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STUDY OF THE SECRETOME OF LEISHMANIA INVOLVED IN THE INFECTION

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RÉSUMÉ

Les parasites protozoaires du genre *Leishmania* sont les agents microbiens responsables d'un groupe de maladies connues sous le nom de leishmanioses. L'infection productive dépend de la capacité de survie du parasite suite au premier contact initial du système immunitaire de l'hôte.

Par conséquent, la prolifération du parasite à l'intérieur des phagolysosomes sera responsable de la pathologie. Les promastigotes stationnaires, récupérés en culture axénique de laboratoire sont semblables aux promastigotes métacycliques. Ces derniers sont fortement immunomodulateurs et sont considérés, traditionnellement comme la forme la plus infectieuse du parasite.

Les protéines sécrétées de différents organismes ont été directement impliquées dans plusieurs pathologies. Donc, il est possible que les protéines sécrétées par *Leishmania*, soient également impliquées dans la capacité du parasite à subvertir le système immunitaire.

Les avancées récentes dans l'étude du sécrétome de plusieurs types de *Leishmania* ont permis d'affirmer que le sécrétome est fort complexe. Nous avons déterminé qu'environ 300 protéines sont sécrétées par le parasite, la plupart d'entre elles ayant aucun signal canonique de sécrétion. Le sécrétome de Leishmania est donc composé surtout de protéines qui sont libérées par sécrétion non conventionnelle,. Afin d'étudier le sécrétome associé à la virulence, nous avons développé et validé une approche qui a permis l'étude des composants de l'exoprotéome des parasites stationnaires et logarithmiques. Cette approche était basée sur la culture continue des parasites dans un milieu de culture sans supplément de sérum de bovin fœtal, cRPMI, dans lequel la virulence des parasites est maintenue. Grâce à cette approche nous avons mis en évidence un exoproteome distinct de ceux jusqu'à date répertorié. La méthode de production et de la récupération de l'exoproteome sont donc très importants. Notre exoprotéome est dominé par la GP63, une glycoprotéine dont l'importance centrale dans l'infection a été déjà validée. La culture continue des parasites est donc essentiel pour avoir un exoprotéome représentatif. Nous avons également déterminé que la cultutre en continu pouvait amener à une diminution de la virulence et quarante divisions sont nécessaires pour une perte de virulence significative. Par conséquent toutes nos études se sont fait chez des parasites comptant moins de 20 divisions. Le principal mécanisme associé à la perte de virulence a été identifié comme une incapacité de se différencier en amastigotes. Le cRPMI a donc permis la culture des parasites pour l'étude de l'exoprotéome tout en maintenant la virulence des parasites.

La présence de vésicules, décrite déjà comme un composant de l' exoprotéome, a été confirmée aussi par notre approche continue et a été confirmé, d'ailleurs, dans l'exoproteome des parasites en phase de croissance logarithmique. Les vésicules récupérées des parasites logarithmiques diffèrent de celles recueillies de parasites en phase stationnaire de croissance. En effet des protéines potentiellement impliquées dans la rénovation et le recyclage du contenu protéique, tels certains composants du ribosome étaient enrichies dans les parasites en phase logarithmique tandis que les vésicules des parasites stationnaires ont un contenu protéomique ayant des caractéristiques similaires

aux corps apoptotiques des cellules de mammifères. En dehors de la GP63, plusieurs autres protéines décrites comme immunomodulatrices ont été retrouvées dans l'exoprotéome des parasites stationnaires, ce qui indique que celui-ci contient un ensemble de protéines avec un potentiel d'interaction directe avec les cellules du système immunitaire.

Immunologiquement, l'exoprotéome récupéré des parasites stationnaires a été capable d'activer les cellules dendritiques, laissant supposer une fonction importante dans la création d'un environnement inflammatoire précoce lors des premières étapes de l'infection.
En conclusion, la recherche développée a contribué à l'avancement des connaissances actuelles sur la biologie du *Leishmania*, grâce au développement et à la validation d'une nouvelle approche afin d'étudier son exoprotéome. L'exoprotéome récupéré était dynamique, il avait une composition spécifique, dépendant du stade du parasite. Cet exoprotéome avait des effets spécifiques sur les cellules dendritiques et il jouait un rôle important dans les étapes précoces de l'infection.

Cette étude a ouvert des nouvelles perspectives sur l'exoprotéome de *Leishmania* spp. permettant la découverte de nouvelles protéines immunomodulatrices et, en corrolaire, de nouvelles cibles pour le contrôle de la maladie associée au parasite.

Mots clés: *Leishmania, sécrétome, vésicules, virulence*.

ABSTRACT

Protozoa parasites of the genus *Leishmania* are the responsible for a group of diseases known as leishmaniasis. The infection is associated with the capacity of these parasites to survive in the phagolysosomes of infected macrophages. Successful infections with pathogenic *Leishmania* spp. are linked to the capacity of the parasite to survive the initial impact of the host immune system and to interfere with the infected cells rendering them incapable of eliminating the parasites. The secreted proteins from the parasite are expected to be in the front line for interactions with the host. Recent advances in the study of the secretome of *Leishmania* spp. depicted it as highly complex with the majority of proteins without any predictable secretion signal. The secretome is composed of proteins that are released by different mechanisms like conventional and unconventional secretion. Several proteins secreted by *Leishmania* spp. are known to interact and influence the outcome of the disease by directly interfering with the host immune cells. The proteomic studies on the *Leishmania* spp. secretome identified more than three hundred proteins released into the exterior. The stationary promastigotes recovered in axenic culture were enriched in the most virulent promastigote form, the metacyclic parasites. Therefore we aimed at evaluating the exoproteome associated with the stationary parasites. To achieve this we developed and validated an approach that would enable the study of the exoproteome components of stationary and logarithmic parasites. This approach was based on the continuous cultivation of the parasites in a medium without any protein supplementation that maintained the basic virulence of the parasites. The continuous approach produced a GP63-rich exoproteome that was distinct from the traditional approaches indicating that the process of recovery induced a significant bias in the study. Furthermore as the continuous approach was chosen, we determined the mechanisms associated with loss of virulence assuring that fully virulent parasites were used. At least forty parasite divisions were required for a short-term loss of virulence. The main mechanism associated with loss of virulence was identified as a growing incapacity to differentiate into amastigotes. The defined time interval of forty divisions enabled us to evaluate the exoproteome without loss of virulence related to the subculture. The protein-free medium developed, cRPMI, retained parasite virulence and morphology similar to that of parasites grown in standard media. The exoproteomes recovered using cRPMI were dominated by proteins without any recognizable secretion sequence, in concordance with reports on other *Leishmania* spp. The presence of vesicles, already reported as a component of the exoproteome, was also confirmed using our continuous approach. Furthermore, the presence of vesicles in the logarithmic parasites exoproteome was confirmed. The protein content of these vesicles presented a dynamic profile that was dependent on the parasite stage. The vesicles recovered from

logarithmic parasites seemed to be related to protein turnover, being significantly enriched in ribosomal components. The vesicles from stationary parasites are of different composition, presenting some characteristics similar to apoptotic bodies. Immunologically the exoproteome recovered from stationary parasites was able to activate dendritic cells suggesting that the exoproteome might have a function in the creation of an early inflammatory environment leading to the recruitment of neutrophils and monocytes that might function as safe heavens for the parasites. In conclusion, our research has contributed to the advance of the current knowledge of *Leishmania* biology, through the development and validation of a novel approach to study the *Leishmania* secretome. The exoproteome recovered from stationary parasites had specific immune-modulating effects on bone marrow derived dendritic cells, indicating that it can play an important role in the precocious steps of infection.

This study opened new perspectives into the *Leishmania* spp. exoproteome that will enable the search of new immunomodulatory proteins that might become the future targets to leishmaniasis control.

Keywords: *Leishmania, secretome, vesicles, virulence.*

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ABBREVIATIONS LIST

(ABC) - ATP-binding cassette

(APCs) - Antigen presenting cells

(BmDCs) - Bone marrow derived dendritic cells

(CR) - Complement receptor

(CL) - Cutaneous leishmaniasis

(DNA) - Deoxyribonucleic acid

(DDT) - Dichloro-Diphenyl-Trichloroethane

(ELISA) - Enzyme linked immunosorbent assay

(ER) - Endoplasmic reticulum

(ESCRT) - Endosomal sorting complexes required for transport

(DCL) - Diffuse cutaneous leishmaniasis

(DAT) - Direct agglutination tests

(fPPG) - Filamentous proteophosphoglycan

(FCS) - Fetal Calf Serum

(GFP) – Green fluorescent protein

(GPI) - Glycosylphosphatidylinositol

(HIV) - Human immunodeficiency virus;

(HASPB) - Hydrophilic acylated surface protein B

(IFNγ) - Interferon-γ

(IL) - Interleukine

(LPG) - Lipophosphoglycan

(MS) - Mass spectrometry

(MHC) - MHajor histocompatibility complex

(Mb) - Mega Base

(mRNA) - Messanger RNA

(moDCs) - Monocytes derived dendritic cells

(MyD88) - myeloid differentiation gene 88

(Mya) - Million years ago

(MVs) - Microvesicles

(MCL) - Mucocutaneous leishmaniasis

(MVBs) - Multivesicular bodies (MVBs)

(NNN) - Novy-MacNeal-Nicolle

(PRRs) - Pattern recognition receptors

(PAMPs) - Pathogen-associated molecular patterns

(PBMCs) - Peripheral blood mononuclear cells

(PCR) - Polymerase chain reaction

(PKDL)- Post kala-azar dermal leishmaniasis

(PSG) - Promastigote secretory gel

(TLRs) - Toll-like receptors

(RFLP) - Restriction fragment length polymorphism

(RNA) - Ribonucleic acid

(rRNA) - Ribosomal RNA

(SL) - Spliced leader

(SHERP) - Small hydrophilic endoplasmic reticulum-associated protein

(TH1) - T helper 1

(Tregs) - T regulatory cells

(TGF-β) - Transforming growth factor beta

(tER) - transitional endoplasmic reticulum

(TNF-α) - tumor necrosis factor alpha

(VDE) - Vesicle depleted exoproteome

(VL) - Visceral leishmaniasis

(WHO) - World Health Organization

"Far better is it to dare mighty things, to win glorious triumphs, even though checked by failure...than to rank with those poor spirits who neither enjoy much nor suffer much, because they live in a gray twilight that knows not victory nor defeat."

- Theodore Roosevelt

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Chapter I

Introduction

1. *Leishmania* spp.

1.1 – Discovery and the first years

In 1900 in British colonial India, at the Army Medical School in Netley, a patient suffering from pyrexia, anemia and enlargement of the spleen, typical symptoms of kala-azar, was admitted for treatment and investigation. William Boog Leishman, at the time Assistant Professor of Pathology at Netley, discovered in this patient a great number of heavily-stained oval bodies in spleenic cells and blood preparations. Later, while studying rats afflicted with trypanosomiasis, he registered similar round bodies in the spleen, blood and liver of the infected rodents (Leishman, 1903). Soon after, Charles Donovan described similar bodies in cells from patients in Madras, in the south of India, and proceeded with the exclusion of the possibility of malaria, a disease that could produce similar symptoms to kala-azar (Donovan, 1903). Finally Ronald Ross, a prominent authority in parasitology at the beginning of the 20th century, attributed to both the discovery of the causative agent of *kala-azar* in India, naming it *Leishmania donovani* and creating the gender *Leishmania* (Ross, 1903).

In less than one decade the etiological agents of *kala-azar* (visceral leishmaniasis) were isolated all over the Mediterranean basin, Sudan, China and Russia (Sen Gupta, 1962). Soon after, several reports associated the gender *Leishmania* with other diseases of unknown origin, like the "delhi sore" in India (Wright, 1903) or the "baurú ulcer" and "espundia" in South America (Lindenberg, 1909; Splendore, 1911). Quickly after the discovery of the causative agent of leishmaniasis it became clear that these microorganisms had an extracellular form that was distinct from the one found inside the diseased patients. In 1904 the extracellular form of the protozoan was isolated and grown in laboratory (Rogers, 1904). The transmission of the parasite became quickly matter of debate with an extensive search for the elusive agent responsible for the transmission of the disease. The correlation between endemic areas and the distribution of certain sandflies species eventually contributed to the discovery of these insects as vectors for the dissemination of the disease (Knowles et al., 1924).

These events were at the genesis for the current definition of *Leishmania* spp. as obligate protozoan parasites that are the etiological agent of a group of diseases collectively known as leishmaniasis.

1.2 - Taxonomy and evolution

The classification of *Leishmania* spp. was initially based on subjective extrinsic characteristics: clinical manifestations, geographic distribution, susceptible vectors and morphological data (Banuls et al., 2007). In the last quarter of the 20th century these criteria were substituted by more accurate intrinsic criteria like patterns of polymorphism exhibited by kinetoplast deoxyribonucleic acid (DNA) (Rodriguez-Gonzalez et al., 2007; Wirth and Pratt, 1982) or antigenic characteristics (Anthony et al., 1985; de Ibarra et al., 1982). These tools eventually lead to the identification of 31 *Leishmania* species of which around 20 are thought to be pathogenic to humans (Figure 1).

Subkingdom	*Protozoa*								
Order	*Kinetoplastida*								
Family	*Trypanosomatidae*								
Genus	*Leishmania*								
Subgenus	*Leishmania*					*Viannia*			
Complex	*L.donovani*	*L.tropica*	*L. major*	*L. aethiopica*	*L. mexicana*	*L. braziliensis*	*L. guyanensis*	*L. naiffi*	*L. lainsoni*
Species	*L. archibaldi*	*L. killicki*	*L. major*	*L. aethiopica*	*L. amazonensis*	*L. braziliensis*	*L. panamensis*	*L. naiffi*	*L. lainsoni*
	L. chagasi	*L. tropica*			*L. garnhami*	*L. peruviana*	*L. guyanensis*		
	L. infantum				*L. mexicana*		*L. shawi*		
	L. donovani				*L. piffanoi*				
					L. venezuelensis				
					L. forattinii				

Figure 1. Taxonomy of pathogenic *Leishmania* spp. Adapted from (Banuls et al., 2007).

Phylogenetically the *Leishmania spp.* protozoa are placed in the genus *Leishmania* belonging to the order Kinetoplastida and the family Trypanosomatidae (Figure 1). Some authors using molecular characterization that included: multilocus enzyme electrophoresis, analysis of the ribosomal ribonucleic acids (rRNA) gene cluster by restriction fragment length polymorphism (RFLP) and sequencing of the small subunit rRNA gene, proposed the separation of the genus *Leishmania* into two divisions, *Euleishmania* and *Paraleishmania*. *Euleishmania* would contain the subgenera *Viannia* and *Leishmania* while *Paraleishmania* would include the *Leishmania hertigi, Leishmania deanei, Leishmania colombiensis, Leishmania equatorensis, Leishmania herreri* in addition to several *Endotrypanum* strains (Cupolillo et al., 2000). This separation is not consensual and traditionally the genus *Leishmania* is divided only into two distinct subgenus *Leishmania (Leishmania)* and *Leishmania (Viannia)* (Lainson and Shaw, 1987). The first contains the species complexes of *L. donovani, Leishmania tropica, Leishmania major* and *Leishmania aethiopica*. The sub-genus *Viannia* is restricted to New World

species of the *Leishmania guyanensis* and *Leishmania braziliensis* complexes and the less known *Leishmania naiffi* or *Leishmania lainsoni* (Figure 1). This division of *Leishmania* genus into two subgenera was initially done using as criteria the area of the sandfly gut (one of the hosts of the protozoan) that was colonized by the parasite (Lainson and Shaw, 1987). This division was later supported by DNA based phylogenetic analyses (Croan et al., 1997).

There is also a group of *Leishmania spp*. related organisms designated as *Sauroleishmania* that traditionally are found in lizards. This exotic group includes the non pathogenic *Leishmania tarentolae*. Mainstream authors place these protozoans in a completely different genus, *Sauroleishmania* (Lainson, 2010). Still, phylogenetic studies present them as closely related to the *Leishmania (Leishmania)* subgenus (Croan et al., 1997; Noyes et al., 1997). *L. tarentolae* is the most carefully studied *Sauroleishmania* species being used as ubiquitous non pathogenic *Leishmania* in several studies (Azizi et al., 2009). The recent sequencing of *L. tarentolae* revealed a high level of synteny and extensive homology and to other pathogenic *Leishmania spp*. (Raymond et al., 2011). This protozoan is also considered as a model organism for the synthesis of recombinant proteins (Basile and Peticca, 2009).

The understanding of the evolution of genus *Leishmania* is hampered by the fact that the phylum *Euglenozoa* lacks a good fossil record (Tuon et al., 2008). Nonetheless, molecular studies place the first direct ancestor of *Leishmania* in the Ordovician more than 488 million years ago (Mya) (Kerr, 2006). This was an epoch where none of the known host for the parasite existed, although the origin of fish and the subsequent evolution of amphibians and reptiles provided probably the first hosts in an environment where hematophagous leeches could functions as the first vectors (Molyneux, 1977). The appearance of winged insects in the Carboniferous (300 Mya) and subsequent radiation into *Diptera* in the Triassic (200 Mya) lead to the appearance of the ancestors of the known insect vectors from the genus *Phlebotomus* and *Lutzomyia* (Tuon et al., 2008). The first evidence of hematophagous insects is recorded at 140 Mya in the Cretaceous (Azar and Nel, 2003) and shortly after the first member of the genus *Leishmania* was found in fossil record from 100 Mya, *Paleoleishmania proterus* (Poinar and Poinar, 2004). It is just after the extinction of the dinosaurs and diversification of the placental mammals that we find the confirmation in the fossil record of the direct ancestors of the vectors *Phlebotomus* and *Lutzomyia* and the direct ancestors of *Leishmania* (Tuon et al., 2008). The global distribution of the parasites has originated a heated debate concerning the geographical origin and the timing of colonization of the different species found today (Kerr, 2000; Noyes et al., 2000). Actually there is more data supporting the origin of the

parasite in the American tropics with migration to the Eurasia by the Bering land bridge (Lukes et al., 2007). This theory also accounts for the distinct evolution of the *Leishmania (Viannia)* subgenus and for the greater variety of species found in south America (Lainson, 2010).

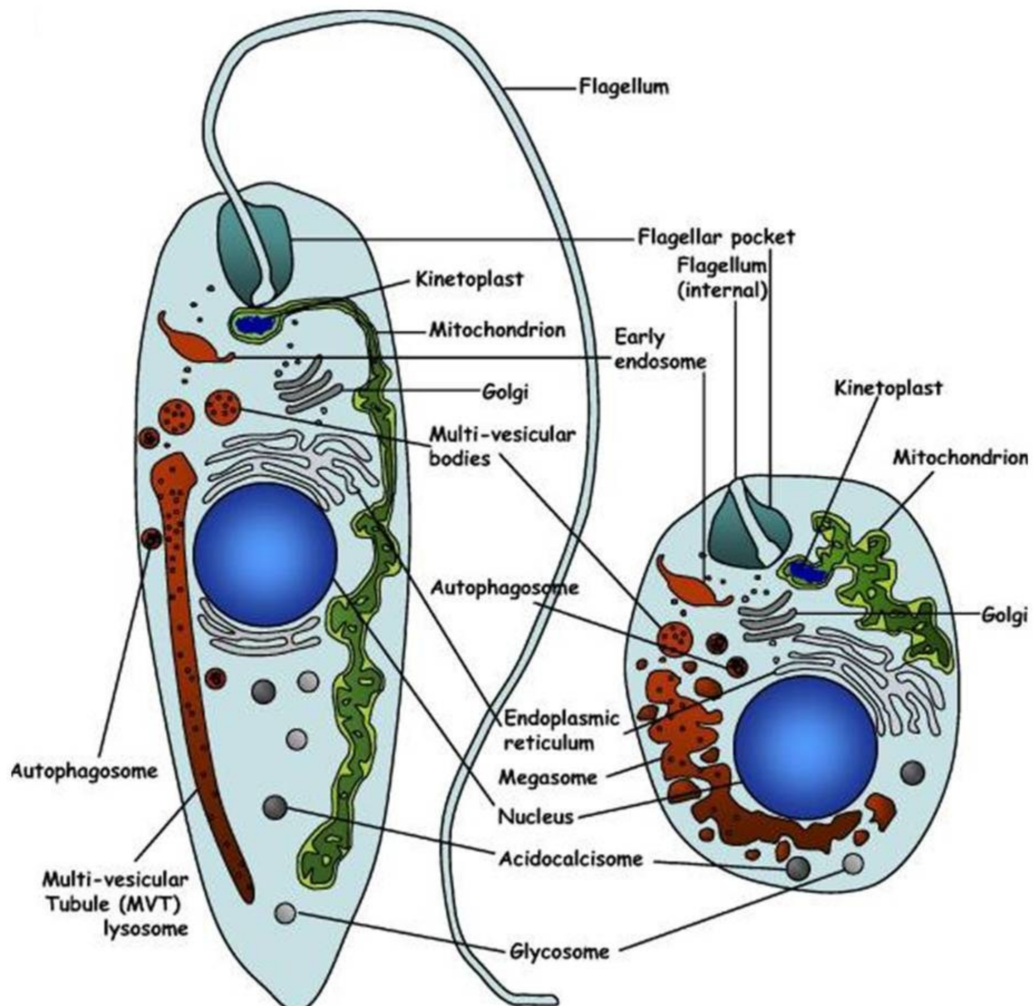

Figure 2. Schematic representation of *Leishmania* spp. developmental forms-Leishmania spp. promastigote (left) or amastigote (right) forms. Adapted from (Besteiro et al., 2007).

1.3 – The life cycle

Leishmania spp. are digenetic parasites with two basic life cycle stages depicted in figure 2: one extracellular stage in an invertebrate host (sandfly) and one intracellular stage within a vertebrate host. In consequence, the parasite exists as two distinct morphological forms, amastigotes and promastigotes, which are adapted for life in vertebrate and invertebrate hosts, respectively.

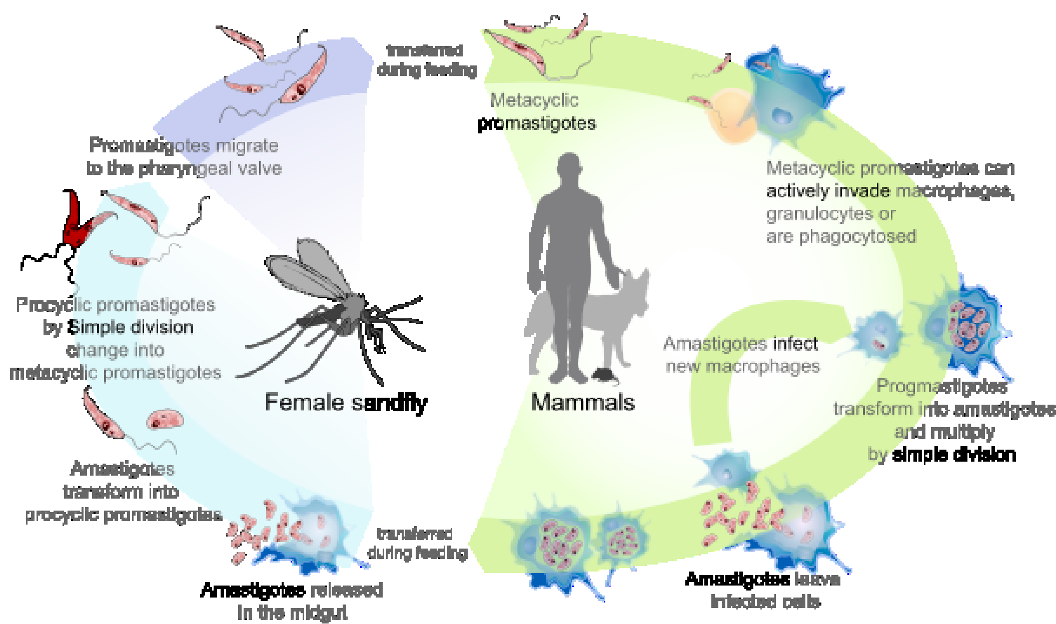

Figure 3. General life cycle of the parasites from the genus *Leishmania*, adapted from http://en.wikipedia.org/wiki/Leishmaniasis.

The typical *Leishmania* spp. life cycle is presented in detail in figure 3. It begins with a female sandfly becoming infected after a blood meal on *Leishmania* spp. infected mammals. In the sandfly, ingested amastigotes differentiate into promastigotes. Inside the insect midgut the promastigotes proliferate and then migrate to the proximity of the foregut where, after morphological and physiological changes, they differentiate into metacyclic promastigotes (Bates, 2007; Bates and Rogers, 2004). These metacyclic promastigotes are non-dividing forms characterized by smaller cell bodies and a bigger flagellum/body size ratio being considered the main infecting form of the protozoan (Bates, 2007). During a subsequent blood meal, metacyclic promastigotes will be regurgitated and injected into the skin to complete the cycle after differentiating into amastigotes establishing themselves in the mammalian host closing the cycle. The

differentiation into amastigotes occurs mostly inside the phagolysome of macrophages. It is the proliferation of the amastigote form in humans that is ultimately responsible for a group of diseases collectively known as leishmaniasis.

Much of the data concerning the life cycle is a compendium of knowledge obtained from different *Leishmania* spp.-vector combinations. Nonetheless the bulk of the obtained knowledge reflects the studies done on *Leishmania (Leishmania)* subgenus.

1.3.1 - The promastigotes stage and the invertebrate hosts

Within the insect host the parasite develops as the promastigote form whose main morphological feature is their needle shape and the long protruding flagellum (Figure 2). The average range of size for *Leishmania* spp. is 12-16 µm length and 1.5 to 3.5 µm width (Bard, 1989).

Only hematophagous females of the sandfly are blood pool feeders, requiring a blood meal for egg development, in consequence only females are transmission active. They use their saw-like mouthparts to puncture the skin creating a pool of blood originating from ruptured superficial capillaries (Bates, 2007). This tissue damage leads to the release of skin macrophages and other phagocytes that can be infected with *Leishmania* spp. The subsequent feeding from the blood pool will lead to the uptake of infected cells. The uptaken blood is stored in the midgut surrounded the peritrophic matrix and digested (Secundino et al., 2005). The changing conditions (decrease in temperature and more basic pH) inside the blood meal involved by the peritrophic matrix lead to the differentiation of the parasite from the amastigote form into the procyclic promastigote form (Kamhawi, 2006). This form of the parasite, characterized by a bulgy body and weak mobility, actively multiply inside the blood meal. After a few days of multiplication, the parasites differentiate from the procyclic form into the highly motile nectomonads parasites characterized by their easily recognizable elongated forms. These nectomonads will accumulate in the posterior end of the blood meal and breakout of the peritrophic matrix in a process that might involve the parasite secretory chitinases (Shakarian and Dwyer, 2000). The freed parasites migrate to the anterior midgut probably driven by sucrose driven chemotaxis (Barros et al., 2006) and eventually become attached to the midgut epithelium avoiding expulsion of the parasites during defecation. Persistence beyond the blood meal is the definitive step for infection of the vector. The epithelium attachment is mainly mediated by the dominant parasite surface

glycoconjugate lipophosphoglycan (LPG) that interacts with epithelium galectins enabling the colonization of permissive sandflies (Kamhawi et al., 2004). The parasites eventually migrate into the vicinity of the stomodeal valve that separates the midgut from the anterior part of the sandfly and differentiate into leptomonads that resume multiplication. These leptomonads secrete the promastigote secretory gel (PSG) that forms a plug like structure extending through the stomodeal valve into the foregut of the sandfly (Rogers et al., 2002). Some of the nectomonads/leptomonads will differentiate into haptomonads, that are found in the cuticular lining of the stomodeal valve and in the foregut (Killick-Kendrick et al., 1974). The function of these haptomonads is not clear, it was proposed that they might function as a scaffold for the formation of the PSG plug by the leptomonads (Bates, 2007). The PSG becomes a semi-solid matrix containing a mesh of a filamentous proteophosphoglycan (fPPG) and promastigotes (Ilg et al., 1996). The promastigotes imbedded in the PSG will be mainly leptomonads and the mammalian infective metacyclic forms that will be mostly located in the apical pole of the PSG plug (Rogers et al., 2002). These metacyclic parasites are the final point of parasite development inside the vector and are considered the main infective form for the mammalian host (Bates, 2007). The most accepted mechanisms for transmission of the promastigotes to the mammalian host are based on either inoculation or regurgitation of the parasites. The inoculation theory implies the inoculation of the parasites present in the saliva by direct infestation of the salivary glands. It is supported mainly by the presence of parasites in the salivary glands and proboscis (Freitas et al., 2002), although this is questioned by some authors due to the difficulty of dissecting glands with total exclusion of contamination of gut parasites (Bates, 2007). The regurgitation theory implies the partial extrusion of the PSG plug to enable the feeding process by clearing the passage to the midgut. This theory is supported by several observations, like damage to the stomodeal valve (Schlein et al., 1992), the actual existence of a blocking PSG, the repetitive non-productive feeding associated to infested sandflies (Rogers et al., 2004), and the fact that most metacyclic parasites are found in the PSG plug with few found in the proboscis (Bates, 2007). During a blood meal, there will thus be the passage of free and PSG imbedded parasites into the blood pool that in conjunction with saliva components will provide the first challenge to the mammalian host immune system. To further support this theory it was shown that saliva and PSG have potent immunomodulatory properties favoring parasite survival and proliferation of the parasites (Kamhawi, 2000; Rogers et al., 2004). Studies in *L. major* demonstrated that sandfly feeding can deliver up to 10.000 parasites, with an average inoculum of less than 1000 parasites (Kimblin et al., 2008; Rogers et al., 2004). The parasites inoculum will be mostly composed of metacyclic parasites and might also include dead or dying parasites. In fact, the presence of apoptotic

parasites was found essential for *in vitro* infections (van Zandbergen et al., 2006), nonetheless their importance in the context of natural infections has not yet been confirmed (Bates, 2008).

The traditional invertebrate hosts for *Leishmania spp*. are small insects of the order *Diptera* belonging to the subfamily *Phlebotominae* (Table 1). This subfamily has five genera, two of them being epidemiologically relevant: *Phlebotomus* in the Old World and *Lutzomyia* in the New World (Killick-Kendrick, 1990, 1999). Only 31 species among the 500 known *Phlebotominae* have been identified as vectors for *Leishmania* spp. (Killick-Kendrick, 1999; Murray et al., 2005). Some sandflies are only capable or harboring specific *Leishmania* spp. while others are permissive to several species enabling the development of parasites from different species (Kamhawi et al., 2000; Pimenta et al., 1994). Parasite surface LPG and sandfly midgut surface lectins were already involved in the host selectivity process in the *L. major-Phlebotomus papatasi* system (Kamhawi, 2006; Kamhawi et al., 2004).

1.3.2 - The amastigote stage and the vertebrate hosts

The parasites residing in the mammalian hosts are mostly found inside the macrophages. They have a distinct circular shape quite different from the promastigote form. These forms, called amastigotes, are non motile with an average diameter of 2.5 to 5 μm (Bard, 1989). The amastigotes have adapted to survive and proliferate inside the inhospitable environment of the macrophage phagolysosome. Remarkably, *Leishmania* and bacterium from the genus *Coxiella* are the only known organisms capable of prospering in fully mature phagolysosomes (Rabinovitch and Veras, 1996).

The protozoan mammalian stage starts in the hemorrhagic blood pool originated by the sandfly feeding. The first cells in contact with the *Leishmania* spp. will be resident skin cells like dermal macrophages, keratinocytes, and Langerhans cells. Phagocytic cells like dermal macrophages eventually will internalize *Leishmania* spp. acting as the first productive host for the parasite, also Langerhans cells were shown to become infected with parasites although their importance in the context of the infection is not clear (Brewig et al., 2009). The rupture of the microvasculature during the feeding process leads to the onset of an inflammatory process with the massive recruitment of neutrophils (Peters et al., 2008). These, although capable of internalizing parasites, are not the definitive hosts for the amastigote form; in fact their importance in the infection is still a

matter of debate (Sibley, 2011). Infection productive cells like dermal macrophages and dendritic cells are not present in sufficient abundance at the site of inoculation to enable the development of the infection, so the recruitment of monocytes is the next step in the colonization of the mammalian host (Peters et al., 2008). Neutrophils can have a pivotal role in the onset of infection inducing the recruitment of these monocytes (Charmoy et al., 2010). The outcome of this first contact with the host is the internalization of the parasites by cells capable of harboring proliferating parasites (macrophages and dendritic cells) and the induction of an immune environment that enables the maintenance of the infection (see section 3). Eventually the parasites proliferate within the macrophages leading to the lysis of the host cells and the release of new amastigotes that infect other cells. Infected cells or free parasites will be taken up by new sandflies during a blood meal completing the life cycle (Sacks and Kamhawi, 2001).

Sporadically, infection can also occur directly via blood (sharing blood infected needles, transfusion, transplacental spread) or organ transplantation (Cruz et al., 2002; Pagliano et al., 2005). In fact in endemic areas for *Leishmania* spp. the blood transfusions are a growing concern due to the lack of adequate control of subclinical infections in blood donors (Cardo and Asher, 2006; Mestra et al., 2011).

Leishmania spp. can be found in different mammals: rodents, canids, edentates, marsupials, procyonids, primates, equines and felines (Table 1) (Lainson, 2010). It is important to state that not all mammals where the parasite was detected are natural reservoirs for the parasites. There are elements like selectivity of the sandfly and susceptibility of the mammalian host that contribute to the distribution of the parasites and the maintenance of the usually zoonotic life cycle (Shaw, 1997). Taking this into consideration, humans are not the natural mammalian reservoir and their infection by zoonotic *Leishmania spp.* is considered sporadic when compared to the sylvatic reservoir (Lainson and Shaw, 1987). The existence of a strictly anthroponotic life cycle is rare, being restricted to *L. donovani* and *L. tropica,* although the later protozoan was also found in canids (Guessous-Idrissi et al., 1997).

1.4 - The genome and gene expression in *Leishmania spp.*

The single most important tool for the study of this protozoan was the genome sequencing of the several *Leishmania* spp. The availability of the nuclear genome sequences enables new insights into the unusual biology of this group of pathogens. Studies integrating full genome analysis brought a wealth of information that enabled

great steps towards the elucidation of *Leishmania* spp. biology. This brought a better understanding of the parasite evolution, immune evasion, enabling new strategies for vaccine and drug development (DebRoy et al., 2010; Downing et al., 2011a; Herrera-Najera et al., 2009). It was only in 2005 that the genome of *L. major* became available (Ivens et al., 2005), two years later, in 2007, the genome *L. infantum* and *L. braziliensis* was also sequenced (Peacock et al., 2007). Currently several other genomes are publically available (Downing et al., 2011b; Rogers et al., 2011). The latest genome to be sequenced was *L. tarentolae* in 2011 (Raymond et al., 2011).

The comparison between *Leishmania* spp. that are etiological agents for different forms of the disease revealed that only 78 genes from the 8300 predicted genes were species specific (Peacock et al., 2007). Even the non pathogenic *L. tarentolae* showed a 90% conservation of the genome with only 250 genes missing from the core *Leishmania* spp. genome (Raymond et al., 2011). Moreover, the comparison of gene content and genome architecture with related pathogens *Trypanosoma cruzi* and *Trypanosoma brucei*, that have distinct disease presentation and life cycle, revealed a conserved core proteome of about 6200 genes in large syntenic polycistronic gene clusters (El-Sayed et al., 2005). This conservation of a core genome once again excludes the possibility of a highly distinct genetic background as the main driving force in the distinct clinical presentations of these related trypanosomatids.

