mechanisms. Target or mechanism of action profiling can be made by comparing the phenotypic profiles of new compounds with reference bioactive compounds (100).

Fluorescence-activated cell sorting (FACS) and microscopy generate descriptors for every cell for each treatment. FACS and microscopy read-out phenotypic profiles of compounds in single cells and generate large amounts of data. So, active compounds in a specific sub-population of cells can be determined. The compound profile is the combination of response parameters of many sub-populations (100).

Proteins and their modifications are cellular activity biomarkers. So, proteomics is an important tool in target and biomarker discovery. Enzyme-linked immunosorbent assays (ELISAs) or immunoblotting-based protein assays are being used to monitor protein changes in HTS. Protein MS enables better fractionation methods and instrument action. Protein-compound interactions can also be monitored by MS. Quantitative methods can determine differential protein binding. The enhancement of multidimensional liquid chromatography (LC) and adaptation of a target-based approach could improve the

sensitivity and reproducibility of proteomic profiling. Proteomics is limited by low throughput of protein detection in complex mixtures by MS and the equipment's high cost (100).

Multi-parameter cell-based assays are screening and profiling tools for target, mechanism of action and toxicity identification. Screening must identify compounds with a desirable profile. These assays should have reasonable throughput, simple analysis matrices and robust criteria to discard toxicity (100). Comparative modeling may be useful to study new drug targets, identify binding sites for small molecules, suggest drug leads and optimize these. There is software that predicts the interaction between large-scale protein structures, known drugs and their ligand binding sites. These can increase the pipeline efficiency in target identification and validation, lead discovery, optimization and clinical trials. This approach can also help to determine the mechanism of action of already known drugs (102).

Another alternative is virtual screening (99). It is based on computational screening of large libraries which target of known structure, and their binding affinity is evaluated. If the molecular structure of a receptor is known, its function can be understood and predicted. New ligands have been discovered using this technology. The structures of known ligands in complex with their receptors are predicted. These docking screens rank the molecules by affinity. Virtual screening avoids syntheses and limits the search to compounds with biological relevance or drug likeness. Virtual screening follows the same guidelines as HTS. It has limited accuracy, but provides screening on a large scale. It produces many false-positive hits. Virtual screening is more accessible than HTS (103).

2.1 Biological Assays for *Leishmania*

The biological assays required to discover new drugs for leishmaniasis are *in vitro* assays with promastigotes and amastigotes of different species in dividing macrophages, which can indicate drugs' activity at achievable concentrations in serum/tissues and *in vivo* assays to study the molecule's PK (70, 78). A huge limitation of the amastigote-macrophage *in vitro* model is the non existence of automation and the need of microscopical evaluation (78).

In drug screening, promastigotes are not as useful as amastigotes. Amastigotes provide information about drugs' ability to permeate through biological membranes. The role played by the host cell on drug-mediated toxicity is important, limiting availability or favoring toxicity. The toxicity data against the host cell must be collected before testing against the amastigote (104).

Amastigotes derived from CL and VL strains are able to multiply *in vitro* in human monocyte-derived macrophages, and this model is closer to *in vivo* situation (27). THP-1 cells are a representative macrophage cell line useful as investigative model, which derive from a human with acute monocytic leukemia. These cells are phagocytes that produce lysosomes and esterases. 4α-phorbol 12-myristate 13-acetate (PMA) is used for THP-1 cell differentiation (105, 106). After differentiation, they become adherent and very similar to natural macrophages (105). THP-1 cells present same morphology, surface membrane receptors, oncogene expression and cytokines production as natural macrophages (107). Nevertheless, they have very different genetic expression from natural macrophages (105).

THP-1 cells undergo monocytic pathway changes with phorbol esters (PEs) incubation. PEs activate PKC, because they mimic diacylglycerol. 1,25-dihydroxyvitamin D_3 (VD_3) can also lead to this differentiation. Comparing both differentiation methods, PMA provides macrophage-like morphology, proliferation loss, CD11b expression, phagocytosis properties and prostaglandin E2 (PGE2)/TNF-α increased release. On the other hand, VD_3 leads to a monocytic-like morphology, no adherence, and it does not interfere with proliferation or PGE2/TNF-α production (108).

Intracellular amastigotes (InAs) (Figure 20) adhere to vacuoles and replicate slowly, so these do not rupture. Axenic amastigotes (AxA) do not show this limitation. InAs promote macrophage's functional and structural integrity maintenance. However, this varies with the infection stage. Inside PVs, amastigotes may degenerate and undergo cytolysis or apoptosis. Infection sites present high levels of InAs and few free extracellular amastigotes. Amastigotes replication is limited, but crucial for infection maintenance (109).

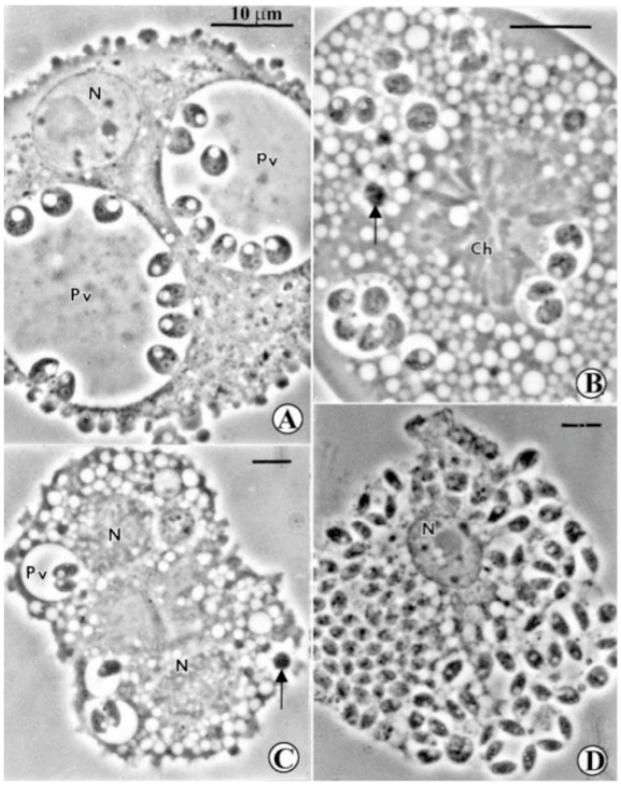


Figure 20. InA inside PV of J774G8 macrophages after long-term infection with *L. amazonensis*. (A) Amastigotes adhered to vacuolar membranes. (B, C) Amastigotes in smaller vacuoles in J774 cells undergoing mitosis. (D) Intact, but degenerating macrophage infected with more than 100 amastigotes. Arrows point to apparently degenerating amastigotes. Bar = 10 µm (109).

Amastigote-macrophage model is considered the “gold standard” for drug screening. Amastigotes can be isolated from short-term *in vitro* cultures in murine peritoneal macrophages (110), human macrophage cell lines (111) or infected tissues (112). Amastigotes isolated from lesions are often contaminated, exist in a limited number and present adsorbed host components (111).

InAs are the best approach for drug screening and their production in THP-1 cells is the most reliable *Leishmania* infection model (113). InAs are less time consuming, show biological parameters more similar to amastigotes isolated from lesions and do not present relevant contaminants (111).

AxAs are an alternative and more practical way for infection, which can be used in drug screening and vaccines research. However, long-term culture may promote promastigote-like features (113). AxAs need a culture medium with lower pH, higher temperature and a specific composition. Many biochemical profiles have shown similar morphological and biochemical features to parasites isolated directly from lesions. AxAs have comparable morphology, biochemistry, infectivity, cyclic transformation and immunochemical molecular characterization. Nevertheless, it must be reminded that AxAs are not in a natural environment, which fully mimics intracellular conditions. This is a huge

limitation comparing to InA. Also, promastigotes stage can be important for axenic differentiation. For instance, *L. amazonensis* and *L. mexicana* require metacyclic promastigotes in order to transform into AxA (111). On the other hand, *L. amazonensis* (35), *L. donovani*, *L. mexicana* (114), *L. peruviana* (106, 111) and *L. pifanoi* do not have this requirement and AxAs are relatively easy to obtain. Though, *L. major* does not provide good AxA production (111).

Promastigotes are easier to cultivate *in vitro* than amastigotes (111, 113), but they should not be used for drug screening purposes. They are not good biological models to screen immunomodulatory compounds. For this purpose, InA are preferred, because they cohabitate with immune system cells, such as macrophages. This way, a natural infection can be mimicked and immune response factors can interact with the screened drugs, providing more reliable data (115).

For drug screening optimal benefits, it is very important to characterize the promastigote population present in a given culture. In order to achieve higher infection rates, metacyclic promastigotes should be used. Standardization is needed, to diminish infectivity rate variation. There are many methods described in the literature for metacyclic promastigotes purification. However, none of them seems to be completely reliable. Metacyclic promastigotes purification by lectins, such as PNA, or monoclonal antibodies is based on oligosaccharides polymorphisms present on the parasites surface (116). Assuming that metacyclogenesis is triggered by lower pH and higher temperature, Luz et al. (2009) studied how preconditioning promastigote could influence infectivity ratio. This group study pH lowering, in order to check intracellular infection and its reliability. They were able to prove higher amount of metacyclic promastigotes in culture after induction, than for the spontaneous culture (117).

3. Cellular Imaging

Cellular imaging techniques are very interesting for target discovery, lead optimization, *in vitro* toxicology and compound profiling. It provides the visualization of a cell population, single cell or sub-cellular structures, with image-analysis tools. Currently, it is used for secondary screening and lead optimization. Generally, a 96- or 384-well plate is used for low-resolution application. The images are collected from each well at different magnifications and processed by integrated software, which analyzes each image, recognizes appropriate cell patterns and measures relevant features. The parameters must be robust, sensitive and reflect the biology. Although these assays are more robust, they are still complex and expensive (95).

The detection systems can be microscopes, fluorescence macroconfocal detectors or fluorometric imaging plate readers with charge-coupled device (CCD) cameras. These systems create a 2D pixel array of information from a biological sample. The imaging system should allow high-resolution analysis of single cells, high throughput, kinetic studies on live cells and efficient data storage and compression systems in user-friendly image-analysis programs. Modern imaging platforms are fast and able to test many conditions simultaneously (95).

Cellular imaging has been used in the analysis of fixed tissue or cell samples. Visible-light microscopy provides non-invasive 3D imaging, and it is used in laboratories to investigate targets in cellular phenotypes. Fluorescence microscopy is very important and confocal microscopy is very used because it is simple to use and allows the imaging of cells in optical sections at high resolution. Laser-scanning microscopes are similar to confocals, but with faster sectioning. Fluorescence resonance energy transfer (FRET) technology detects the transfer of energy from donor to fluorophore acceptor, providing better spatial resolution. This transfer can be captured by confocal or multiphoton microscopy. Temporal resolution of protein-protein interactions can be done by fluorescence lifetime imaging (FLIM). FLIM monitors detect changes in the fluorescence lifetime and analyze dynamic changes. FLIM and FRET can show evidence for physical interactions between proteins (95).

In flow cytometry, suspended cells are individually passed by a focused light source, and these are labeled with fluorescent antibodies. Flow cytometry can be used to isolate cells from a mixed population, identifying sub-populations. Flow cytometers are being increasingly used in biomarkers assays, to identify markers able to measure drug efficacy in preclinical and clinical stages. Automated microscopy and high-throughput flow cytometry make quantitative measurements for single-cell using multiple fluorescent

channels. Flow cytometry provides data on more cells because of its speed, is highly sensitive and easier to multiplex because of its simpler optical set-up. On the other hand, microscopy-based read-outs provide more information on each cell because it has superior spatial resolution. These differences are fading, because flow cytometers with imaging capability are being developed and microscopy read-outs have become faster. Still, it is easier to analyze non-adherent cells by flow cytometry and adherent cells by microscopy. Microscopy-based assays are better for HTS than cytometry, because fixed and stained cells can be stored.

Fluorescence microscopy has great potential to become a high-throughput and highly multiplexed profiling tool, because only a small number of cells are needed. HCS can measure many cell parameters, generating huge amounts of data. Nevertheless, live cell microscopy remains an interesting area in which HTS and image analysis are under development. Automated microscopy enables automated cell image acquisition and analysis, with high throughput and spatial resolution.

The challenges in phenotypic profiling are: combining data generated by HTS; implementing profiling on cell types; using data-mining methods and to get information; and developing technologies to acquire and handle single-cell and time-dependent information (100). Cytometric bead arrays and flow cytometry or fluorescence macro-confocal imaging allow seeing how disease phenotypes can be modulated. At low resolution, it is possible to quantify phenotypic changes, and at high-resolution mode, sub-cellular changes can be quantified (95).

3.1 Confocal microscopy

Several types of confocal microscopes are available. The most commonly used for fluorescence microscopy are laser-scanning fluorescence microscopy (LSM). These microscopes use lasers as light sources and collect images by scanning the laser beam. Lasers provide intense illumination within a narrow range of wavelengths (118). LSM can be classified as two photons LSM (TPLSM) or confocal LSM (CLSM). TPLSM has a better spatial resolution, and provides 3D reconstruction based on image stacking. This technology allows imaging in UV spectrum and deeper sample penetration (119). Two-photon excitation occurs when a fluorophore simultaneously absorbs 2 photons, each having half the energy needed to raise the fluorophore to the excited state. The light intensities required for simultaneous absorption occur at focal point, so only fluorophores at focal point are excited. It allows optical sectioning without spatial filter in front of the detector. The wavelengths needed to excite standard visible light fluorophores by two-

photon absorption are longer and penetrate tissue better than the wavelengths used for one-photon excitation, making it possible to look deeper into a specimen (118).

CLSM detects fluorescence point-by-point or line-by-line. Point scanning leads to higher contrast and resolution, but low scan speed. Spinning disk confocal microscope has a multibeam excitation or whole field imaging detection system, with higher speed, and less damaging for biosamples. There is a limited penetration depth, and there is a significant difference between axial and lateral resolution (120). LSM enables visualization deep in live and fixed cells and tissues, is 3D with high-resolution and is non-destructive (119). One of the major applications of CLSM is multiple label imaging. Confocal microscopy can merge a non-confocal transmitted light image with fluorescence images. Fluorescent proteins can be used as reporters of different spectral properties, providing multicolor labeling. Brainbow technique allows the use of 90 labels, being all these detected in a single sample with a standard laser scanning confocal microscope equipped with a spectral detector (121).

Confocal microscopes (Figure 21) are light microscopes that use lasers and very small apertures to produce images of thin layers of samples. It provides higher resolution and optical sectioning without out of focus light degrading the image. After, these layers can be reconstructed and a 3D image can be recreated. Confocal microscopy is often used with fluorescent techniques. Fluorescence can highlight and allow visualization of naturally or derivatized fluorescence.

Confocal microscopy can give qualitative information about topography, morphology and composition of the sample, in μm. Quantitative information is possible with counting methods. Confocal microscopy has the ability to control depth of field and ability to collect serial optical sections from thick specimens (119). Light is collected from thin optical sections, representing single focal planes. Focal plane structures are better defined (118). Thin section images can be produced, avoiding physical sectioning or compression. Confocal microscopy is very popular due to its high-quality images (119).

The light source is a laser, which uses the objective lens in the discriminator to focus it onto different planes on the sample. The sample is placed on a slide and on a stage. The objective lens is the discriminator, and it forms a real intermediate image that is detected by the photomultiplier tube (PMT). The PMT gets the image and transfers it to a computer for magnification. Then, the images are displayed digitally on a monitor and can be manipulated (119).

Fluorescent molecules excited by incident light emit fluorescence in all directions. The fluorescence collected by the objective comes to focus in image plane, which conjugates with the focal plane. A pinhole aperture in the image plane allows fluorescence from the

illuminated spot in the specimen to pass to the detector, but blocks out of focus light. The separation of in focus signal from out of focus background is done by pinhole aperture (118).

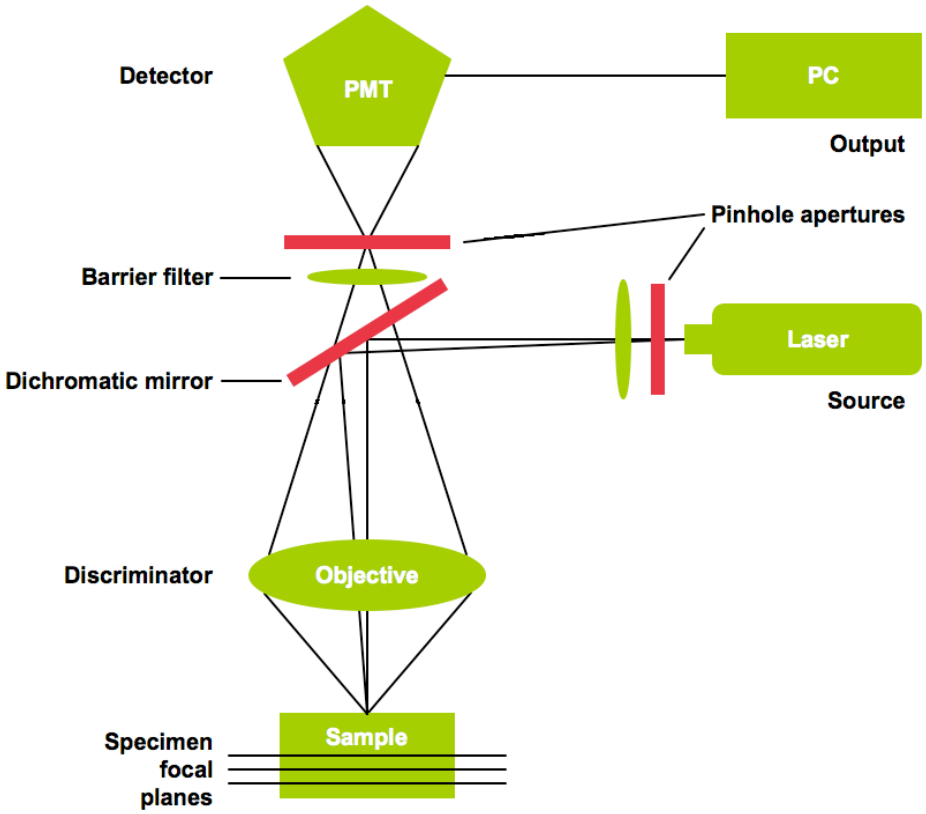


Figure 21. Confocal microscope schematic figure (118, 119).

V – Materials and Methods

1. Cells Culturing

Parasite's culture

Leishmania spp. promastigotes were cultivated in axenic M199 culture medium (Sigma-Aldrich™), supplemented with 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco®), 4.6 mM $NaHCO_3$ (Sigma®), 40 mM HEPES (Sigma®; pH 7.5), 0.1 mM adenine (Sigma®), 4 nM biotin (Sigma®) and 10% heat-inactivated fetal bovine serum (FBS) (Gibco®) for *L. major* (MHOM/IL/81/FRIEDLIN), *L. donovani* (MHOM/ET/67/HU3) and *L. amazonensis* (MHOM/BR/73/M2269), and 20% FBS for *L. braziliensis* (MHOM/BR/2903).

L. major, *L. amazonensis* and *L. braziliensis* cultures were started at $5x10^5$ parasites/mL and *L. donovani* cultures at 10^6 parasites/mL. Promastigotes were counted on C-Chip hemocytometers (InCyto) after being fixed with a 4% paraformaldehyde (PFA) (Sigma-Aldrich™) solution.

Maintenance cultures were grown in T75 flasks (Nunc™) at 28 °C with closed caps in a final volume of 10 mL. These were sub cultured every 3 to 4 days. Cultures used for macrophage's infection were grown in T175 flasks (BD Falcon) at 28 °C with closed caps in a final volume of 100 mL. For infection assay number 2 and 3, 24 hours before each infection, 5 mL of *L. donovani* culture were incubated at 37 °C and the other *Leishmania* species at 34 °C.

Macrophage's culture

THP-1 cells were cultivated in suspension at a density of $5x10^5$ cells/mL and Raw 264.7 cells at $8x10^5$ cells/flask in RPMI 1640 medium (WelGene) supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin and 10% heat-inactivated FBS. Both cell lines were incubated at 37 °C with 5% CO_2. Subcultures were made every 3 to 4 days. THP-1 cells differentiation into macrophages was induced by PMA addition (Sigma-Aldrich™), and then incubated for 48 hours, in a final solution of 50 ng/mL.

2. Infection Assays

Before infection, all cells were counted, centrifuged (promastigotes at 2500 rpm and macrophages at 1500 rpm for 5') and resuspended in RPMI 1640 medium. Then, 10 µL of promastigotes suspension were added to 40 µL of THP-1 or Raw 264.7 cells (50 parasites for 1 macrophage host cell) in Greigner 384-wells Plate (Bio-One).

Infection with *L. donovani*, *L. major*, *L. amazonensis* and *L. braziliensis* was made both for THP-1 and Raw 264.7 cells. One infection plate was done for each day of parasite's culture, from the 3rd day until the 10th. Then, the infection was kept for 6 days and the plates were incubated at 34 or 37 °C, in a 5% CO_2 atmosphere.

24 hours after, infection was washed with a phosphate buffered saline (PBS) 1x solution (Welgene). First infection day columns were fixed with 2% PFA solution and the remaining infection day's columns were added 50 µL of RPMI 1640 medium. After, every 24 hours, the corresponding infection day column was washed and fixed.

When all columns were fixed, the plates were again washed with PBS 1x solution and stained with 5 µM Draq5 (Biostatus), in 4% PFA solution. Infection plates were then read in Evotec Technologies Opera™ (Perkin Elmer) and in ImageXpress Ultra (Molecular Devices). The images acquired were analyzed. Parasite infection ratio and number of amastigotes per infected macrophage were determined.

In total, 4 whole infection assays were done. Below, there is an overview of what was done for each experiment set.

Infection assay number 1

All parasites were incubated at 28 °C and infected cells at 37 °C. Infection plates were read in Evotec Technologies Opera™, with 20x magnification. The plate format used is represented on Figure 22

PX		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
		THP-1												Raw 264.7											
		1		2		3		4		5		6		1		2		3		4		5		6	
		28	37	28	37	28	37	28	37	28	37	28	37	28	37	28	37	28	37	28	37	28	37	28	37
A	NI																								
B																									
C	LD																								
D																									
E																									
F	LM																								
G																									
H																									
I	LA																								
J																									
K																									
L	LB																								
M																									
N																									
O	NI																								
P																									

Figure 22. Plate format used for infection assay number 1, incubation made at 37 °C.

Infection assays number 2 and 3

For each infection day, promastigotes from 2 different incubation temperatures were used, in order to mimic vector and host temperature. Vector incubation temperature was 28 °C for all *Leishmania* species, but host temperature was 37 °C for *L. donovani* and 34 °C for the other remaining species. The plates were incubated with 5% CO_2 at 37 °C, for *L. donovani*, and at 34 °C for the other *Leishmania* species.

Parasites were incubated at 37 °C 24 hours prior to infection. Cells infected by *L. amazonensis*, *L. braziliensis* and *L. major* (CL and MCL strains) were incubated at 34 °C, and cells infected by *L. donovani* (VL strain) at 37 °C. After, all infection plates were read in Evotec Technologies Opera™, with 20x magnification and, only for infection number 3, also with 40x. The plate formats used are represented below. On Figure 23, there is the plate format representation used for incubation at 34 °C and on Figure 24, the one used at 37 °C.

PX		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
		THP-1												Raw 264.7											
		1		2		3		4		5		6		1		2		3		4		5		6	
		28	34	28	34	28	34	28	34	28	34	28	34	28	34	28	34	28	34	28	34	28	34	28	34
A B	NI																								
C D E	LM																								
F G H	LA																								
I J K	LB																								
L M	NI																								
N O P																									

Figure 23. Plate format used for infection assays number 2 and 3, incubation made at 34 °C.

PX		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
		THP-1												Raw 264.7											
		1		2		3		4		5		6		1		2		3		4		5		6	
		28	37	28	37	28	37	28	37	28	37	28	37	28	37	28	37	28	37	28	37	28	37	28	37
A B C D																									
E F	NI																								
G H I	LD																								
J K	NI																								
L M N O P																									

Figure 24. Plate format used for infection assays number 2 and 3, incubation made at 37 °C.

Infection assay number 4

All parasites were incubated at 28 °C and infected cells at 37 °C. Infection plates were read in Evotec Technologies Opera™, with 40x magnification, and in ImageXpress Ultra, with 20x magnification. The plate format used is represented on Figure 25.