Up until the 1990s, the gene specific functions within the organism itself were difficult to study. With the advent of DNA transfection in *Leishmania spp.*, it became possible to overexpress proteins in the protozoan, to knockout genes and complement the null mutants, switching off and on essential functions, permitting significant advances in the study of the *Leishmania* spp. biology and disease pathology (Laban and Wirth, 1989; LeBowitz, 1994).

Like all other eukaryotes, the Kinetoplastida order present two different genomes: nuclear and mitochondrial (kinetoplast). The nuclear genome of *Leishmania spp.* has an approximate size of 34 Mb (e.g. 32.8 Mb and 35.5 Mb in the case of *L. major* and *L. infantum*, respectively) and the chromosomes size ranges from 0.3 to 2.8 Mb (Bastien et al., 1992b; Wincker et al., 1996). The *Leishmania* spp. base karyotype varies in number, from 36 chromosomes for Old World species (Wincker et al., 1996) to 34 or 35 for New World species (Britto et al., 1998).

Leishmania spp. nuclear genome is also known for its plasticity (Bastien et al., 1992b). In fact a high level of spontaneous chromosomal polymorphism was recently reported in *L. major* (Sterkers et al., 2011). In addition to this chromosomal plasticity, *Leishmania* spp. can have additional genetic material resulting from amplification of

genomic sequences. These can occur spontaneously or happen as a consequence of parasite exposure to adverse conditions such as drug selection (Grondin et al., 1996; Navarro et al., 1994; Olmo et al., 1995; Segovia, 1994; Ubeda et al., 2008). As the core genome of the *Leishmania* spp. is quite similar it is probable that this genomic plasticity contributes significantly to phenomena such as the distinct virulence between clinical isolates and also drug resistance (Sterkers et al., 2011; Ubeda et al., 2008).

Several reports of interspecies *Leishmania* spp. hybrids (Chargui et al., 2009; Odiwuor et al., 2011; Ravel et al., 2006) lead to the possibility of genetic exchange between parasites. This was highly controversial because it was accepted that *Leishmania* spp. multiply by binary fission and sexual crossing under laboratory conditions failed (Banuls et al., 2007). However, a 2009 report clearly showed the formation of hybrids in the sandfly (Akopyants et al., 2009). More recently the same phenomena was described with *L. donovani* (Sadlova et al., 2011) indicating that sexual reproduction might be a general phenomenon in *Leishmania* spp. during the life cycle. This was further supported by genetics analysis done in *L. braziliensis* and *L. guyanensis* that demonstrated that the percentage of homozygotes was much higher than what expected for a clonal population (Rougeron et al., 2011; Rougeron et al., 2009).

Like in all eukaryotes, DNA content in *Leishmania spp.* is not restricted to the nucleus. The parasite has a single mitochondrion, containing the mitochondrial DNA condensed in a disk-shaped structure, the kinetoplast. This structure is the defining feature of these protozoans and was at the origin of the designation Kinetoplastida for the order (Shapiro and Englund, 1995). This second genomic organelle of the kinetoplastid contains two classes of DNA rings, maxicircles and minicircles. The maxicircles represent the actual mitochondrial genome. Only a few dozen different maxicircles exist, ranging in size from 35 to 50 kb, being present at about 10-30 copies per cell. The gene products of the maxicircles are similar to those of mitochondrial DNA in higher eukaryotes, including rRNA and several respiratory chain components (Liu et al., 2005). Each kinetoplast also contain a few thousand minicircles, ranging in size from 0.8 to 1.6 kb, present at about 30.000-50.000 copies per cell. These minicircles contain the genes for guide RNAs. These guide RNAs are necessary to edit the maxicircle transcripts into functional messanger RNA (mRNA) (Simpson, 1997; Stuart et al., 1997). Unlike higher eukaryotes, the kinetoplastid mitochondrial genomes contain no transfer RNA constituting the single greatest difference in mitochondrial genetic structure (Schneider and Marechal-Drouard, 2000).

The mechanism of transcription initiation in kinetoplastid protozoa is remarkably different from the remaining eukaryotes. The near absence of promoters for RNA polymerase II is the most notable difference in gene expression (Clayton, 2002). The most accepted theory for the RNA polymerase II activity in these organisms is that transcription starts upstream of most 5' genes of each cluster, originating polycistronic transcripts (Martinez-Calvillo et al., 2003). These polycistronic transcripts will then be processed at the 5' end by *trans*-splicing. A common 39 nucleotide methylated mini-exon sequence or spliced leader (SL) is *trans*-spliced upstream of the ATG start codon of the mRNA replacing the capping system found in the majority of eukaryotic organisms (Clayton, 2002; Sturm et al., 1999). The SL gene in *Leishmania* spp. is present as a tandem array of about 150 copies comprising 0.1 % of parasite genome (Lamontagne and Papadopoulou, 1999; Miller et al., 1986). In contrast to most protein encoding genes in trypanosomatids, the SL precursor RNA has clearly identifiable promoters with a short consensus initiator element (Luo et al., 1999) being transcribed by the RNA polymerase II (Gilinger and Bellofatto, 2001).

As a consequence of the almost complete absence of promoters for RNA polymerase II (Clayton, 2002) there is a loss of an entire level of gene expression control at the transcription level. This fact in an organism that thrives in two distinct environments, with different temperatures, nutrients and distinct survival requirements might be problematic. In fact *Leishmania* does not have any significant qualitative control at the transcription initiation level but seems to have adopted a system where post-transcriptional mechanisms, such as mRNA stability and processing, are the major determinants in the regulation of mRNA abundance, and ultimately of protein production (Requena, 2011). The existence of stage specific proteins, like the amastigote specific amastin family, are indicative of the efficiency of these mechanisms (Moore et al., 1996; Nourbakhsh et al., 1996; Souza et al., 1992; Wu et al., 2000). In the genomic era, using a combination of proteomic and genomic approaches, it was possible to confirm that an almost constitutive expression of the genome exits in both promastigote and amastigote stages with low levels of stage specific variability. However, when using a quantitative proteomic analysis, a higher heterogeneity was found in protein expression between both stages (Leifso et al., 2007; McNicoll et al., 2006). Documented mechanisms through which *Leishmania* spp. regulate gene translation include changes in mRNA stability (Brittingham et al., 2001; Kelly et al., 2001; Mishra et al., 2003; Muller et al., 2010a; Muller et al., 2010b) and rate of translation (Boucher et al., 2002; Zeiner et al., 2003). These gene regulation mechanisms are remarkably efficient, enabling the smooth passage between the different life forms of the protozoan.

1.5 – *Leishmania* spp. in the lab: axenic culture

In the wild, a steady population of *Leishmania* spp. is maintained by the sylvatic life cycle. To enable the study of this protozoan in a laboratory environment a stable reproducible source of microorganisms is required. Although the use of sandflies for the growth of the *Leishmania* spp. is possible, issues related to biosecurity and the lack of adequate facilities negates the generalized utilization of the sand fly model (Volf and Volfova, 2011). As a consequence the bulk of the available information about *Leishmania* spp. is obtained from the study of axenic promastigotes. The promastigote form can be easily cultivated at temperatures below 28ºC in different types of media (Hendricks et al., 1978; Sadigursky and Brodskyn, 1986). Years of development of axenic culture permitted the definition of generalized nutritional requirements which include: heme (Chang and Chang, 1985); 6-hydroxymethylpterine and related pteridines (Bello et al., 1994); high levels of folic acid (Kar, 1997); the vitamins thiamine, nicotinic acid, pantothenate, riboflavin, and biotin (Schuster and Sullivan, 2002) and also several essential amino acids required by other eukaryotes (Schuster and Sullivan, 2002). The first medium used for *in vitro* maintenance of *Leishmania* spp. was Novy-MacNeal-Nicolle (NNN) medium (Nicolle, 1908), still remaining a medium of choice for the isolation of field strains (Schuster and Sullivan, 2002). Since the development of the NNN medium several other media formulations were created for the growth of *Leishmania* spp. in a continuous effort to develop more affordable and defined media (Grekov et al., 2011; Rodrigues Ide et al., 2010; Schuster and Sullivan, 2002; Sharief et al., 2008). The media formulations can be either diphasic or monophasic. The diphasic media contains a solid/semi-solid phase and a liquid phase. The solid phases are enriched with a variety of supplements, including peptone, beef infusion, glucose, tryptose, liver extract, brain heart infusion, individual amino acids, nutrient or trypticase soy agars and blood in concentrations from 2.5 to 50% with the rabbit preferred to other sources of blood (Schuster and Sullivan, 2002). The liquid phases can be as simple as water or as complex as RPMI (Schuster and Sullivan, 2002). These biphasic media are usually not very amenable for routine lab utilization usually being cumbersome for use. In consequence, their application resides mostly in the isolation of field strains. Therefore monophasic (liquid) media are preferred for the growth and maintenance of *Leishmania* spp. in the laboratory environment. These liquid media can be undefined (with many of the components found in the solid phase of diphasic media), semidefined (using commercially available insect and mammalian tissue culture media, supplemented normally with fetal calf serum) or defined (all components are known). Undefined media are usually less expensive and enable a broader use (Schuster and Sullivan, 2002). Semidefined media are usually the media of choice for lab

use enabling a combination of high yield and reproducibility of growth. In these media fetal calf serum (FCS) is the most used supplement in concentrations between 5-20%. These defined media are not amenable to use in studies that involve the detection of released proteins from the parasites because of the dominant contribution of serum related proteins (Silverman et al., 2008). In 1999 a report, using completely defined medium, enabled a better understanding of the nutritional requirements of these protozoan (Merlen et al., 1999). In addition, *Leishmania* spp. grown in this medium maintained infectivity, proteomic and antigenic profiles of the control semidefined media in a clear indication that serum complementation can be replaced without apparent metabolic cost to the parasites (Merlen et al., 1999).

The metacyclic parasite is considered the endpoint of promastigote development inside the sandfly (Sacks and Perkins, 1985). Metacyclogenesis can be conveniently reproduced during *in vitro* culture: the procyclic forms correspond to promastigotes in the exponential phase of growth, eventually these parasites enter into stationary phase and ultimately a fraction of these stationary parasites differentiates into the metacyclic form (Sacks and Perkins, 1984). The *in vitro* characterization of the metacyclic parasites is traditionally based on morphological information and biological criteria. Metacyclic parasites are of a smaller size than stationary parasites, with a flagellum at least twice the body length (Sacks and Perkins, 1984). Moreover they are more resistant to complement (Zakai et al., 1998) and express stage specific genes like the small hydrophilic endoplasmic reticulum-associated protein (SHERP) or hydrophilic acylated surface protein B (HASPB) (Knuepfer et al., 2001; Sadlova et al., 2010). These characteristics resemble those found in sand fly promastigotes (Sacks and Perkins, 1984, 1985). Still at the molecular level, a study in *L. major* revealed some differences between metacyclic *in vitro* and *in vivo* with the protein HASPB showing a different pattern of expression in the vector metacyclic stage (Sadlova et al., 2010). Albeit, the *in vitro* metacyclic parasites are the reference promastigote form for most experimental procedures (Zakai et al., 1998). However, this form of the parasite is present in variable percentage in cultures. Stationary phase cultures represent a heterogeneous population with distinct virulence profiles (da Silva and Sacks, 1987). Therefore several techniques were developed for the purification of *Leishmania* spp. metacyclic forms. The oldest methods are based on LPG. LPG of procyclic parasites is smaller than their stationary counterpart, furthermore there are also species specific differences related to the sugar moiety (Sacks, 2001). In consequence, the use of LPG specific monoclonal antibodies has enabled the recovery of metacyclics from several species (Courret et al., 1999; Lira et al., 1998; Sacks et al., 1995). Also, an affinity based method that relies on the lectin binding to the LPG of non metacyclic parasites was used

succesfully for *L. major* (Pinto-da-Silva et al., 2002; Soares et al., 2002). Still, these LPG based techniques are not applicable to several species because they are highly dependent on the knowledge of species specific changes in LPG composition (Spath and Beverley, 2001). In 2001 a more general technique, based on the buoyant density of the parasites, enabled the purification of metacyclics using a Ficoll-based gradient centrifugation (Spath and Beverley, 2001). Although stationary-phase promastigotes with undefined *in vitro* passages are commonly used without limitations, it has been demonstrated that continuous culture over time induces loss of virulence. In fact, long-term *in vitro* culture of promastigotes was one of the first empirical approaches to development of attenuated strains enabling the study of virulence associated genes (Mitchell et al., 1984). In fact it was described that continuous culture of *L. donovani* induced virulence loss was associated with reduced expression of LPG and Kinetoplast membrane protein 11 (Mukhopadhyay et al., 1998). Also disadvantageous adaptations to the media may result in loss of virulence (Segovia et al., 1992). Either way, alterations in the physiology of the parasite induced by long-term growth in media may lead to misinterpretation and contradictory results.

The study of the amastigote form is essential because this is the stage whose proliferation is *de facto* responsible for the pathology. Amastigotes can only be obtained from experimentally infected animals or host macrophages infected *in vitro*. This limitation prevents the obtainment of large numbers of amastigotes free of host cell contaminants, a fact that has hampered the investigations of their metabolic, biochemical and biological properties (Gupta et al., 2001). An alternative model is to keep amastigote-like parasites in axenic cultures. Several *Leishmania* species can be maintained as amastigotes in axenic laboratory conditions (Bates et al., 1992; Doyle et al., 1991; Eperon and McMahon-Pratt, 1989; Hodgkinson and Soong, 1997; Nasereddin et al., 2010; Puentes et al., 2000; Rainey et al., 1991; Saar et al., 1998; Sereno and Lemesre, 1997). In laboratory conditions, axenic amastigotes can be obtained by increasing the temperature as a trigger, either alone or in combination with a reduction of pH (Hodgkinson and Soong, 1997). However, careful optimization of culture conditions is strictly necessary, since the conditions used in one species/strain might not be appropriate for others species (Gupta et al., 2001). For *L. infantum* the differentiation occurs within 3-4 days after the transfer of stationary-phase promastigotes to a cell free culture medium at an acidic pH and at 37ºC (Sereno and Lemesre, 1997). The axenic amastigotes have morphological, biological, biochemical and immunological characteristics that are similar to "true" intramacrophagic amastigotes (Bates, 1993; Saar et al., 1998). Axenic amastigotes express amastigote specific genes like amastin and cysteine protease B, have specialized structures

called megasomes, thrive at temperatures and pH compatible with the macrophage phagolysosome and are capable of productive infections (Bates, 1993; Saar et al., 1998). In spite of these similarities, gene expression analysis in *L. infantum* axenic amastigotes revealed that gene expression was distinct from intramacrophagic amastigotes (Rochette et al., 2009). The differences in gene expression are related to specific metabolic process, like fatty acid metabolism, intracellular transport and membrane vesicular fusion, proteolysis, and the response to oxidative stress (Rochette et al., 2009). In general and despite controversy about the biological properties (Holzer et al., 2006; Rochette et al., 2009), the axenically cultured amastigotes are a useful tool in several studies being commonly used in drug screening (Gupta, 2011). These axenic amastigotes are more likely to be abnormal promastigotes than *bona fide* amastigotes but the lack of a better model still justify their use.

2 – Leishmaniasis

Leishmaniasis is a typical example of an anthropozoonosis. The majority of the infections are originally zoonotic, with dogs, cats, lizards, gerbils, squirrels and other rodents as their primary vertebrate hosts (Table 1). The exceptions are visceral leishmaniasis (VL) caused by *L. donovani* and cutaneous leishmaniasis (CL) caused by *L. tropica* in India and Iran, where humans are the main hosts of infection.

Table 1. Major epidemiological characteristics of pathogenic *Leishmania* spp. Adapted from (Dedet, 2008)

Parasite species	*Reservoir hosts*	*Sandfly vector*	*Clinical form*	*Distribution and ecological patterns*
L. donovani	Humans	*P. argentipes*	Kala azar PKDL	Rural disease
L. infantum	Dogs	*P. (Larroussius)* spp.	Infantile VL	Rural disease
L. major	Gerbilline rodents	*P. papatasi, P. duboscqi*	Localized CL	Rural disease of Old World arid and perarid areas
L. tropica	Humans	*P. sergenti*	Localized CL	Urban disease of Near and Middle East
L. aethiopica	Hyracoïds	*P. longipes, P. pedifer*	LCL; DCL	Rural disease, Ethiopia
L. killicki	Unknown	Unknown	LCL	
L. braziliensis	Wild mammals	*Lu. wellcomei; Lu. intermedia; Lu. gomezi; Lu. ylephiletor; etc.*	CL and MCL	Sylvatic zoonosis of New World primary rain forest
L. guyanensis	Sloths, anteaters, opossums	*Lu. umbratilis; Lu. whitmani*	Localized CL	Sylvatic zoonosis of New World primary rain forest
L. panamensis	Sloths, monkeys	*Lu. trapidoi; Lu. gomezi; Lu. ylephiletor*	Localized CL	Sylvatic zoonosis of New World primary rain forest
L. amazonensis	Echimyd rodents	*Lu. flaviscutellata*	LCL; DCL	Sylvatic zoonosis of New World primary rain forest
L. mexicana	Rodents	*Lu. olmeca*	LCL	Sylvatic zoonosis of New World primary rain forest
L. naiffi	Armadillos	*Lu. ayrozai; Lu. paraensis; Lu. squamiventris*	LCL	Sylvatic zoonosis of New World primary rain forest
L. lainsoni	*Agouti paca*	*Lu. ubiquitalis*		Sylvatic zoonosis of New World primary rain forest
L. peruviana	dogs	*Lu. peruensis*	LCL	Arid valleys of western slopes of Peruan Andes

Leishmaniasis is one of the most significant neglected tropical diseases, the estimated disease burden places it second in mortality and fourth in morbidity among tropical infections (Bern et al., 2008). It is prevalent in tropical and subtropical regions of the world and endemic in 88 countries (See figure 4 for global distribution of the disease) with a total population at risk of 350 million resulting in two million new cases reported annually (Kedzierski, 2010). Like many other tropical diseases leishmaniasis is associated with poverty making the research for better treatments or vaccines less attractive to industry. Unlike other tropical diseases there has been an increase on incidence of leishmaniasis in the last 20 years. Factors related to population mobility, expansion of housing in highly endemic zones (Gramiccia and Gradoni, 2005) and also increased resistance to first line anti-*Leishmania* drugs contribute to this increase (Ouellette and Papadopoulou, 1993). Also in non endemic areas a steady rise on the number of cases has also been reported as a consequence of global mobility. In fact the number of British travelers with leishmaniasis more than quadrupled between 1995 and 2003 (Lawn et al., 2004) and in just two years between 2003-2005 more than 600 US soldiers were diagnosed with leishmaniasis (Zapor and Moran, 2005). The appearance of opportunistic co-infections with human immunodeficiency virus (HIV) as result of the overlap between endemic areas of leishmaniasis and HIV foci in suburban areas also contributed to the increase of the registered cases (Alvar et al., 1997; Desjeux, 2004; Tremblay et al., 1996). The impact of the co-infection is probably underestimated due to constraints in surveillance and in the actual detection of the co-infection (Desjeux, 2004). In south-western Europe more than 70% of all adult cases of visceral leishmaniasis are associated with HIV/AIDS and up to 9% of HIV-infected individuals are infected with *Leishmania* spp. (Cruz et al., 2006).

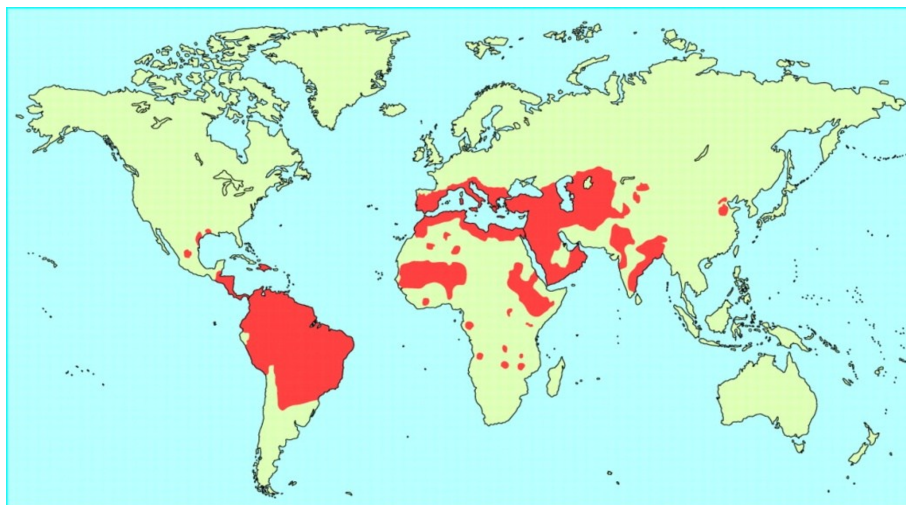

Figure 4. Global geographic distribution of leishmaniasis, adapted from (Davies et al., 2003)

2.1 - Disease presentation

Leishmaniasis can have distinct presentations (Depicted in figure 5) generally depending of the infecting species (around 20 are thought to be pathogenic – see table 1) and the immunological status of the host. The disease can present itself in the form of chronic and often scarring ulcerations representing the non-visceralizing presentation of the disease: cutaneaous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL) or diffuse cutaneous leishmaniasis (DCL). These forms of the disease are usually non-fatal, self healing although socially stigmatizating due to the development of lesions in the skin or mucosal surfaces. The visceralizing form of the disease (VL) has a more severe presentation, involving dissemination of the parasite to internal organs such as bone marrow, liver or spleen originating a lethal progressive disease if untreated. This form of the disease is fatal if not treated and is responsible for 70.000 yearly deaths (Kedzierski, 2010).

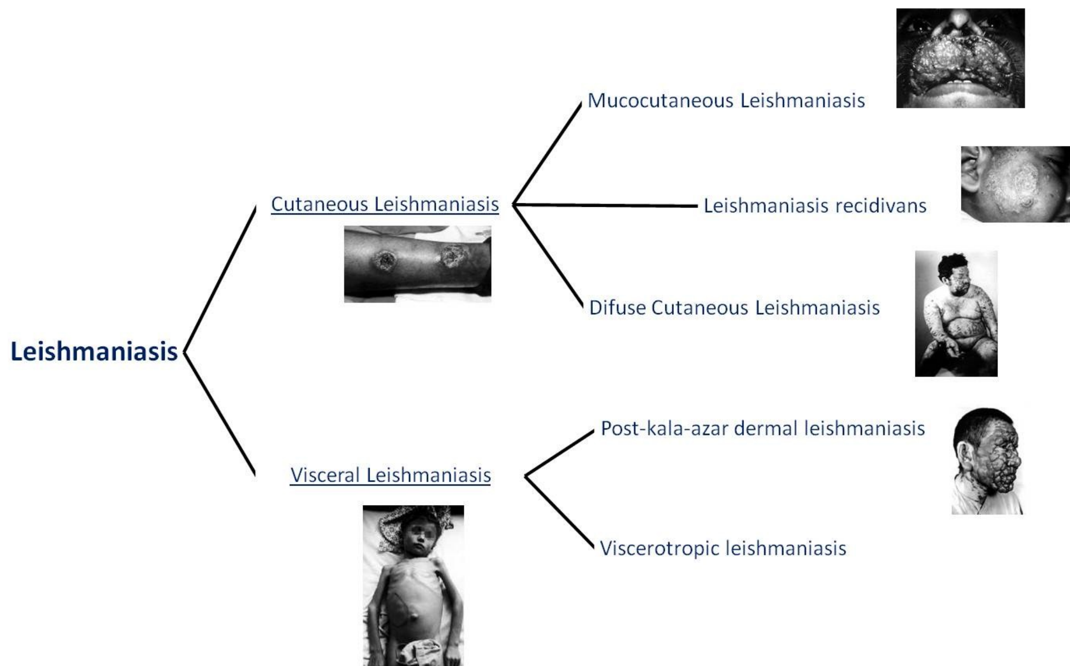

Figure 5. Disease presentation and major clinical signs of Leishmaniasis. Pictures adapted from http://emedicine.medscape.com/article/1108860-clinical#a0217

2.1.1 - Cutaneous leishmaniasis

The cutaneous form of the disease accounts for more than half of the total leishmaniasis cases in the world. CL may result from infection by any of the *Leishmania* spp. that infects humans. However, it is traditionally associated with several species of the *Leishmania* subgenera of both the Old World (*L. tropica, L. major* and *L. aethiopica*) and New World (*L. mexicana* complex, especially *L. mexicana* and *L. amazonensis*). Also, several species of the *Viannia* subgenera (most notably *L. braziliensis*, *L. panamensis, L. guyanensis* and *L. peruviana)* are also causative agents of CL (Murray et al., 2005). With an incubation period that can last from a few days to months, the disease is characterized by single or multiple localized lesions on exposed areas of the body, face, neck, arms and legs that typically ulcerate or persist as nodules or plaques (Dowlati, 1996). The initial lesion is often a small and red papule in the site of inoculation, showing localized cell infiltration. Usually, this disease manifestation will be resolved spontaneously, with the recovery time depending both on the species and on the immune status of the individuals. However, secondary infections are common, leading to permanent disfiguration (Murray et al., 2005). A more severe form of CL is DCL caused by *L. aethiopica*, *L. amazonensis* and *L. mexicana*. This is a particular form of CL, which occurs only in hosts with deficient

immune responses. The infection is characterized by a primary lesion, which spreads to involve multiple areas of the skin with numerous parasites present in each lesion (Roberts and Janovy, 2000). Another CL variation is leishmaniasis recidivans, a rare chronic infection caused essentially by *L. tropica*. This unusual form of leishmaniasis is characterized by new lesions appearing around old healed ones (Murray et al., 2005).

2.1.2 - Mucocutaneous leishmaniasis

Mucocutaneous leishmaniasis or "espundia" is a form of CL in which mucosal dissemination of the infection occurs. This disease is usually caused by *L. braziliensis, L. panamensis* and *L. guyanensis* (rarely the latter two species), which are found in Central and South America. After a cutaneous self-healing episode 1-10% of the patients develop in the space of 5 years mucocutaneous complications (Machado-Coelho et al., 2005). Mucocutaneous complications may also originate upon inadequate treatment of infections by some *Leishmania* spp. such as *L. mexicana* (Roberts and Janovy, 2000). In MCL the infection spreads to the mucosal system of the nasal and oral cavities, where metastatic lesions develop leading to partial or total destruction of those membranes. Unchecked the infection might lead to severe disfiguration or even death, usually due to secondary bacterial infections or malnutrition (Osorio et al., 1998; Santrich et al., 1990).

2.1.3 - Visceral leishmaniasis

Species of the *L. donovani* complex (*L. donovani*, *L. infantum* and *L. chagasi*) are the principal causative agents of VL, also known as kala-azar or Dum-Dum fever. Also, *L. tropica*, in the Old World and *L. amazonensis* in the New World can sometimes be viscerotropic. Usually, VL has an incubation period that varies from 2 to 4 months leading to several clinical presentations: asymptomatic, acute and chronic (Murray et al., 2005). In highly endemic areas, more than 60% of the local population can have circulating anti-*Leishmania* antibodies, or present a positive skin test for the disease. However, only about 15% of the population report having the active disease (Bern et al., 2007). The remainder will retain a state of subclinical (asymptomatic) leishmaniasis contributing to the perpetuation of the life cycle in anthroponotic areas. The usual course of the disease involves targeting the visceral organs resulting in a myriad of symptoms that include fever, cachexia, hepatosplenomegaly and polyclonal hypergammaglobulinemia (Davidson,

1998). It is the most severe form of leishmaniasis and, in the absence of treatment, leads to multisystemic disease, with increased susceptibility to secondary infections and ultimately resulting in death (Boelaert et al., 2000; Davidson, 1998). Patients, who have recovered from VL, usually have lifelong immunity to reinfection but occasionally relapses may occur under immunosuppressive conditions (Sundar et al., 2002a). In east Africa and in India a secondary reminiscent complication of VL is the post kala-azar dermal leishmaniasis (PKDL). The PKDL may develop in 5-10% of patients several months or years after an apparent successful kala-azar treatment (Zijlstra et al., 2003). It is characterized by the development of nodules over the face and limbs. This form of the disease is considered to be a major source of parasites for new infections due to the large number of organisms present in the skin lesions rendering them more accessible to the vector (Zijlstra et al., 2003).

2.1.4 - Particular cases of infection

2.1.4.1 - *HIV-Leishmania* co-infection

In HIV-infected patients, VL accelerates the onset of AIDS by cumulative immunosuppression leading to increased viral replication. More than 90% of the VL-HIV co-infections do not represent a novel infection event but are reactivations of latent parasitic infections (Morales et al., 2002). Moreover, leishmaniasis patients are highly susceptible to HIV infection, and may change from an asymptomatic status to symptomatic leishmaniasis developing internal and external clinical signs of the disease (Desjeux et al., 1999). The global burden of the co-infection is thought to be on the rise with the exception of Southern Europe where the very effective Highly Active Antiretroviral Therapy for HIV is responsible for a decrease in the number of cases (Fernandez Cotarelo et al., 2003). HIV infections associated with cutaneous leishmaniasis are rare (Rabello et al., 2003).

2.1.4.2 - Atypical presentations, hybrid and mixed infections, and other co-infections

It is important to state that the above mentioned disease presentations are not always related to the traditional etiological agents. For instance in South America, there are cases of VL caused by traditional cutaneous species like *L. amazonensis* (Aleixo et al.,

2006; Almeida et al., 1996) and *L. tropica* (Alborzi et al., 2006). Also MCL caused by *L. donovani* and *L. major* was described in Sudan (el-Hassan et al., 1995). Another level of complexity in disease presentation is related to the existence of *Leishmania* hybrids that often have increased virulence with pathologies typical of both original species/strains (Dujardin et al., 1995). Also the existence of mixed infections can occur and contribute to complex diagnosis and treatment options, in fact very few infections with mixed species have been described (Antoniou et al., 2004; Bastrenta et al., 2003; Martinez et al., 2002). However, the existence of disease foci with several endemic *Leishmania* species (Lucas et al., 1998) lift the possibility that real numbers of mixed infections may be masked by incomplete diagnosis (Bastrenta et al., 2003).

Concerning co-infections with other pathogens, besides the already mentioned HIV (Cruz et al., 2006), abundant literature can be found. Pathogens like *Mycobacterium tuberculosis* (Delobel et al., 2003), *T. cruzi* (Bastrenta et al., 2003) or *Salmonella* (Djidingar et al., 1997) often exist associated with *Leishmania* infections. These co-infections can lead to very unusual forms of the disease (Calza et al., 2004) with sometimes complicated treatment options.

2.1.5 – Experimental models for leishmaniasis

The progress in controlling leishmaniasis requires an improved appreciation of the biology of the parasite to design novel prevention and treatment strategies.

The use of animals as models in medical research started in the 19th century with the application of Koch's Postulates (Croce, 1996). Since then several animal models were developed for the study of numerous biological agents. *Leishmania spp.* are no exception: several animal models exist that enable the study of the parasite and the disease. Curiously the majority of the experimental animals used to study leishmaniasis are not natural hosts for any *Leishmania* spp. (Sacks and Melby, 2001). Furthermore, for a long time, inoculation of animals was the only possible way to obtain amastigotes (Gupta et al., 2001) making them even more important.

A useful experimental animal model must have several prerequisites such as the capacity to mimic the pathological features and immunological responses in comparison to what happens into humans. Although many experimental animal models are used, none of them reproduces accurately the disease development in humans. Rodents, such as mice and hamsters are the most common used animal models for *Leishmania* spp. research (Garg and Dube, 2006; Sacks and Melby, 2001). The different forms of leishmaniasis have

distinct outcomes in rodents. Mice are considered an excellent model for cutaneous leishmaniasis. Notwithstanding, for visceral strains the situation is different. Visceral species develop a self healing or subclinical form that is different from the disseminated visceral disease, found in humans. Furthermore even susceptible strains manage to control their infections unlike the human infection that is fatal if not treated (Barbosa Junior et al., 1987). Several inbred murine models for experimental leishmaniasis are well documented. Infections in BALB/c mice induce a progressive disease being therefore considered susceptible strains. Other strains like C57BL/6, CBA or C3H develop transient lesions, controlling parasite multiplication and developing immunity to re-infection, being considered resistant strains. Studies on these inbred mouse models have permitted the characterization of the immune mechanisms that play a role in the development of the infection. Subtle differences in the genetics of these mice lead to dramatic changes in disease outcome demonstrating that host genetic factors play a major role in the susceptibility or resistance (Depledge et al., 2009; Silva et al., 2011; Wei et al., 2004). Hamsters are considered a better model for VL, since they mimic the progressive fatal disease characteristic of humans, with the parasite disseminating to the liver, spleen and bone marrow (Ghosh and Ghosh, 1987). Still, the lack of immunological reagents and inbred strains of hamsters has limited the exploitation of this animal model (Garg and Dube, 2006). Other less often used models, normally used in later stages of vaccine or drug development (Garg and Dube, 2006), like dogs and monkeys have the advantage of resembling more the pathology in humans but are more expensive models.

Animal models also showed the importance of the route of inoculation in parasite virulence (Menon and Bretscher, 1998; Paranhos-Silva et al., 2003; Rolao et al., 2004). Reproduction of natural infection using laboratory colonies of sand flies is possible but the parasite inoculum is highly variable (100-10000 parasites) making standardized infections impossible (Kimblin et al., 2008; Rogers et al., 2010). The alternative was to develop other infection procedures. Traditionally these vary in parasite number (10^4-10^8 parasites) and administration routes (intradermic, intravenous or intraperitoneal) (Mbati, 1995). Most of the studies in VL use the endovenous or intraperitoneal routes to inoculate the parasites, the latter being preferred since it induces a higher homogeneity of infection (Rolao et al., 2004). In recent years, there has been a tendency to mimic as much as possible the natural infection. Thus, lesser numbers of infective parasites (10-1000 metacyclic promastigotes) can be used, being intradermaly or subcutaneously injected in the experimental animals (Belkaid et al., 2000; Courret et al., 2003; Lira et al., 2000). To further improve experimental conditions, several infection relevant components present in the sand fly vector like salivary proteins, salivary glands or parasite derived

proteophosphoglycan like the promastigote secretory gel have been tested as complements to the infection inoculum (Milleron et al., 2004; Monteiro et al., 2007; Rogers et al., 2010; Rogers et al., 2004).

2.2 – Diagnosis and control of the disease

The early detection and control of the disease is essential. Leishmaniasis is characterized by a non specific clinical presentation that requires an accurate differential diagnosis to decide the treatment options. The diagnostic tests must be sensitive, especially for VL that is fatal, and highly specific because treatment is not risk free involving often considerable toxicity. The control of the vectors and sylvatic reservoirs is also an important aspect in disease control. The absence of vaccine prevents the eradication of the disease.

2.2.1 – Leishmaniasis diagnosis

Leishmaniasis can induce a broad spectrum of clinical manifestations. Moreover, the majority of clinical symptoms associated with leishmaniasis are non pathognomonic. Hence, they can mimic diseases of other pathologies rendering difficult the clinical diagnosis (differential diagnosis includes, among others, superinfected insect bites, leprosy, tuberculosis, skin cancer for CL and malaria, African trypanosomiasis, brucellosis, schistosomiasis for VL). Consequently, a differential diagnosis is essential to confirm the clinical suspicion (Herwaldt, 1999). The most common methods for diagnosis for all leishmaniasis forms include: direct parasite observation in infected tissues; immunological detection of parasite antigens or anti-*Leishmania* antibodies and direct parasite DNA or RNA detection in tissue samples by molecular approaches.

2.2.1.1 – Parasitological diagnosis

Direct visualization of amastigotes in clinical specimens is the gold standard for leishmaniasis diagnostic (Murray et al., 2005). Tested biological material can range from skin biopsies (for CL and MCL) to aspirates from the spleen, bone marrow and lymph nodes (for VL). Diagnostic sensitivity will be dependent on the parasite number and their

dispersion on the specific biological samples. For aspirates from spleen, bone marrow, and lymph nodes the sensitivity is 95%, 55-97%, and 60%, respectively. For skin biopsies the sensitivity is lower but using combined microscopy and culture it should be in the 85% range (Guerin et al., 2002; Ramirez et al., 2000). In fact when the combination of adequate biological material and microscopy and technical skills are available, this remains the method *par excellence* for confirming leishmaniasis (Murray et al., 2005). The parasites can be observed in stained preparations (with Giemsa, Romanovsky, Hematoxilin & Eosin) or visualized upon recovery in NNN medium or animal inoculation (Herwaldt, 1999). Culture based diagnosis of MCL has been shown to have very low sensitivities, since the parasites in the lesions are often in low numbers (Rosbotham et al., 1996). Similarly, for PKDL diagnosis, parasitological diagnostic often lacks sensitivity in skin smears or biopsy specimens, since the parasite number in the skin can sometimes be low (el Hassan et al., 1992). Major limitations for the use of parasitological diagnosis are the requirement of often invasive procedures for sample recovery, and the high dependency on the specific technical skills of the microscopic operator rendering it impossible to implement outside the hospital/laboratory environment.

2.2.1.2 – Immunological diagnosis

Immunological diagnosis methods include traditional serological tests and also the determination of *Leishmania*-specific cell-mediated immunity (e.g. skin testing for delayed-type hypersensitivity reactions). These methods are unable to distinguish between resolved and active infections, since in some cases *Leishmania*-specific antibodies can persist after cure, hence leading to false positives (Santarem et al., 2005). Also in immunocompromised patients the serological methods are not adequate and can yield false negative results due to the immunosuppression. Immunological approaches to the diagnosis are more commonly used in VL than either CL or MCL, since non-viscerotropic leishmaniasis usually presents a low number of *Leishmania* specific circulating antibodies (Herwaldt, 1999). Among the serological methods, the agglutination tests (direct agglutination tests and fast agglutination screening tests) are considered highly specific and sensitive tests, suitable for both laboratory and field uses (Schallig et al., 2001; Schoone et al., 2001). These tests use whole trypsinized, coomassie-stained promastigotes either as a suspension or in a freeze-dried form. The indirect fluorescent antibody test, immunoblotting and enzyme linked immunosorbent assay (ELISA) are other classical methods used in the detection of *Leishmania*-specific antibodies. The ELISA based techniques are one of the most used serological approaches for the diagnosis of

leishmaniasis. Its specificity and sensitivity depend greatly on the antigen used (Singh et al., 2005). The protein rK39 is the most widely used antigen for diagnosis of VL and PKDL in India (Salotra et al., 2002; Singh et al., 1995a), being commercially available as a dipstick test (Sundar et al., 2002b). Moreover, rk39 has proven to be a good prognostic test for monitoring VL patients submitted to chemotherapy and in detecting VL in immunocompromised patients (Houghton et al., 1998). Serological testing should be considered in blood banks and pre-transplantation screening for latent *Leishmania* spp. infection, since these constitute a possible route of transmission. When *Leishmania*-specific antibody titers are low, an alternative diagnosis method consists in detecting parasite antigens directly. Recently, a latex agglutination test for the detection of *Leishmania spp.* antigens in the urine of VL patients was developed (KATEX), which is considered as a good marker of infection in immunocompromised patients. It is a simple and rapid test, however no similar system has yet been developed for CL and MCL (Sundar et al., 2005). Diagnoses based on the determination of *Leishmania*-specific cell-mediated immunity are usually used as an indicator of the prevalence of CL and MCL in human and animal populations, as it develops during the active disease. Also, since VL patients only develop strong *Leishmania*-specific cell-mediated immunity when cured, these methods serve as an indicators of a successful cure of VL (Bensoussan et al., 2006). The leishmanin skin test or Montenegro reaction measures delayed-type hypersensitivity reactions to intradermal injection of the leishmanin antigen (killed *L. donovani* parasites) in the patient's arm, in a highly sensitive and specific manner (Weigle et al., 1991).

Although the immunological based techniques are invaluable tools in the diagnosis of the disease, they are indirect methods of detection, presenting real problems in endemic areas where presence of antibodies or parasite material can be synonymous of contact with the parasite and not active disease. Nonetheless the immunological tests based on freeze-dried antigen DAT tests and the immunochromatographic dipstick tests are becoming the reference tests for field use because they have high sensitivity and specificity and require minimal technological expertise or laboratory setup (Chappuis et al., 2006).

2.2.1.3 – Molecular diagnosis

Another direct method for the detection of parasites is based on the use of molecular biology techniques. The detection of DNA or RNA sequences unique to the protozoan enables a high degree of specificity and sensitivity. The polymerase chain

reaction (PCR) based assays currently constitute the main molecular diagnostic approach in developed countries. The PCR allows a highly sensitive and specific (up to 100%) detection of the *Leishmania* spp. In fact a positive PCR is often required for differential diagnosis in order to initiate therapy (Reithinger and Dujardin, 2007). The PCR based parasite detection have consistently been shown to be better than microscopy or parasite culture, particularly in samples with low parasite loads such as blood (Cruz et al., 2002). The contribution of PCR also appears to be particularly relevant for the diagnosis of leishmaniasis in patients co-infected with HIV with detection being done from minimally invasive blood samples of infected patients (De Doncker et al., 2005). Also confirmation of successful therapy often involves PCR confirmation, especially in VL (Maurya et al., 2005). In CL this confirmation is not required because 80% of patient scars remain PCR positive even several years after clinical cure (Schubach et al., 1998).

The predictive power of the PCR based assays is very high. They are capable of detecting DNA or RNA from the parasite just a few weeks after the appearance of the first clinical symptoms allowing even *Leishmania* spp. identification when combined with restriction fragment length polymorphism (RFLP) sequencing. Different *Leishmania*-specific DNA and RNA sequences can be targeted. Commonly used regions include the gp63 gene locus, telomeric sequences, both conserved or variable regions in mitochondrial minicircles, mini-exon derived DNA and rRNA genes, among others (Osman et al., 1997). Several distinct PCR based techniques are used. These techniques include: PCR-restriction fragment length polymorphism analysis (PCR-RFLP), real-time PCR (RT-PCR), PCR-Single strand conformational polymorphism (PCR-SSCP) (for detection of ribosomal RNA genes) and PCR-ELISA (Reithinger and Dujardin, 2007). These different techniques in combination or alone enable: the detection of the parasite; quantification of parasite in tissue (Mary et al., 2004); detection of parasite viability using RNA instead of DNA (van der Meide et al., 2005); species identification (Singh et al., 2005) and also the detection of parasite specific features, such as resistant strains, depending on finding adequate phenotypical genetic markers (Reithinger and Dujardin, 2007).

However, the use of PCR as a routine diagnostic method is a technique restricted to laboratory use. In fact, for the direct protozoan detection in clinical laboratories of countries where the parasite is not endemic the trend is to prefer molecular diagnosis. The cause for this is the lack of microscopists with extensive experience in detecting amastigotes in microscopy slides and also the existence of well equipped laboratories. In contrast, in countries where the parasite is endemic, microscopists skills are maintained due to routine laboratory practice, microscopy tends to be preferred as the first-line parasite detection method (Reithinger and Dujardin, 2007).

2.2.2 – Treatment of the disease

Leishmaniasis is consistently characterized as one of the most neglected diseases in terms of coordinated drug development, requiring new, safe, affordable and easy-to-use treatment options (Modabber et al., 2007; Vanlerberghe et al., 2007). The first efficient drug treatments for leishmaniasis were introduced over 60 years ago (Croft et al., 2006). Nowadays although several treatment options are available they present some limitations concerning toxicity, cost and requirement of parenteral administration (See table 2).

Table 2. Standard treatment regimens for visceral leishmaniasis**.** Adapted from (van Griensven and Boelaert, 2011)

	Manufacturer* (trade name of drug)	Regimen	Clinical efficacy	Resistance	Toxicity	Cost of drug course (US$)	Disadvantages
Pentavalent antimonials (sodium stibogluconate, meglumine antimoniate)	Sodium stibogluconate: Albert David (SSG), GlaxoSmithKline (Pentostam). Meglumine antimoniate: Sanofi Aventis (Glucantime)	20 mg antimony per kg bodyweight daily for 20–30 days (depending on geographic area), intravenous or intramuscular	35–95% (depending on geographic area)	Treatment failure up to 60% (Bihar, India)	Moderately toxic: cardiac effects, pancreatitis, nephrotoxicity, hepatotoxicity	53 (generic) to 198 (branded SSG)	Quality control; length of treatment; painful injection; toxicity; resistance in India
Amphotericin B	Bristol Meyers Squibb (Fungizone); generic companies	1 mg/kg every other day for up to 30 days (15 mg/kg total dose), intravenous	>97% for all regions	Not documented	Moderately toxic: nephrotoxicity (inpatient care required)	About 21 (generic)	Need for slow intravenous infusion; dose-limiting nephrotoxicity; heat instability
Liposomal amphotericin B	Gilead (AmBisome)	5–20 mg/kg total dose in 4–10 doses over 10–20 days, intravenous	Asia: >97%; India, single dose: 91%; Africa: not fully established	Not documented	Nephrotoxicity (limited)	280 (preferential) for 20 mg/kg dose; about 3000 (non-preferential)	Price; need for slow intravenous infusion; heat stability (needs to be stored below 25°C)
Miltefosine	Paladin, Montreal, Canada (Impavido)	2–2·5 mg/kg daily over 28 days (India only), oral	Asia: 94% (India); Africa: 60% (single field study), 93% in patients not infected with HIV and excluding those lost to follow up	Only in laboratory isolates	Gastrointestinal effects (20–55% of patients, usually mild), nephrotoxicity, hepatotoxicity, possibly teratogenic	About 74 (preferential), about 150 (non-preferential)	Price; possibly teratogenic; potential for resistance (half-life); poor patient compliance
Paromomycin sulphate	Institute for OneWorld Health;† Gland Pharma, Hyderabad, India	15 mg/kg daily for 21 days (India only), intramuscular	Asia: 94% (India); Africa: under evaluation	Only in laboratory isolates	Nephrotoxicity, ototoxicity, hepatotoxicity (all relatively rare)	About 15	Efficacy varies between and within regions; potential for resistance?

Generic pentavalent antimonials were introduced in the 1940s and still are the recommended first line drugs for the treatment of all forms of leishmaniasis (Frezard et al., 2009; Vanlerberghe et al., 2007). Pentavalent antimonials include the commercially available stibogluconate (Pentostam®) and meglumine antimoniate (Glucantime®) preparations. Pentavalent antimonials exact mechanism of action in leishmaniasis is still

unknown. It is generally accepted that the Sb^{v} acts as a pro-drug being converted to Sb^{III}, the accepted active form of the drug (Frezard et al., 2009). The reduction of Sb^{V} can potentially happen *in vivo* in the phagolysosome or inside the parasites. Glutathione, cysteine or cysteinyl-glycine thiols can actively reduce Sb^{V} (Ferreira Cdos et al., 2003). Glutathione is found mostly in the cytosol of macrophages while the two latter thiols are mostly found inside the lysosomes. The most abundant thiol in the parasite is the glutathione-spermine conjugate, trypanothione. This thiol was shown to be capable of reducing Sb^{V} (Ferreira Cdos et al., 2003). Also the parasite proteins thiol-dependent reductase 1 and antimoniate reductase have been involved in the reduction process inducing susceptibility to Sb^{V} (Denton et al., 2004; Zhou et al., 2004). The entry of Sb^{III} into the parasite was shown to be mediated by an aquaglyceroporin named AQP1 (Gourbal et al., 2004). Upon entry into the parasite, the Sb^{III} will eventually lead to the killing of the parasite. Although DNA fragmentation suggest a role for apoptosis, the exact mechanisms of action of the antimonials is still unclear (Sereno et al., 2001; Sudhandiran and Shaha, 2003). Some evidence was found to support that possible molecular target for Sb^{III} are trypanothione reductase or zinc-finger proteins (Cunningham and Fairlamb, 1995; Demicheli et al., 2008). Also Sb^{V} was shown to have potential antiparasitic properties in the context of the macrophage. Sb^{V} is capable of forming stable complexes with ribonucleosides. These complexes might function as inhibitors to *Leishmania* spp. purine transporters or interference directly with the purine salvage pathway (dos Santos Ferreira et al., 2006). Antimonial resistance is a severe problem in some endemic areas (Lira et al., 1999). Antimonial unresponsive clinical isolates suggest a multifactorial mechanism of resistance (Decuypere et al., 2005; Singh et al., 2003). In fact, several specific antimonial resistance mechanisms are already described. These include loss of AQP1 activity (Gourbal et al., 2004), extrusion of trypanothione SB^{III} complexes by ATP-binding cassette transporters or the sequestration of SB^{III} in vacuoles (El Fadili et al., 2005; Legare et al., 2001). Antimonial use in clinical practice also presents other important disadvantages: long duration of treatment, parenteral administration, frequent toxic effects (Rijal et al., 2003) and also unpredictable outcome of therapy in HIV/VL co-infected patients (Delgado et al., 1999). Nevertheless, in endemic areas where no resistance phenomena to antimonials are reported, it still remains a highly effective drug (Vanlerberghe et al., 2007).

Pentamidine is an aromatic diamine used as a second line drug in leishmaniasis. It was initially used for antimonial unresponsive patients in India (Berman, 1997). Although, its precise mode of action is not known, it is reported that the drug enters inside the *L. donovani* promastigote through arginine and polyamine transporters (Kandpal and

Tekwani, 1997). Although the pentamidine mechanism of action remains unclear, it was reported that upon uptake it accumulates in the mitochondrion and might interfere with the mitochondrial respiratory chain (Basselin et al., 1996). Although no resistant field isolates were reported, resistance related to ATP-binding cassette (ABC) transporters have already been described (Coelho et al., 2007). The use of pentamidine to treat VL is hampered by the higher toxicity of pentamidine when compared to antimonials and reports of lab acquired resistance phenomena (Jha, 1983). However, this drug is still considered as an attractive alternative for CL, since it demonstrates high cure rates with short periods of treatment (Berman, 1997) and is capable of performing better than Glucantime for some species in CL (Soto et al., 1994).

In the areas of antimonial resistance, amphotericin B is highly regarded for the remarkable efficacy in VL (Thakur et al., 1993). Amphotericin B was not developed as a leishmanicidal drug: in fact it is a common polyene antifungal drug widely used to treat systemic fungal infections (Bern et al., 2006). The use of formulations of amphotericin B for the treatment of leishmaniasis is biochemically sound because the target of amphotericin B in fungi are ergosterol-like sterols (Bern et al., 2006) and ergosterol is the major membrane sterol of *Leishmania* species (Berman et al., 1986). Despite its high efficacy, amphotericin is toxic with side effects, including nephrotoxicity (Laniado-Laborin and Cabrales-Vargas, 2009). Some adverse effects of amphotericin B have been circumvented by using liposomal formulations like AmBisome to reduce the toxicity and to extend the plasma half-life of amphotericin B. Though, the high cost of AmBisome limits its use in developing endemic countries (Vanlerberghe et al., 2007). The mechanism of AmBisome is unknown but it is thought to be related to the interaction with sterols. AmBisome can also actively prevent the entry of *L. donovani* in macrophages by binding with the sterols on the macrophage membrane (Paila et al., 2010). Moreover it can also induce the formation of aqueous pores in promastigote cell membranes inducing cell lysis (Ramos et al., 1996). The acquisition of resistance to amphotericin B is possible in the laboratory although it was reported to select against virulence (Mbongo et al., 1998). Laboratory acquired resistance to amphotericin B is associated with ergosterol deficiency in the parasite membrane (Mbongo et al., 1998). In *L. donovani* the enzyme S-adenosyl methionine transferase (important for the biosynthesis of ergosterol) is associated with increased resistance to amphotericin B (Pourshafie et al., 2004). Despite similar cure rates to antimonials, amphotericin B is not the first line treatment in highly endemic countries because it is considered to be too expensive to compete in cost-effectiveness with the other regimens. In the treatment of VL caused by *L. infantum* in Southern Europe, liposomal amphotericin B is the preferred treatment regimen (Vanlerberghe et al., 2007).

Paromomycin, an aminoglycoside antibiotic, constitutes another parenteral alternative treatment to leishmaniasis (Thakur et al., 2000). In phase III studies performed in India and Sudan, paromomycin demonstrated to be not inferior to amphotericin B for VL treatment (Musa et al., 2010; Sundar et al., 2009). Moreover, it may even be advantageous because of shorter duration in its administration and increased safety in pediatric patients (Sundar et al., 2007). Topical application of paromomycin derivatives also have shown encouraging results for CL treatment (Armijos et al., 2004; Ben Salah et al., 2009). The leishmanicidal mechanism of action of paromomycin is still not clear. Several evidences point to an action related to the translation process. Interference with protein synthesis and mitochondrial interference were demonstrated to occur upon exposure to the aminoglycoside (Jhingran et al., 2009). In fact, direct interference with ribosomes seems to occur preventing ribosomal function and continuous recycling of ribosomal machinery (Hirokawa et al., 2007; Jhingran et al., 2009). Laboratory resistance to paromomycin can be induced in the laboratory being associated with a reduced uptake of the drug (Jhingran et al., 2009; Maarouf et al., 1998). Despite the potential for development of resistance associated with aminoglycosides, paromomycin cures both, VL and CL (the latter more effectively) but the limited availability of the drug still restricts its use in endemic regions. Furthermore, combination therapy of paromomycin with antimony generally results in a regimen that is highly efficacious for visceral disease (Berman, 1997) reducing the side effects associated with both drugs.

All the aforementioned drugs have to be delivered in a clinical environment because parenteral administration is required. Therefore one of the main goal of drug development in leishmaniasis is to find efficient oral agents (Herwaldt, 1999). The imidazole derivatives that inhibit ergosterol biosynthesis were the first oral agents used in leishmaniasis. Ketoconazole was in fact the first oral compound used for leishmaniasis in a controlled environment (Urcuyo and Zaias, 1982). Although it showed some promise in some forms of CL (Navin et al., 1992) it was demonstrated to be completely ineffective for others (Navin et al., 1992; Singh et al., 1995b). The use in a monotherapy regime in VL is not recommended due to the suboptimal healing rates described in several works (Rashid et al., 1994; Sundar et al., 1990). Also itraconazole and fluconazole were tested with variable success for CL with variable cure rates varying from no effect to 100% (Alrajhi et al., 2002; Dogra and Saxena, 1996; Laffitte et al., 2005; Nassiri-Kashani et al., 2005; Sousa et al., 2011). The interest in these imidazole derivatives has been rekindled with reports of successful combination therapy in VL (Barragan et al., 2010; Shakya et al., 2011). The hypoxanthine analog allopurinol, inhibits the purine anabolism in *Leishmania spp.* (LaFon et al., 1985). As with the imidazole derivatives it was shown to have reduced

value in a monotherapy setting (Velez et al., 1997). Combination therapy with allopurinol was tried with some success in CL (Esfandiarpour and Alavi, 2002; Martinez and Marr, 1992; Momeni and Aminjavaheri, 1995; Momeni et al., 2002) but in VL results are not as promising showing reduced efficacy even in combination with other drugs (Ramesh et al., 2010). Still, a report on unresponsive patients to antimony showed an improvement in pentamidine performance when coupled with allopurinol treatment (Das et al., 2001).

The first major breakthrough in the use of an oral agent was miltefosine. This compound, originally developed as an antineoplasic drug, presents a remarkable reported efficiency (>82%) in several phase IV reports for VL (Bhattacharya et al., 2007; Rahman et al., 2011). It also showed a remarkable efficacy for the treatment of different types of CL and MCL (Berman, 2008; Killingley et al., 2009; Soto et al., 2009; Tappe et al., 2010). Moreover, it can be safely used in children (Palumbo, 2008; Sundar et al., 2003) and treats with high success rates, patients who were refractory to antimonial drugs (Berman, 2008). However, miltefosine teratogenicity constitutes still a major limitation to its use for women of reproductive age (Berman, 2008). Furthermore miltefosine as an oral drug, is more prone to non-supervised treatment regimes that may lead more easily to the non compliance of treatment leaving the patients with sub-therapeutical doses. This latter fact in conjunction with miltefosine long half-life, subtherapeuthical quantities of miltefosine are still detected in the patients blood 5 months after the therapeutical regime (Dorlo et al., 2008), constitutes a high risk of development of resistance (Berman, 2008). In fact several laboratory isolates were obtained (Moreira et al., 2011; Perez-Victoria et al., 2003a), but to this date no field isolate for miltefosine resistance was recovered. The mechanism of action of miltefosine and the corresponding molecular targets are still unknown (Berman, 2008). Nonetheless it is clear that the activity of miltefosine is related to intracellular accumulation of the drug, which is regulated by two transporters, LdMT and its b-subunit LdRos3, a P-type ATPase, belonging to the aminophospholipid translocase family (Perez-Victoria et al., 2003b). The accumulation of the drug leads to the development of an apoptosis like death in *L. donovani* (Paris et al., 2004). Resistance to miltefosine is associated to changes in the efflux of the drug. Mutations in the above mentioned transporters or over expression of the glycoprotein MDR1 leads to miltefosine resistance (Perez-Victoria et al., 2003b; Perez-Victoria et al., 2001). In addition the diminution of unsaturated phospholipid alkyl chains in the parasite membrane was reported in resistant strains (Rakotomanga et al., 2004). Miltefosine is the most cost-effective option of treatment in areas of detected antimonial resistance, but its use as a first-line drug in monotherapy is limited by its teratogenicity and the high potential for resistance development (Vanlerberghe et al., 2007).

Sitamaquine (8-aminoquinoline), is the only drug that was specifically developed for treatment of VL (Yeates, 2002). The phase II trials of sitamaquine in India and Kenya demonstrated its efficacy against VL (>83%), but the latter presented a case of renal complication associated with sitamaquine (Jha et al., 2005; Wasunna et al., 2005). In CL the drug presented a disappointing performance with a failure to contain the disease even in the mouse model (Garnier et al., 2006). Its mechanism of action seems to involve electrostatic interactions leading to drug insertion within biological membranes (Coimbra et al., 2010). After binding to the membrane, sitamaquine translocation is mediated by the action of a still unknown transporter. It accumulates in *Leishmania spp.* cytosolic acidic compartments, acidocalcisomes, however correlation between its action and this reported accumulation is not clear (Lopez-Martin et al., 2008). Nonetheless it is clear that high concentration of sitamaquine affects parasite motility, morphology and growth (Duenas-Romero et al., 2007). Although field resistance against this drug has not been reported, *in vitro* resistance against the *L. donovani* promastigote has been reported (Bories et al., 2008). Sitamaquine still requires more studies to evaluate efficacy, mode of action and toxicity to become a viable alternative to the already established VL treatments (Loiseau et al., 2011).

A therapeutical approach using different combinations of some of the above mentioned drugs in the treatment of leishmaniasis is expected to decrease therapy cost, reduce toxicity and avoid resistance (Meheus et al., 2010; Shakya et al., 2011). In spite of several case reports of success combination therapy in treatment for leishmaniasis (Collini et al., 2009; Kumar et al., 2011), the lack of standardized clinical data makes monotherapy still the reference approach. Only recently, significant steps have been taken to standardize and evaluate the effectiveness of combination therapy. In 2011 a published study with more than 600 VL patients in India demonstrated that combination treatments are efficacious and safe, decreasing the duration of therapy (Sundar et al., 2011). Therefore, upon independent rigorous validation, combination therapy might become an important tool for the control of the disease (van Griensven et al., 2010).

The successful chemotherapy is very much dependent on a competent immune system (Murray et al., 1989), therefore the treatment options for HIV co-infection will be dependent on the immune status of the patient (Cruz et al., 2006). In general, these patients have lower cure rates, higher drug toxicity rates, and suffer higher fatality level for leishmaniasis than do immunocompetent patients (Alvar et al., 2008). The treatment of co-infected patients is made more difficult by the lack of uniform clinical data. In consequence optimal treatment regimes have not been established. In fact relapses with complicated parasitological and clinical data are common making secondary prophylaxis

an option to consider (Pasquau et al., 2005). In a 12 month follow up study with the use of AmBisome, secondary prophylaxis decreased the probability by 50% of having relapse (Lopez-Velez et al., 2004). Albeit, in settings where medical care is insufficient for monitoring toxicity or VL is anthroponotic, there is the increase risk of inducing drug resistance with secondary prophylaxis (Croft et al., 2006).

2.2.3 – Control of vectors and reservoirs

For the direct control of leishmaniasis the World Health Organization (WHO) prioritizes the use of efficient diagnosis tools, the application of adequate treatment options and the mandatory report of all leishmaniasis cases as minimal control measures. In the absence of vaccines the eradication of the disease includes broad disease control directives. These involve the control of reservoirs and vectors of the disease. Three different approaches exist for the indirect control of the disease transmission: vector and reservoir control, and also personal protective measures. Within sylvatic environments the sandflies generally rest in dark moist places usually within a few hundred meters of their breeding site, promoting the existence of very active infection foci. The increased urbanization on the periphery of towns, invading the traditional breeding grounds of the vector represents one of the major causes for increase of disease incidence. Measures like widespread deforestation near urbanized terrains or the use of insecticides are feasible alternatives to control the vector. In fact, the extensive use of Dichloro-Diphenyl-Trichloroethane (DDT) had a dramatic effect on the peridomestic sand fly vector of VL in endemic areas of India, resulting in a considerable reduction in the number of cases in some localities (Vanlerberghe et al., 2007). Due to its environmental and human hazardous effects, DDT was substituted by synthetic pyrethroids, like deltamethrin and cyhalotrin, which showed to be effective against all vector species (Davies et al., 2000; Marcondes and Nascimento, 1993). Another major measure against both cutaneous and visceral leishmaniasis is the control of reservoir hosts. The control of sylvatic animals requires difficult environmental decisions, such as forest cleaning or destruction of rodent burrows around human activities. For zoonotic VL, the restriction of the canine domestic reservoir is essential. The control of transmission of canine leishmaniasis has two major purposes: protect the dogs themselves from VL and reduce the availability of parasite reservoir. The systematic euthanasia of infected dogs showed limited impact in the transmission of the parasite (Gramiccia and Gradoni, 2005). Several insecticide-based preparations have been specifically registered for dog protection against sand fly bites, including deltamethrin-impregnated collars and topical application of permethrin or

deltamethrin (Courtenay et al., 2009; Halbig et al., 2000; Maroli et al., 2001; Reithinger et al., 2004). Also several vaccine candidates are under study for canine leishmaniasis prevention and one has already been registered for veterinary use in Brazil (Borja-Cabrera et al., 2002). However, the efficiency of these preventive measures is dependent on their adoption by the whole canine population and the impact will be reduced if a still unknown sylvatic reservoir maintains a population of infected animals sufficient to perpetuate the infectious cycle. Ultimately, personal protective measures including the use of mechanical barriers such as bed-nets, sometimes impregnated with insecticides, the avoidance of outdoor activities when sand flies are most active (dusk to dawn), wearing protective clothing and the application of insect repellent to exposed skin are highly recommended WHO directives against leishmaniasis.

3 – Immunopathogenesis of *Leishmania spp.* infection

Leishmania spp. infections are excellent examples of complex host-parasite interactions. The parasite developed a remarkable range of sophisticated adaptive mechanisms, which not only allow to evade and inhibit normal macrophage functions, but also subvert innate and acquired (both cell and humoral) immunity to their own advantage.

3.1 - Establishment of *Leishmania* spp. in the host

The early recognition and subsequent triggering of a proinflammatory response to invading pathogens is associated with the innate immune system (Medzhitov and Janeway, 2000). The adaptive branch of the immune system is responsible for the subsequent elimination of pathogens and the generation of immunological memory. The adaptive immune response is characterized by the specificity developed in clonal gene rearrangements from a broad repertoire of antigen-specific receptors on lymphocytes. The innate immune response, on the other hand, is mediated mostly by phagocytic cells and antigen presenting cells (APCs), such as dendritic cells (DCs), granulocytes and macrophages being considered as somewhat nonspecific (Mogensen, 2009). Traditionally the innate immune system was considered as a primitive, nonspecific system involved only in destroying and presenting antigen to cells of the adaptive immune system. Nowadays it is considered as a highly developed system capable of discriminating between self and

foreign with a much greater specificity than previously thought. In fact it now accepted that innate and adaptive immune responses are much more entwined than initially believed. Several important findings support the idea that the innate immune system, besides being essential for early pathogen recognition, is also involved in the activation and shaping of adaptive immunity (Iwasaki and Medzhitov, 2004).

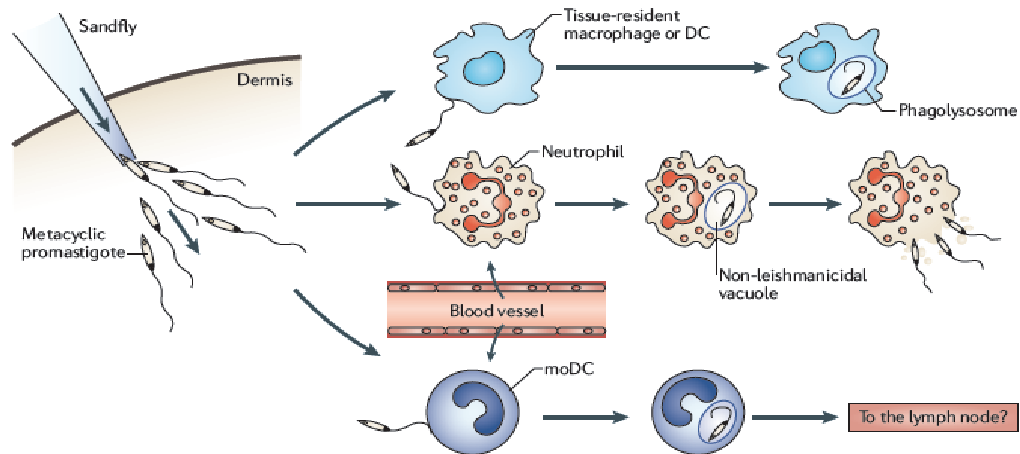

Figure 6. Cells involved in the first steps of infection by *Leishmania* spp. Metacyclic promastigotes are deposited in the dermis in a mixture of immunomodulatory salivary secretions and parasite-derived proteophosphoglycans. Promastigotes from the initial inoculum are phagocytosed by tissue-resident macrophages and dermal dendritic cells (DCs). Inflammatory monocyte-derived DCs (moDCs) are recrutited to the local site by local inflammatory signaling. These infected inflammatory moDCs may facilitate parasite traffic to the draining lymph node. Long-term replication and perpetuation of the pathogen principally involves either macrophages or moDCs, depending on the parasite species. Adapted from (Kaye and Scott, 2011).

Upon entry into a susceptible mammalian host, *Leishmania* promastigotes must survive the impact of the innate immune system. The first immediate challenge before entering the first cells is to survive the complement system. Metacyclic parasites are highly resistant to complement mediated lysis. This resistance is a multifactorial event, being associated with several parasite components, like LPG (Puentes et al., 1990), GP63 (Brittingham et al., 1995) or surface kinases (Hermoso et al., 1991). The parasites that survive the complement system quickly interact with resident cells (Figure 6). Among the first cells interacting with the promastigotes are the epidermal keratinocytes, that may play an important role in the early stages of infection because *in vitro L. major* infection of these cells enable the creation of a cytokine environment leading to the onset of a T helper 1 (Th1) response mainly by Interleukine (IL)-6 secretion (Ehrchen et al., 2010). Also

resident dendritic cells like Langerhans are known to interact with the parasite on the first four hours of infection, mainly by uptaking promastigotes and inducing the activation of cytotoxic CD8-T cells (Brewig et al., 2009). The first cells to be recruited to the infection site are neutrophils (Peters et al., 2008), probably due to the action of alarmins, molecules associated with tissue damage (Kaye and Scott, 2011). The neutrophils are thought to function as an intermediate non productive host, with some authors considering them as a vehicle for infection facilitating a silent entry into the macrophages (Laskay et al., 2003). Therefore the importance of these cells in the infection it is not clear, because neutrophils were shown to be capable of eliminating the promastigotes therefore their role as a transient host was questioned (McFarlane et al., 2008). The debate goes on with contradictory results that often are species dependent (Novais et al., 2009; Peters et al., 2008). Dermal macrophages are expected to be the first infection productive cells. In fact they become the main infected population after 24 hours (Peters et al., 2008). There are several reported uptake receptors and phagocytic mechanisms, still, little evidence of *in vivo* relevant mechanisms exists (Rittig et al., 1998). Nonetheless several receptors were shown *in vitro* to promote parasite uptake. The mannose/fucose receptors, complement receptor (CR) 1 also known as CD35, CR3, CR4 and the receptors for the C-reactive protein and fibronectin have been described as interacting directly or indirectly with surface promastigote molecules for further attachment and uptake (Culley et al., 1996; Kane and Mosser, 2000; Mosser and Edelson, 1987; Russell and Wright, 1988; Talamas-Rohana et al., 1990; Wilson and Pearson, 1986). Still it is generally accepted that *Leishmania* uptake by macrophages is mostly CR3 dependent (Kane and Mosser, 2000). Upon the entry in macrophages *Leishmania* spp. are engulfed by the parasitophorous vacuole that matures into a functional phagolysome. This maturation in some species is delayed by an LPG dependent mechanism that seems to disrupt lipid microdomains in the membranes (Dermine et al., 2005; Dermine et al., 2000). This delay in maturation enables the differentiation of the parasite into the amastigote form. Other species like *L. mexicana* and *L. braziliensis* do not seem to delay phagosome maturation. Instead they form large phagosomes containing multiple parasites. These phagosomes will have a diluted hydrolytic content enabling the differentiation of the parasite (Duclos et al., 2000). As the numbers of resident macrophages and DCs in the skin are too limited to sustain parasite multiplication and the establishment of an infection, the progression of infection requires the recruitment of monocytes. These can differentiate into monocytes derived dendritic cells (moDCs). These moDCs are described as permissive host cells for the parasite (De Trez et al., 2009). Albeit in mice lacking the chemokine receptor CCR2 (incapable of recruiting monocytes to the lesions), a non-protective TH2 response is induced by the infection leading to increased susceptibility (Sato et al., 2000). This may

seem to be a contradiction because although the bone marrow derived DCs (BmDCs) are permissive, their absence induces a worst prognosis. In fact the parasite seems to further disrupt the activity of these cells, not just using them as a productive host. DCs are pivotal cells in the definition of the immune response for example, they are capable of secreting IL-12, that leads to a host-protective Th1 type response (Leon et al., 2007). The bmDCs Infected *in vitro* with *L. braziliensis* promastigotes do not upregulate MHC (major histocompatibility complex) class II (limiting the capacity to present antigens) nor secrete IL-12 (Carvalho et al., 2008). Eventually DCs or macrophages will transport parasites from the initial site of infection to the lymph node that drains that site and acquired immune responses are initiated that will lead to the elimination or survival of the parasite as summarized in figure 7 (Ehrchen et al., 2008).