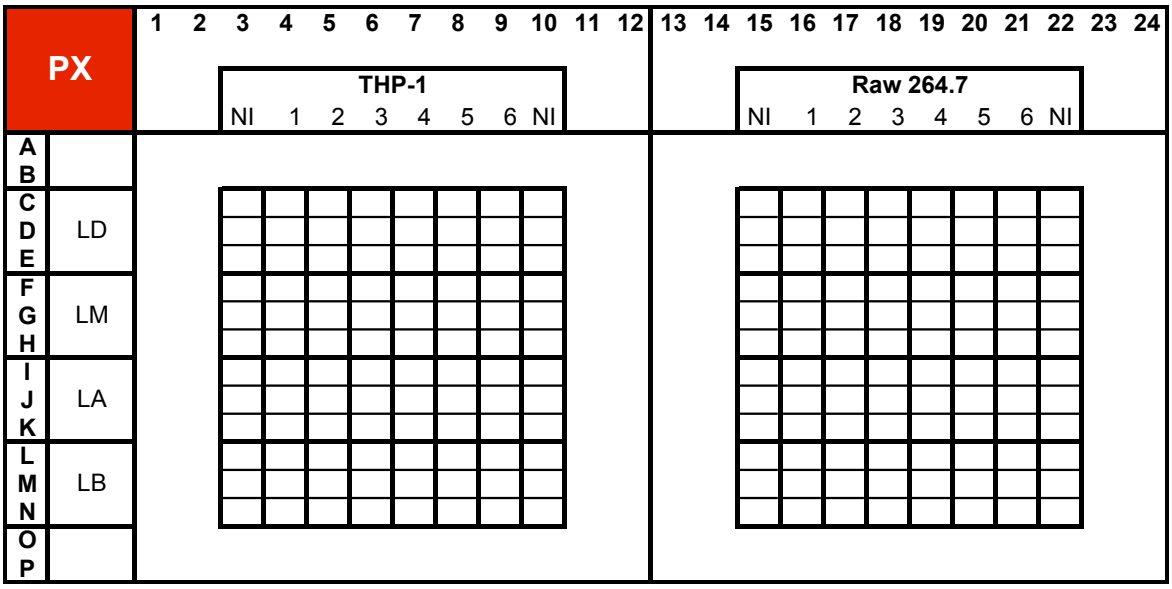


Figure 25. Plate format used for infection assay number 4, incubation made at 37 °C.

3. Replication Assays

Infection was carried on 24-wells plates (Corning) using coverslips and done as the infection assay described for 384-wells plate. Wells supernatant was rejected 24 hours post-infection and coverslips were incubated 12 hours with 1 mM bromodeoxyuridine (BrdU; Sigma®) and deoxycytosine (dC) (GE Bioscience) solutions, in a final volume of 200 µL.

Then, coverslips were washed 3 times with PBS 1x solution, incubated with absolute methanol (4 °C, 10 min; Merck), HCl 1.5 M (4 °C, 15 min; Fluka, Sigma®) and Triton X-100 0.1% (4 °C, 10 min; Sigma-Aldrich™). All solutions were prepared in sterile PBS 1x solution.

In order to show cellular replication, the fixed infection was probed with primary antibody anti-Br (1:400 dilution in 4% bovine serum albumin (BSA); Molecular Probe, Gibco®) and secondary antibody anti-IgG Alexa Fluor 488 (1:400 dilution in 4% BSA, 4 °C, 45 min; Molecular Probe). Then, DNA was stained with DAPI, (1:2000 dilution in 4% BSA, 4 °C, 45 min; Sigma®). Between steps, coverslips were washed twice with PBS 1x solution.

In the end, probed coverslips were placed in glass slides with 2 µL of Vectashield (VectorLabs). Analysis was carried in Nikon Eclipse 90i epifluorescence microscope.

4. Image and Data Analysis

Our aim in developing an image-processing pipeline is to detect cell infection ratio under the conditions described in previous sections of this chapter. In doing so, we must detect intensity patterns of *Leishmania* parasites within the cytoplasm of cells in images. This requires 2 distinct detection methods for cytoplasm regions and for a number of parasites within the detected regions. For the detection of cytoplasm regions, the following preprocessing steps are applied to the images (Figure 26):

- Background removal;
- Resizing;
- Gaussian blurring and local intensity maxima.

The purpose of the first preprocessing step is twofold. A successful removal of the background greatly improves the processing time. Secondly, it reduces the number of false positive detections of cytoplasm. In order to carry out the first preprocessing step, we must first obtain a range of background intensity values from the collection of acquired images. Also, since the images were acquired under the same conditions, we may safely assume that there exists such range of values we may apply across the whole collection of images. For our screening purposes, 3 scientists were assigned to 3 different subsets of images for random sampling of background intensities. All 3 reported that the background intensities range, from which we applied a threshold for background removal step. The second preprocessing step is also to reduce the processing time of the detection method. Originally 2000 by 2000 images are reduced to 1000 by 1000 by bilinear interpolations of pixels values. The final step of the preprocessing is to detect intensity local maxima with a given window size where we locate the positions of nuclei. The assumption is that there exists only one nucleus per cell such that the locations of the local maxima coincide with the center of the nuclei. These local maxima positions are used as a discrete set of points for Voronoi tessellation for detection of cytoplasm areas. By overlapping the detected areas of the cytoplasm with the parasite detection, which we will explain in the following paragraph, we may compute infection ratio.

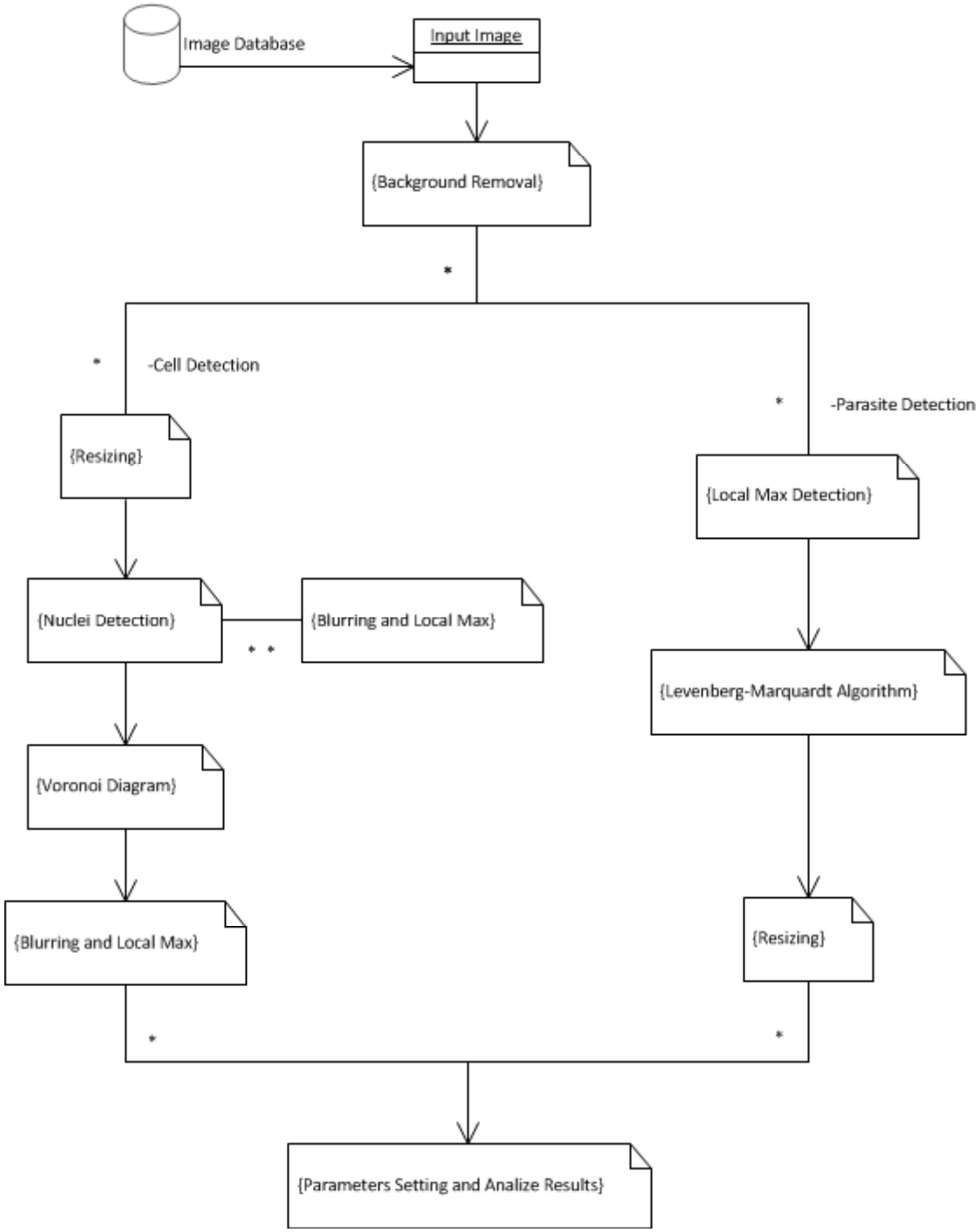


Figure 26. A diagram of the pipeline for image processing unit of leishmaniasis infection rate detection algorithm.

Parasite detection method shares the first preprocessing step as the cell detection algorithm. It also initiates detection by locating local maxima. However, the window size of the local maxima detection is fit to model parasite sizes, rather than the sizes of nuclei. As the 3D plot of intensity values of cell body and parasite in Figure 27 shows, the number of connected pixels that compose parasites is much smaller than that of the nuclei. Therefore, we may obtain salient points, which may potentially be the center of parasites

by detecting the local maxima. Once the salient points are obtained, the method builds a parasite model from a randomly selected set of point locations. The procedure of building of the model broadly involves the following sequential steps:

- A manual user selection of random point locations for training set data;
- A bidirectional decomposition of neighboring pixels at the selected points;
- Computations of a range of means and standard deviations by Levenberg-Marquardt algorithm.

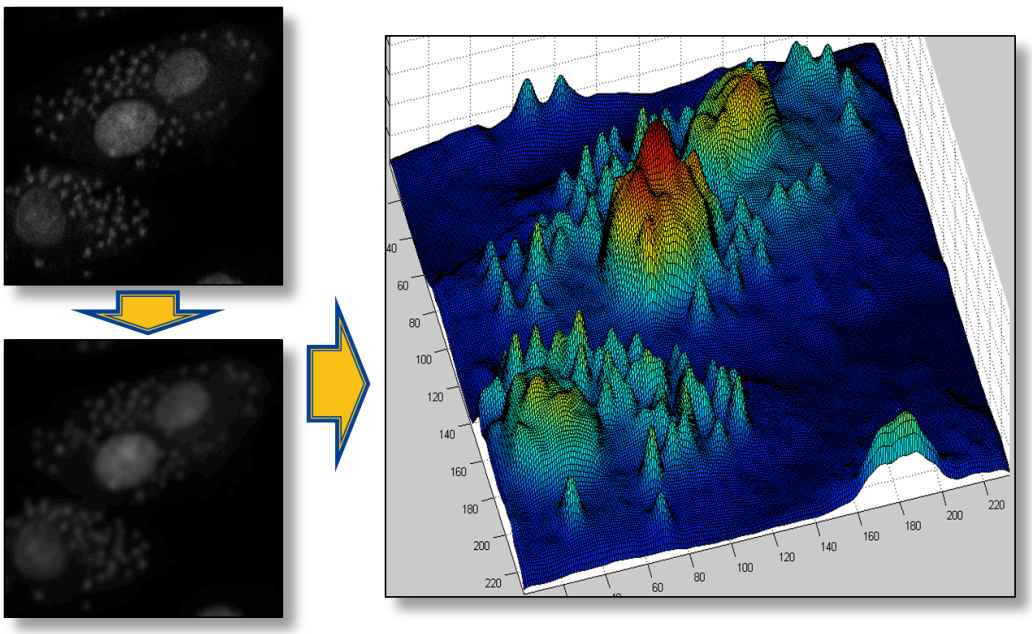


Figure 27. Intensity patterns of *Leishmania* parasites compared to the patterns of nuclei.

In essence, the method determines the Gaussian distribution (Figure 28) fitting shapes of parasites through solving least squares curve fitting problems with the user supplied local data. By selecting a wide range of different shapes of parasite, the method is ensured to obtain a reliable representation of parasite model across the collection of acquired images. The method then measures how well the locations of the local maxima represent the model acquired by computing the distance between the decomposed distributions at the locations and the model we built from the train set data.

The 2 different detection methods described above are diagramed in Figure 26. The 2 processes run in parallel then overlap at the last stage of the overall process to compute the infection ratios by counting the detected parasites inside each detected cell region.