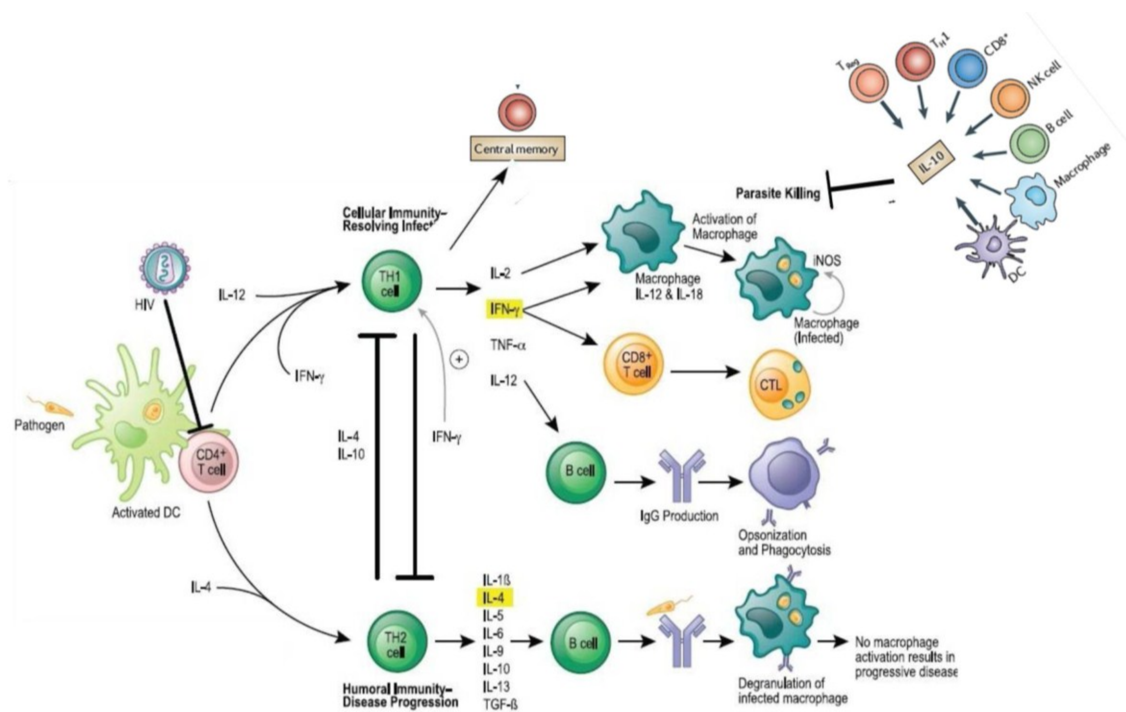

Figure 7. Immune response associated with *Leishmania* spp. infection. Monocytes infiltrate the site of infection and differentiate into dendritic cells (DCs). In disease progression DCs become infected but fail to become activated, results in a default pathway of naive T-cell differentiation into IL-4-secreting TH2 cells. The elimination of the parasite requires CD4+ T cells activation and differentiation into T helper (TH1) cells, which produce interferon-γ (IFNγ). This cytokine promotes parasite killing by infected cells and also further induces the development of TH1 cells. Some CD4+ T cells fail to become TH1 cells, and adopt a central memory T cell phenotype. CD8+ T cells recognizing leishmanial antigens are also activated and also produce IFNγ. Control of the inflammatory response associated with parasite elimination is largely mediated by the production of interleukin-10 (IL-10), which can come from several different cell types, including regulatory T (TReg) cells, TH1 cells, CD8+ cells, natural killer (NK) cells, B cells, macrophages and DCs leading to disease persistency. Adapted from (Ezra et al., 2010; Kaye and Scott, 2011)

The CD4+ T lymphocytes can be detected in draining lymph nodes as early as 16h post-infection. These cells will expand and differentiate into cells with distinct immunological phenotypes depending on the mouse strain (Heinzel et al., 1989; Stetson et al., 2002). Ultimately the ongoing interactions between the agents of acquired immunity and the parasites will lead to the characteristic immunopathology associated with the parasite. It is generally accepted that control and clearance of *Leishmania* infection in the mammalian host requires an active Th1 type immune response, driven by the production of interleukin (IL)-12 by antigen presenting cells and interferon gamma (IFN-γ) by T cells. Eventually IFN-γ in conjunction with tumor necrosis factor alpha (TNF-α), leads to the activation of macrophages and the destruction of the internalized parasites (Kaye and Scott, 2011).

The bulk of knowledge related to the immunopathology comes from the study of different murine models. Murine cutaneous leishmaniasis is characterized by a clear Th1/Th2 polarization that defines disease outcome. In fact, Balb/c mice infected with *L. major* display a marked Th2 immune response (IL-4, IL-5 and IL-13), with the consequent inhibition of a Th1 response with the diminished production of IFN-γ leading to the establishment of the infection (Fowell et al., 1998; Gumy et al., 2004). The opposite happens in resistant C57BL/6 where a strong Th1 response leads to the resolution of *L. major* infection (McMahon-Pratt and Alexander, 2004). Remarkably, despite the elimination of symptomatology associated with the infection, 1% of viable organisms were shown to still persist in C57BL/6 mice, leading to a state of sub-clinical parasite persistence. IL-10 seems to be responsible for this parasite persistence because sterilizing immunity could be obtained in the absence of IL-10, albeit this immunity was not accompanied by the traditional protection against re-infection associated with this model (Belkaid et al., 2002). Therefore IL-10 has a central role in the pathogenesis. For some time a candidate for the production of IL-10 was a specific subset of *Leishmania*-specific CD4(+)CD25(+)Foxp3(+) T regulatory cells (Tregs). This was supported by the production of IL-10 *ex vivo* (Belkaid et al., 2002). Later, using Rag-/- (T cell deficient) mice, compelling evidence was presented showing that dual IL-10 and IFN-γ-producing Th1 cells, CD4(+)CD25(-)Foxp3(-), were required to inhibit the acquired immune responses against *L. major*, while CD4(+)CD25(+)Foxp3(+) T regs, were not (Anderson et al., 2007). In fact, IL-10 producing CD4(+)CD25(+)Foxp3(+) T regs were not sufficient to maintain chronic infection in Rag-/- mice , while introduction of CD4(+)CD25(-)Foxp3(-), dual IL-10 and IFN-γ-producing Th1 cells, resulted in persistent infection (Anderson et al., 2007). Data, from mice models of acute visceral leishmaniasis (*L. donovani* infections in

C57BL/6 mice), suggests that the IL-10 and TGF-β (transforming growth factor beta) are responsible for inhibition of a curative Th1 response and not Th2 cytokines IL-4 or IL-13 as is observed in CL, (Wilson et al., 2005). Syrian hamsters, unlike mice, are unable to clear infections by *Leishmania* spp. that are responsible for VL. Upon infection, Syrian hamsters develop a fatal severe disseminated disease representing a better model for active human visceral disease. Despite the differences in disease phenotype, the cytokine profiles in *L. donovani* infected hamsters are similar to those in mice. They both have high levels of Th1 cytokines IFN-γ, IL-12 and TNF-α along with high levels of IL-10 and TGF-β; remarkably Th2 cytokines seem to be absent (Wilson et al., 2005). Immune responses in human leishmaniasis are still not clear and quality data is scarce. Peripheral blood mononuclear cells (PBMCs) from individuals with subclinical infections or cured patients, proliferate and produce large amounts of IL-2, IL-12 and IFN-γ in response to *Leishmania spp*. antigens (Wilson et al., 2005). In contrast, PBMCs from acute VL patients are not responsive to *Leishmania* antigens, neither proliferating nor producing IFN-γ (Carvalho et al., 1992). Moreover, the antigen-specific responses of PBMCs from cured individuals were seen to be inhibited when co-cultured with PBMCs from the same patient prior to healing, indicating that these cells are capable of mediating immunosuppression, perhaps through the release of immunosuppressive factors (Carvalho et al., 1989). The cytokine environment seems mainly composed of pro-inflammatory cytokines with high levels of IL-1, IL-6, IL-8, IL-12, TNF-α, IFN-γ in the plasma (Kurkjian et al., 2006). Furthermore in contrast to murine VL, the prominent Th2 cytokine IL-4 can be detected in the plasma (Sundar et al., 1997). As stated above IL-10 has a pivotal role in the immunopathology contributing to immunosuppression associated with parasite persistence. In fact, IL-10 has been found elevated in the plasma of active VL, and also in bone marrow and spleen (Ghalib et al., 1993; Nylen et al., 2007; Sundar et al., 1997). Interestingly CD4(+) CD25(-) Foxp3(-) activated T cells constituted the primary source of IL-10 in Indian VL patients, also being capable of producing IFN-γ (Nylen et al., 2007). Furthermore the proinflammatory environment acting on macrophages (associated with IL-1 and IFN-γ) have the potential to upregulate IL-27, which in turn can induce IL-21 to expand IL-10 producing T cells (Ansari et al., 2011). The existence of this expansion of IL-10 producing T-cells was also supported by the presence of both RNA transcripts and protein expression for IL-10 that were only reduced upon resolution of the disease (Ghalib et al., 1993). In addition to IL-10, high circulating levels of TGF-β, soluble IL-4 receptor, IL-6 and soluble IL-2 receptor are thought to potentially play immunosuppressive roles in human visceral leishmaniasis though the mechanisms have not been clearly elucidated (van der Poll et al., 1995; Wilson et al., 2005). The CD4(+)CD25(-)Foxp3(-), dual IL-10 and IFN-γ-producing Th1 cells subset was found to be the primary source of IL-10 in patients with VL (Nylen et

al., 2007). Also no correlation between CD4(+)CD25(+)Foxp3(+) T regs frequency in the site of infection was found in VL excluding once again these cells as the source of IL-10 (Maurya et al., 2010). Although these findings were in VL patients, the immunopathology associated to VL does not clearly follow a Th1/Th2 dichotomy and the causative mechanisms of immunosuppression in VL not clear.

It is clear that *Leishmania* can induce specific responses from the infected cells that harbor it manipulating the immune system to enable the survival of the parasite. Macrophages and dendritic cells, central elements in the definition of the immune response, were clearly shown to be modulated by the presence of the parasite (Blanchette et al., 1999; Carvalho et al., 2008; Gregory et al., 2008; Neves et al., 2010). Chang et al. have proposed a model to explain *Leishmania* virulence that conceptualizes two different groups of parasite proteins, secreted and intracellular components (Chang et al., 2003). Thus, according to this model, parasite surface and secreted molecules (infection-related molecules) are considered as a prerequisite for virulence, helping the parasite to successfully establish itself inside the host macrophage phagolysosome and contributing to the maintenance of the infection by interfering with macrophage functions. The other group consists on non-secreted highly conserved parasite molecules classified as 'pathoantigens' or 'panantigens' (pathology-related molecules), which are only visible upon amastigote cytolysis and contribute to the immunopathology associated with leishmaniasis inducing a non-productive immune response (Chang et al., 2003; Santarem et al., 2007).

3.2 – The importance of Toll-like receptors in *Leishmania* spp. infection

The pivotal concept behind the innate immune response is the recognition of evolutionarily conserved structures on pathogens designated as pathogen-associated molecular patterns (PAMPs). This recognition is mediated by a limited number of pattern recognition receptors (PRRs), of which the family of Toll-like receptors (TLRs) has been studied the most (Akira et al., 2006; Medzhitov and Janeway, 2000; Mogensen, 2009). PAMPs are defined as being invariant among classes of pathogens, essential for the survival of the pathogen, and distinguishable from "self" (de Diego et al., 2007). The PPRs are capable of recognizing conserved structures in widely different pathogens including viruses, bacteria, fungi, and protozoa (Akira et al., 2006). The PPRs do not recognize only PAMPs they can also recognize host factors. These host factors are usually found in atypical localizations or with unusual conformations as a consequence of infection,

inflammation or extreme cellular stress (Beg, 2002). The PRRs, present at the cell surface or intracellularly, upon recognition by PAMP trigger a proinflammatory and antimicrobial response activating a myriad of intracellular signaling pathways (Akira and Takeda, 2004). The activation of PRRs by PAMPs results in the production of cytokines, chemokines, cell adhesion molecules and immunoreceptors that induce the early host response to pathogens and also make the connection with the adaptive immune response (Akira et al., 2006). In mammalian species the PRRs and the signal transduction pathways associated to them belong to an evolutionarily conserved system responsible for responding and surviving to microbial infection (Mogensen, 2009).

Since 1963, consistent reports attributed to pathogen derived structures like DNA and RNA the capacity to activate cells by unknown mechanism (Jensen et al., 1963). Although the existence of receptors that could recognize these conserved microbial structures was proposed in 1989 (Janeway, 1989) it was only in 1997 that human homologues to the *Drosophila melanogaster* Toll protein were discovered and implicated in the activation induced by several microbial components (Medzhitov et al., 1997). Since then ten TLRs have been identified in humans each recognizing distinct PAMPs derived from various microbial pathogens (Mogensen, 2009). All TLRs are membrane associated glycoproteins characterized by an extracellular or luminal ligand-binding domain containing leucine-rich repeat motifs and a cytoplasmic signaling Toll/interleukin-1 receptor homology domain (O'Neill and Bowie, 2007). TLRs can be divided into groups recognizing similar PAMPs for distinct origins (see figure 8). The TLR1, TLR2, TLR4, and TLR6 recognize lipids, while TLR3, TLR7, TLR8, and TLR9 recognize nucleic acids (Akira et al., 2006).

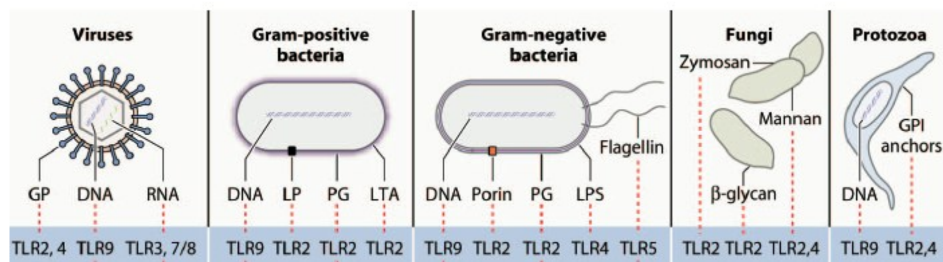

Figure 8. Recognition of PAMPs from different classes of microbial pathogens. Viruses, bacteria, fungi, and protozoa display several different PAMPs, some of which are shared between different classes of pathogens. Major PAMPs are nucleic acids, including DNA, dsRNA, ssRNA, and 5-triphosphate RNA, as well as surface glycoproteins (GP), lipoproteins (LP), and membrane components (peptidoglycans [PG], lipoteichoic acid [LTA], LPS, and GPI anchors). Adapted from (Mogensen, 2009).

This general division is not strict, as some TLRs can recognize such divergent structures as LPS, the fusion protein of respiratory syncytial virus, and cellular heat shock proteins (Akira et al., 2006). This capacity to recognize several structures using a limited number of specific receptors can be explained by the binding of ligands to different regions of the extracellular portion of TLRs or the involvement of different PAMP-binding molecules, such as the accessory molecule MD2 that mediates LPS binding to TLR4 (Kim et al., 2007). Ligand-receptor specific interactions can also be achieved through the formation of heterodimers between TLR2 and either TLR1 or TLR6 (Ozinsky et al., 2000). TLRs can also be grouped according to their cellular distribution, TLRs (TLR1, -2, -4, -5, -6, and -10) are expressed at the cell surface, while others (TLR3, -7, -8, and -9) are located almost exclusively in intracellular compartments, such as endosomes and lysosomes. This latter group is specialized in recognition of nucleic acids, with self versus nonself discrimination provided by the distinct localization of the ligands rather than differences in molecular structure (Iwasaki and Medzhitov, 2004). Although TLRs have been identified in most cell types, the most frequently studied TLRs are present in APCs such as macrophages, DCs and B lymphocytes (Iwasaki and Medzhitov, 2004). The ultimate result of the TLR activation by a PAMP is the triggering of downstream signaling pathways resulting in the generation of an antimicrobial proinflammatory response. The binding to TLRs by individual PAMPs, leads to the activation of several different signaling pathways. Signal transduction is initially mediated by adaptor molecules that will be also responsible for the specificity of the response (O'Neill and Bowie, 2007). The recruitment of adaptor molecules to a given TLR is followed by activation of downstream signal transduction pathways via phosphorylation, ubiquitination, or protein-protein interactions, inducing the activation of transcription factors that regulate the expression of genes involved in inflammation and antimicrobial host defenses (Akira et al., 2006) The TLR-induced signaling pathways can be classified according to the adaptor molecules involved (See figure 9). Broadly they are divided into MyD88 (myeloid differentiation factor 88) dependent or independent TLRs (O'Neill and Bowie, 2007). The subsequent signaling cascade can involve NF-κB, mitogen-activated protein kinases or IFN regulatory factors (Akira et al., 2006; O'Neill and Bowie, 2007). The first two have a prominent role in the induction of a proinflammatory response while the latter is involved in IFN production (Kawai and Akira, 2007; Mogensen, 2009).

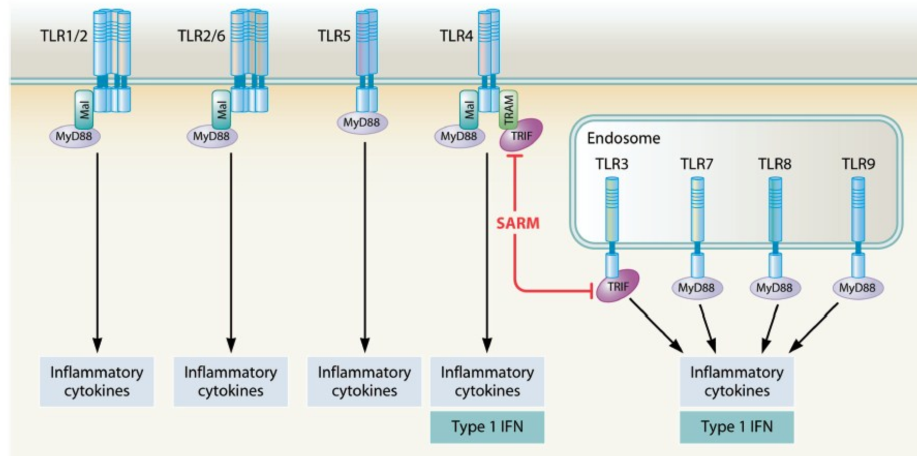

Figure 9. TLRs associated immune response. TLR1/2 and TLR2/6 utilize MyD88 and Mal as adaptors. TLR3 is dependent on TRIF for signaling. In the case of TLR4, four different adaptors, i.e., MyD88, Mal, TRIF, and TRAM, are involved, whereas TLR5, -7, -8, and -9 utilize only MyD88. The fifth adaptor, SARM, negatively regulates TRIF-dependent signaling. Overall, MyD88-dependent signaling induces proinflammatory cytokine production, whereas TRIF-dependent signaling stimulates a type I IFN response. Adapted from (Mogensen, 2009).

3.2.1 – Toll like receptors and *Leishmania*

The bulk of the knowledge on the role of TLRs during infection is concentrated on virus and bacteria. Traditionally gram-negative bacteria are recognized by TLR4 via the lipid A portion of LPS (Poltorak et al., 1998), whereas lipoteichoic acid, lipoproteins, and peptidoglycan of gram-positive bacteria are detected by TLR2 (Schwandner et al., 1999; Yoshimura et al., 1999). TLRs activation by bacteria is not restricted to LPS. Other surface or intracellular PAMPs can activate distinct TLRs (Mogensen et al., 2006). A recurrent example in the literature is flagelin in flagellated bacteria that is specifically recognized by TLR5 inducing TNF-α production in response to flagellated bacteria (Hayashi et al., 2001). Nucleic acids in intracellular compartments is associated with viral infection and recognized by TLR3 for double-stranded RNA, TLR7 and TLR8 for single-stranded RNA (Alexopoulou et al., 2001; Diebold et al., 2004; Heil et al., 2004). TLR9 recognizes unmethylated CpG DNA present in viruses and bacteria (Hemmi et al., 2000).

The importance of PRRs during infection with protozoan pathogens is still a matter of debate because, unlike virus and bacteria infections, few studies exist on protozoan infections. Major PAMPs identified in protozoa depicted in figure 9, include glycosylphosphatidylinositol (GPI) anchors (Almeida et al., 2000; Almeida and Gazzinelli,

2001; Campos et al., 2001), which activate TLR2 and TLR4, as well as unmethylated DNA activating TLR9 (Debierre-Grockiego et al., 2003; Gazzinelli and Denkers, 2006; Shoda et al., 2001). The intracellular protozoan *Toxoplasma gondii* causes asymptomatic infection in normal hosts but can be fatal in immunocompromised individuals, particularly in the absence of IL-12 production (73). In response to *T. gondii* infection, IL-12 is produced through a mechanism dependent on MyD88 (Scanga et al., 2002; Sukhumavasi et al., 2008). This production was shown to be dependent of TLR2 and TLR4 activation by GPIs anchors (Debierre-Grockiego et al., 2007). Also the potent IL-12 inducer profilin-like protein from *T. gondii* tachyzoites is recognized by murine TLR11 (Yarovinsky et al., 2005), a nonfunctional TLR in humans (Akira et al., 2006). Also the hemozoin, a heme degradation product from *Plasmodium falciparum* infection was able to activate TLR9 indirectly by becoming coated with parasite protozoan DNA and targeting into the endosome (Parroche et al., 2007). Also heat-shock protein 70 (HSP 70) from different trypanosomatids are potent activators of the immune system by myeloid differentiation primary response gene 88 (MyD88) pathway (Qazi et al., 2007). The opportunistic intestinal parasite *Encephalitozoon cuniculi* can also bind *in vitro* to TLR2 from macrophages inducing the production of inflammatory cytokines (Fischer et al., 2008). The TLRs are also known to be essential for *T. cruzi* infection control as MyD88 -/- and TRIF -/- mice show increased susceptibility to infection (Koga et al., 2006). In *Leishmania* spp. few TLR activators were described. LPG, but not other surface glycolipids, is a TLR2 agonist capable of activating mouse macrophages and human NK cells, in a MyD88-dependent manner (Becker et al., 2003; de Veer et al., 2003; Kavoosi et al., 2010). Also *Leishmania infantum* Sir2 (silent information regulator 2 protein) was shown to induce the maturation of DCs in a TLR2-dependent manner with the secretion of IL-12 and TNF-α (Silvestre et al., 2009b). Evidence so far point to a multiple TLRs orchestrated defense against *Leishmania* spp. The importance of TLRs in *Leishmania* spp. infection was initially demonstrated by the ability of *L. major* parasites to activate the IL-1 promoter in macrophages via a MyD88-dependent pathway (Hawn et al., 2002). Later it was shown that mice lacking MyD88 have increased susceptibility to infection (de Veer et al., 2003). Also TLR4-deficient mice had higher parasite burdens and were less efficient in the resolution of cutaneous lesions caused by *L. major*, suggesting a role for TLR4 in host defense against *L. major* (Kropf et al., 2004; Kropf et al., 2003). TLR2 and TLR3 were also shown to be necessary for NO and TNF-α secretion in *L. donovani* infected-macrophages (Flandin et al., 2006). Paradoxally TLR-2 -/- mice was shown to be less susceptible to infection with reduced parasite loads with a diminished recruitment of inflammatory cells during the first two weeks after *L. amazonensis* infection (Guerra et al., 2010). Also TLR-4 mediates the effects of *L. mexicana* promastigotes contributing to the

inhibition of host macrophage IL-12 production (Shweash et al., 2011). The expression of TLR9 and IL-12 release by myeloid DCs was shown to be required for NK cell activation in mice infected with *L. infantum* (Haeberlein et al., 2010). Another remarkable example of TLR interaction in *Leishmania* spp. involves *L. guyanensis*. This parasite is itself infected by a virus, *Leishmania* RNA virus-1 (LRV-1) (Guilbride et al., 1992). The presence of LRV-1 is associated with increased TLR3 dependent secretion of IFNβ and other pro-inflammatory cytokines from macrophages. Moreover, cutaneous lesion is reduced in *Tlr3*−/− mice compared with wild-type mice when both are infected with *L. guyanensis* strains presenting high levels of LRV-1, but no difference is observed when both are infected with *L. guyanensis* strains that present low levels of or lacked LRV-1. Hence, the inflammatory potential of *L. guyanensis* is dependent upon the virus load (Ives et al., 2011) in a remarkable example of manipulation of the immune host. Traditionally, mounting an inflammatory response through PRRs is a prerequisite for containment and eradication of invading pathogens, still it is not clear the importance of these PRR-mediated responses in the *Leishmania* spp. because the inflammatory environment promoted by their activation may contribute to the immunopathogenesis by inducing the recruitment of neutrophils and other APCs required to establish the infection.

3.3 – APC deactivation induced by *Leishmania* spp.

The capacity of the *Leishmania* spp. to survive inside APCs is not limited to the early activation of TLRs. It is known that infected macrophages have a reduced capacity to kill the parasites upon activation (Vannier-Santos et al., 2002). Much attention has been given to this remarkable capacity of *Leishmania* spp. to modulate and survive in APCs. This action requires live and active parasites because fixed or irradiated parasites while inducing TLR activation, they cannot proceed with the subsequent APC inactivation as was shown in DCs using *L. infantum* (Neves et al., 2010). Therefore agents for this effect must be components actively released from living parasites. In fact several *Leishmania* spp. such as, *L. donovani, L. major, L. mexicana,* and *L. braziliensis*, induce NF-kappaB cleavage, in a GP63 dependent mechanism, in infected macrophages leading to the induction of chemokine gene expression (Gregory et al., 2008). A similar phenomena effect is seen in DCs using *L. infantum* (Neves et al., 2010) indicating that the mechanisms for APC deactivation are probably overlapping. The same metalloprotease is also capable of cleaving the protein tyrosine phosphatases leading to the deactivation of downsteam signaling and ultimately leading to attenuation of host innate inflammatory responses and microbicidal macrophage functions (Contreras et al., 2010; Gomez et al., 2009). In 2011

mTOR, a serine/threonine kinase that regulates the translational repressor 4E-BP1, was also presented as a target for GP63. The mTOR cleavage leads to activation of the translational repressor 4E-BP1 altering dramatically gene expression enhancing parasite survival. These results were further validated using 4E-BP1 knock out mice which showed increased susceptibility to the parasite, a clear demonstration of the capacity of the parasite to manipulate the host machinery (Jaramillo et al., 2011). It has also been shown that the LPG molecule attenuates multiple macrophage functions (54-59) including phagosome maturation delay, inhibition of oxygen radical formation, and cell signaling through effects on protein kinase C (Dermine et al., 2005; Descoteaux et al., 1992; Lodge and Descoteaux, 2005). Also *Leishmania* elongation factor-1α was shown to be capable of deactivating macrophage functions by activating the Src homology 2 domain containing tyrosine phosphatase-1 (SHP-1) (Nandan et al., 2002). These studies showed the contribution of different parasite components to the pathogenesis. Interestingly also promastigote culture supernatants, retain the characteristics seen in many of the above mentioned studies, in fact culture supernatants were shown to be capable of reproducing the characteristics of the live parasites (Contreras et al., 2010). They were also capable of activate latent TGF-β leading to enhanced parasite persistence in infected macrophages (Gantt et al., 2003) reproducing what was later seen for cathepsin-B (Somanna et al., 2002). In 2009 it was shown that *Leishmania* can release exosome-like vesicles that can also interact with the host cells (Silverman et al., 2010a; Silverman et al., 2010b).

These observations are excellent examples of the complex host-parasite interactions existing during the infection. The parasite seems to have developed a remarkable range of sophisticated adaptive mechanisms, which not only allow to evade and inhibit normal macrophage functions, but also subvert innate and acquired (both cell and humoral) immunity to their own advantage. Chang et al devised a model to explain *Leishmania* virulence that conceptualizes two different groups of parasite proteins (Chang et al., 2003). Thus, according to this model, parasite surface and secreted molecules (infection-related molecules) are considered as a prerequisite for virulence, helping the parasite to successfully establish itself inside the host macrophage phagolysosome and contributing to the maintenance of the infection by interfering with macrophage functions. The other group consists of non-secreted highly conserved parasite intracellular molecules classified as 'pathoantigens' or 'panantigens' (pathology-related molecules), which are only visible upon amastigote cytolysis and contribute to the immunopathology associated with leishmaniasis diverting the immune response towards intracellular components, rendering them less productive (Chang et al., 2003; Santarem et al., 2007). Additional support for such a model came from two studies in which *Leishmania* were

shown to have reduced survival in macrophages when secretion was disrupted. Expression of either a mutated *Leishmania* calreticulin, a chaperone of the endoplasmic reticulum (ER) which is essential for a functional N-terminal secretion system (Debrabant et al., 2002), or a dominant negative mutant of the AAA ATPase Vps4 (Besteiro et al., 2006), which has been shown to disrupt protein secretion from various eukaryotic cells (Yang et al., 2004), greatly reduced *Leishmania* virulence both *in vitro* and *in vivo* (Besteiro et al., 2006; Debrabant et al., 2002).

4 – Exoproteome

4.1 – Definition

The collective extracellular proteins released from an organism under defined conditions is known as secretome (Hathout, 2007). Recently, the definition was further refined to the use in the context of *Leishmania* spp., secretome refers to proteins actively secreted from the cell using a classical or a nonclassical mechanism of secretion, including exosome release. The term 'exoproteome' is then defined as all the proteins present in the extracellular space, including extracellular proteins released from the surface or originating from cell lysis, and the above mentioned secretome (Corrales et al., 2010).

4.2 - Mechanisms of protein release in the *Leishmania spp.* exoproteome.

The exoproteome of *Leishmania* spp. is composed of proteins released from the surface, secreted, and originating from cell lysis. For many years proteins originating from cell lysis provided a plausible explanation for the presence of intracellular proteins in the exoproteome (Chang et al., 2003). Recent studies with *L. donovani* indicated that vesicle structures with exosome like characteristics were also responsible for delivering intracellular components into the exoproteome. Conventional and unconventional secretion is also known to contribute to the exoproteome (see figure 10 for details) with several proteins already characterized (Corrales et al., 2010).

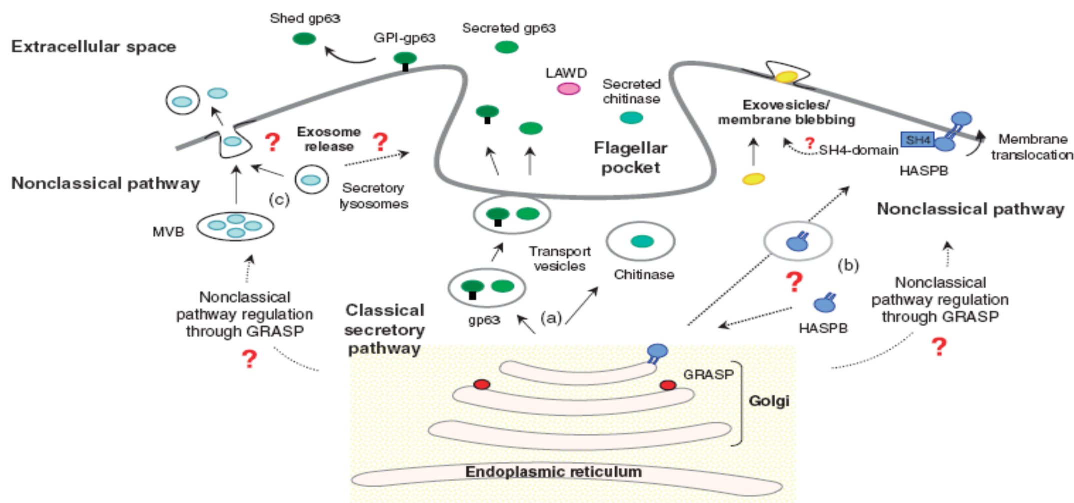

Figure 10 - Schematic diagram summarizing the different secretion mechanisms in *Leishmania* spp. (a) Proteins secreted through the classical secretory pathway, such as gp63 or chitinase, are exported by secretory vesicles and released into the extracellular space of the flagellar pocket by fusion of the vesicles with the plasma membrane. Glycosylphosphatidylinositol-anchoring proteins are swept out of the flagellar pocket to the cell body and attached to the external surface of the membrane by their glycosylphosphatidylinositol moiety. Gp63 is released in a secreted soluble form and a glycosylphosphatidylinositol-anchored form. Some proteins devoid of a signal peptide are localized in the flagellar pocket (e.g. LAWD: *Leishmania* antigenic tryptophan-aspartic acid. (b) HASPB is synthesized on free ribosomes in the cytoplasm and may be transferred to the outer leaflet of the Golgi membrane and would use conventional vesicular transport to reach the plasma membrane, where translocation could occur. The SH4 domain of HASPB induces the production of nonapoptotic membrane blebbing. (c) Proteins may be released into the extracellular space through exosomes originating from either lysosomes or multivesicular bodies (MVB) of endosomal origin. Whether GRASP, essential to the unconventional secretion pathway of Dictyostelium during development, plays a role in the nonclassical secretion pathway in *Leishmania* spp. remains to be clarified. Adapted from (Corrales et al., 2010).

4.2.1 - The surface of the promastigote

Proteins released or shed from the surface of the promastigote are present in the exoproteome. These can be released by the action of proteases or phospholipases that release GPI anchored proteins (Yao et al., 2003). The surface of *Leishmania* spp. is composed of a few dominant components that are present in all species (see figure 11).

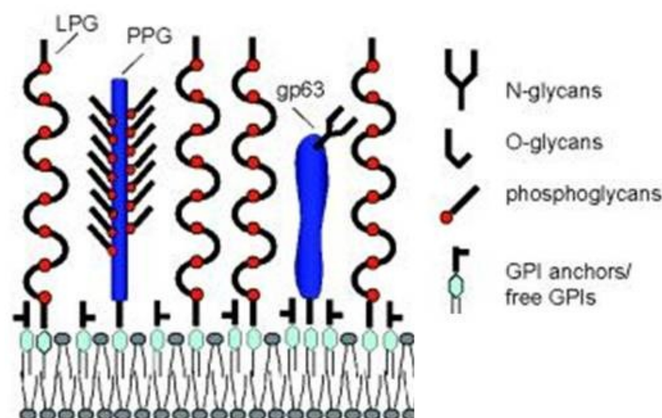

Figure 11. Surface coats of *Leishmania* ssp. The surface coats of *Leishmania* spp. parasites are dominated by LPG, GPI proteins and/or non-protein linked GPI glycolipids. Adapted from (McConville et al., 2002).