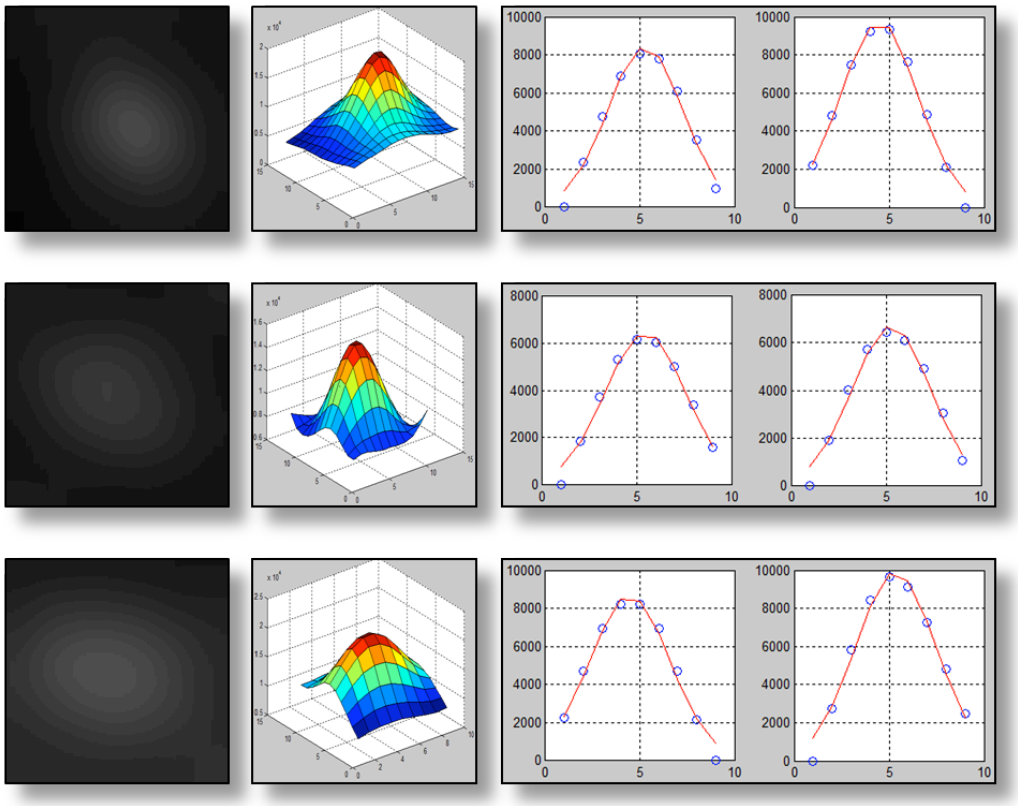


Figure 28. A Gaussian modeling of *Leishmania* parasites pattern on HTS acquired images.

VI – Results and Discussion

1. Infection Images

The results presented on this dissertation derive from a phenotypic interpretation of infection development in 4 *Leishmania* species, in 2 macrophage cell lines. Some images acquired during infection assays are shown as examples in Figure 29 and Figure 30. These figures respectively illustrate THP-1 and Raw 264.7 cells infected with *Leishmania* parasites throughout 6 infection days.

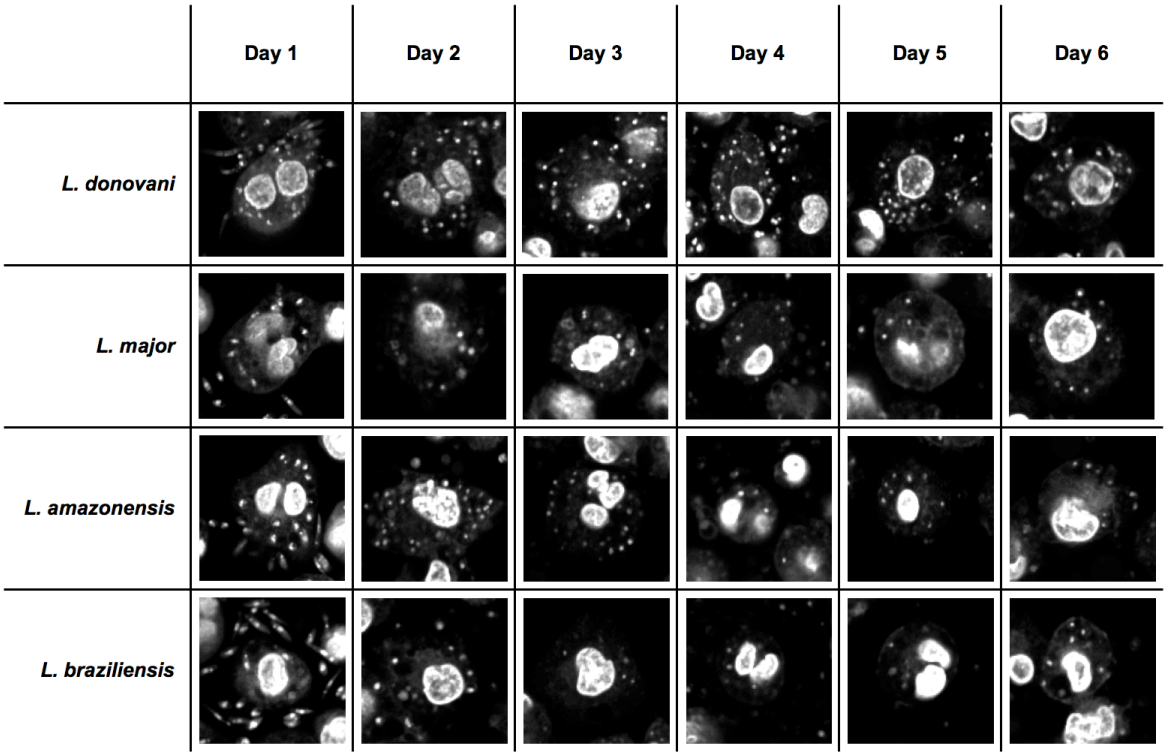


Figure 29. THP-1 cells infection with promastigotes from 6th day of culture.

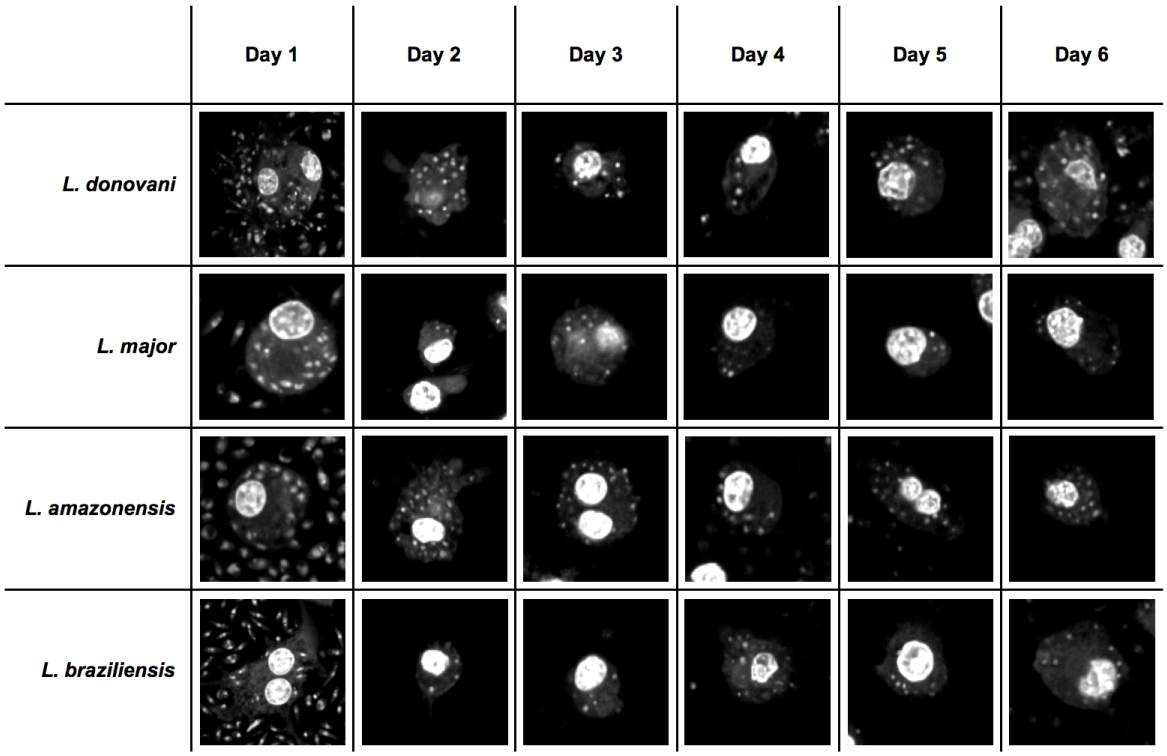


Figure 30. Raw 264.7 cells infection with promastigotes from 6th day of culture.

From these pictures overview, it could be hypothesized that THP-1 cells showed higher infection ratios, and InA were easier to detect. THP-1 cells stop dividing after PMA differentiation. So, there was always a constant cells' confluence on the well's bottom, proving better and more consistent results. On the other hand, Raw 264.7 cells kept replicating and on the last days of infection, cellular debris could be seen. The cell monolayer was not uniformly distributed and led to more variable results.

In order to confirm visualized impressions and hypothesis, software was especially developed to allow automated and unbiased image mining analysis of the data.

2. Image Mining

Infection assays' images were acquired by confocal microscopy. So, it must be taken in consideration that only one single focal cell plane was acquired. This fact implicates that not all parasites on the well bottom were detected.

Analyses were performed in an automated fashion by an algorithm developed for image analysis. Automated analysis from software detection was susceptible to misinterpretation of few images, such as identification of false parasites (artifact or chromatin condensation) and not identification of true parasites. Software was optimized in order to reduce as much as possible these false positives and false negatives. It was possible to adjust and control some parameters for data analysis, optimizing software accuracy in the phenotypic interpretation (Figure 31).

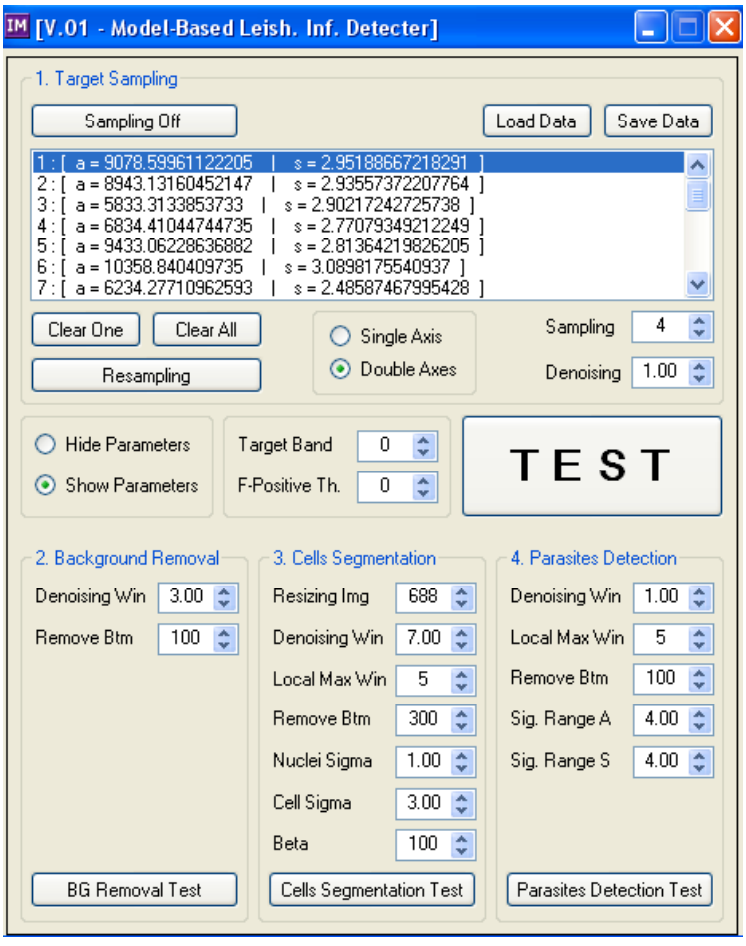


Figure 31. Software tuning interface. It enables control of background removal, cell segmentation and parasite detection parameters. After careful tune, these parameters can be saved and loaded later for another experiment analysis.

Figure 32 illustrates a confocal microscope image and how the software developed at IPK analyzes it. This image results from all the tuning made by interacting with the software, and it could be improved by constant testing at anytime.