Promastigotes are covered by glycoproteins that are anchored to the protozoan membrane by a GPI anchor. These proteins form a glycocalix that is about 7 nm thick in procyclic promastigotes and it thickens in later stages of development being at least twice as thick in metacyclic parasites. The dominant surface molecule of the promastigote is LPG. It is composed of repetitive units consisting of a disaccharide and a phosphate, linked to the membrane by a GPI anchor. The LPG differs from species to species by the presence of glycan side chains. LPG of *L. major*, for example, is highly branched, whereas that of *L. donovani* is not (McConville et al., 1995). Furthermore the structure of LPG differs between procyclic and metacyclic promastigotes, being significantly longer in the latter, and is almost completely absent from amastigotes (McConville et al., 1992; Pimenta et al., 1991). Studies using purified LPG or parasite strains defective in LPG production have shown that LPG can play important roles in parasite survival and modulation of the immune response. Another abundant surface molecule is the glycoproteinGP63 (McConville and Ralton, 1997; Pimenta et al., 1991). This is a zinc-dependent metalloprotease with a wide range of substrates (McMaster et al., 1994). GP63 is hidden under the 10-fold more abundant LPG that actually towers over all other surface components. GP63 expression is down-regulated in amastigotes (Schneider et al., 1992). The GP63 is one of the most prominent proteins in the parasite and is composed of two distinct forms one that is conventionally secreted and another that is shed (released from the surface) probably by processing of the GPI anchor. The dynamics of GP63 release are complex and known to be responsive to environmental stimuli (Yao et al., 2007). Free or vesicle-bound GP63 will contribute to immediate protection against complement mediated lysis (Joshi et al., 2002) and might even enter the host cell cytoplasm through a

lipid raft-dependent mechanism (Gomez et al., 2009), to cleave the cytosolic host protein tyrosine phosphatases (Gomez et al., 2009), contributing to macrophage energy (Contreras et al., 2010). The most abundant molecules in the promastigote surface are the glycosylinositol phospholipid (GIPL). These GPI-linked glycolipids are 10 times more abundant than LPG. Being quite small compared to LPG they exist close to the parasite membrane. Unlike LPG, which is continually shed, GIPL have a long half-life in the membrane so it is believed to have a protective role at the promastigote surface (Ferguson, 1997; Proudfoot et al., 1995). In fact GIPLs recovered from *L. major* where capable of inhibiting nitric oxide production in macrophages by an unknown mechanism diminishing their capacity to eliminate the parasites *in vitro* (Proudfoot et al., 1995). Nonetheless it is still a matter of debate of what is their exact role in their interactions with the host (Ferguson, 1997; McConville and Ralton, 1997).

4.2.2 - The classical secretory pathway

The use of the secretion pathway to deliver effector molecules by microbial pathogens is almost a trademark in pathogenesis. Still, little is known concerning the exact secretory pathway in *Leishmania* (McConville et al., 2002). Most of the organelles involved in secretion and the early endocytic pathway are organized around the flagellar pocket. In fact this organelle was identified to be the endpoint of the secretion apparatus as was elegantly shown for the variant surface glycoprotein in *T. brucei*. Using immunogold labeling, it was demonstrated that variable surface glycoprotein (VSG) was sequentially present in ER, Golgi cisternae, accumulated in the *trans*-Golgi network, transport vesicles, flagellar pocket and ultimately cell surface (Duszenko et al., 1988). In fact, despite being a very divergent eukaryotic lineage the basic features of the classical secretory pathway are conserved (McConville et al., 2002) with the remarkable exception of the highly polarized delivery system (Field et al., 2007). Similarly to higher eukaryotes, secretory proteins are associated with N-terminal signal peptides that direct the proteins through the endoplasmic reticulum (ER) (Schatz and Dobberstein, 1996). The ER comprises the nuclear envelope and a connected system of cisternal or tubular membranes that can be associated with the plasma membrane (Ilgoutz et al., 1999; Weise et al., 2000). Depending on the physiological need of the cell, the ER can expand or diminish, in dividing *T. brucei* the ER can account for 60% of the internal membrane content (Coppens et al., 1987). The ER network in trypanosomatids, besides the traditional classical rough and smooth domains, also contains a specialized transitional ER (tER) directly opposite to the single Golgi apparatus that is only present in dividing parasites (Mullin et al., 2001;

Weise et al., 2000). The space between the tER and the *Cis*-Golgi is packed with transport vesicles (Weise et al., 2000). The transport of these vesicles is maximized by the intimate association between the Golgi and the tER (100 nm apart) enabling the high level of protein and lipid transport required by the cell surface (Mullin et al., 2001; Weise et al., 2000). The trypanosomatids have a single Golgi apparatus comprised of a stack of 3 to 10 cisternae (Figueiredo and Soares, 1995; Weise et al., 2000). Intriguingly brefeldin A, a compound traditionally used in the disruption of secretion, by preventing transport between ER and Golgi, has no effect on trypanosomatids (Figueiredo and Soares, 1995; McConville et al., 2002). Traditionally transport through the Golgi apparatus is mediated by two mechanisms: vesicular transport between cisternae or the maturation and progressive movement of cisternae towards the *trans* face of the Golgi (Glick, 2000). The latter seems to be preferred in trypanosomatids because ultrastructural evidence point to the formation of new cisternae in the *cis*-Golgi (Weise et al., 2000). The Golgi cisternae appear to be functionally differentiated (McConville et al., 2002), quite likely as a result of complex modifications associated with O-linked glycans or phosphoglycans that require specific sets of enzymes (Ha et al., 1996; Moss et al., 1999). The last step of secretion is the passage of proteins from the *trans*-Golgi to the flagellar pocket. The study of dominant glycoproteins in the trypanosomatids revealed some information related to, the nature of the exact process leading to this last event. The variant surface glycoprotein in *T. brucei* is transported to the flagellar pocket by a system of cisternal and tubovesicular membranes (Duszenko et al., 1999; Webster and Grab, 1988). The GP63 in *L. mexicana* is transported by a distinct process, being delivered to the flagellar pocket in large vacuoles (Weise et al., 2000). Furthermore ultrastructural studies consistently show small vesicles between the Golgi and the flagellar pocket indicating a possible role for these vesicles (McConville et al., 2002). It is then possible that the transport to the flagellar pocket involves distinct mechanisms. In fact the nature of the main transport mechanisms is yet to be characterized. The flagellar pocket is a highly specialized structure present in all trypanosomatids. The known functions for this structure include secretion of proteins, addition of integral membrane proteins and endocytic activity (Landfear and Ignatushchenko, 2001). The membrane of the flagellar pocket represents between 0,4-3% of the parasite surface (Landfear and Ignatushchenko, 2001) and is the most active endocytic organelle being capable of internalizing an equivalent membrane area every 2 minutes (Coppens et al., 1987) (Overath et al., 1997).

A few studies exist on the properties of individual secreted proteins. They have been shown to play specific roles in the survival and proliferation of the parasite in both the insect stage and in the mammalian host. In the insect vector, it is clear that several secreted elements have a significant role in the survival and proliferation of the parasite.

The sand fly stomodeal valve is clogged with promastigote secretory gel (Rogers et al., 2002). This PSG is secreted in such an abundance that it forms a semisolid plug in which the parasites are embedded, forcing the stomodeal valve open enabling foregut colonization and transmission (Rogers et al., 2004). The PSG is mainly composed of filamentous secreted phosphoglycans (Rogers and Bates, 2007). These phosphoglycans where also shown to confer resistance to the fly midgut digestive enzymes (Secundino et al., 2010). These glycoproteins also seem to have a role in the establishment of the infection in the mammalian host (Rogers et al., 2010), being themselves immunomodulatory they are attractive targets for vaccine approaches. In fact the use of the N-terminal domain of dominant proteophosphoglycan of *L. donovani* led to a protective lasting Th1 response in golden hamster that conferred protection to infectious challenges (Samant et al., 2009). Beside these filamentous proteophosphogycans, this family of proteoglycans includes the promastigote secreted acid phosphatases and other non filamentous proteophosphoglycans from amastigotes and promastigotes. The promastigote secreted acid phosphatases were among the first proteins studied in *Leishmania* (Gottlieb and Dwyer, 1982), in fact for a long time acid phosphatases activity was considered a marker of virulence (Katakura and Kobayashi, 1988; Singla et al., 1992). The acid phosphatases have species specific characteristics. In *L. mexicana* it appears to be filamentous but in *L. donovani* it is not filamentous, although oligomeric (Ilg et al., 1991). Furthermore this protein seems to be resistant to several proteases indicating that in the proteolytic environment of the sandfly midgut it can have a long functional life (Joshi et al., 2004). In addition, also the amastigote non filamentous proteophosphogycans have shown *in vitro* interesting properties, inducing vacuole formation in macrophages and is also being an activator of the mannose-binding lectin pathway (Peters et al., 1997a; Peters et al., 1997b). Another secreted protein important in the sand fly stage is the secreted chitinase (Schlein et al., 1991). This protein is mostly studied in *L. mexicana* and was shown to be important in the survival inside the host. Its overexpression leads to a faster sandfly colonization and damage to the stomodeal valve (Rogers et al., 2008). Interestingly the activity of this protein does not seem to be restricted to the sandfly stage. In fact amastigotes present 2-4 fold higher levels of chitinase activity during their growth *in vitro* than promastigotes hinting at a putative function in the amastigote stage (Joshi et al., 2005). Indeed the overexpression of this protein leads to increased pathology in mice, due to a higher intramacrophagic survival rate of the overexpressors (Joshi et al., 2005). GP63 also is expected to have a significant role in the sandfly vector, because it is mostly produced in the promastigote stage (Davies et al., 1990) where it should have a protective and nutritional role (Santos et al., 2006). GP63 has been extensively studied in the interaction with the host cell (Yao et al., 2003).

Free or vesicle-bound GP63 will contribute to immediate protection against complement mediated lysis (Joshi et al., 2002) and might even enter the host cell cytoplasm through a lipid raft-dependent mechanism (Gomez et al., 2009), to cleave the cytosolic host protein tyrosine phosphatases (Gomez et al., 2009), contributing to macrophage deactivation (Contreras et al., 2010).

4.2.3 - Unconventional secretory pathway

The classical secretory pathway is not the only way for the delivery of proteins into the extracellular space. Several proteins were reported to be exported without being involved the ER-Golgi apparatus. The main features of unconventional secretion are: (1) the lack of known conventional signal peptides; (2) not requiring passage through ER-Golgi apparatus; (3) secretion resistance to brefeldin A, a specific inhibitor of classical secretion (Nickel and Rabouille, 2009). The later point has been used by some authors to support unconventional secretion in *L. donovani* (Silverman et al., 2008), but several reports indicate that brefeldin A is not capable of disrupting conventional secretion in trypanosomatids (Figueiredo and Soares, 1995; McConville et al., 2002). Several proteins that can be classified as unconventionally secreted, were already characterized in eukaryotes (Prudovsky et al., 2008). These proteins include IL-1 β (Qu et al., 2007), galectin 3 (Mehul and Hughes, 1997), the antiviral protein Mx1 (Toyokawa et al., 2007) and fibroblast growth factor 2 (Seelenmeyer et al., 2008). Two main mechanisms have been proposed to explain the release of these proteins, the export mediated by direct translocation across plasma membranes or the transport through uncharacterized intracellular transport intermediates (Nickel, 2010). Components like the caspase 1 and Golgi reassembly stacking protein (GRASP) and the autophagy machinery were recently implicated in the unconventional secretion process (Abrahamsen and Stenmark, 2010; Duran et al., 2010; Keller et al., 2008; Kinseth et al., 2007; Manjithaya and Subramani, 2010, 2011; Pfeffer, 2010). A well characterized unconventionally secreted protein, the HASPB, has been described in *L. major* (Stegmayer et al., 2005). This protein is associated with the plasma membrane of metacyclic promastigotes and amastigotes (Flinn et al., 1994) being essential in the metacyclogenesis process (Sadlova et al., 2010). Although the exact translocation process is still unknown there are some important requirements for translocation. Using green fluorescent protein (GFP) SH4 chimeras, it was shown that it is the N-terminal SH4 domain phosphorylation that regulates the subcellular localization of the protein (Tournaviti et al., 2009). Also the anchoring of HASPB to the membrane is dependent of specific myristoylation and palmitoylation of the

same N-terminal SH4 domain (Denny et al., 2000). There is now mounting evidence that these N-terminal SH4 domains indeed have a role in the translocation process. In 2007 it was shown that heterologous expression of these domains led to the reorganization of the membrane inducing non-apoptotic membrane blebs (Tournaviti et al., 2007). This later fact is reminiscent of the membrane blebbing involved in the translocation of the nonclassical secretion of IL-1 β (Qu et al., 2007). A particular case of unconventional secretion, the release of vesicle similar to exosomes, was described in *L. donovani* (Silverman et al., 2010a).

4.2.3.1 – Vesicle release

The systematic exoproteome studies in *Leishmania* spp. consistently reported the massive presence of proteins that have no recognizable signal for export (Silverman et al., 2008). Although some of them might be delivered through unconventional secretion, most of them were found to be delivered by exosome like vesicles (Cuervo et al., 2009). The release of microvesicles (MVs) is accepted as a key factor of eukaryotic and prokaryotic cell biology (Cocucci et al., 2009; Ellis and Kuehn, 2010; Mathivanan et al., 2010). For many years electron microscopy data consistently revealed the existence of small vesicles in the intercellular space that were considered to be either artifacts from damaged cells or the result of necrotic events. It was only after the acknowledgement that these vesicles were the result of specific cellular processes (Pan and Johnstone, 1983), that the importance of these extracellular organelles was recognized. In the last 30 years the work on MVs in eukaryotes enabled the identification and characterization of at least three distinct vesicle populations (see figure 12), exosomes, that originate in the exocytosis of MVBs (Multivesicular Bodies), "Shedding Microvesicles" that originate in the budding of the plasma membrane and apoptotic bodies released upon apoptotic death (Thery et al., 2009).

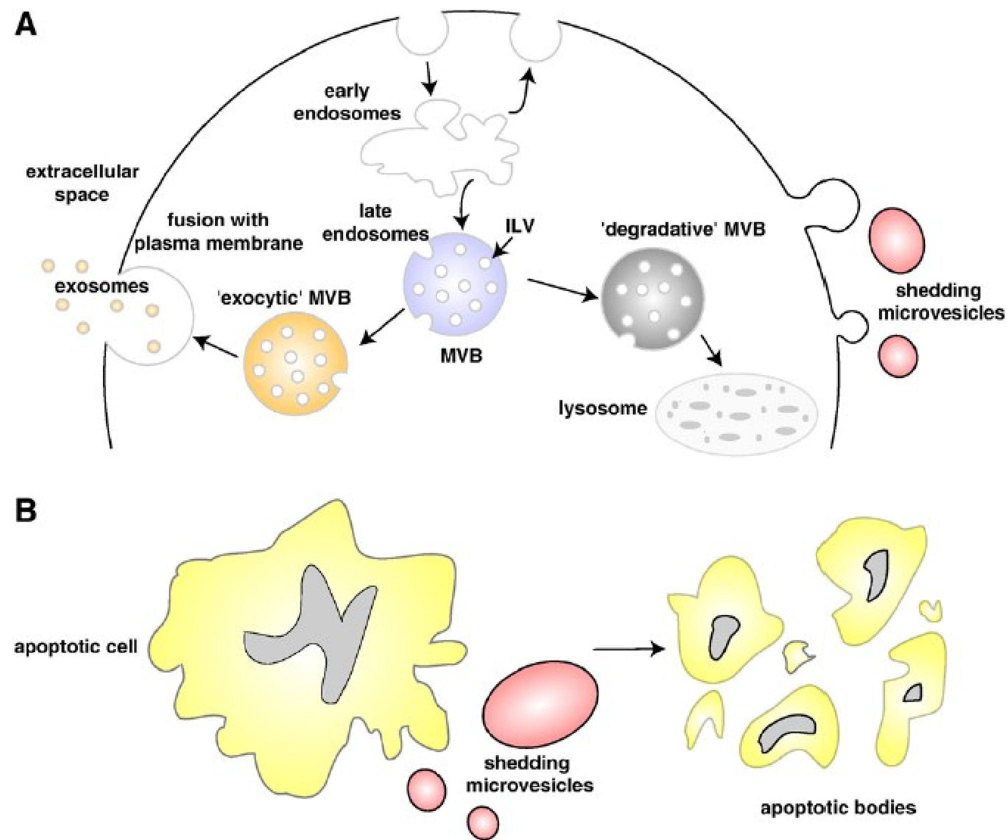

Figure 12. Release of extracellular microvesicles into the extracellular space. A, Release of exosomes and shedding microvesicles is shown. In early endosomes, proteins are either recycled to the plasma membrane (PM) or sequestered in Intraluminal vesicles (ILVs) of the larger Multivesicular Bodies (MVBs). ILVs of MVBs are generated by budding from the limiting membrane into the lumen of endosomes. Concerning the biophysical properties, MVBs either can be degradative (evolving into lysosomes) regulated by ESCRT or ubiquitination or can be exocytic (i.e., fuse with PM with sub sequel release of their contents— exosomes). Shedding microvesicles are released by the process of blebbing or shedding from the cell membrane. B, Apoptotic or dying cells with cell shrinkage, a hallmark of apoptosis, leads in generation of apoptotic bodies. These vesicles are remnants of the degrading apoptotic cell with nuclear and cytoplasmic content. Adapted from (Mathivanan et al., 2010)

The vesicles identified in *Leishmania* spp. have properties similar to exosomes (Silverman et al., 2010a). Little is known concerning their formation and function in *Leishmania* spp. As depicted in figure 12, in higher eukaryotes, exosomes are formed within endosomes by invagination of the limiting membrane, resulting in the formation of a multi-vesicular body (Thery et al., 2009). The "endosomal sorting complexes required for transport" ESCRTs I, II, and III function sequentially in the generation of multivesicular bodies (MVBs) in many eukaryotic cells. The multimeric AAA ATPase Vps4 is thought to bind the ESCRT III complex and catalyze its disassembly in an ATP dependent manner, thus completing the final stages in intraluminal vesicle fission and

MVB formation. Work on Vps4 mutants in mammalian cells showed that functional Vps4 is required for both MVB formation and normal trafficking of MVBs to lysosomes (Fujita et al., 2003; Yoshimori et al., 2000). Exosomes release into the extracellular environment occurs by fusion of MVBs with the plasma membrane (Keller et al., 2006). Vesicle trafficking is a complex process involving a multitude of regulatory proteins. The Rab family of small GTPases is associated with distinct membrane-bound compartments. The localization of the Rab proteins is thought to be related to the targeting and fusion of transport vesicles to the correct destination membrane (Brennwald, 2000). Thus far, two Rab GTPase family members, Rab11 (Savina et al., 2002) and Rab27 (Haddad et al., 2001; Mizuno et al., 2007; Stinchcombe et al., 2001), have been established as having direct roles in exosome release. Rab11 mutants have less exosome release, to disrupt MVB trafficking (Savina et al., 2005), in the K562 reticulocyte model (Savina et al., 2002). Rab27a was shown to directly control the fusion of secretory lysosomes with the cell membrane (Stinchcombe et al., 2001). In addition, Rab27b was shown to regulate exocytosis of mast cell granules by a still unknown mechanism (Mizuno et al., 2007). In the *Leishmania* spp. genome a few orthologs exist for the Rab proteins (Ivens et al., 2005). Also several other proteins whose orthologs are known to be involved in MVB formation and vesicle trafficking were found in the *Leishmania* genome strengthening the possibility of active machinery for exosome trafficking in *Leishmania* spp. (Ivens et al., 2005).

Upon release, the half lives of these vesicles can vary, sometimes they are broken down in a few minutes, circulating only in the extracellular space adjacent to the release, but they can also travel long distances appearing in many biological fluids like urine or blood (Thery et al., 2009). This potential for persistency enables close range and long range signaling in a way that soluble agents cannot. They share many proprieties with soluble/surface agents. Indeed, for example, DC-derived exosomes displayed at the surface functional class I and II MHC molecules and accessory molecules, having the capacity to prime specific cytotoxic T lymphocytes (Zitvogel et al., 1998). Nonetheless, the functions of MVs are not exhausted in direct interactions via specific receptors, they can also function as central platforms for the coordination of multisignaling processes such as coagulation (Muller et al., 2003). Furthermore due to their membranous nature they can induce retrograde signaling enabling the fusion with the recipient cell or induce active engulfment of the entire vesicle, this sort of interactions enables horizontal transfer of membrane and/or cargo molecules (specific proteins and mRNAs) (Thery et al., 2009). Therefore these MVs play a major role in the interaction between immune cells (See figure 13). The subversion of these vesicle mediated interactions might be exploited by pathogens

using their own produced MVs to interfere with the functions of the immune cells (Ellis and Kuehn, 2010)

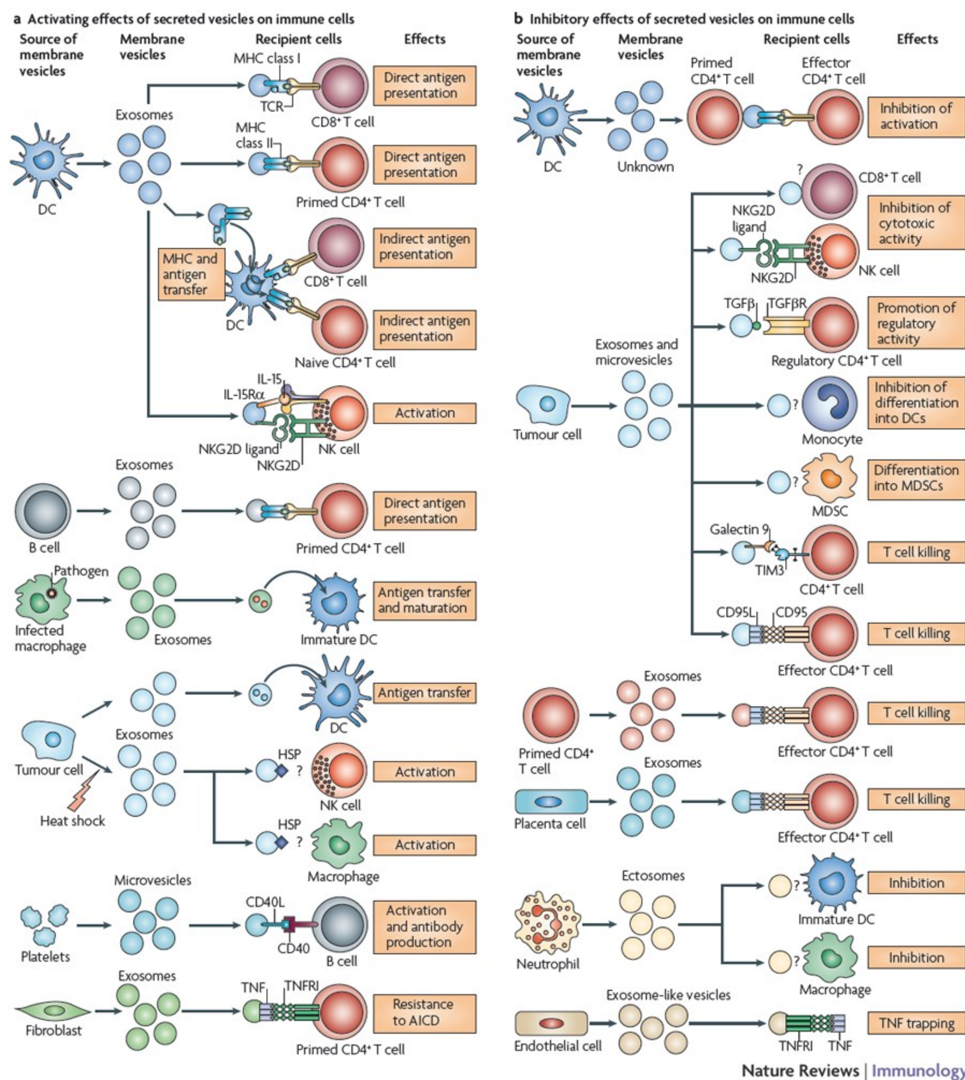

Figure 13 Involvement of secreted vesicles in interactions of immune cells. This figure summarizes the main effects of secreted vesicles on immune cells observed *in vitro*. **a** | Vesicles secreted from various cellular sources have numerous activating effects on immune cells, including direct peptide–MHC complex presentation to T cells, antigen and/or peptide–MHC complex transfer to dendritic cells (DCs) leading to indirect antigen presentation, activation of natural killer (NK) cells, macrophages and B cells and maturation of DCs, and tumour necrosis factor (TNF)-dependent protection of T cells against activation-induced cell death (AICD). **b** | Inhibitory functions of vesicles derived from various cellular sources include: inhibition of T cell activation, inhibition of NK cell and CD8+ T cell cytotoxicity, promotion of regulatory T cell activity inhibition of monocyte differentiation into DCs and promotion of monocyte differentiation into myeloid-derived suppressor cells (MDSCs), T cell killing through ligation of CD95 by CD95 ligand (CD95L) or T cell immunoglobulin domain and mucin domain protein 3 (TIM3) and TNF trapping. HSP, heat shock protein; IL-15Rα, IL-15 receptor α-chain; NKg2D, NK group 2, member D; TgFβR, transforming growth factor-β receptor; TNFRI, TNF receptor superfamily, member I. Adapted from (Thery et al., 2009).

The release of microvesicles (MVs) by eukaryotic microorganisms was considered to be biologically releveant only recently. For a long time it was thought that these vesicles found in culture supernatant were artifacts resulting from indirect processes like mechanical damage or death (Silverman and Reiner, 2011). Only a few eukaryotic pathogens have been shown to actively secrete MVs. Microvesicles were detected in pathogenic fungus, like *Cryptococcus neoformans* (Rodrigues et al., 2008) and *Histoplasma capsulatum* (Albuquerque et al., 2008), and in protozoan parasites like *L. donovani* (Silverman et al., 2010a) and *Trypanosome cruzi* (Goncalves et al., 1991). These pathogen derived vesicles are a new and exciting field, because they have the potential to mimic the characteristics of the host vesicles. In fact there are several ways by which these pathogen derived vesicles might interact with the host cells. These vesicles are expected to interact directly with surface receptors of target cells, functioning in fact as extensions of the pathogen. The potential action of these vesicles is not limited to surface interactions, the direct delivery of virulence factors to the host cells may play an important role in the pathogenic process. Prokaryote membrane vesicles, a naturally secreted product of Gram-negative bacteria, have in fact been found to be the agents of delivery of several toxins and other bacterial associated virulence factors to host cells (Ellis and Kuehn, 2010). As an example, *Legionella* vesicles were shown to inhibit the fusion of bacteria containing phagosomes (Galka et al., 2008), interestingly this seems reminiscent of LPG driven delay in phagosome maturation (Dermine et al., 2005; Dermine et al., 2000). For lower eukaryotic MVs few studies were done. Nonetheless some interesting reports exist in the literature. The single most interesting fact is the evidence that vesicles recovered from *L. donovani* expressing GFP are taken up by macrophages (Silverman et al., 2010a). This fact, added to a proteomic analysis of the MVs of *L. donovani* that revealed the presence of several known virulence factors (Silverman et al., 2010a) enhances the potential of MVs as a system for delivery of virulence factors (Ellis and Kuehn, 2010). The virulence factors detected in *L. donovani* included the prominent metaloprotease GP63 whose immune modulatory properties enabled, for example, direct modulation of host signaling (Halle et al., 2009; Jaramillo et al., 2011). Also other immunomodulatory proteins like elongation factor 1-α, aldolase and kinetoplastid membrane 11 (Carvalho et al., 2005; Nandan et al., 2007; Nandan et al., 2002) were found among the cargo proteins of *L. donovani* (Silverman et al., 2010a). In fact the potential for cargo delivery into the host cells may require the reevaluation of several proteins as potentially involved in the pathogenesis. Actually all the *Leishmania* proteins shown to translocate into the macrophage cytosol were shown to be part of the *Leishmania* exosome cargo (Silverman et al., 2010a). The definition of the role of these vesicles in the context of infection is still in its infancy. *Leishmania* MVs exhibit some anti-inflammatory properties. Using human monocytes in

the context of *L. donovani* infection, MVs were shown to inhibit the pro-inflammatory cytokine production (TNF-α) while promoting immunosuppressive cytokine production (IL-10). Furthermore MVs from *L. major* also induced down regulation of monocytes-derived dendritic cell maturation (Silverman et al., 2010b). Interestingly this capacity was cargo dependent because exosomes from *L. major* HSP100 (-/-) had distinct protein cargo and induced a pro-inflammatory profile in the same cells (Silverman et al., 2010b). Nevertheless the few reports that addressed the *in vivo* properties of these vesicles seem to be consistent with a permissive infection. *L. donovani* and *L. major* MVs treatment prior to infection exacerbated the infection and increased parasite load in susceptible and resistant mice (Silverman et al., 2010b). A similar report with *T. cruzi* vesicles also resulted in increased parasite loads and exacerbated infection (Trocoli Torrecilhas et al., 2009). Little is still known about the biogenesis of these vesicles in *Leishmania*, although some orthologs to endosomal machinery related to exosomal biogenesis were identified (Ivens et al., 2005). There are still doubts relating to the exact nature of these vesicles, still some general facts related to vesiculation are already known. It seems to be induced by physiological stress agents like temperature shift to 37ºC (Hassani et al., 2011; Silverman et al., 2010a) and acidic pH (Silverman et al., 2010a).

4.3 – Exoproteome in *Leishmania spp.* immunopathology

The survival of the parasite is dependent on "convincing" the immune system components to divert their activity towards a non productive response. Therefore if the cross talk between the parasite and the immune host could be compared to actual communication, the surface and released components would be the actual language enabling specific interactions with their biological targets, like receptors or transcription factors.

The *Leishmania* exoproteome is highly immunogenic eliciting strong immunity and some protection against infection in mice and dogs (Lemesre et al., 2007; Tonui et al., 2004; Tonui and Titus, 2007). Intriguingly there was some species specific cross protection, because *L. major* exoproteome was capable of protecting from *L. donovani* challenge but not from *L. braziliensis* (Tonui and Titus, 2007). The high immunogenicity of the exoproteome makes it an attractive source of immunogenic antigens. In fact among the components of the possible recombinant vaccine Leish-111f, the thiol-specific antioxidant was identified in the exoproteome (Coler et al., 2007). This vaccine has shown some potential in the context of human and canine leishmaniasis (Coler et al., 2007;

Nascimento et al., 2010; Trigo et al., 2010). Therefore, the exoproteome has potential for the discovery of new immunogenic proteins. In fact a vaccine approach for the intracellular pathogen *M. tuberculosis* is based on the immunogenicity of a defined exoproteome component, the 30 kDa major secretory protein (Horwitz, 2005). Although, two recent reports from 2011 showed a different facet of the exoproteome. *L. major* secreted antigens were shown to be immunosuppressive inducing IL-4 production in lymphocyte culture (Tabatabaee et al., 2011) while *L. mexicana* exoproteome induced inhibition of macrophage functions by cleavage and activation of the host protein tyrosine phosphatases, specifically SHP-1 and PTP1-B (Hassani et al., 2011).

4.4 – Approaches for the study of the exoproteome

The knowledge on the *Leishmania* exoproteome is in its infancy if we compare it with higher eukaryotes or prokaryotes (Corrales et al., 2010).

In recent years great progress in mass spectrometry (MS), bioinformatics, and analytical techniques have enabled broad scale analysis of proteins in complex biological samples, like whole tissue or culture media (Lane, 2005). The quick development of these techniques combined with a growing interest in the exoproteome of *Leishmania* and related trypanosomatids led to several studies resulting in a list of a few hundreds of proteins that are found in the exoproteome from several species (Cuervo et al., 2009; Geiger et al., 2010; Hassani et al., 2011; Silverman et al., 2008; Silverman et al., 2010a; Trocoli Torrecilhas et al., 2009). Although the proteomic techniques developped are highly efficient, the detection of exoproteome components in culture supernatants presents specific challenges. Standard culture media for the growth of *Leishmania spp.* is a complex mixture dominated by the serum supplement components. The presence of serum proteins represents a specific problem for the detection of the parasite exoproteome because it will mask the parasite derived components. Also proteins released into the medium upon parasite death might contribute very significantly to the exoproteome, masking the secretome. The low abundance of secreted proteins poses another challenge because concentration steps are required before subsequent proteomic analysis. Two major techniques to resolve the concentration problem include selective precipitation or direct concentration using ultrafiltration. To overcome the above mentioned limitations for exoproteome recovery the standard procedure consists in washing the parasites to eliminate serum components and then incubate them in a serum-free medium for a defined time (Corrales et al., 2010). There is a short interval between the time of culture

required for obtaining detectable amounts of secreted proteins and the cell survival in a serum-free medium avoiding cell lysis, therefore short culture times are preferred. Studies with *Leishmania* spp. tend to use less than 6 hours of incubation in serum-free media (Cuervo et al., 2009; Hassani et al., 2011; Silverman et al., 2008).

The most traditional technique used in proteomic approaches to study the exoproteome is two-dimentional gel electrophoresis combined with MS (Cuervo et al., 2009), but the first true systematic study of the *Leishmania* exoproteome was made using stable isotope labeling with amino acids in cells - SILAC. This study by Silverman and colleagues confirmed the specific enrichment of several intracellular proteins that could not be explained by simple parasite lysis. The above mentioned proteomic study clearly demonstrated that *Leishmania* exoproteome contains a myriad of proteins with distinct cellular origins, most lacking a clear amino-terminal secretion signal (Silverman et al., 2008). This fact was later confirmed in subsequent studies with other species (Cuervo et al., 2009; Hassani et al., 2011; Silverman et al., 2010b). Eventually the release of exosome like vesicles from the *L. donovani* surface was identified as a major contributor for non classical secretion in the exoproteome (Hassani et al., 2011; Silverman et al., 2010a). Genome based computational predictions have also been used to mine the *Leishmania* spp. genome using computer algorithms to screen for conserved N-terminal signal sequences. Several bioinformatic applications have been developed for the detection of secreted proteins from eukaryotes (Klee and Sosa, 2007). These programs are based on different algorithms and lead often to distinct predictions. To further increase the difficulty associated with *in silico* prediction none of these tools has been created specifically for *Leishmania* spp. In fact there is only one report on the validation of some of these bioinformatics tools in the related trypanosomatid *T. cruzi* (Ferella et al., 2008). The value of these individual approaches was limited but combining several prediction servers allowed to have almost 50% prediction rate when compared to the detection of the proteins in specific sub-cellular fractions (Ferella et al., 2008). Therefore to improve the *in silico* prediction power several distinct tools can be combined: presence of transmembrane domains, glycosylphosphatidylinositol-anchoring signal and mitochondrial targeting signals can be combined to screen for classically secreted proteins. To date there was only one major study done for *in silico* prediction of potential secreted proteins in *L. donovani* (Silverman et al., 2008). Silverman *et al.* analyzed the secretome searching for protein sequences with a signal peptide, lacking a transmembrane domain and glycosylphosphatidylinositol-anchoring signal, and found 217 potentially secreted proteins. Among these only 14 were detected in the subsequent proteomic approach (Silverman et al., 2008). In fact the study of the *Leishmania* spp. secretome is

impaired by the complexity of the exoproteome therefore experimental validation is required in order to evaluate actual secretion of the predicted proteins. Furthermore, taking into consideration the relative minor differences in gene content and RNA gene expression among the different *Leishmania* spp. (Lynn and McMaster, 2008), the value of these *in silico* approaches is always relative because an applied proteomic study will always be necessary when performing comparative or stage specific studies.

Due to the importance of the extracellular components of the parasite in the interaction with surrounding environment, it is of vital importance to improve and validate appropriate guidelines for exoproteome recovery. This should lead to the understanding of the exoproteome dynamics through the parasites life cycle and to define the mechanisms associated with protein release in the different developmental forms. This should allow a better understanding of the parasite biology and the mechanisms that enable it to survive.

Chapter II

Results

CHAPTER II

1. Objectives

This project was originally focused on studying the *Leishmania* secretome with the objective of understanding the contribution of individual secreted proteins towards virulence. To identify secreted proteins responsible for virulence we proposed to use virulent and avirulent strains/species and compare their secretomes. The complexity of the secretome prevented the use of different strains/species to look for secreted proteins in a basic comparative approach between virulent and avirulent strains. Therefore we refocused the objective of the project into the development and validation of an approach that would enable the study of the exoproteome components of stationary and logarithmic parasites. To achieve this we proposed to:

- Define the experimental time frame where parasites maintain optimal virulence;
- Develop a protein-free medium that would enable the study of the secretome;
- Validate and characterize the exoproteome from stationary and logarithmic parasites;
- Evaluate the effect of the exoproteome in the dendritic cells (DCs).

The achievment of these goals will enable the creation of new tools for the study of the exoproteome components ultimately contributing to a better understanding of *Leishmania* spp. pathogenesis.

2. Article name: Impact of continuous axenic cultivation in *Leishmania infantum* virulence

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# and ‡ Both authors contributed equally to this work

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In the present study we evaluated the phenomena of virulence loss associated with continuous growth in RPMI.

Main Results:

-Metacyclic parasites are highly immunomodulatory.

-Virulence loss is associated with continuous subpassages and appears as early as after 10 passages.

-The virulence loss is associated to a defect in differentiation into amastigotes and can be reverted upon differentiation into amastigotes, both *in vitro* and *in vivo*.

Conclusions:

L. infantum upon continuous subculture rapidly looses virulence through a process related to its incapacity to differentiate into amastigotes.

Article title:

Impact of continuous axenic cultivation in *Leishmania infantum* virulence

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Short Title: Virulence loss in long term axenic promastigotes

Keywords: Leishmania infantum; Metacyclogenesis; Axenic *in vitro* cultures; Virulence loss, differentiation

Abstract

Experimental infections with visceral *Leishmania* spp. are frequently performed referring to stationary parasite cultures that are comprised of a mixture of metacyclic and non-metacyclic parasites often with little regard to time of culture and metacyclic purification. This may lead to misleading or irreproducible experimental data. It is known that the maintenance of *Leishmania* spp. *in vitro* results in a progressive loss of virulence that can be reverted by passage in a mammalian host. In the present study, we aimed to characterize the loss of virulence in culture comparing the *in vitro* and *in vivo* infection and immunological profile of *L. infantum* stationary promastigotes submitted to successive periods of *in vitro* cultivation. To evaluate the effect of axenic *in vitro* culture in parasite virulence, we submitted *L. infantum* promastigotes to 4, 21 or 31 successive *in vitro* passages. Our results demonstrated a rapid and significant loss of parasite virulence when parasites are sustained in axenic culture. Strikingly, the parasite capacity to modulate macrophage activation decreased significantly with the augmentation of the number of *in vitro* passages. We validated these *in vitro* observations using an experimental murine model of infection. A significant correlation was found between higher parasite burdens and lower number of *in vitro* passages in infected Balb/c mice. Furthermore, we have demonstrated that the virulence deficit caused by successive *in vitro* passages results from an inadequate capacity to differentiate into amastigote forms. In conclusion, our data demonstrated that the use of parasites with distinct periods of axenic *in vitro* culture induce distinct infection rates and immunological responses and correlated this phenotype with a rapid loss of promastigote differentiation capacity. These results highlight the need for a standard operating protocol (SOP) when studying *Leishmania* species.

Author summary

Protozoan of the genus *Leishmania* undergo several developmental transitions during its life cycle. *Leishmania* alternates between two morphologically distinct forms, promastigotes (insect stage) and amastigotes (vertebrate stage). Most of the available information about *Leishmania* spp. has been obtained from studying *in vitro* cultured promastigotes, an excellent experimental model for the different developmental stages present in the insect vector. Although promastigotes are grown in a controlled environment, the maintenance of long term culture results in loss of virulence, which can lead to a misinterpretation and often contradictory experimental results. It is then of great interest to unravel the defects arising from sustained axenic parasite culture in laboratory settings. The authors demonstrate a correlation between the maintenance of parasite culture with a growing defect of the promastigote form to differentiate in the mammalian amastigote form. This research provides a biological explanation for the loss of virulence due to sustained parasite culture and discusses the impact for all experimental work done with visceral *Leishmania* species.

Introduction

Protozoan parasites of the genus *Leishmania* undergo several developmental transitions during their life cycle. Ingestion of infected macrophages during a blood meal by the sandfly vector leads to the release of intracellular amastigotes into the vector's midgut. This abrupt change in environment induces the transformation into extracellular procyclic promastigotes. The procyclic form within the vector midgut replicates and ultimately transforms into virulent metacyclic promastigotes, in a complex process that encompass parasite migration towards the upper gut of sandfly vector (Bates, 2007). In laboratory conditions, it is possible to achieve indefinite promastigote growth outside the sandfly using several established media; the procyclic forms correspond to promastigotes in exponential phase of growth that will eventually pass into a stationary phase, a fraction of these stationary parasites differentiates into the metacyclic form, with properties resembling those of sand fly promastigotes (Sacks and Perkins, 1984, 1985). Although stationary-phase promastigotes with undefined *in vitro* passages are commonly used without limitations, it has been demonstrated that continuous culture over time induces loss of virulence. In fact, long-term *in vitro* culture of promastigotes was one of the first empirical approaches to efficiently identify parasite virulence genes leading to the experimental development of attenuated strains (Mitchell et al., 1984). Similarly, long-term *in vitro* growth of drug-resistant parasites was suggested to mediate a loss of the resistance phenotype (Hadighi et al., 2006). This can be due to either loss of virulence factors induced by the lack of a survival pressure or due to disadvantageous adaptations to the media resulting in phenomena similar to clonal selection (Segovia et al., 1992). Either way, alterations in the physiology of the parasite that are induced by long-term growth in these media may lead to misinterpretation and contradictory results. Thus, one must carefully consider the influence of the different laboratorial factors in order to minimize these variables.

The current study is based upon the hypothesis that maintenance of *Leishmania* spp. in axenic *in vitro* culture results in a progressive loss of virulence quickly generating a significant bias towards the experimental data. We have compared the *in vitro* and *in vivo* infections and focused on the influence that axenic parasite growth and long-term maintenance can have on *in vitro* infections outcome. Our results demonstrate, for the first time, that the loss of virulence caused by the maintenance of axenic promastigotes in culture can be the result of a growing inability to differentiate into amastigote forms. Moreover, the induction of differentiation from promastigote to amastigote and then back to promastigote forms both *in vitro* and *in vivo* was capable to restore parasite virulence. Overall, our study demonstrated the need of a standard operating protocol (SOP) to study

visceral *Leishmania* spp. highlighting the crucial importance for proper control of parasite cultures in studies focusing on the mammalian stage, such as drug development or vaccine trials.

Materials and methods

Animals and parasites

Ten to twelve-week-old female Balb/c mice were obtained from Instituto de Biologia Molecular e Celular (IBMC; Porto, Portugal) animal facilities. Under laboratory conditions, the animals were maintained in sterile cabinets and allowed food and water *ad libitum*. Animal care and procedures were in accordance with institutional guidelines. All conducted experiments were done in accordance with the IBMC.INEB Animal Ethics Committee and the Portuguese Veterinary Director General guidelines. RS has an accreditation for animal research given from Portuguese Veterinary Direction (Ministerial Directive 1005/92). A cloned line of virulent *L. infantum* (MHOM/MA/67/ITMAP-263) was grown at 26°C in RPMI 1640 medium (Lonza, Swtzerland) supplemented with 10% heat-inactivated Fetal Bovine Serum - FBS (Lonza, Switzerland), 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin and 20 mM HEPES buffer. The MHOM/MA/67/ITMAP-263 clone (zymodeme MON-1) was originally isolated from the bone marrow of a human patient in Morocco and cloned by micromanipulation. In some experiments, a previously uncharacterized field attenuated *L. infantum* strain was used (species confirmed by pteridine reductase 1 sequencing and currently under ongoing characterization in our laboratory). To minimize the possibility of clonal bias, we have performed three independent recoveries of parasite from Balb/c mice for these experiments. All cultures were initiated at 10^6 parasites/ml and passed each 5 days. Promastigote to amastigote differentiation was achieved by culturing 10^7 stationary phase promastigotes/ml at 37ºC in a cell free culture medium (MAA20) (Sereno and Lemesre, 1997). Amastigote to promastigote differentiation was performed by culturing 10^7 axenic amastigotes/ml in complete RPMI medium for 4 days at 27 ºC. In alternative, spleens of infected Balb/c mice were placed in similar culture conditions for 7 days.

Ficoll density purification assay

Metacyclic promastigotes were purified from cultures with 3, 5 or 9 days or from 5-day cultures with 4, 21 and 31 (P4, P21 and P31) *in vitro* passages by Ficoll density gradient, as previously described (Yao et al., 2008). Briefly, 6 ml of 40% Ficoll was overlaid by 6 ml of 10% Ficoll in RPMI base. Then, 6 ml of PBS containing 1.2×10^9 parasites was placed at the top of the Ficoll gradient. The step gradient was centrifuged for 10 minutes at 370 g at room temperature without brake. The metacyclics promastigotes were recovered from the layer between 0% and 10% Ficoll solution. Metacyclic

promastigotes, identified by morphological criteria, *i.e.*, short and slender with a long flagellum twice the body length using phase contrast on a Nikon Eclipse 80i.

qPCR analysis

Total RNA was isolated from cells with the Trizol® reagent (Invitrogen, Barcelona, Spain), according to the manufacturer's instructions. Briefly, parasites were washed with ice-cold phosphate-buffered saline (PBS), harvested and homogenized in 800 µl of Trizol by pipetting vigorously. After addition of 160 µl of chloroform, the samples were vortexed, incubated for 2 min at room temperature and centrifuged at 12.000 g, for 15 min, at 4ºC. The aqueous phase containing RNA was transferred to a new tube and RNA precipitated with 400 µl of isopropanol for at least 10 min at room temperature. Following a 10 min centrifugation at 12.000 g, the pellet was washed with 1 ml of 75% ethanol and resuspended in 10 µl of 60ºC heated RNase free water. The RNA concentration was determined by using a Nanodrop spectrophotometer (Wilmington, DE, USA) and quality was inspected for absence of RNA degradation or genomic DNA contamination, using the Experion RNA StdSens Chips in the ExperionTM automated microfluidic electrophoresis system (BioRad Hercules, CA, USA). RNA was stored at -80ºC until use. RT was performed with equal amounts of total extracted RNA (1 µg) obtained from parasites recovered from different experimental conditions by using Superscript II RT (Gibco BRL) and random primers (Stratagene). Real-Time quantitative PCR (qPCR) reactions were run in duplicate for each sample on a Bio-Rad My Cycler iQ5 (BioRad, Hercules, CA, USA). Primers sequences were obtained from Stabvida (Portugal) and thoroughly tested. qPCR was performed in a 20 µl volume containing 5 µl of complementary cDNA (50 ng), 10 µl of 2x Syber Green Supermix (BioRad, Hercules, CA, USA), 2 µl of each primer (250 nM) and 1 µl H_2O PCR grade. Specific primers for histone H4 (forward: 5' ACACCGAGTATGCG -3'; reverse: 5'- TAGCCGTAGAGGATG-3'; LinJ35.1400 histone H4: Gene ID 5073031), Small Hydrophilic Endoplasmic Reticulum-associated Protein (SHERP) (forward: 5' CAATGCGCACAACAAGAT -3'; reverse: 5'- TACGAGCCGCCGCTTA-3'; LinJ23.1190 SHERP: Gene ID 5069222) and rRNA45 (forward: 5'CCTACCATGCCGTGTCCTTCTA -3'; reverse: 5'- AACGACCCCTGCAGCAATAC -3') (Ouakad et al., 2007) were used for amplification. After amplification, a threshold was set for each gene and cycle threshold-values (Ct-values) were calculated for all samples. Gene expression changes were analyzed using the built-in iQ5 Optical system software v2.1 (Bio-Rad laboratories, Inc). The results were normalized using as reference gene the rRNA45 rRNA sequence (Ouakad et al., 2007).

Viability analysis

Purified and non-purified promastigotes at a density of 10^5/ml were washed and suspended in Annexin V binding buffer. Parasites were incubated at room temperature for 15 minutes with AnnexinV-Cy5 (BD Pharmingen, San Diego, CA) and 7-AAD (Sigma). Parasites subjected to Ultra Violet light during 30 minutes and kept in culture for 4 hours were used as positive control. In amastigote differentiation, $2x10^6$ cells with 1 µM of propidium iodide (PI) were used. Data were collected in a BD FACScalibur cytometer (20.000 gated events) and analyzed by FlowJo software (Ashland, OR).

Promastigote CFSE-labeling

Purified and non-purified promastigotes ($6x10^7$/ml) were washed two times, suspended in PBS containing 5 µM of carboxyfluorescein succinimidyl ester (CFSE) (Invitrogen Molecular probes, Eugene, Oregon) and incubated at 37ºC for 10 minutes. Labeled parasites were washed, incubated at 4ºC for 5 minutes. Parasites were washed again to remove the exceed CFSE dye and suspended in culture medium before proceeding to macrophage infections. For promastigote to amastigote differentiation and proliferation analysis, 10^7 CFSE-labeled promastigotes were placed on 1 ml of MAA20. Each day, 100 µl of culture added with 1 µM of PI was analyzed by flow cytometry. Axenic amastigotes, identified by the absence of visible flagella and oval shape body, were observed in phase contrast on a Nikon Eclipse 80i.

In vitro macrophage infection

Cell suspension of bone marrow was obtained by flushing the femurs of susceptible Balb/c mice. The cell suspension was cultured in Dulbecco's modified Eagle's medium (DMEM) (Lonza, Switzerland), supplemented with 10% heat-inactivated FBS (Lonza, Switzerland), 2 mM L-glutamine, 100 U/ml penistreptomycin and 1 mM sodium pyruvate. After overnight incubation at 37ºC, non-adherent cells were recovered (300 g for 10 min, at room temperature) and cultured in 24-well culture dishes at $2x10^5$cells/ml in supplemented DMEM. For bone-marrow derived macrophages (BMMø) differentiation 10% L-929 cell conditioned medium (LCCM) was added at days 0 and 4. At day 7 of culture, CFSE labeled promastigotes were incubated with the BMMø at a 10:1 ratio. After 4 hours, infection was stopped the infection rates were determined at 4, 24 and 48 hours

post-infection by a BD FACScalibur cytometer and analyzed by FlowJo software. In some experiments, BMMø were infected and submitted to lipopolysaccharide (LPS) stimulus. Briefly, four hours after infection, infection was stopped and 1 µg/ml of LPS (Sigma) added. Twenty-four hours post-infection, BMMø culture supernatants were collected for cytokine quantification by Enzyme-Linked Immunosorbent Assay - ELISA (TNF-α, IL-12p40, IL-6 and IL-10), using commercial sandwich immunoassay kits (Biolegend and BD, San Diego, CA). Also, BMMø were recovered at 24 hours post-infection for surface co-stimulatory markers analysis. Thus, BMMø were stained with CD40-PE and MHCII-APC at 4ºC, during 30 minutes in the dark. The cells were then washed in PBS and suspended in 200 µl of PBS-2% FBS. Data were collected by a BD FACScalibur cytometer and analyzed by FlowJo software.

Animal experiments and parasite quantification

Promastigotes recovered from stationary culture with 4, 21 and 31 *in vitro* passages stationary-culture were collected, washed and suspended in sterile PBS. A volume of 200 µl of PBS containing 10^8 parasites was injected intraperitoneally. Mice of each group were sacrificed at 56 days post-infection. The parasite burden in the spleen and liver was determined by limiting dilution as previously described (Silvestre et al., 2007).

Statistical analysis

The data was analyzed using the non-parametric Kruskal-Wallis test followed by Dunn posttest for multiple comparisons when necessary.

Results

The maintenance of *L. infantum* promastigotes in axenic cultures results in diminished virulence

We started by clarifying our *in vitro* model of *L. infantum* infection in relation to the parasite development stage. *L. infantum* parasites recovered from the spleen of infected Balb/c mice were used to start axenic cultures at a 10^6 parasites/ml. The first task was to clearly define the culture time frame in which we can recover stationary parasites. Performing basic cell cycle analysis we excluded the use of the parasites until 2 days of culture because there was still significant active division (Fig. S1A). In order to evaluate the infectivity of stationary *L. infantum*, we used CFSE-labeled stationary promastigotes recovered at 3, 5 and 9 days of *in vitro* growth and BMMø as infection cellular target. Our data demonstrate that 3rd culture-day *L. infantum* promastigotes were significantly less infectious when compared with the 5th and 9th days of culture (Fig. S1B). These differences were already observed at 4 hours post-infection indicating a deficient parasite uptake with 3rd culture-day *L. infantum* promastigotes. Intraphagolysosomal adaptation mechanisms do not appear to be involved in the infection differences since similar infection percentages reductions were found between 4 and 24 hours (15.6 ± 0.4 for 3rd culture-day; 15.0 ± 1.2 for 5th culture-day; 18.1 ± 1.1 for 9th culture-day, when comparing 4 with 24 hours post-infection). Many reports now relate virulence with parasites culture viability (Wanderley et al., 2006; Wanderley et al., 2009). In order to lay down the hypothesis that differences in infectivity could be attributed to non-viable parasites, we evaluated the percentages of apoptotic or necrotic parasites by AnnV/7AAD labeling (El-Fadili et al., 2010). Nevertheless, no significant differences were found between all culture days (data not shown).

Several groups have already reported that long-term *in vitro* cultivation (more than 12 months) of *Leishmania* spp. leads to a totally avirulent promastigote population (Grimm et al., 1991; Segovia et al., 1992). According to these findings, we decided to evaluate if the sustained maintenance of *L. infantum* promastigotes in axenic culture at shorter time periods lead to distinct BMMø *in vitro* infection rates with distinctive immunologic phenotypes. In order to accomplish this, we maintained *L. infantum* promastigotes recovered from the spleen of infected mice for 4, 21 and 31 passages, which are equivalent to 20, 105 and 155 division events, considering simple exponential growth. The long-term maintenance of *L. infantum* in culture did not modify the promastigote growth behavior (Fig. 1A) neither their viability that was always superior to 90% (data not shown). Taking into account the distinct infection profiles depicted in Fig. S1B, we chose 5-day culture

promastigotes to compare infectivity. When non-purified parasite cultures were used to *in vitro* infect BMMø, a marginal but significant loss of infectivity at 48 hours for P21 and P31 when compared to P4 was observed (Fig. 1B). Metacyclic forms have been understood to be the most infective parasite form (Louassini et al., 1998; Yao et al., 2008). Therefore, we enriched the promastigote culture recovered from P4, P21 and P31 in metacyclics recovered by Ficoll density gradient, herein referred as Ficoll-purified promastigotes (Spath and Beverley, 2001), and analyzed their infectivity in primary BMMø cells (Fig. 1C). When Ficoll-purified metacyclic promastigotes from these cultures were used, differences were abrogated irrespective of the passage used (Fig. 1C). To confirm the enrichment of metacyclic promastigotes in this fraction and as an internal control of our experimental conditions, we analyzed the expression of two genes, SHERP and histone H4 that can be used to evaluate metacyclogenesis. SHERP gene is found to be up-regulated in infective metacyclic promastigotes (Knuepfer et al., 2001). On the other hand, higher expression of histone H4 is associated with exponential phase promastigotes (Soto et al., 1997). Indeed, the qPCR analysis demonstrated a significant increase in the SHERP mRNA transcripts in Ficoll-purified promastigotes when compared with non-purified parasite cultures (Fig. S2). These results suggested that the maintenance of *L. infantum* in axenic cultures resulted in a virulence leakage affecting their infectivity probably by the loss of metacyclic parasites. Nevertheless, no significant differences were found between the percentage of Ficoll-purified promastigote recovery from each culture (P4: 6.6 ± 0.1%; P21: 3.4 ± 1.6%; P31: 4.0 ± 0.9%).

Maintenance of long-term axenic *L. infantum* cultures decrease parasite capacity to modulate host cell functions in an inflammatory context

We have above demonstrated that the BMMø infection by *L. infantum* promastigotes depends not only upon the days of culture but is significantly modulated by their axenic culture period. However, we were unable to detect any major changes on macrophage activation status when submitted to *L. infantum* infection (data not shown), which can be explained by the *Leishmania* silent entry mechanism (Silvestre et al., 2009a). Therefore, we hypothesized that, when facing an inflammatory stimulus, axenic cultures with high passage number should be less successful in subverting macrophage effector functions being less capable of promoting infection. In order to investigate this hypothesis, we incubated BMMø cells with Ficoll-purified or non-purified parasites from distinct culture periods, which were 4 hours later submitted to LPS stimulation. As before, we observed a decrease of the infection rate with the augmentation of parasite *in vitro* passages (Fig. 2A).

This difference was minimized if Ficoll-purified promastigotes were used instead, although it was still statistically significant at 48 hours post-infection (Fig. 2B). LPS stimulation rapidly induces a surface up-regulation of MHCII molecules and co-stimulatory marker CD40. The analysis of these markers demonstrated that high passage number parasites had lower capacities to counteract the LPS activation stimulus (Fig. 2C). Once again, these differences were abrogated when metacyclic-enriched populations were used (Fig. 2C). We have also evaluated the levels of secreted IL-6, IL-12p40 and TNF-α and of the anti-inflammatory IL-10 cytokine. We found that the capacity to control LPS-induced cytokines was variable depending on the number of parasite passages, likely reflecting its distinct virulence. While significant differences were found with P31 parasites in the BMMø secretion levels of IL-6 and TNF-α (Fig. S3A and S3B, respectively), the major modifications were observed at the IL-12p40 and IL-10 levels (Fig. S3C and S3D, respectively). Indeed, P4 parasites were more capable to down-regulate IL-12p40 secretion induced by LPS stimulus, while increasing IL-10 cytokine secretion. This demonstrates that high passage parasites failed to counteract the secretion of pro-inflammatory cytokines induced by LPS in a similar manner. Moreover, if a pro-inflammatory/IL-10 ratio is constructed, a strong correlation was observed between shorter axenic culture maintenance periods and lower pro-inflammatory/IL-10 ratios (Table 1). Since the metacyclic enrichment diminished the differences observed in parasite infection rate and co-stimulatory markers found with stationary-phase promastigotes in different passages, we further investigated whether the cytokine bias was similarly altered. Indeed, the use of Ficoll-purified metacyclic enriched promastigotes, whatever their source, shifted the cytokine environment towards an anti-inflammatory ratio. Although some statistical differences were found between Ficoll-purified promastigotes from different passages, all displayed lower pro-inflammatory/IL-10 ratios when compared with LPS stimulation (Table 1). Overall, these data suggests that when *Leishmania*-infected BMMø are faced with an inflammatory stimulus, there is a specific overall loss of modulatory capacity that seems to be related to the highly immunoregulatory population of metacyclic parasites.

Sustained culture of *L. infantum* promastigotes results in an *in vivo* loss of virulence

We have above demonstrated that sustained axenic parasite culture results in a rapid loss of *in vitro* virulence. Previous reports demonstrating an *in vivo* loss of virulence were based on long-term, usually more than 1 year, parasite maintenance (Grimm et al., 1991; Segovia et al., 1992). However, we have observed a clear *in vitro* defect after only 21

passages. Therefore, we decided to validate the observed phenotype by performing *in vivo* infections using the susceptible Balb/c mice model. Six weeks after the infectious challenge with non-purified stationary-phase promastigotes recovered from P4, P21 or P31 cultures, a significant difference was found between P4 and high passage number parasite infections in the liver (Fig. 3A). Similarly, we have observed a significant lower parasite burden in the spleen of P31 infected mice that P4 infections (Fig. 3B). These results confirm the observed *in vitro* loss of virulence with parasite culture maintenance.

Loss of virulence originates from inadequate capacity to differentiate into amastigote forms

To elucidate the biological mechanisms that account for the loss of virulence due to long term parasite culture, we started by hypothesizing two major potential reasons: decrease number of metacyclic promastigotes or inadequate differentiation into amastigote forms. The quantification of Ficoll-purified promastigotes described above did not show any significant differences among the passages suggesting similar metacyclic quantities. However, the Ficoll density gradient assay is not a specific and sensible test for the quantification of metacyclic promastigotes in a culture but rather a method for its enrichment. Thus, to evaluate the hypothetical deficit on the generation of metacyclic promastigotes, we have performed *in vitro* infections using BMMø as targets, where we substitute 5% or 10% of non-purified stationary-phase promastigotes from each passage with similar percentages of Ficoll-purified fractions of P4 cultures to increase the total percentage of metacyclic promastigote. As a positive control, we used a naturally attenuated *L. infantum* from which we were unable to recover metacyclic promastigotes. Indeed, although the promastigotes of this *L. infantum* strain presents a similar axenic growth curve (Fig. S4), we were always unable to recover by Ficoll density gradient relevant number of promastigotes (lower that 0.1% of initial culture) from stationary-phase cultures. The quantification of CFSE-positive BMMø demonstrated that increasing the percentage of Ficoll-purified promastigotes did not significantly enhance, at any time point, the percentage of infected BMMø for P4 (Fig. 4A), P21 (Fig. 4B) or P31 (Fig. 4C) promastigotes. However, the opposite was observed with the naturally attenuated strain, where a significant increase of infected macrophages was observed at 48 hours post-infection (Fig. 4D). These results demonstrated that the lack of virulence originated from sustained parasite culture cannot be reverted by the addition of enriched-metacyclic fractions. This excludes a defect in the capacity to generate metacyclic promastigotes as the inherent biological cause for the virulence loss. Therefore, we investigated the

potential role of inadequate capacity to differentiate in the amastigote form. CFSE-labeled stationary-phase promastigotes recovered from each passage were placed on MAA20 (Sereno and Lemesre, 1997) and followed for three days. We evaluated the promastigote differentiation by light microscopy, axenic amastigotes proliferation by CFSE labeling and overall viability by PI staining. All cultures presented axenic amastigotes-like cells after 3 days of differentiation (data not shown). However, high passage number promastigotes displayed a striking decrease of differentiated cells. To quantify these differences, we have assessed the progressive diminution in the intensity of CFSE staining after the differentiation process. In fact, while P4 promastigotes progressively diminished CFSE fluorescence (Fig. 5A and B), high passage number promastigotes exhibit a severe defect to proliferate as amastigotes forms, as observed in both histogram curves (Fig. 5A) and quantification of mean fluorescence CFSE intensity (Fig. 5B). Moreover, this defect was not correlated with a difference on cell death, since similar percentages of viable parasites were found for all cultures during the differentiation process (Fig. 5C). Interestingly, the naturally attenuated strain did not display any significant change to differentiate when compared with P4 promastigotes, suggesting a distinct mechanism of loss of virulence that is not related with the capacity to generate axenic amastigotes.

L. infantum virulence is restored after full differentiation to amastigote forms

It is a current empirical methodology to pass *Leishmania* spp. promastigotes in experimental models to maintain virulence. We have hypothesized that the differentiation process from promastigotes to amastigotes forms would select the most virulent parasites in a heterogeneous culture assuring the continuity of competent and adapted parasites. Therefore, to explore this assumption we have differentiated promastigotes from each passage number in amastigotes both in axenic and *in vivo* conditions. Axenic amastigotes were obtained by differentiating promastigotes in MAA20 medium (Sereno and Lemesre, 1997) for a period of 3 days. The viable axenic amastigotes were maintained axenically in culture for 10 days, after which were re-differentiated to promastigotes forms. In alternative, we recovered *L. infantum* parasites from the spleen of infected Balb/c mice by allowing amastigote to promastigote differentiation for a period of 7 days. All these promastigotes were sub-cultured for 4 passages and used to infect BMMø. Remarkably, we did not observe any difference between infections whatever the initial parasite source used (Fig. 6A and B). Again, we used as a control the naturally attenuated *L. infantum* strain. Although an increase of virulence was observed after the differentiation protocol (Fig. 6A), when compared to non-differentiated parasites (Fig. 4D), we observed a general lower

infection percentage with the exception of 4 hours post-infection. Overall, these results demonstrate that the defect on virulence due to sustained parasite maintenance can be recovered either by *in vitro* or *in vivo* full differentiation to amastigote and back to promastigotes forms.

Discussion

Visceral *Leishmania* infections studies have been the center of some controversy which can occasionally be traced back to the use of distinct *in vitro* promastigotes culture conditions. The plasticity of the *Leishmania* genome (Bastien et al., 1992a) is an important variable to consider when axenic promastigotes are used for *in vitro* or *in vivo* studies. Thus, parasite phenotypic plasticity allows it to adapt to the environment generating discrepancies between studies in different laboratories even when using the same *Leishmania* strain.

In the current work, we have started by investigating in our *in vitro* model of *L. infantum* infection the relation between the parasite development stage and its infectivity. The first step was to discard logarithmic parasites because they are not ultimately responsible for the infection (Sacks and Perkins, 1985), so we used a basic cell cycle analysis to discard multiplying parasites (less than 10% of total population are in S/G2 phase after the third day of culture). This data correlated clearly with basic morphological visualization. Stationary *L. infantum* cultures in day 5 and 9 induced higher BMMø infection rates than day 3 parasites. This difference in infectivity might translate the time frame required for becoming truly metacyclic parasites (Sadlova et al., 2010), which was also corroborated by the less amount of metacyclic recovered (data not shown). Since the presence of apoptotic parasites is essential for a virulent inoculum of *Leishmania* promastigotes (van Zandbergen et al., 2006), we decided to quantify the percentage of apoptotic and dead parasites in each case to remove this possible bias from our analysis. In fact, for all time frames tested, the differences in infectivity were not related to apoptotic or dead parasites in the non-purified or Ficoll-purified populations, with culture viability always higher than 90%.

Some authors described that *in vitro* maintenance for long periods constitute an important factor for the loss of virulence in *L. infantum* (Grimm et al., 1991) and *L. major* (Segovia et al., 1992) promastigotes. Nonetheless, this loss of virulence is a reversible phenomenon, since serial passages on susceptible mice allow the parasite to recover a virulence phenotype (Katakura and Kobayashi, 1985). In the present study, we complemented the previous observations by comparing the impact of continuous *in vitro* culture on *Leishmania* promastigote virulence and also into the capacity of host macrophage manipulation. Our data clearly demonstrated a loss of *L. infantum* virulence related to the augmentation of *in vitro* culture periods although no modification was observed in the axenic promastigote growth behavior. This significant loss of infectivity was observed as soon as 105 days of successive (21 passages) culture and worsened with parasite maintenance in culture. In fact, 20 passages was the soonest time point where we

could have a significant reproducible loss of infection. There is a grey area between passage 9 and passage 20 where we can have variation of infection in a manner that probably reflects the initial parasite inoculum recovered from the mammal. This was also observed after *in vivo* infection where we had a significant decrease in parasite burden after 21 and 31 passages, confirming the *in vitro* observations.

The percentage of metacyclic in a heterogeneous stationary-phase is an important factor in the parasite infectivity since they are significantly more infective than the non-purified population. We used a Ficoll density gradient methodology to enrich the percentage of metacyclic promastigotes (Saraiva et al., 2005). Beyond the morphological changes, during the *Leishmania* spp. differentiation process, modifications also occur in gene expression and in the composition of parasite surface that help to characterize metacyclic promastigotes. Thus, we have evaluated the enrichment of metacyclic promastigotes in the Ficoll-purified fraction by microscopy (data not shown) and through qPCR analysis of the SHERP and histone H4 gene expression. The augmentation of SHERP gene expression in Ficoll-purification supported an enriched metacyclic population. The use of Ficoll-purified promastigotes abolished the differences found among the different passages. Yet, significant differences were found for P21 and P31, at 48 hours post-infection when facing an inflammatory stimulus, showing that the phenomenon of loss of virulence, although less prominent in the metacyclic-enriched parasites, was not restricted to the unpurified culture. Since the differences at the infection level were significant, we examined if there was a potential effect on the macrophage activation status. In the presence of a strong inflammatory stimulus, *L. infantum* is able to suppress certain LPS-derived pro-inflammatory cytokine responses in an active parasite-specific process while it augments the production of some anti-inflammatory cytokines (Silvestre et al, unpublished data). Indeed, the addition of LPS to *Leishmania* spp. infected cells was demonstrated to synergistically induce the secretion of the anti-inflammatory IL-10 cytokine in monocytes (Meddeb-Garnaoui et al., 2009) and in macrophages (Lapara and Kelly, 2010). The functional polarization of macrophages into IL-10 producers characterized as M2 cells (Mantovani et al., 2004) has been long understood to play a crucial role in the success of parasite infection process (Cunningham, 2002). Our results demonstrated a growing defect of high passage parasites to modulate the LPS stimulatory effect. Furthermore, it is clear from the inflammatory profile depicted in Table 1 that metacyclic enriched fractions are always significantly more effective in abrogating a macrophage response to the inflammatory stimuli than their non-purified counterparts revealing the metacyclic parasites as a highly immunomodulatory population with a distinct profile from the non-purified population. There is a distinct and significant loss of immunomodulatory properties from P4 to P21 that becomes stable after P21. This

loss of immunomodulatory properties seems reminiscent of phenomenon of transient gene expression similar to what happens under drug pressure (Ubeda et al., 2008), being lost upon the terminus of immunological pressure. Indeed, this might be happening in just a few passages of axenic culture. In an attempt to explain the loss of virulence mechanism, some authors referred to a reduction of metallo and cysteine peptidases activity, important for virulence, in *L. braziliensis* (Bates et al., 1994; Lima et al., 2009) and in *L. amazonensis* (Chaudhuri and Chang, 1988) or mitochondrial defects (Nasyrova et al., 1993) during long-term culture. However, others have been unable to detect any differences in the parasite enzymatic profile with long *in vitro* periods of cultivation (Cuervo et al., 2008; Soares et al., 2002).

One can speculate that the overall loss of immunomodulatory properties over time, and in consequence loss of virulence might reflect a diminution of the number of metacyclic parasites in the population. Although the percentage of Ficoll-recovered promastigotes was quite similar among the three tested passages, these fractions do not constitute a pure metacyclic population, so we decided to complement older passage parasites with Ficoll recovered metacyclic promastigotes to access if the loss of virulence could be reverted by exogenous addition of metacyclic parasites from an early passage. No improvement in the overall infection was observed, although, when these same metacyclic parasites were added to an avirulent field strain, from which we were unable to recover metacyclics, there was an improvement on the infection.

Another possibility to explain the virulence loss was the possibility of a defective promastigote to amastigote differentiation. Our data clearly demonstrated that high passage number promastigotes displayed decrease capacity in differentiating, which was not correlated with decreased cellular viability. This incapacity translates into fewer parasites able to differentiate leading to a less capable population to face host cell response. Ultimately, this results in lower parasite burdens *in vitro* and *in vivo*. Moreover, these axenic amastigotes recovered after differentiation were morphologically indistinguishable and retained similar growth capacity (data not shown). To ultimately state and confirm the importance of promastigote to amastigote differentiation as a driving selective force for virulence, we showed that parasites passed through the amastigote stage, either *in vitro* or *in vivo*, revert the loss of virulence. This fact in conjunction with the remarkable loss of immunomodulatory properties leads to the early loss of virulence detected in our model.