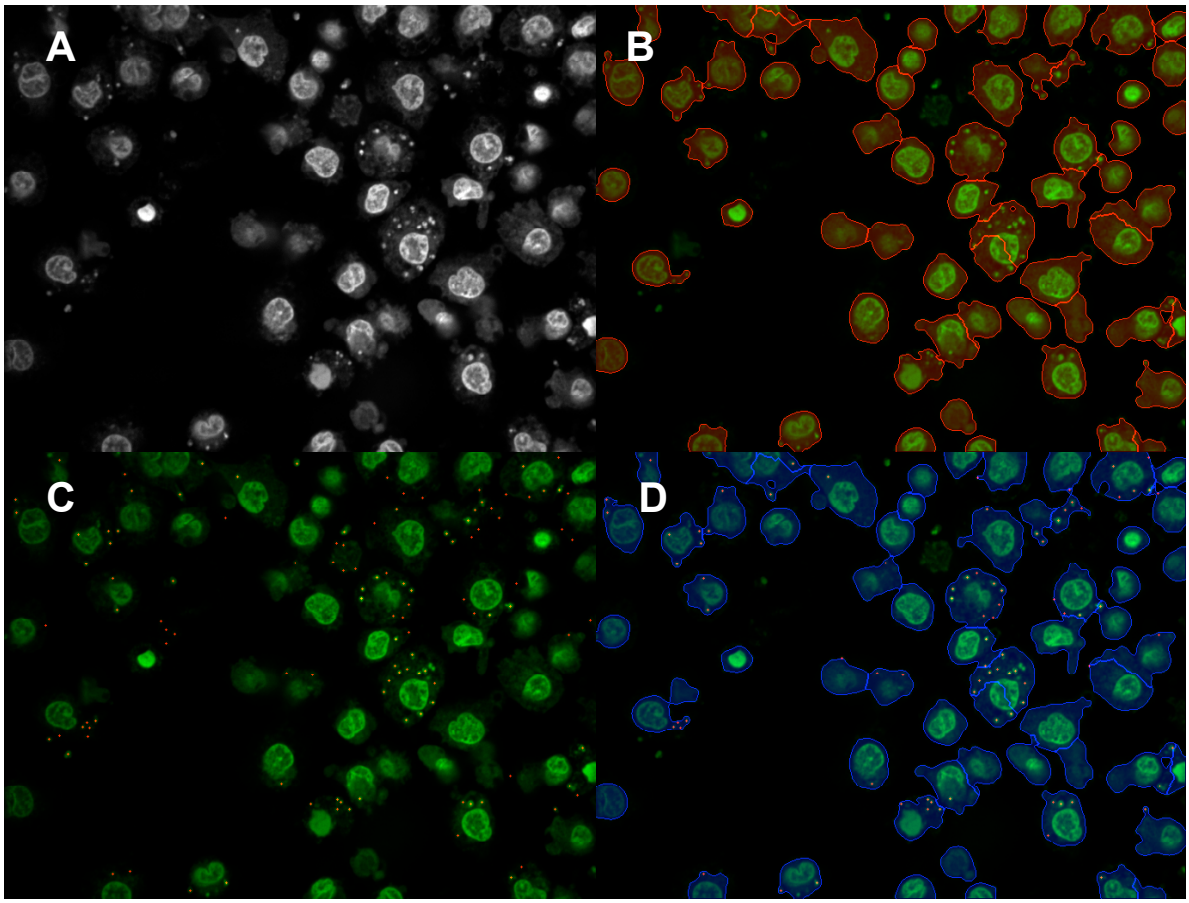


Figure 32. Software analysis of confocal microscope images. The original image (A) is tested for cell segmentation (B) and parasite detection (C). Then, software analysis image D.

The original images were subjected to many different analysis parameters, in order to mine as much information as possible. As a first step, sampling of the image was done, in order to determine the expected amastigote's intensity inside host cells. After background removal, the cells were identified and segmented (Figure 32 – B). Then, parasites were detected, as shown in red dots from Figure 32 – C. Figure 32 – D illustrates the interpretation of software analysis, after algorithm implementation over the raw image.

Figure 33 shows non-infected macrophages. The software detects some few false parasites. Results can be normalized after the knowledge of (false) infection ratio of non-infected macrophages, the negative control.

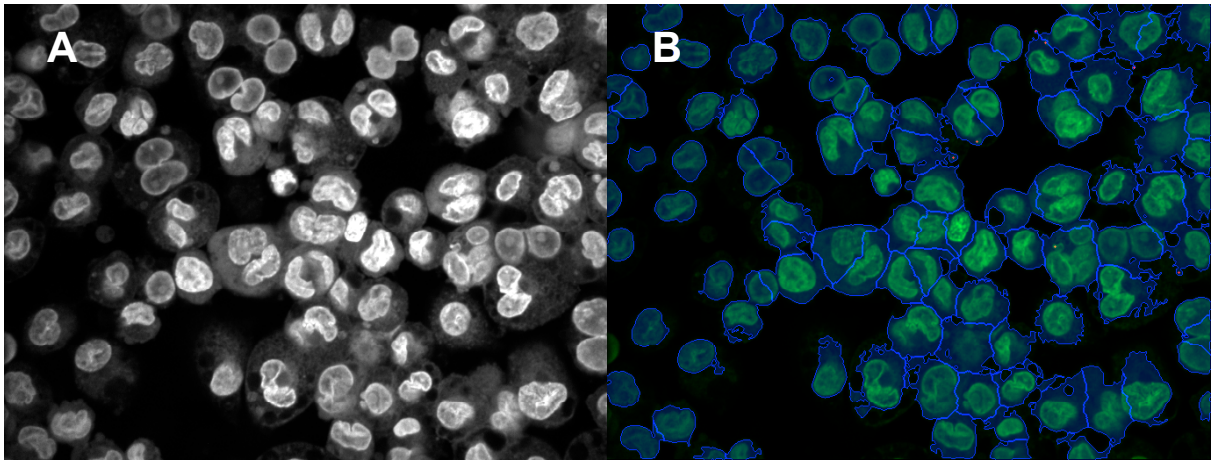


Figure 33. Negative control original (A) and processed image (B).

3. Infection Optimization

3.1 Promastigote fitness optimization

This project's first aim was to establish the best day of promastigote *in vitro* culture that led to the highest macrophage (human and murine) infection ratio. *In vivo* infection and disease establishment is very complex, involve several factors from host immune system, and is not comparable to the results obtained in any *in vitro* work. Our purpose was to compare different species of the parasite and host cells, in order to get optimal infection conditions for each species. So, an optimal *in vitro* infection system could be established and used as a study disease model or as an infection model applied to drug screening.

To perform the analysis, we assumed that after the extracellular parasites were washed from the wells 24 hours post-infection, there would not be an increase in the infection ratio as there would be no more extracellular promastigotes to infect new host cells. The 6th post-infection day was then used as a common factor in all comparisons ahead, for the 4 *Leishmania* species both in THP-1 (Figure 34) and Raw 264.7 (Figure 35) cells.

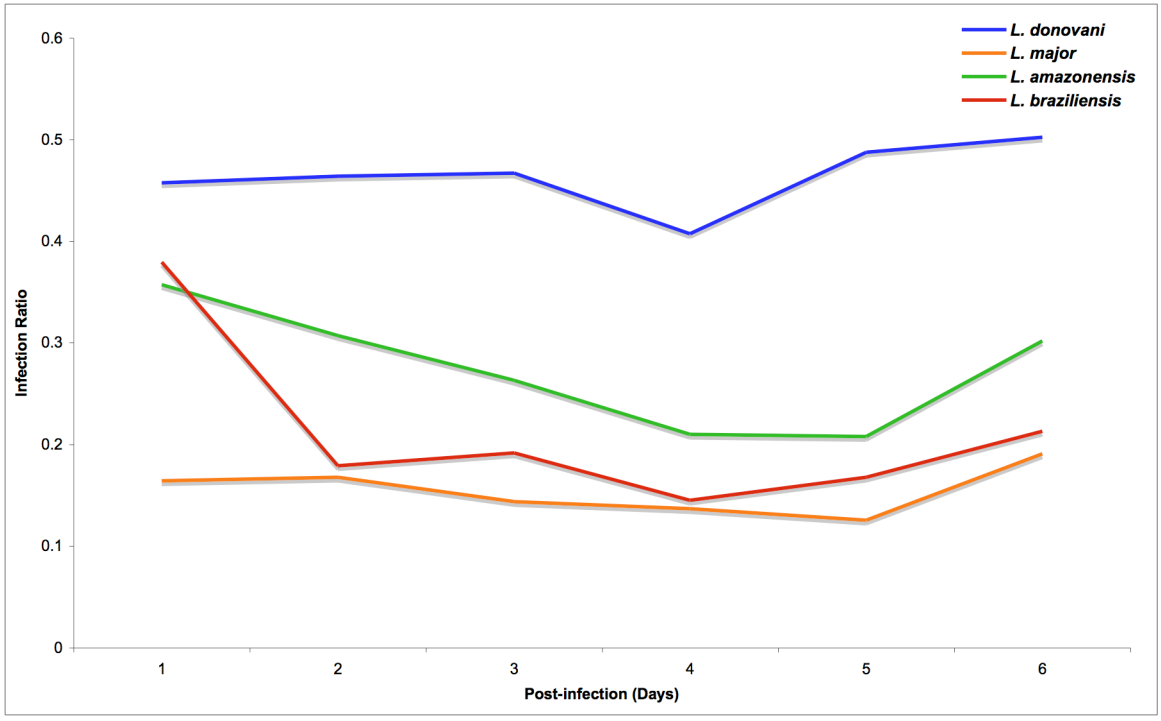


Figure 34. THP-1 cells infection ratio overview during post-infection (6 days), with promastigotes from the 5th day of culture.

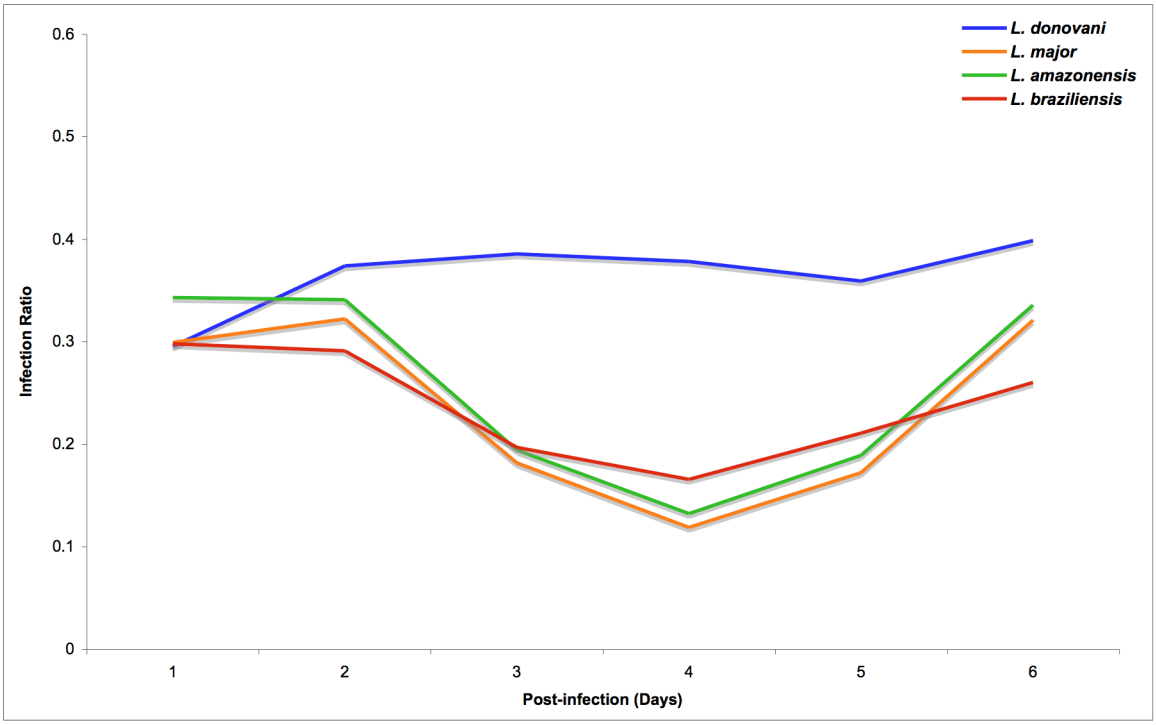


Figure 35. Raw 264.7 cells infection ratio overview during post-infection (6 days), with promastigotes from the 5th day of culture.

As was told on Chapter II, one key factor for infection establishment is the differentiation of *Leishmania* promastigotes into metacyclic state, enabling proper adaptation to the macrophage phagocytic vacuole (32). After recent findings, it is also known that a successful infection establishment depends on the ratio of apoptotic promastigotes versus the metacyclic ones (34). In this sense, to estimate the best time from the *in vitro* promastigote culture, we promoted infection of macrophages from different days of promastigote culture. In order to understand if the best promastigote culture condition for infection depends also on the macrophage properties, we tested infection using 2 different macrophage cell lines: differentiated THP-1, which is a human macrophage from an acute monocytic leukemia, and Raw 264.7 cells, which is a mouse leukemia macrophage.

We also tested if *in vitro* cultures preconditioning improved promastigotes infection ability, as mentioned, for example, in Luz et. al (2009). For that purpose, we did infection assays with promastigotes incubated at 28 °C, which is the usual *in vitro* culture incubation temperature, and at 34 °C or 37 °C, only 24 hours prior to infection. The 28 °C incubation temperature mimics the insect vector temperature. Therefore, it has been hypothesized that increasing this temperature to values obtained in the human host (34 °C for cutaneous strains and 37 °C for visceral strains), infection ratio could be enhanced.