We do not rule out metacyclogenesis related defects as a driving force for a virulence. In relation to metacyclogenesis it has been argued that a successful and complete differentiation is dependent on the presence of large amount of metacyclic promastigotes (Cysne-Finkelstein et al., 1998). However, it is still not clear whether this process is an

essential step in the differentiation *in vitro*, since procyclic promastigotes appear to differentiate with equal efficiency as metacyclics (Barak et al., 2005; Debrabant et al., 2004; Goyard et al., 2003; Saar et al., 1998). Indeed, our results with the naturally attenuated strain support this notion. Our data do not rule out that P21 or P31 metacyclic promastigotes could not display any sort of biochemical or protein expression defect that may impact the differentiation process. Similarly, we cannot reject the idea of longer periods of sustained cultured originating defective metacyclic cultures. However, during our study, the loss of virulence was related to a specific defect on promastigote to amastigote differentiation.

Overall, our data demonstrated that the loss of virulence is linked with decreased capacity to differentiate in amastigote forms, which may probably be originated from the absence of a complete life cycle. Therefore, special care must be taken when performing experiments with axenic *Leishmania* promastigotes. The systematic and rigorous control of *Leishmania* culture conditions should be considered as a keystone for each experimental protocol. The differences found in infectivity accompanied by disparate effects at the macrophage activation levels point to significant differences at biochemical and structural level, enlarging the effects of careless parasite maintenance to other experimental fields. This information is extremely relevant especially for those developing new drug and vaccine approaches. In such cases the immune response to the parasite is the essence of the experimental procedure.

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Figure 1. Long-term *in vitro* maintenance of *L. infantum* promastigotes does not alter parasite growth. *L. infantum* growth curves were performed by neubauer chamber counting **(A)**. BMMø were infected at a 1:10 (cell/parasite) ratio with non-purified **(B)** and Ficoll-purified **(C)** promastigotes labeled with CFSE. Data were acquired by FACScalibur cytometer and analyzed by FlowJo software. Three independent experiments were performed; one representative experiment is shown. The mean and standard deviation are shown. **P* < 0,05, ***P* < 0,01, ****P* < 0,001 statistical significance relatively to P4.

Figure 2. Long-term *in vitro* maintenance of *L. infantum* promastigotes results in loss of virulence *in vitro*. BMMø were submitted to LPS stimulation 4 hours after infection with non-purified (**A**) or Ficoll-purified (**B**) CFSE-labeled promastigotes at a 1:10 (cell/parasite) ratio. The percentage of infected cells was determined by the number of CFSE-positive cells in a FACScalibur cytometer. Expression of MHCII and co-stimulatory molecule CD40 at 24 hours post-infection. Thick line – P31, dotted line - P4, thin line - LPS and shadded hystogram - isotype control **(C)**. Two independent experiments were performed; one representative experiment is shown. The mean and standard deviation are shown. **P* < 0,05; ***P* < 0,01 statistical significance relatively to P4.

Figure 3. Long-term *in vitro* maintenance of *L. infantum* promastigotes results in loss of virulence *in vivo*. Balb/c mice were infected with stationary phase promastigotes submitted to 4, 21 or 31 successive *in vitro* passages. After 6 weeks post-infection, the parasite load was determined in liver (**A**) and spleen (**B**) by limiting dilution. The mean and standard deviation are shown. **P* < 0,05; ***P* < 0,01; ****P* < 0,001

Figure 4. Decreased capacity to infect is not correlated with a loss of metacyclic promastigotes. BMMø were infected at a 1:10 (cell/parasite) ratio with non-purified promastigotes submitted to 4 (**A**), 21 (**B**) or 31 (**C**) successive *in vitro* passages with 5% or 10% of Ficoll-purified parasites or without (Mock). As a control, BMMø were infected with a naturally attenuated strain in the same conditions (**D**). Data were acquired by FACScalibur cytometer and analyzed by FlowJo software. Two independent experiments were performed; one representative experiment is shown. The mean and standard deviation are shown. ***P* < 0,01

Figure 5. Diminished capacity of long-term cultured *L. infantum* promastigotes to differentiate and proliferate as axenic amastigotes. CFSE-labeled non-purified promastigotes submitted to 4, 21 or 31 successive *in vitro* passages or from a field recovered naturally attenuated strain (Aten) were cultured in MAA20 for 3 days to induce differentiation into the amastigote form. (**A**) Parasite multiplication was followed by FACScalibur quantification of CFSE fluorescence. (**B**) For each time, the mean fluorescence intensity (MFI) was calculated. The mean and standard deviation are shown. $^{**}P < 0{,}01$; $^{***}P < 0{,}001$ (**C**) At the same time, parasite viability was followed by propidium iodide (PI) incorporation.

Figure 6. Virulence is recovered after *in vitro* or *in vivo* promastigote-amastigote differentiation process. BMMø were infected at a 1:10 (cell/parasite) ratio with non-purified promastigotes differentiated from *in vitro* axenic amastigotes (**A**) or *ex-vivo* intracellular amastigotes (**B**). As a control, a naturally attenuated strain (Aten) was submitted to the same experimental conditions. Data were acquired by FACScalibur cytometer and analyzed by FlowJo software. Three independent experiments were performed; one representative experiment is shown. The mean and standard deviation are shown. $^{*}P < 0{,}05$; $^{**}P < 0{,}01$.

Figure S1. **Cell cycle analysis and *in vitro* virulence of *L. infantum* recovered in distinct culture days.** *L. infantum* promastigotes were cultured at a 10^6/ml. Each day, $2x10^6$ promastigotes were recovered and the cell cycle analyzed by PI staining (**A**). BMMø were incubated with non-purified CFSE labeled *L. infantum* promastigotes at a ratio of 1:10 (cell/parasite). The percentage of infected cells was obtained by quantifying the number of CFSE-positive cells (**B**). Data were acquired at 4 and 24 hours post-infection in a FACScalibur cytometer and analysed by FlowJo software. Three independent experiments were performed; one representative experiment is shown. The mean and standard deviation are shown. $^{*}P < 0{,}05$, $^{**}P < 0{,}01$ statistical significance relatively to 3rd day of parasite growth.

Figure S2. Indirect quantification of metacyclic promastigotes in heterogenous and Ficoll-purified cultures by gene transcription analysis. Transcription profile of SHERP and Histone H4 genes obtained by qPCR, for non-purified and Ficoll-purified promastigotes. Normalizations were made against the reference gene rRNA45. Three independent experiments were performed, each performed in duplicate;

one representative experiment is shown. The mean and standard deviation are shown. $^*P < 0{,}05$.

Figure S3. Long-term cultured *L. infantum* promastigotes show decrease capacity to modulate an inflammatory stimulus *in vitro*. BMMø were submitted to LPS stimulation 4 hours after infection with non-purified promastigotes. The levels of IL-6 (**A**), TNF-α (**B**), IL-12p40 (**C**) and IL-10 **(D)** were quantified 24 hours post-infection on BMMø supernatants by ELISA. Three independent experiments were performed; one representative experiment is shown. The mean and standard deviation are shown. $^*P < 0{,}05$; $^{**}P < 0{,}01$; $^{***}P < 0{,}001$ statistical differences relative to LPS unless depicted by a bar.

Figure S4. Naturally attenuated *L. infantum* strain has a similar axenic growth in comparison to WT strain. *L. infantum* growth curves were performed by neubauer chamber counting. The mean and standard deviation are shown.

Figure 1

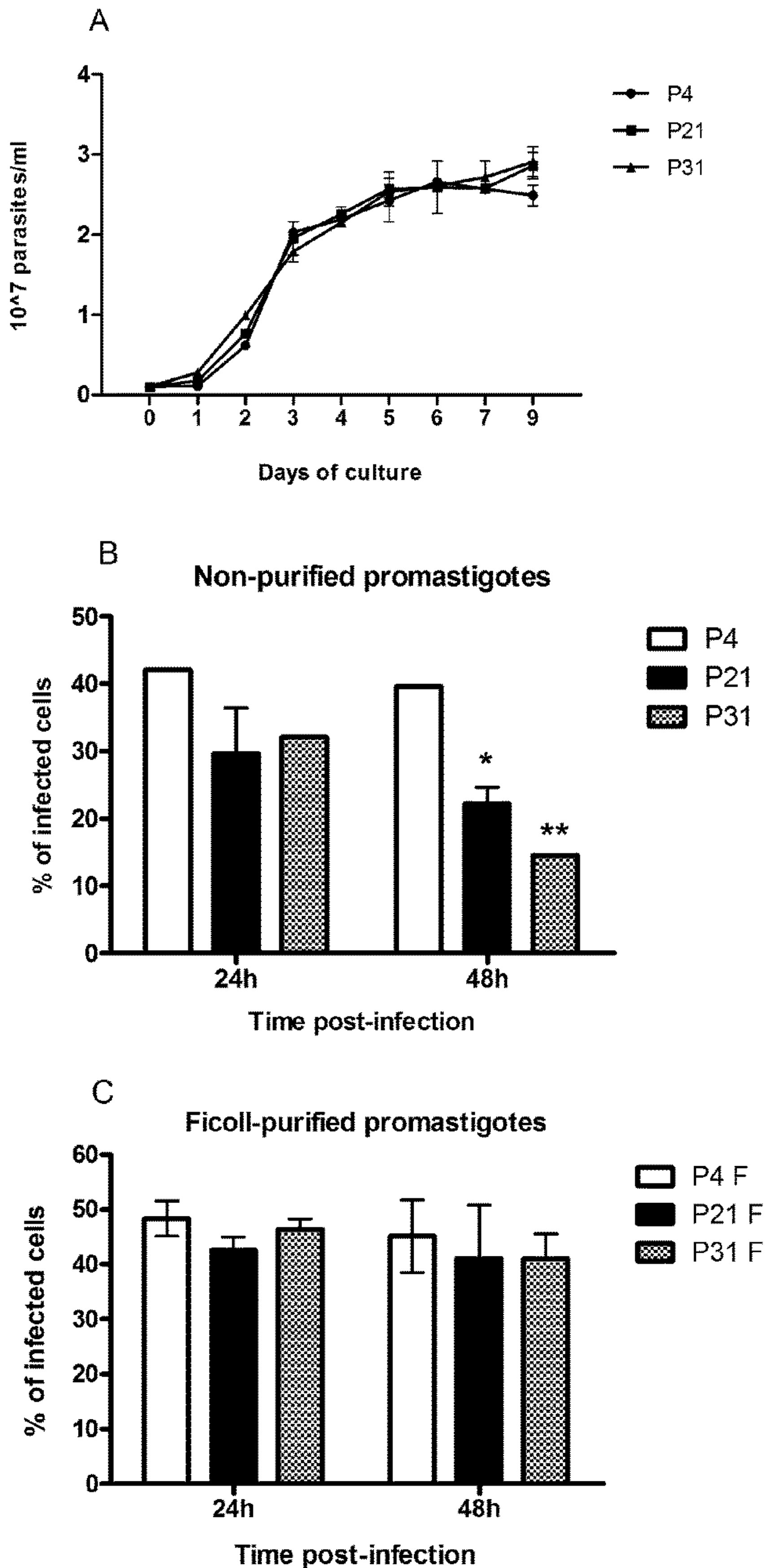


Figure 2

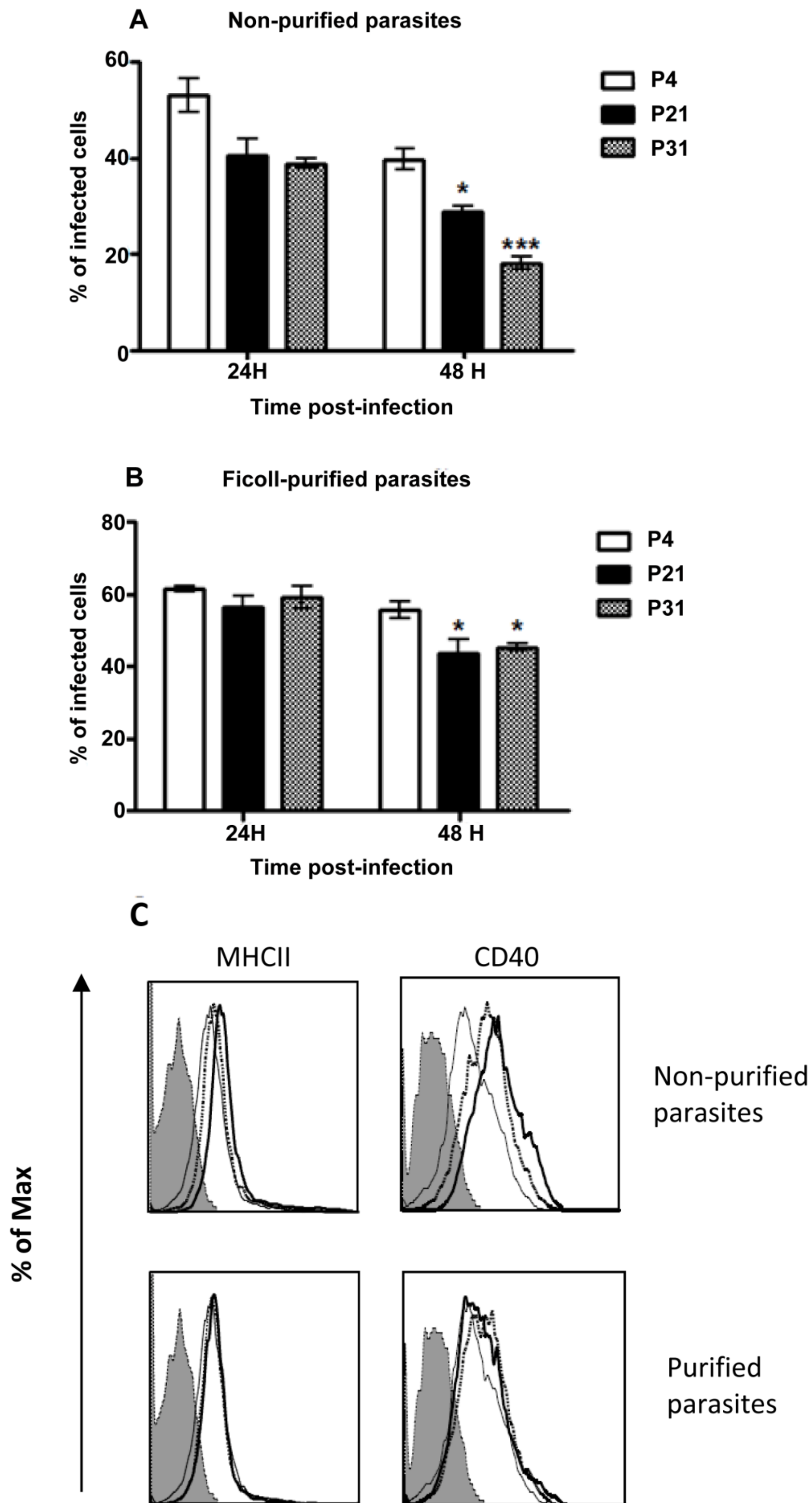


Figure 3

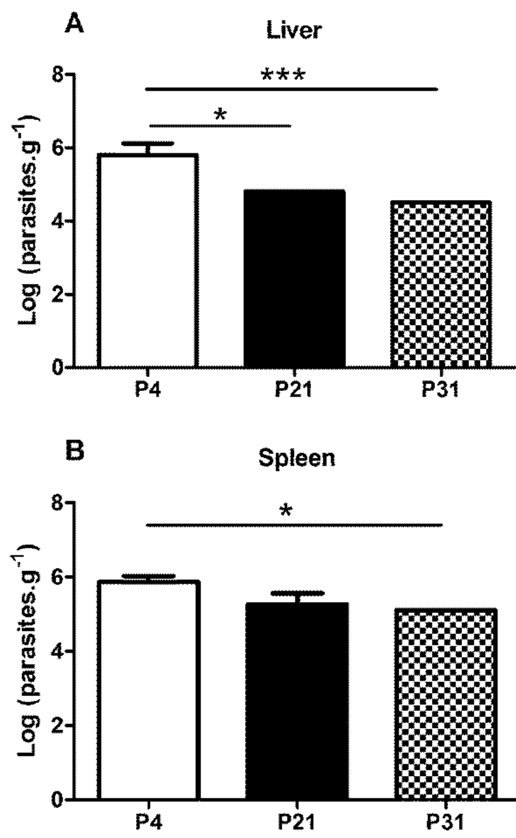


Figure 4

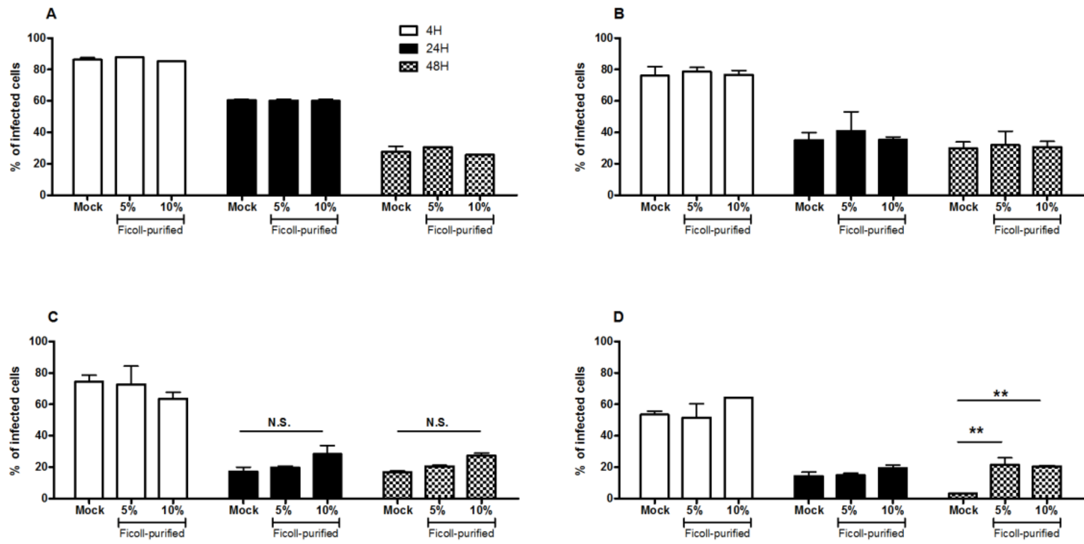
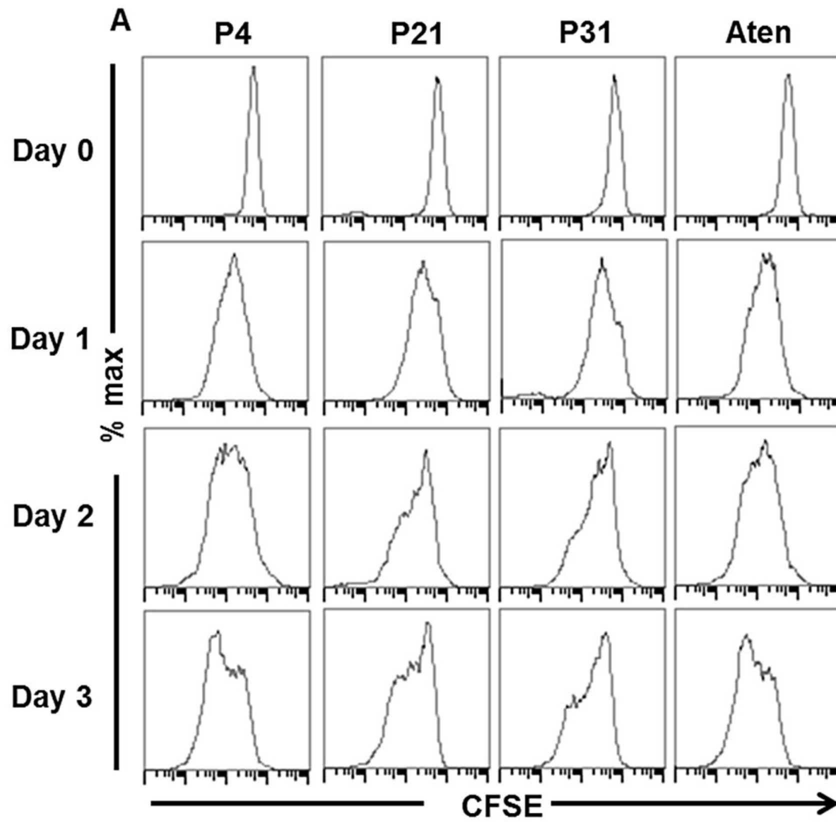
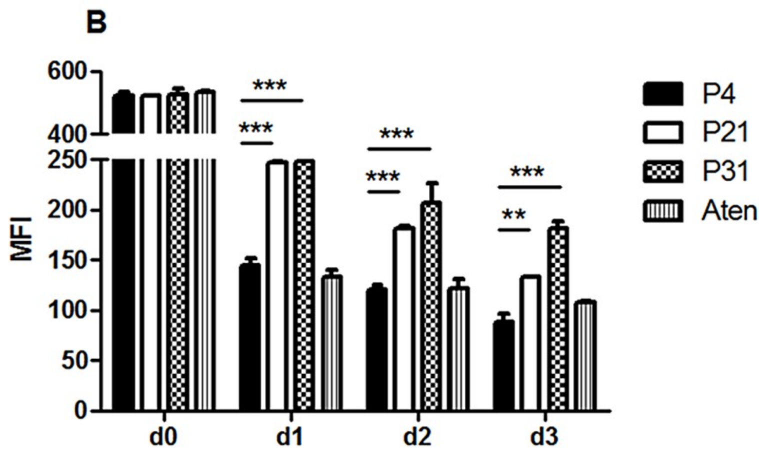


Figure 5





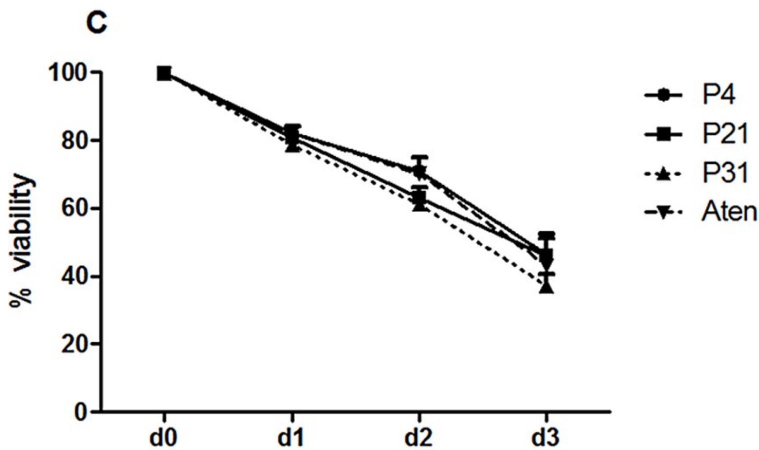


Figure 6

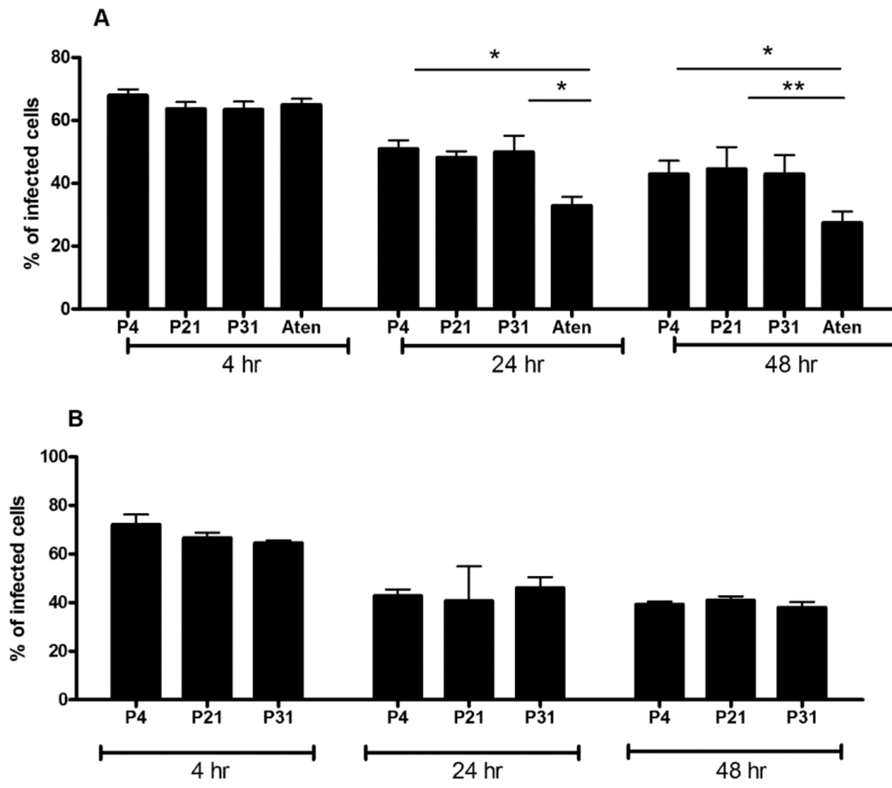


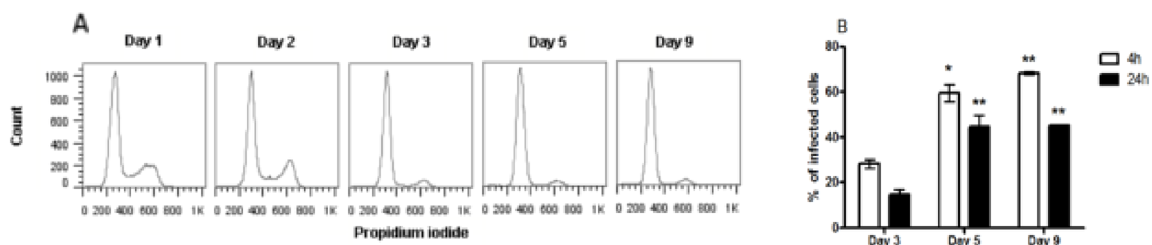
Table 1 Ratio of several pro-inflammatory cytokines/IL-10 quantified by ELISA on cell supernatants of 24 hours infected BMMø.

		IL-12p40/IL-10	IL-6/IL-10	TNF-α/IL-10
Non - purified	LPS	1,15 ± 0,13	3,46 ± 0,14	6,74 ± 0,40
	LPS P4	0,80± 0,01	0,40 ± 0,003 ***	0,30 ± 0,016 ***
	LPS P21	2,41 ± 0,04 *	1,39 ± 0,10*	1,09 ± 0,06 *
	LPS P31	1,95 ± 0,72	1,15 ± 0,13*	0,84 ±0,07 *
Ficoll - purified	LPS P4	0,18 ± 0,00 ***	0,15 ± 0,01 ***	0,09 ± 0,01 ***
	LPS P21	0,45 ± 0,02 ***	0,39 ± 0,01 **	0,19 ± 0,03 **
	LPS P31	0,40 ± 0,01 ***	0,40 ± 0,01 **	0,18 ± 0,01 **

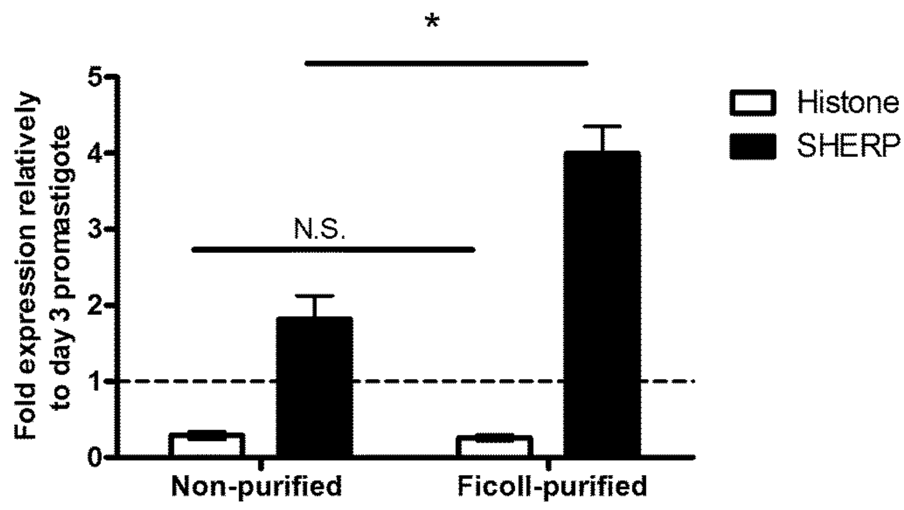
Significance markers printed between rows/brackets: Non-purified — IL-12p40/IL-10: ***, *; IL-6/IL-10: **, * *, *; TNF-α/IL-10: *, *. Ficoll-purified — IL-12p40/IL-10: *, *; IL-6/IL-10: ***, *, ***, ***, *; TNF-α/IL-10: *, *.

The values are expressed in arbitrary units. One representative experiment out of two is shown. The mean and standard deviation are shown. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P<0.001$ statistical significant relatively to LPS and between passages of culture presented. All Ficoll-purified ratios are significantly different (at least $^{*}P < 0.05$) from the related non-purified population.

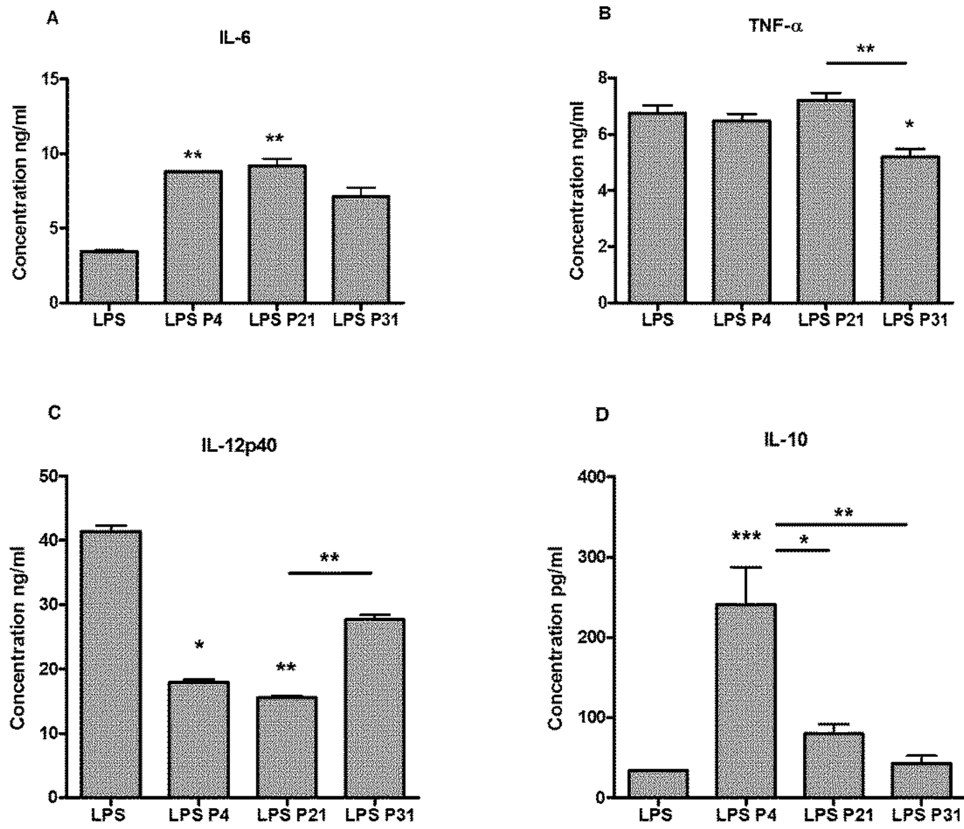
Suplemmentary figure 1



Suplemmentary figure 2



Suplemmentary figure 3



Suplemmentary figure 4

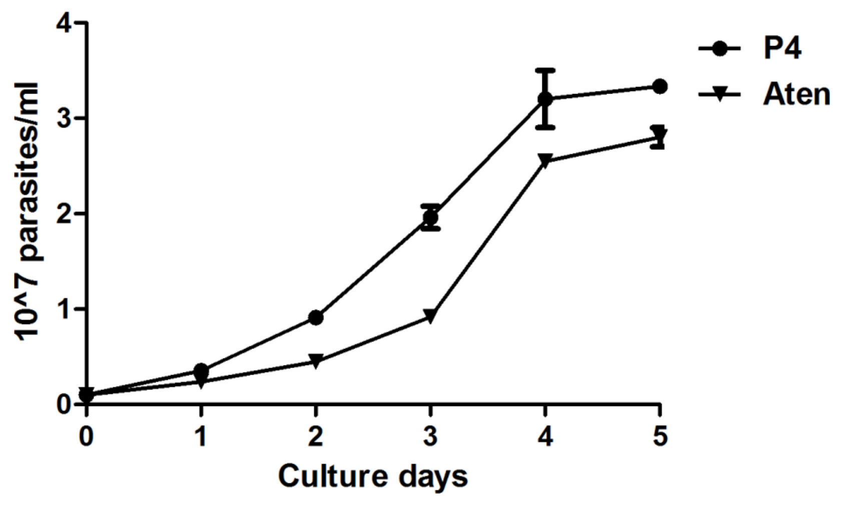
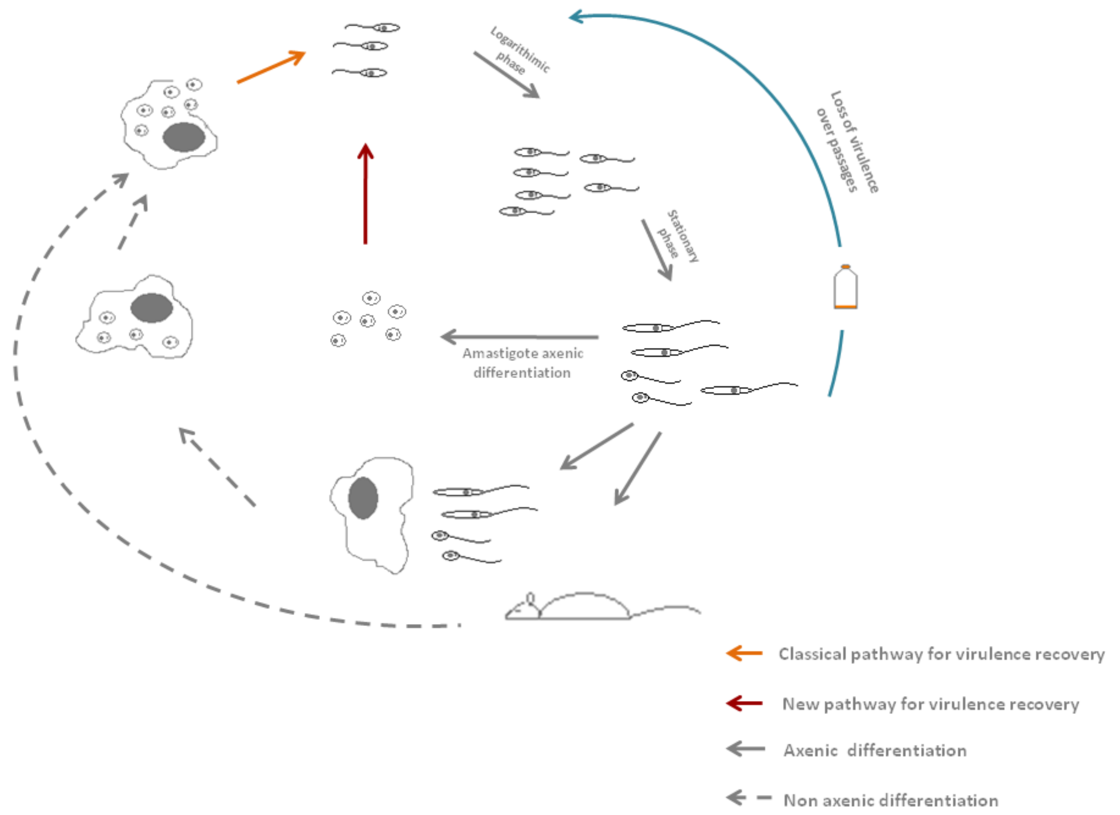


Diagram with the results presented in the article


Logarithimic phase
Stationary phase
Loss of virulence over passages
Amastigote axenic differentiation
Classical pathway for virulence recovery
New pathway for virulence recovery
Axenic differentiation
Non axenic differentiation

3. Article name: The development of a semi-defined media for growth of *Leishmania infantum*

Nuno Santarém, Ricardo Silvestre, Diana Moreira, Marc Ouellette & Anabela Cordeiro-da-Silva

In the present study we developed a protein-free medium for the cultivation of *L. infantum*

Main Results:

-The low molecular weight components of FCS could be substituted by the SDM base.