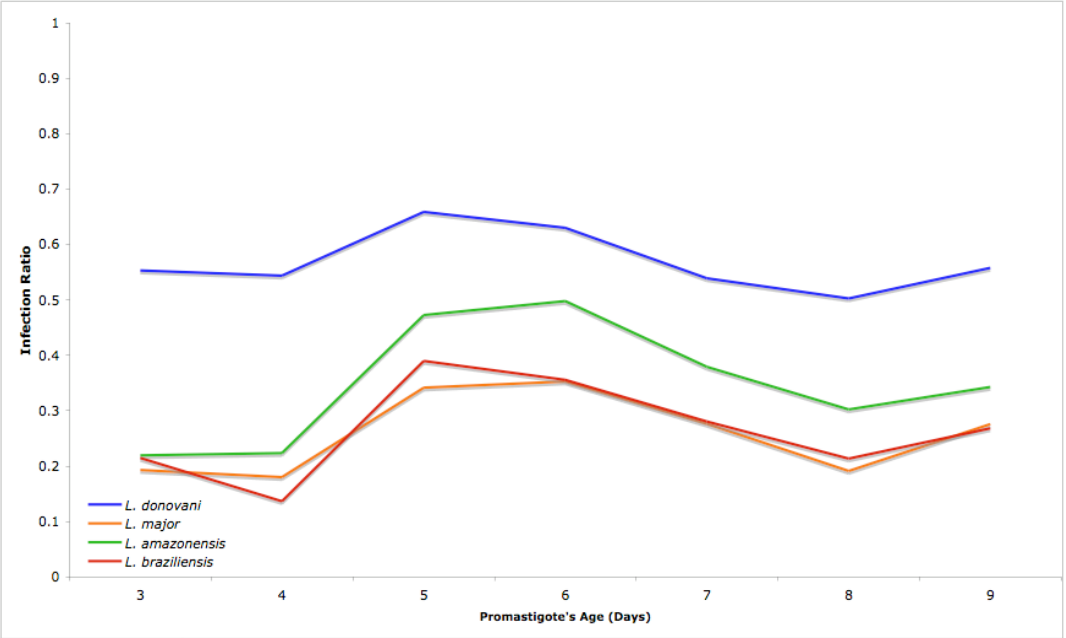
The first measured parameters were the ratio of infected macrophages in the period of 24 hours exposition to the parasites (infection ratio), and the average number of parasites phagocytized per macrophage during this time. These parameters are in fact more related to the ability of the macrophage to phagocyte parasites, once the infection can be considered passive from the parasite point of view. For this reason, we evaluated parameters more associated to parasite's fitness. The extracellular parasites (non phagocytized parasites) were washed from the wells 24 hours after the infection moment, to avoid late infection. We measured again the infection ratio and the average number of parasites per infected macrophage 24 hours after the first measurement (48 hours after the infection moment), elucidating how many of the phagocytized parasites could survive inside the macrophage, and be able to establish an infection (Table 6).

Table 6. 3rd day of *in vitro* culture promastigotes infection in THP-1 cells.

	Infection Ratio (%)		**Average Number of Parasites per Cell**	
	Time after infection			
	24 h	**48 h**	**24 h**	**48 h**
L. donovani	56	55	2.8	2.6
L. major	40	30	2	1.6
L. amazonensis	43	32	2.1	1.7
L. braziliensis	33	28	1.8	1.4

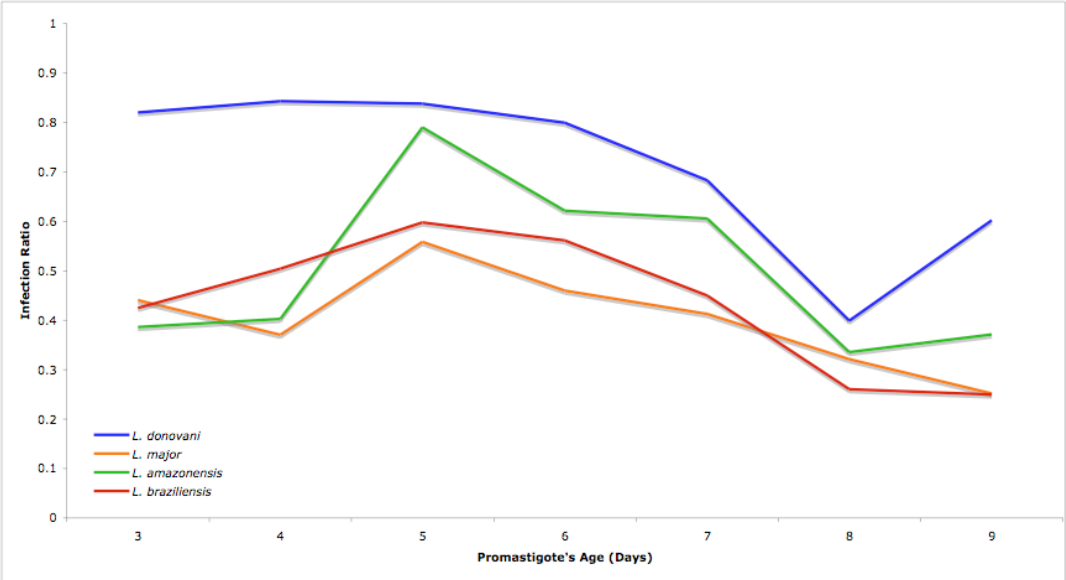
L. major, *L. amazonensis* and *L. braziliensis* promastigotes from the 3rd day of *in vitro* culture demonstrated not to establish infection in efficient way as the infection ratio decreased 5% to 10% from 24 to 48 hours post-infection. The average number of parasites per cell also decreased in the same time period. Differently from other species, *L. donovani* promastigotes established stable infection, keeping same infection ratio and average number of parasites per infected cell from 24 to 48 hours post-infection.

The infection ratio from 48 hours post-infection can be considered a good measurement for stable infection after washing extracellular parasites 24 hours post-infection, as the phagocytized parasites unable to establish the infection would have been eliminated by the macrophage. This elimination of the non-adapted parasites explains the decrease in the infection ratio from 24 to 48 hours post-infection and the decrease in the number of parasite per infected cell. For all the 4 *Leishmania* species tested in this experiment, the promastigotes from days 5 and 6 (Figure 36 and Figure 37) were the most efficient to establish macrophage infection.



	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9
L. donovani	55%	50%	64%	65%	56%	46%	54%
L. major	30%	21%	35%	36%	29%	16%	24%
L. amazonensis	32%	35%	50%	46%	45%	31%	33%
L. braziliensis	28%	18%	46%	34%	30%	18%	26%

Figure 36. Infection ratio from promastigote *in vitro* culture from day 3 to day 9, 48 hours after the infection of THP-1 cells.



	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9
L. donovani	76%	84%	84%	78%	70%	37%	47%
L. major	42%	36%	54%	47%	39%	32%	21%
L. amazonensis	36%	45%	68%	68%	50%	34%	28%
L. braziliensis	38%	50%	62%	63%	39%	29%	26%

Figure 37. Infection ratio from promastigote *in vitro* culture from day 3 to day 9, 48 hours after the infection of Raw 264.7 cells.

L. donovani was the specie with the highest infection ratio and the specie that demonstrated more stability and resistance to macrophage elimination from 24 to 48 hours post-infection. All the other 3 species (*L. major*, *L. amazonensis* and *L. braziliensis*) showed a decrease in the infection ratio from 24 to 48 hours post-infection, indicating that macrophages were able to eliminate part of the phagocytized parasites. This fact suggests that the parasites were not in optimal conditions for infection. The results were consistent both in THP-1 and Raw 264.7 cells, corroborating the hypothesis that the best parasite condition for infection establishment does not depend on the host cell. The ratio of parasites used in infection was 50 parasites per cell, both for THP-1 and Raw 264.7 cells. Because this ratio was only optimized for *L. donovani* infection, maybe, if a different ratio was tested, different results could be observed.

The graphs below (Figure 38) show the different infection ratios obtained with promastigote's incubated at 28 °C and at 34 °C (*L. major*, *L. amazonensis* and *L. braziliensis*) or at 37 °C (*L. donovani*). These temperatures were chosen accordingly to insect vector and mammal host temperatures.

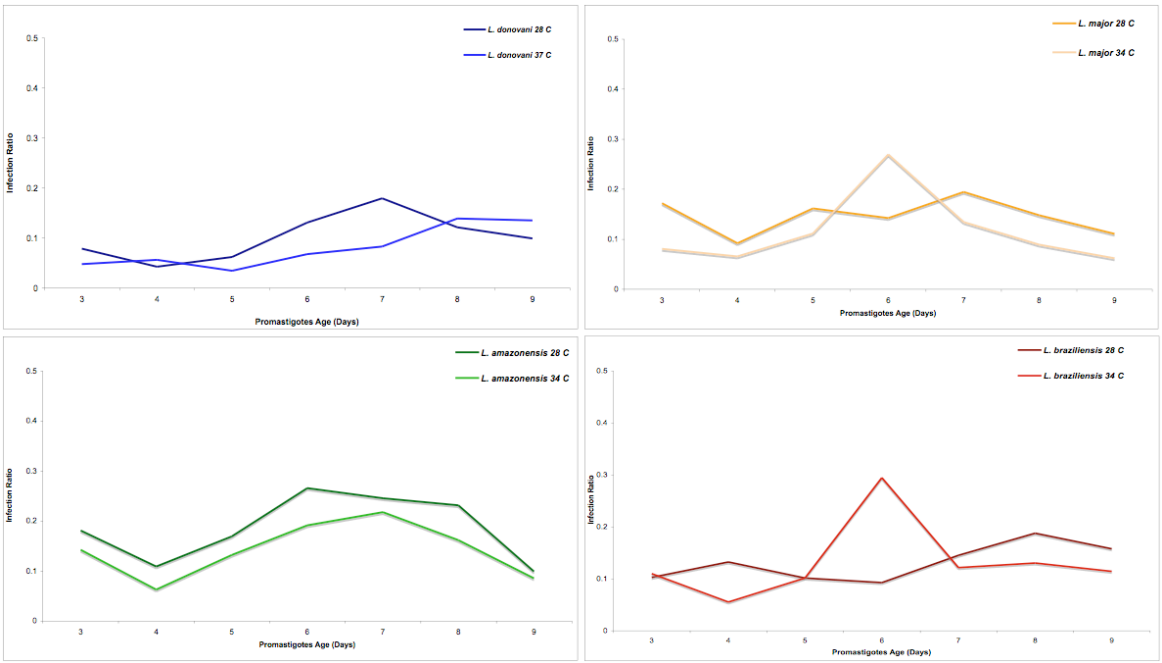


Figure 38. Infection ratio comparison between different incubation temperatures, in THP-1 cells.

As we can see on the graphs, the highest infection peaks for all species occur between the 5th to the 7th day of promastigote's cultures. We must state that obtaining precisely only one day of promastigote's age with the highest infection is imprecise, because the culture growth curve is estimated and can be affected by many factors. Nevertheless, results were reproducible throughout all experiments.

Comparing promastigotes' culture incubation temperatures, it did not seem to exist a correlation between incubation temperature and consequent infection ratio. The highest infection ratio peaks for *L. major* and *L. braziliensis* occur for promastigotes incubated at 34 °C. Interestingly, apart from the highest infection day, the other days tended to show higher infection ratios for promastigotes incubated at the insect vector host temperature, 28 °C. For *L. amazonensis*, the infection ratio was always higher for 28 °C incubated parasites, but there was a clear parallelism with infection obtained with 34 °C promastigotes. *L. donovani* showed similar results for both temperatures, but had better infection ratios for cultures incubated at 28 °C.

Figure 39 shows the same comparison above, but for Raw 264.7 cells. *L. donovani* results are not very clear, but it seems that promastigotes incubated at 28 °C led generally to higher infection ratios. *L. amazonensis* also showed the same trend as *L. donovani*, better infection ratio for promastigotes incubated at 28 °C. Once again, *L. major* and *L. braziliensis* showed higher infection ratios with parasites incubated at 34 °C. Such as for THP-1 cells, infection ratios had the same range of values and similar trends for both incubation temperatures.

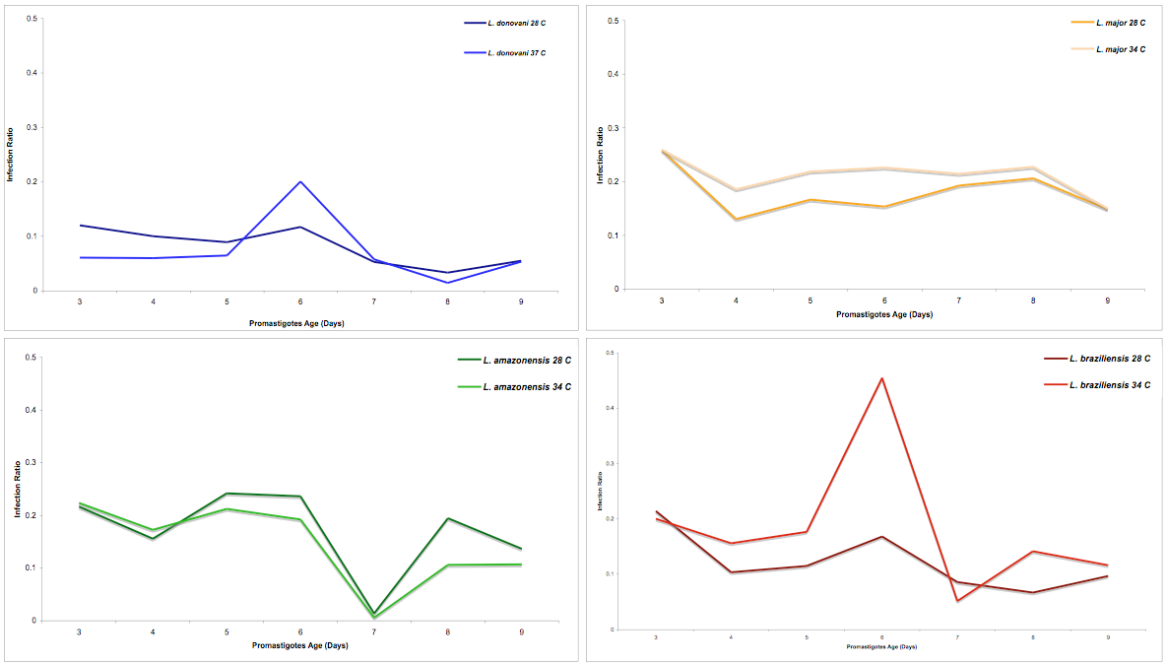


Figure 39. Infection ratio comparison between different incubation temperatures, in Raw 264.7 cells.

3.2 Optimal infection day

Another aim of this project was to shed some light into, still unclear, infection development during the parasite cycle inside the host cell. Figure 40 and Figure 41 correlate the number of parasites per infected cell with post-infection day.

In order to choose the optimal post-infection day for each cell line, we must consider some key points. Because THP-1 cells do not multiply after differentiation, and because infection is washed 24 hours post-infection, we considered infection to be constant throughout all post-infection. Only the number of parasites inside infected cells (amastigotes) should vary. The same does not occur with Raw 264.7 cells. These cells can multiply during the infection assay, and, therefore, infection ratio can vary during the post-infection period.

According to Figure 40, for THP-1 cells, any day during post-infection provided, theoretically, the same infection ratio value. However, the number of InA is expected to be higher on the last infection days, as the parasites replicate continuously inside the host cell. The results did not confirm this hypothesis. This fact could be due to either non-development of the intracellular amastigote parasite or to an artifact of the image analysis algorithm.

To understand better this issue, some images were visually analyzed and the results were compared to the software analysis. It was observed that when the macrophage was infected with multiple parasites with short distance between them, the software was unable to detect all the parasites. In this sense, the data regarding the infection ratio could be still considered precise, as at least one parasite is always detected. Although, the ratio number of parasites per infected cells probably underestimated the number of intracellular parasites. To study the phenomenon of amastigote dynamics inside the macrophage, replication assays will be performed, so multiplication ratio of the intracellular parasites can be accurately observed.

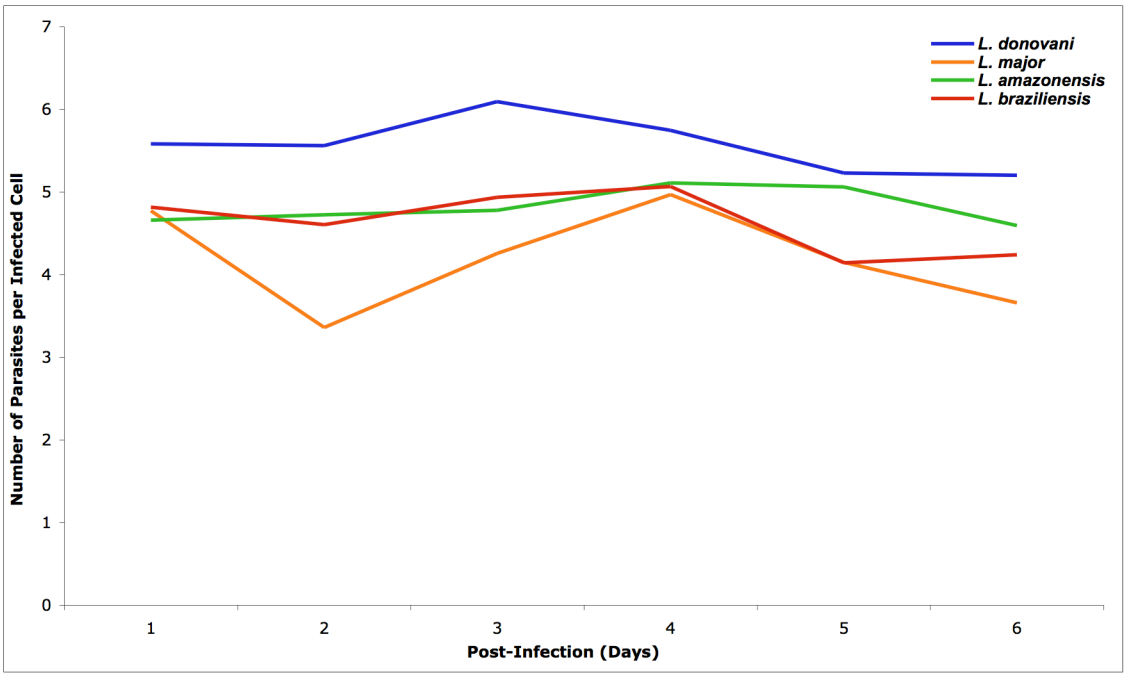


Figure 40. Amastigotes' growth dynamic. This graph shows parasite number per infected THP-1 cell over all post-infection days, with promastigotes from the 5th day of promastigotes' culture.

For Raw 264.7 cells, Figure 41, infection ratio should be read on the first days of infection, while there is still a reliable confluence of host cells in the well's bottom. Also, number of parasites per infected cells should be quantified in 5th or 6th day post-infection, because, theoretically, it will have higher values.

The optimal day for reading depends on the project's purpose. For instance, if the aim is to evaluate a drug efficacy by measuring infection ratio, any post-infection day can be chosen. On the other hand, if the aim is to check if any given drug interferes with the amastigote's replication ability inside the host cell, then, the final days of infection should be read from the 4th day on.

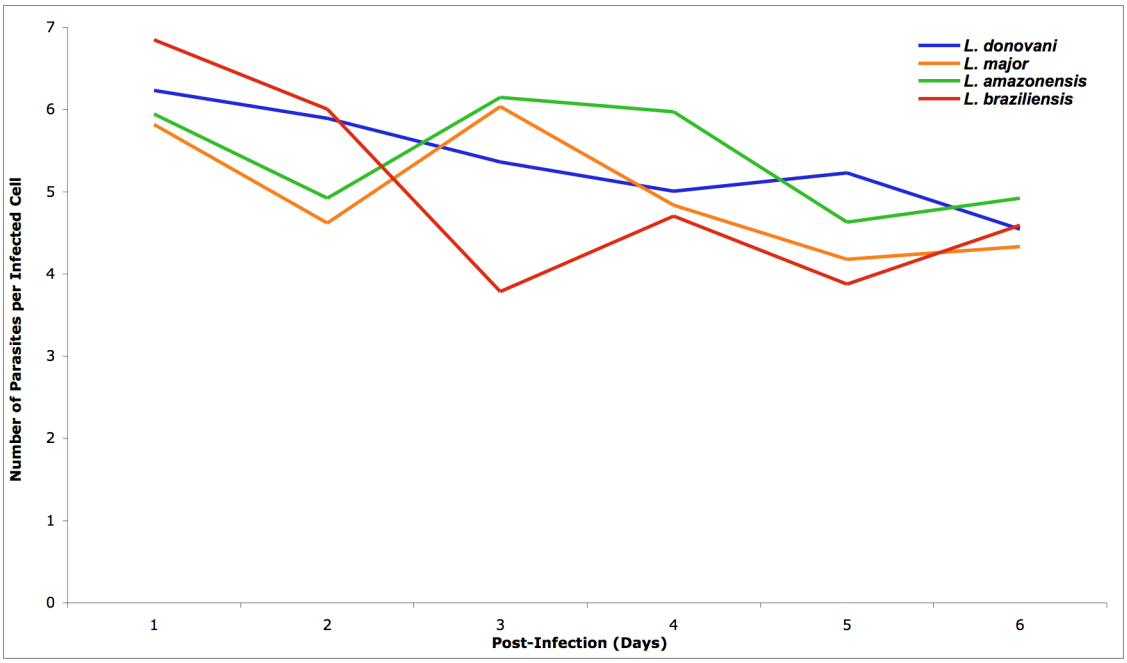


Figure 41. Amastigotes' growth dynamic. This graph shows parasite number per infected Raw 264.7 cell over all post-infection days, with promastigotes from the 5th day of promastigotes' culture.

Figure 42 and Figure 43 show promastigote's infection fitness on both cell lines. In these graphs, promastigote's age is correlated simultaneously to parasite number per infected cell and infection ratio. The infection ratio marker represents a percentage of the total length of the bar itself, and the number of parasites is represented by the total bar length. The data indicates, once more, that the number of InA did not significantly increase along the infection course.

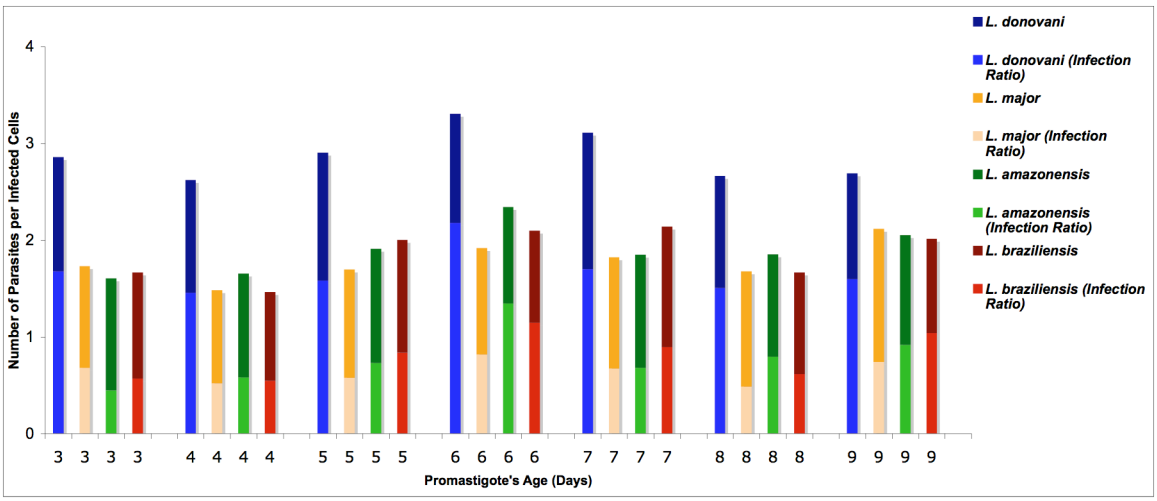


Figure 42. Promastigote's infection fitness in THP-1 cells.

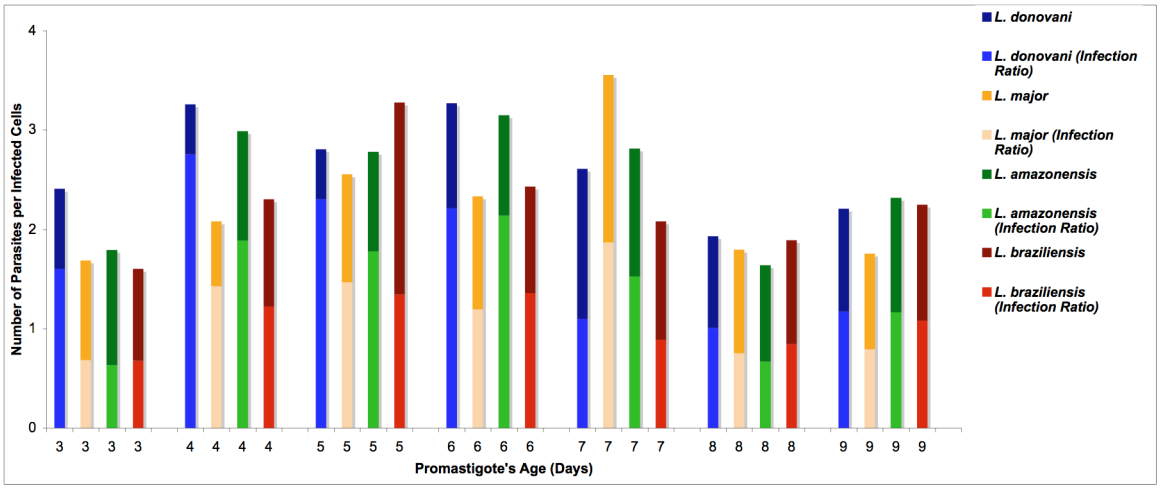


Figure 43. Promastigote's infection fitness in Raw 264.7 cells.

Even though the results do not reflect entirely the amastigote's replication dynamics, it can be seen some trend in the values obtained. Therefore, for THP-1 cells, the highest parasite number of parasites per infected cell in *L. donovani*, *L. amazonensis*, and *L. braziliensis* is the 6th day of promastigote's *in vitro* culture, and in *L. major* is the 9th day. For Raw 264.7 cells, the highest parasite number of parasites per infected cell in *L. donovani* is the 4th day of promastigote's *in vitro* culture, in *L. major* is the 7th day, in *L. amazonensis* is the 6th day, and *L. braziliensis* is the 5th day.

The following graphs (Figure 44, Figure 45, Figure 46 and Figure 47) show infection differences between THP-1 and Raw 264.7 macrophage cell lines. They correlate parasite number per infected cell with promastigote culture day. There is one graph per species. Infection ratio is represented by a percentage of the total bar length, and the number of parasites by the total bar length.

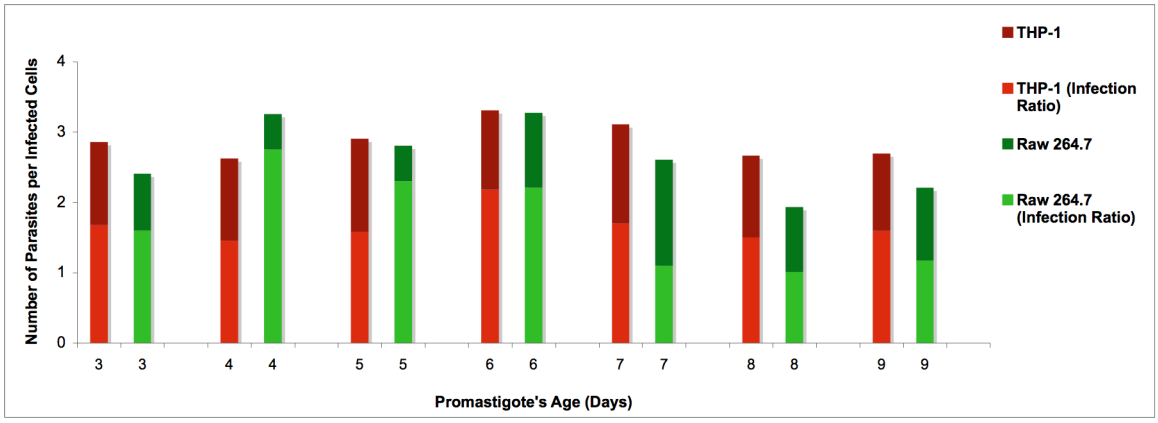


Figure 44. *L. donovani* infection.

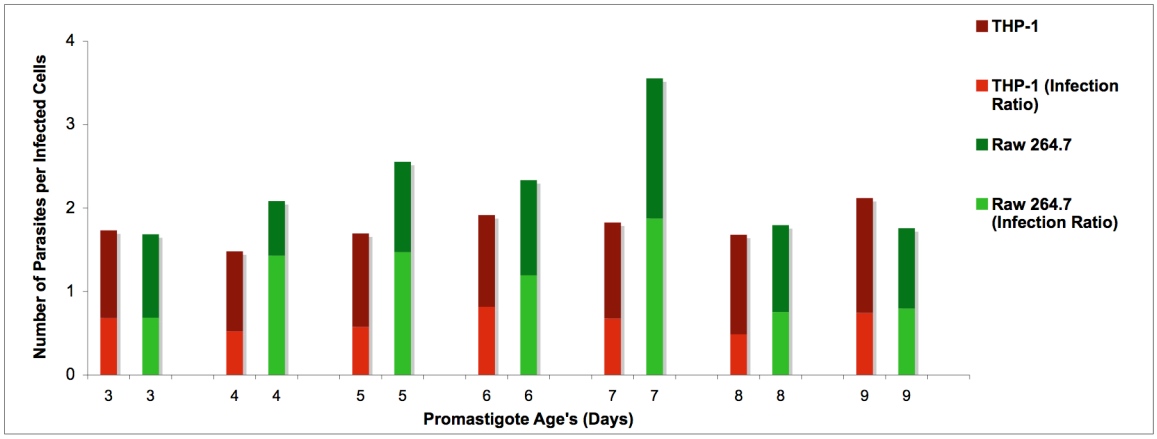


Figure 45. *L. major* infection.

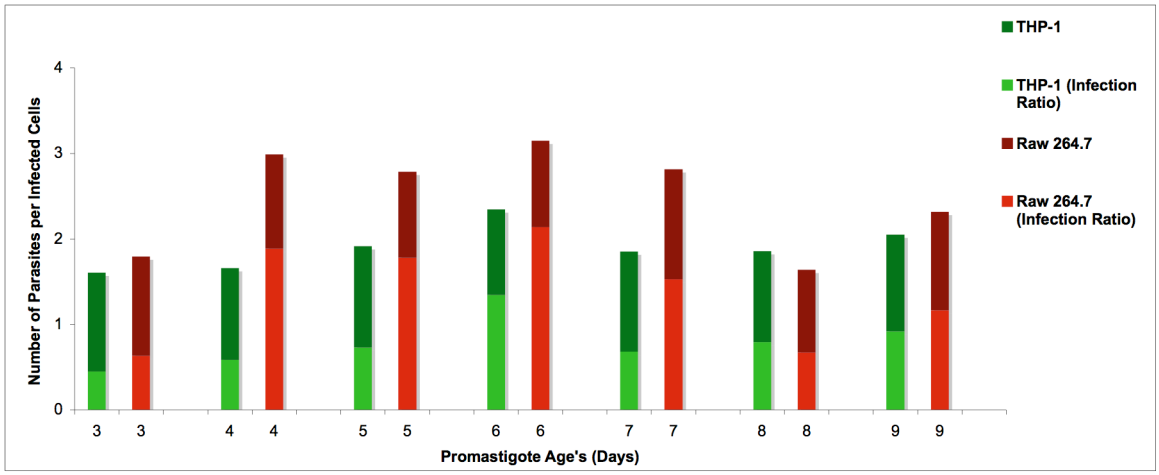


Figure 46. *L. amazonensis* infection.

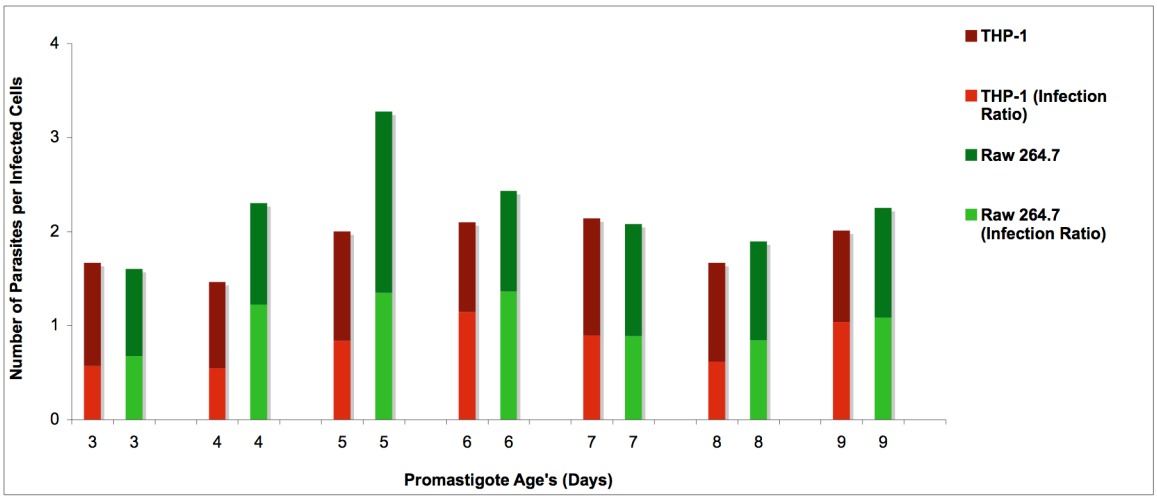


Figure 47. *L. braziliensis* infection.

From these figures, we can conclude that Raw 264.7 cells tend to lead to higher number of parasites inside the host cell. Nevertheless, as it was mentioned before, these results are not as reliable or as consistent as THP-1 results. Raw 264.7 cells keep on multiplication, even after infection, and after reaching the maximum density cells start to die, leading to the appearance of cellular debris or nuclear artifacts that end up being wrongly identified as parasites. These results stress once more the need to estimate InA with some other technical means, in order to have more accurate and precise results.

However, for all species, THP-1 and Raw 264.7 cells provide the same range of infection ratio and parasite number per infected cell, suggesting that the process is more parasite than host cell dependent.

4. Replication Assays

On this subchapter, we show preliminary results obtained during replication assays. The aim was to establish amastigote doubling population time using this method. This way, it will be possible to overcome limitations imposed by software-automated analysis. These experiments lack automatization, but provide better resolution images and are consequently more accurate to measure amastigote's replication dynamics during post-infection period. The replication assay was based in immunofluorescence. After infection was stopped, BrdU was added to infection coverslips for 12 hours. Due to its similarity to thymidine, cells incorporate BrdU bases during replication and new DNA strands will bear them. Then, incubation with anti-Br antibodies primes these newly incorporated DNA bases. Alexa Fluor 488 antibody mark anti-Br sites and provide a green fluorescence signal. DAPI stains DNA strands both in host cell and parasites.

In Figure 48, preliminary images from replication assay are shown. These images were taken 24 hour post-infection. In blue, host cell and parasite DNA is stained with DAPI, and in green replication sites are tagged. 24 hours post-infection, there is a clear overlap of parasite nuclei and replication sites. Therefore, we can state that all *Leishmania* species used in this project, started replicating inside host cells, THP-1, in a period inferior to 24 hours after infection.

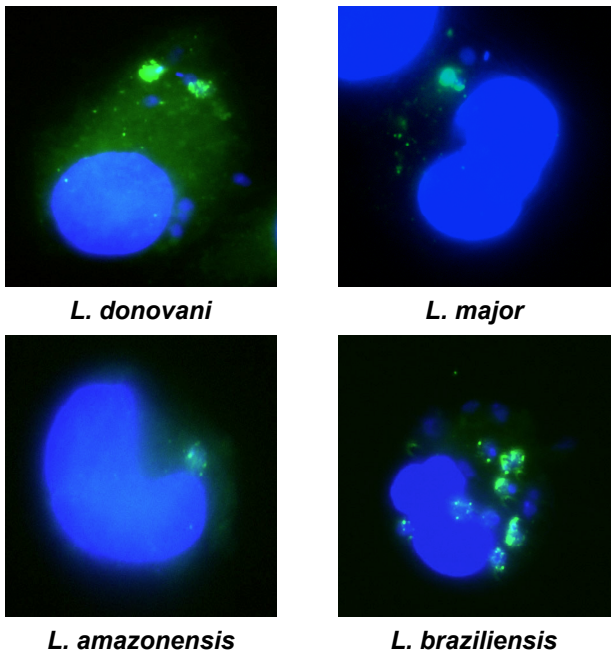


Figure 48. Replication assay images for 24 hours post-infection in THP-1 cells.

VII – Conclusion

This optimization carried in this project was never done before and it has a great scientifical value for many fields of research. Because our group is focused in drug discovery, obviously, these results will be very useful for this purpose. Now, we will be able to have optimized infection models, in order to get better results during HTS. As was mentioned before, HTS success relies deeply in a consistent, reproducible and robust biological model.

On the other hand, these infection dynamics differences identified between species can point to very important biological behavior strategies. *Leishmania* parasites can in fact promote a wide range of disease clinical manifestations. Our results might correlate some tropism preferences with infection conditions. For instance, *L. donovani* parasites replicate and infect in higher rates at 37 °C, more than the other species. This is because *L. donovani* is viscerotropic and the other species studied have higher skin tropism. Therefore, these first results can be the basis and origin of many other studies, in HTS or in pure biology research fields.

This project results provide better infection protocols for 4 *Leishmania* species in 2 macrophage cell lines. The parameters optimized for each possible infection are represented on Table 7.

Table 7. Optimal infection protocol for *L. donovani*, *L. major*, *L. amazonensis*, and *L. braziliensis* in THP-1 and 8. Raw 264.7 cells.

		Promastigote's Age	Promastigote's Incubation Temperature	Post-Infection Day
L. donovani	**THP-1**	5	28 °C	4 to 6
	Raw 264.7	5	28 °C	3 or 4
L. major	**THP-1**	5	34 °C	4 to 6
	Raw 264.7	5	34 °C	3 or 4
L. amazonensis	**THP-1**	5	28 °C	4 to 6
	Raw 264.7	5	28 °C	3 or 4
L. braziliensis	**THP-1**	5	34 °C	4 to 6
	Raw 264.7	5	34 °C	3 or 4

Regarding the amastigote dynamics, it is not yet entirely possible to conclude some solid result, due to analysis limitations. However, further studies are already going on and will hopefully answer our questions. Other optimized parameters showed very consistent results and our further experiments will use them as starting point.

We shall also choose THP-1 over Raw 264.7 cells. In all results we obtained, THP-1 cells always showed better phenotypic characteristics and, therefore, they always led to more precise and accurate results. Also, THP-1 cells are derived from human macrophages. So, infection obtained with this cell line will be closer to real human leishmanial infection. This way, HTS will be driven to get new drug scaffolds, targeting, more accurately, towards human infection and its specificities.

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