-The cRPMI medium sustain the growth of *L. infantum* which retain similar growth characteristics to parasites grown in standard RPMI (morphology, cell cycle, viability, metacyclogenesis).

-The parasites grown in cRPMI were able to infect to the same extent as parasites in standard medium both *in vitro* and *in vivo*.

Conclusions:

The medium cRPMI is a viable alternative to grow *L. infantum* retaining basic biological characteristics like morphology and virulence.

Article title:

The development of a semi-defined media for growth of Leishmania infantum

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Keywords: Leishmania infantum; Axenic *in vitro* cultures; Virulence loss, Culture Media

Abstract

The elimination of serum or of serum-derived macromolecules from *Leishmania* culture media could decrease culture variability and enable the development of experimental approaches that require the absence of proteins in the culture media. We report a defined medium, derived from RPMI 1640 and SDM 79 without serum-derived proteins and/or macromolecules that can be used as a substitute of serum-supplemented media for the continuous *in vitro* cultivation of promastigote forms of *Leishmania*. This medium, cRPMI enables continuous subculture of *Leishmania* maintaining a typical promastigote morphology, viability, cell cycle profile, metacyclic profile and the capacity to differentiate into amastigotes. The absence of serum and macromolecules in the cRPMI medium did not markedly change the *in vitro* infectivity in bone marrow-derived macrophages and their virulence in animals compared with parasites cultivated in standard non-defined medium. Serum-free culture will be increasingly important in providing stability and reproducibility in promastigote cultures enabling applications such as exoproteome recovery and metabolic studies.

Introduction

Leishmania spp. are parasites with a digenetic life cycle. Extracellular promastigotes proliferate within the digestive tract of sandflies until a subsequent blood meal, which enables the transmission of the parasite to a mammalian host. In the host, the parasite differentiates into the intracellular amastigote form that thrives in the phagolysosome of macrophages [1,2]. More than 20 species of these parasitic protozoa are responsible for a group of vector borne neglected tropical diseases collectively known as leishmaniasis. The disease is responsible for 2 million new cases each year and 350 million people distributed throughout 88 countries are at risk. Leishmaniasis is characterized by different clinical manifestations that can range from self healing cutaneous presentations to a fatal visceralizing ailments [3]. Moreover, the clinical manifestations are often species specific and influenced by the immune status of the host, becoming a serious issue in the case of HIV co-infections [1]. No effective human vaccine exists and disease treatment is often toxic, prone to developing resistance or expensive for the majority of the infected population [3]. There is thus a growing need for better understanding of these protozoa to allow the development of better and affordable therapeutical approaches.

Most of the available information about Leishmania spp. has been obtained from studying axenic promastigotes cultures. The promastigote forms can be easily cultivated in different types of media under laboratory conditions [6]. The unrestricted use of these media has an inherent biological cost. It was described by several authors that continuous in vitro growth of parasites induces virulence loss [7]. This decrease in virulence can be due to several factors: a growing incapacity to differentiate into amastigotes (Diana et al, submited), or through hypothetical adaptations to the medium that lead to loss of infecting capacity mediated by the loss of virulence factors [7]. Conventional media are not appropriate for several biological studies such as exoproteome recovery or metabolic studies because most media are complemented with fetal calf serum (FCS) or other protein supplements. FCS is a complex, highly variable, and difficult to characterize reagent. It is particularly expensive when used to produce large culture of cells for protein purification. An ideal culture medium must enable indefinite parasite maintenance upon subculturing, retain the basic characteristics of promastigotes (morphology and infectivity), be affordable, have a simple preparation and ultimately be as defined as possible to enable metabolic studies [6]. With these criteria in mind, we developed a functional and inexpensive medium based on two distinct media routinely used for growing., namely RPMI and SDM 79 [8,9]. The cRPMI is a FCS-free medium, or free of proteins and peptides that readily supports the growth and maintenance of promastigote forms of various Leishmania infantum strains and also other species of *Leishmania*.

Morphologic and biologic properties of cRPMI grown promastigote forms are presented and their virulence was evaluated.

Matherials and methods:

Parasites and cell culture

A cloned line of virulent L. infantum (MHOM/MA/67/ITMAP-263) was grown at 26°C in SDM-79 [9] supplemented with 10% fetal calf serum – FCS - (Lonza), 5 µg/ml hemin (Sigma), and 5 µM biopterin (Sigma) at pH 7.0 - standard SDM - or RPMI 1640 medium (Lonza) supplemented with 10% FCS, 2mM L-glutamine (Lonza), 100 U/ml penicillin (Lonza), 100 mg/ml streptomycin (Lonza) and 20 mM HEPES buffer (Lonza) – standard RPMI or cRPM (RPMI base complemented with 10% SDM base and 2,5 µg/ml of hemin). To minimize the possibility of clonal bias, we have performed three independent recoveries of the parasite from Balb/c mice for these experiments. All cultures were initiated at either 10^6 parasites/ml (standard RPMI and cRPMI) or 2,5x105 for standard SDM and passed every 5 days. Promastigote to amastigote differentiation was achieved by culturing $1x10^7$ stationary phase promastigotes/ml in a cell free culture medium at an acidic pH and 37ºC (MAA20) [10].

Complementation experiments

FCS was passed through a centriprep Ultracel YM-3 filtering unit (Millipore) to remove the bulk of the protein content from the filtrate. The filtrate was then passed through the filtering device once again and then sterilized by filtration through a 0,2 µm filter (Milipore) and stored at 4ºC, until use (this fraction will be herein referred as <3 kDa). The retentate was dialyzed twice against phosphate buffer saline (PBS) to completely remove the low molecular weight components and stored at 4ºC until use (this fraction will be herein referred as >3 kDa). For the complementation experiments *L. infantum* promastigotes growing in complete RPMI or SDM79 were washed twice in PBS and used at a density of 1x106/ml in the respective base medium without hemin. These were subcultured for 2 passages complemented with none or one of the following supplements: 10% FCS; 10% >3kDa; 10% <3kDa; 2.5 µg/ml hemin; 5 ng/ml hemin and 10% >3Kda; 5 ng/ml hemin and 10% <3Kda. After two passages the growth enabling situations were placed at a density of $1x10^6$/ml and evaluated for growth after 4 days using a Neubauer chamber. Morphology of the parasites was registered using phase contrast on a Nikon Eclipse 80i.

Supplementation experiments

For the supplementation experiments *L. infantum* promastigotes growing in complete RPMI were washed twice in PBS and used at a density of $1x10^6$/ml in the respective base with 2.5 µg/ml of hemin. These parasites were then complemented with one or none of the following SDM 79 supplements: 10% SDM; 20% SDM; 50% SDM or 100% SDM. After 4 days, growth was determined using a Neubauer chamber.

Growth curves

Promastigotes in cRPMI, standard RPMI or standard SDM growing for 24 hours in a startup culture with the above defined starting inoculums were used as starter for the growth curves. At defined time points the parasites were counted using a Neubauer chamber.

Cell cycle analysis

Promastigotes, $2x10^6$, were recovered from cultures in the required medium at defined time points, washed twice and resuspended in 1 ml of PBS 2% FCS. This was followed by the addition of 3 ml of cold absolute ethanol (Panreac) with continuous vortexing. Cells were fixed for at least 1 hour at 4ºC and then washed twice in PBS, 1200 g for 5 minutes. Before analysis, cells were resuspended in 50 µg/ml propidium iodide (Sigma) staining solution with 0.5 ng/ml RNase A (Sigma) and incubated 30 min at 4ºC. Data was collected in a BD FACScalibur cytometer (20.000 gated events) and analyzed by FlowJo software (Ashland).

Viability analysis

Promastigotes were recovered washed and resuspended at 10^5/ml in Annexin V binding buffer (BD Pharmingen). Parasites were then incubated at room temperature for 15 minutes with AnnexinV-Cy5 (BD Pharmingen) and 7-AAD (Sigma). Parasites subjected to ultra violet light during 30 minutes and kept in culture for 4 hours were used as a positive control. Data were collected in a BD FACScalibur cytometer (20.000 gated events) and analyzed by FlowJo software.

Ficoll density purification assay

Metacyclic promastigotes were purified after 3, 5 or 9 days of culture or from 5-day cultures with 4, and 21 in vitro passages by Ficoll density gradient, as previously described [11]. Briefly, 6 ml of 40% Ficoll was overlaid by 6 ml of 10% Ficoll in RPMI. Then, 6 ml of media containing 1.2x109 parasites was placed at the top of the Ficoll gradient. The step

gradient was centrifuged for 10 minutes at 370g at room temperature without brake. The metacyclics promastigotes were recovered from the layer between 0% and 10% Ficoll solution. Metacyclic promastigotes were identified by morphological criteria, i.e., short and slender with a long flagellum twice the body length using phase contrast on a Nikon Eclipse 80i microscope.

Real time RT-PCR

Total RNA was isolated from cells with the Trizol® reagent (Invitrogen, Barcelona, Spain), according to the manufacturer's instructions. Briefly, parasites were washed with ice-cold phosphate-buffered saline (PBS), harvested and homogenized in 800 µl of Trizol by pipetting vigourously. After addition of 160 µl of chloroform, the samples were vortexed, incubated for 2 min at room temperature and centrifuged at 12.000 g, for 15 min, at 4ºC. The aqueous phase containing RNA was transferred to a new tube and RNA precipitated with 400 µl of isopropanol for at least 10 min at room temperature. Following a 10 min centrifugation at 12.000 g, the pellet was washed with 1 ml of 75% ethanol and resuspended in 10 µl of 60ºC heated RNase free water. The RNA concentration was determined by using a Nanodrop spectrophotometer (Wilmington, DE, USA) and quality was inspected for absence of degradation or genomic DNA contamination, using the Experion RNA StdSens Chips in the ExperionTM automated microfluidic electrophoresis system (BioRad Hercules, CA, USA). RNA was stored at -80ºC until use. RT was performed with equal amounts of total extracted RNA (1 µg) obtained from parasites recovered from different experimental conditions by using Superscript II RT (Gibco BRL) and random primers (Stratagene). Real-Time quantitative PCR (qPCR) reactions were run in duplicate for each sample on a Bio-Rad My Cycler iQ5 (BioRad, Hercules, CA, USA). Primers sequences were obtained from Stabvida (Portugal) and thoroughly tested. qPCR was performed in a 20 µl volume containing 5 µl of complementary cDNA (50 ng), 10 µl of 2x Syber Green Supermix (BioRad, Hercules, CA, USA), 2 µl of each primer (250 nM) and 1 µl H2O PCR grade. Specific primers for histone H4 (forward: 5' ACACCGAGTATGCG -3'; reverse: 5'- TAGCCGTAGAGGATG-3'), Small Hydrophilic Endoplasmic Reticulum-associated Protein (SHERP) (forward: 5' CAATGCGCACAACAAGAT -3'; reverse: 5'- TACGAGCCGCCGCTTA-3') and rRNA45 (forward: 5'CCTACCATGCCGTGTCCTTCTA -3'; reverse: 5'- AACGACCCCTGCAGCAATAC -3') [12] were used for amplification. After amplification, a threshold was set for each gene and cycle threshold-values (Ct-values) were calculated for all samples. Gene expression changes were analyzed using the built-in iQ5 Optical system software v2.1 (Bio-Rad laboratories, Inc). The results were normalized using as reference gene the rRNA45 rRNA sequence [12].

Staining of parasite promastigotes with CFSE

Stationary-phase promastigotes from each culture medium at a concentration of $1.2*10^7$/ml were used for carboxyfluorescein succinimidyl ester (CFSE) labeling. Leishmania promastigotes were washed twice with PBS and labeled with 5µM of CFSE for 10 minutes at 37ºC with shaking for 3 minutes. Then, 9mL of complete DMEM was added and tubes centrifuged for 10 minutes at 1200g. The supernatant was discarded and the pellet was resuspended in 5mL of complete DMEM and incubated at 4ºC for 5 minutes. Finally, promastigotes were resuspended in complete DMEM before proceeding to macrophage infections.

In vitro macrophage infection

Cell suspension of bone marrow was obtained by flushing the femurs of susceptible Balb/c mice. The cell suspension was cultured in Dulbecco's modified Eagle's medium (DMEM) (Lonza, Switzerland), supplemented with 10 % heat-inactivated FBS (Lonza, Switzerland), 2 mM L-glutamine, 100 U/ml penistreptomycin and 1 mM sodium pyruvate. After overnight incubation at 37ºC, non-adherent cells were recovered (300g for 10 min, at room temperature) and cultured in 24-well culture dishes at 2x105cells/ml in supplemented DMEM. For macrophage differentiation 10% L-929 cell conditioned medium (LCCM) was added at days 0 and 4. At day 7 of culture, CFSE labeled promastigotes were incubated with the adherent bone-marrow derived macrophages (BMMø) at a 10:1 ratio. After four hours, infection was stopped and the infection rates were determined at 4, 24 and 48 hours post-infection by a BD FACScalibur cytometer and analyzed by FlowJo software.

Animal experiments and parasite quantification

Ten to twelve-week-old female Balb/c mice were obtained from Instituto de Biologia Molecular e Celular (IBMC; Porto, Portugal) animal facilities. Under laboratory conditions, the animals were maintained in sterile cabinets and allowed sterile food and water ad libitum. Animal care and procedures were in accordance with institutional guidelines. All experiments were approved by and conducted in accordance with the IBMC.INEB Animal Ethics Committee and the Portuguese Veterinary Director General guidelines. RS has an accreditation for animal research given from Portuguese Veterinary Direction (Ministerial Directive 1005/92). Promastigotes recovered from stationary culture with 4 in vitro passages stationary-culture were collected, washed and suspended in sterile PBS. A volume of 200 µl of PBS containing 108 parasites was injected

intraperitoneally. Mice of each group were sacrificed at 56 days post-infection. The parasite burden in the spleen and liver was determined by limiting dilution as previously described [13].

Statistical analysis

The data was analyzed using the non-parametric Kruskal-Wallis test followed by Dunn posttest for multiple comparisons when necessary.

Results:

Development of a FCS-free medium

The use of FCS as a supplement in most standard media, makes them unfit for studies involving the parasite exoproteome or basic metabolic pathways. Therefore, our strategy was to create a protein free medium that enabled the continuous growth of the parasites conserving basic biological characteristics and infectivity.

The first objective was to determine the importance of the 10% FCS complementation in two different media (SDM and RPMI). To achieve this we used ultracentrifugation devices with 3kDa membranes to separate the FCS into two distinct fractions: the retentate composed of protein associated components with a molecular weight higher than 3 kDa (>3 kDa) and the filtrate composed of components with less than 3 kDa (<3 kDa). These two distinct fractions were then used to complement the defined SDM and RPMI bases (bSDM and bRMPI). The growth of parasites in these media was evaluated after 4 days of culture (Fig 1 A-B). Parasite growth was determined after 3 passages in the same medium to enable adaptation to the media and evaluate the true capacity of sustaining culture. All growth capable situations enabled subsequent subpassages (data not shown). Without hemin complementation, bSDM and bRPMI, were not able to promote growth even when complemented with <3kDa. This lack of growth was shown to be partially reverted by the addition of hemin. The concentration of hemin was adjusted to 2,5 ug/ml, since lower amounts were growth limiting (data not shown). The bSDM when complemented with hemin presented 60% of normal growth while bRPMI did not grow significantly when complemented with hemin though the parasites were alive and presented normal morphology. The complementation of bSDM or bRPMI with >3kDa enabled total recovery of growth for both media (Fig 1A-B). The complementation of both bases with the >3kDa

enabled the homogeneous needle shaped morphology of stationary parasites indistinguishable from the standard media. The complementation with <3kDa in bSDM induced similar growth and morphological features similar to hemin complementation, while in bRPMI it induced a relative growth of 27% with cultures of stationary morphology (Fig 1B). The maintenance of parasite morphology similar to the one existing in both standard media is a good indication of the usefulness of the medium. Therefore we proceeded with the mixture of RMPI and SDM to obtain a compromise between yield and morphology. The combination that enabled the maintenance of the parasite morphology indistinguishable from the standard medium was obtained supplementing RPMI with 10% bSDM (Fig 1C). The medium developed was called cRPMI and enabled the continuous maintenance of *L. infantum* cultures with sequential passages for at least 6 months. Also the growth of other strains and species was achieved upon adjusting the RMPI/SDM ratio for optimal growth (data not shown). As one of the characteristics that we considered during medium development was the price, we also compared the cost of production of the media used (Sup table 1-3). The most expensive to produce was SDM with more than 60 €/L, while cRPMI was the cheapest with 29 €/L. When adjusted for parasite yield the most profitable was SDM with only 0,43 € for a mass of 10^9 parasites (Sup table 4).

Characterization of cRPMI parasites

The growth curve for the parasites grown in cRPMI was lower than in the original media. Furthermore the growth pattern resembled more the RPMI standard media than the SDM counterpart (Fig 2A). The cRPMI enabled a continuous and reproducible growth to a maximum of $\approx 1.2 \times 10^7$ parasites/ml with a doubling time of around 11h. Furthermore as much as 20 consecutive passages had no effect on the parasite growth (Sup Fig 1). Also the basic morphology of the parasite was similar when comparing cRPMI with standard RPMI or SDM (Fig 3). Culture viability for cRPMI was similar to the one obtained in RMPI enabling longer times of cultures for up to 9 days (Fig 4A). Cell cycle analysis showed that cRPMI growing parasites were mostly equivalent to RPMI with actively multiplying parasites only at day 1, as shown by more than 30% of the parasites in G1/S (Fig 4B-D). In standard SDM the percentage of G1/S parasites remains high for at least 2 days, and then at the third day decreases to a lower basal level. The capacity to generate metacyclic parasites and to differentiate into amastigotes was also evaluated. Using a Ficoll gradient for the enrichment of metacyclic parasites we recovered similar percentages of metacyclic in the three media (Between 4-6%). Also the expression of the metacyclic specific genes meta1 and SHERP when compared to Histone 4 revealed (Fig 5 A-B) an enrichment indicating the increase of expression of the metacyclic specific markers and the concomitant decrease in Histone 4 expression. The differentiation to amastigotes was

evaluated verifying the capacity of these parasites to differentiate into axenic amastigotes in MAA20. In fact no differences were detected in the capacity to differentiate using an equal starting inoculum. After day 3 all cultures had a similar number of parasites and all were capable of being sub cultured (Data not show). The in vitro virulence of the cRPMI parasites was tested in bone marrow derived macrophages. The cRPMI parasites where capable of infecting at similar levels to parasites grown in RPMI, while the SDM parasites presented higher capacity to infect with infections significantly higher than the other two media (Fig 6A). To evaluate the virulence loss associated with these media we performed infections on parasites with 20 passages in each medium and showed that the parasites that lose more infective capacity are the ones grown in SDM, while the ones in cRPMI maintain their infectivity (Fig 6B). The in vivo infections reflected the in vitro profiles with similar behavior for RPMI and cRPMI (Fig 6C-D).

Discussion:

The use of sandflies to grow Leishmania promastigotes although feasible is not a viable approach for routine promastigote growth [14]. In consequence, several different media were developed for routine growth of Leishmania making the use of axenically grown parasites an accepted model for the procyclic and stationary forms of the promastigote parasite [15,16]. The presence of FCS (or any other protein supplements) in most commonly used media prevented their use in studies that required the recovery and identification of parasite derived proteins from the culture media such as exoproteome studies. Therefore we developed an approach to create a medium using two established media for the growth of Leishmania [6]. Both RPMI and SDM79 are usually supplemented with 10% FCS, making them unfit for exoproteome and metabolic studies. In the absence of FCS both media bases were unable to promote parasite growth or survival (Fig 1). This was not unexpected as Leishmania spp. are heterotrophic for heme requiring an exogenous heme source [17], therefore the removal of the serum leads to the loss of hemoglobin as the main usable iron/heme source [18,19]. To overcome this limitation we used hemin as an iron/heme source [20,21]. In fact the supplementation of bRPMI and bSDM with hemin enabled the recovery of 61% of the growth in bSDM (Fig 1B) while in bRPMI the parasites were remaining viable (higher than 85%) although no significant growth was observed. To better understand the contribution of FCS to parasite growth we fractionated it into two distinct parts. The >3kDa fraction that included proteins and associated components was dialyzed twice with PBS to remove all low molecular weight

components. While the < 3kDa was composed mostly of low molecular weight components associated with FCS, like vitamins and other small molecules. The >3kDa fraction was able to sustain parasite growth in both media used (Fig 1). In fact it is known that bovine serum albumin, a major component of serum, can be added as a supplement to defined medium improving the doubling times of the cultures [6]. The <3kDa fraction, when supplemented with hemin enabled a marginal improvement in growth with the bRPMI but did not enable any difference in bSDM (Fig 1). This might be explained because the bSDM base is richer in nutrients than bRPMI (as shown by the higher parasite yield of SDM medium when compared to RMPI – Fig 2). In consequence, the nutrients supplied by the <3kDa were not able to supplement the growth in bSDM (Fig 1A). This lead to the conclusion that the <3kDa fraction was not required for growth in bSDM, either because the components present in this fraction are present in exceeding quantity in the bSDM composition or due to the presence of alternative nutrients in amounts that make the contribution of the <3kDa redundant. In bRPMI the <3kDa fraction was able to induce a marginal growth, 32% of normal growth (Fig 1B) because the bRPMI is more restricted in nutrients. Therefore some nutrients present in the <3kDa fraction where required for growth in this base and their availability in the <3kDa fraction limited the growth. Therefore the SDM should have all the basic nutrients required for growth because it does not require the <3kDa. Nevertheless growth was not the only requisite that was sought. The morphology of the parasites was considered an immediate quality control as parasites with awkward morphology are known to be stress induced therefore such a characteristic in the developed media would be unwanted [22]. The supplementation with >3kDa always enabled normal needle shape morphology (data not shown). In the bSDM, the supplementation with <3kDa or hemin lead to the multiplication of parasites with abnormal morphology (data not shown). In the bRPMI medium the complementation of hemin itself was enough to have the traditional needle shaped parasites, although with poor yields. Remarkably, it seemed to be medium specific elements that enabled acquisition and maintenance of the stationary form. Although interesting, the unraveling of the biology behind this specific difference was not within the scope of the reported work. From the complementation study, it was concluded that the bSDM contained the basic components required for parasite multiplication, and if they could be diluted in RPMI base we could improve the yield of the bRPMI hopefully maintaining the morphology of the parasites. Therefore we found that 10% SDM base supplementation of RPMI base enabled the growth of parasites that were morphologically indistinguishable from parasites grown in complete medium (Fig 1C). This mixture was adjustable to be strain and species specific. We were able to grow several different species/strains in this

media including the non-pathogenic *L. tarentolae* that had optimal growth with 20% SDM (data not shown).

The capacity to sustain growth per se was not the exclusive characteristic of cRPMI that was sought for. The parasites grown in this medium should have similar properties to their counterparts in the standard RPMI medium. The morphology of the parasites grown in these cRPMI was indistinguishable from the standard media (Fig 3). Furthermore, as a result of cRPMI being mostly composed of bRPMI we found that it retained characteristics similar to RMPI. It sustained a high viability that was maintained over time leading to a longer life span of the culture when compared to the SDM standard medium (Fig 4A). Also the cell cycle analysis was similar to RPMI with the log phase evident on the first day of culture, but then a very low percentage of parasites remained in G1/S (Fig 4B and D). This was distinct from the SDM culture that at day 2 still had a high percentage of multiplying parasites (Fig 4C). The existence of metacyclic parasites was also an important aspect that we looked to preserve in cRPMI, because these parasites are highly immunomodulatory (Diana et al, submitted) and shown to be the most infective forms of the promastigotes [4,5]. Using Ficoll gradient we recovered similar percentages of metacyclic in all media tested. As Ficoll recovery is poorly quantitative, we used the expression of metacyclic genes to confirm the existence of metacyclic parasites. Using SHERP, META1 and Histone 4 we concluded that the profile is once again similar to RPMI being higher at day 5. The capacity to differentiate into amastigotes is an essential step in the life cycle of the parasite, therefore we confirmed that cRPMI derived parasites were able to differentiate into axenic amastigotes and originate axenic forms that could be maintained in culture (Data not shown). The cost of a medium is ranked among the characteristics valued in a developed medium. Although the most expensive medium to produce was standard SDM the price adjusted to yield made the most cost efficient medium (Sup Table 4). The cRPMI had the least cost but the poor yield associated to it transformed it in the least cost efficient medium (Sup table 4). This is not a significant limitation for the use of cRPMI, it makes it less attractive for continuous growth of the parasites.

Although the biological characterization was important the defining characteristic that was looked for in the medium was the maintenance of parasite infectivity. In effect in vitro infectivity was once again similar to standard RPMI, enabling stable infections both in vivo and in vitro reinforcing the idea that this medium is quite similar to the standard RPMI medium (Fig 6A). Also in vitro short term loss of virulence was evaluated using parasites with twenty passages and in fact cRPMI was able to retain most of the virulence while SDM lost more than 50% of virulence over 48h (Fig 6B). This might be to the

recently described lost of capacity to differentiate into amastigotes reported (Diana et al). The loss of virulence is consistent with a lesser number of generations passed since recovery of the parasites from the mice, at least 80, 120 and 200 generations passed for cRPMI, standard RPMI and standard SDM, respectively. This is an interesting characteristic because as consequence of the lesser yield obtained when using cRPMI the parasites could be maintained for longer time in culture without visible significant loss of virulence. Ultimately the capacity to infect in vivo was the defining characteristic of cRPMI, because it enabled infection levels similar to the other media (Fig 6 C and D). Overall these findings clearly indicate that promastigotes grown in cRPMI are able to grow indefinitely upon subculture, have metacyclogenesis, transform into amastigote, and infect both in vivo and in vitro to the same extent as standard media supplemented with FCS.

Conclusion:

The medium developed, cRPMI, is capable of sustaining the growth of parasites that are indistinguishable from grown in conventional media and conserve the virulence associated to parasites grown in standard medium. The access to a serum-free medium for culturing promastigote forms could provide information leading to a better understanding of the nutritional and metabolic requirements of these organisms. It will also become a valuable tool for studying the exoproteome involving continuous growth of the parasite.

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Figures:

Figure 1 – Complementation studies with bSDM and bRPMI

1A - Growth of L. infantum promastigotes after four days of culture in bSDM complemented with : no supplement (NS); 10% of the lower fraction of the serum (<3 kDa); 10% of high molecular components of serum (>3kDa); 10% of high molecular components of serum with 2.5 μg/ml hemin (Hem; > 3 kDa); 2.5 μg/ml hemin (Hem) and 10% of lower molecular components of serum with 2.5 μg/ml hemin (Hem; <3 kDa). The number of parasites was normalized in relation to standard SDM. One representative experiment of five.

1B - Relative growth of L. infantum promastigotes after four days of culture in bRPMI complemented with: no supplement (NS); 10% of the lower fraction of the serum (<3 kDa); 10% of high molecular components of serum (>3kDa); 10% of high molecular components of serum with 2. 5 μg/ml hemin (Hem; > 3 kDa); 2.5 μg/ml hemin (Hem) and 10% of lower molecular components of serum with 2.5 μg/ml hemin (Hem; <3 kDa). The number of parasites was normalized in relation to standard RPMI. One representative experiment of three.

1C - Growth of L. infantum promastigotes after four days of culture in bRPMI complemented with 2.5 μg/ml of hemin and different percentages of bSDM. One representative experiment of five made.

Figure 2 – Growth curves of L. infantum in the different media

Promastigotes were cultured with an initial concentration of 1x10 6 /ml parasites in standard RPMI (RPMI) or cRMPÌ (cRPMI) and 2,5x105 in standard SDM (SDM). The growth curve was done by counting the parasites at different time points in a hemocytometer. One out of at least three independent experiments is shown.

Figure 3 – Dominant morphology of stationary L. infantum

Promastigotes were cultured with an initial concentration of 1x10 6 /ml parasites in standard RPMI or cRMPÌ and 2,5x105 in standard SDM . After 5 days the general aspect of the cultures was registered. Panel (A) SDM; panel B (RPMI); panel C (cRPMI).

Figure 4 – Viability and cell cycle analysis

A – Promastigotes viability, at the defined time points, as defined by Annexin V (Ann V) and 7-aminoactinomycin D (7-AAD) negative parasites growing in standard RPMI (RPMI), cRMPÌ (cRPMI) or standard SDM (SDM). The viability analysis was done using FACSCalibur cytometer and analyzed by FlowJo software. One out of at three independent experiments is shown.

B-D - Percentage of parasites in G1, G2 or S at the defined time points in standard RPMI (B), standard SDM (C) and cRPMI (D). The cell cycle analysis was done using FACSCalibur cytometer and analyzed by FlowJo software. One out of at least three

Figure 5 - Indirect quantification of metacyclic promastigotes in the different media.

Promastigote relative gene expression of SHERP and META1 at the defined time points, as determined qPCR, when compared to histone h4. Basic normalizations for the three genes were made using the reference gene rRNA45. Three independent experiments were executed, each performed in duplicate; one representative experiment is shown.

Figure 6 – In vitro and in vivo virulence of promastigote growing in cRPMI

BMMø were infected at a 1:10 (cell/parasite) ratio with non-purified promastigotes submitted to 4 (A) or 21 (B) successive in vitro passages in cRPMI, standard RPMI and standard SDM. Data were acquired by FACScalibur cytometer and analyzed by FlowJo software. The mean and standard deviation are shown. Three independent experiments were performed; one representative experiment is shown. Balb/c mice were infected with stationary phase promastigotes submitted to 4 successive in vitro passages in cRPMI, standard RPMI and standard SDM. After 6 weeks post-infection, the parasite load was determined in liver (D) and spleen (C) by limiting dilution. The mean and standard deviation are shown. Two independent experiments were performed; one representative experiment is shown.*$P < 0,05$; **$P < 0,01$; ***$P < 0,001$

Supplemental data:

Supplemental figure 1 - Growth curves of L. infantum in cRPMI after 20 passages.

Promastigotes with 4 or 20 passages were cultured with an initial concentration of 1x10 6 /ml parasites in standard cRPMI (RPMI). The growth curve was done by counting the parasites at different time points in a hemocytometer. One out of at least three independent experiments is shown.

Supplemental table 1 – Composition and cost of standard SDM

Supplemental table 2 – Composition and cost of standard RPMI

Supplemental table 3 – Composition and cost of cRPMI

Supplemental table 4 – Comparative cost of the media used in the study

Figure 1

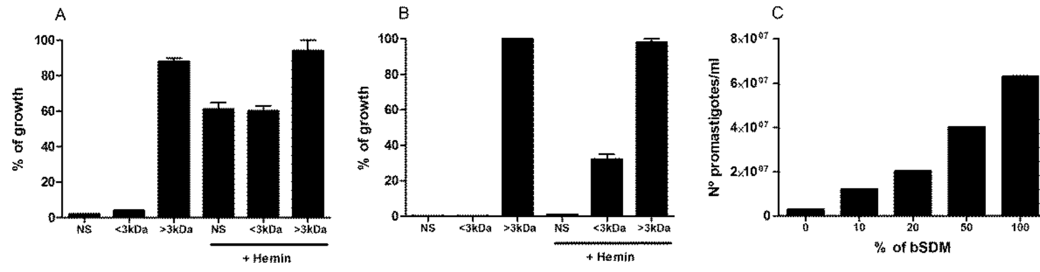


Figure 2

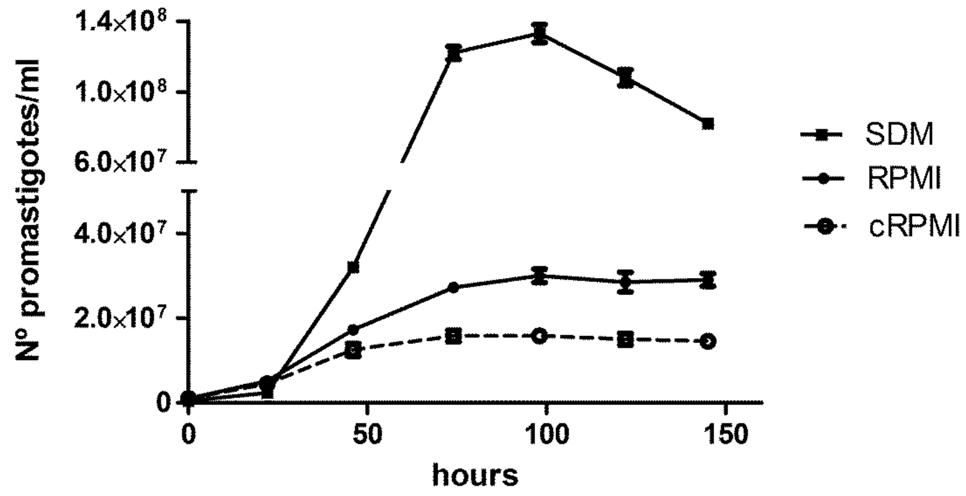


Figure 3

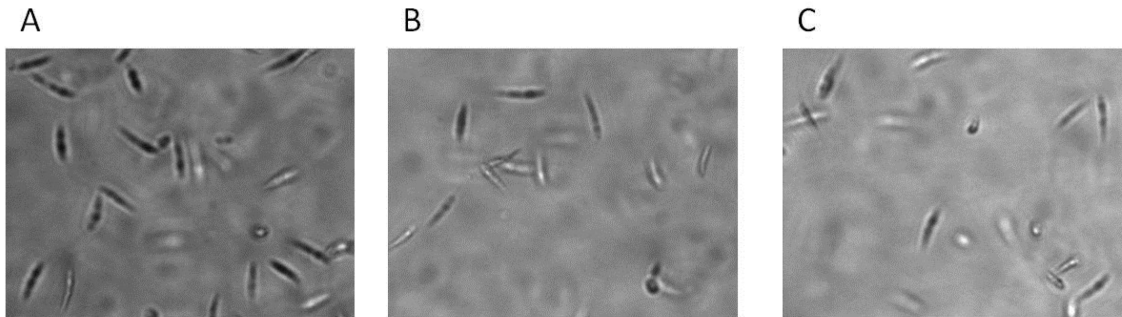


Figure 4

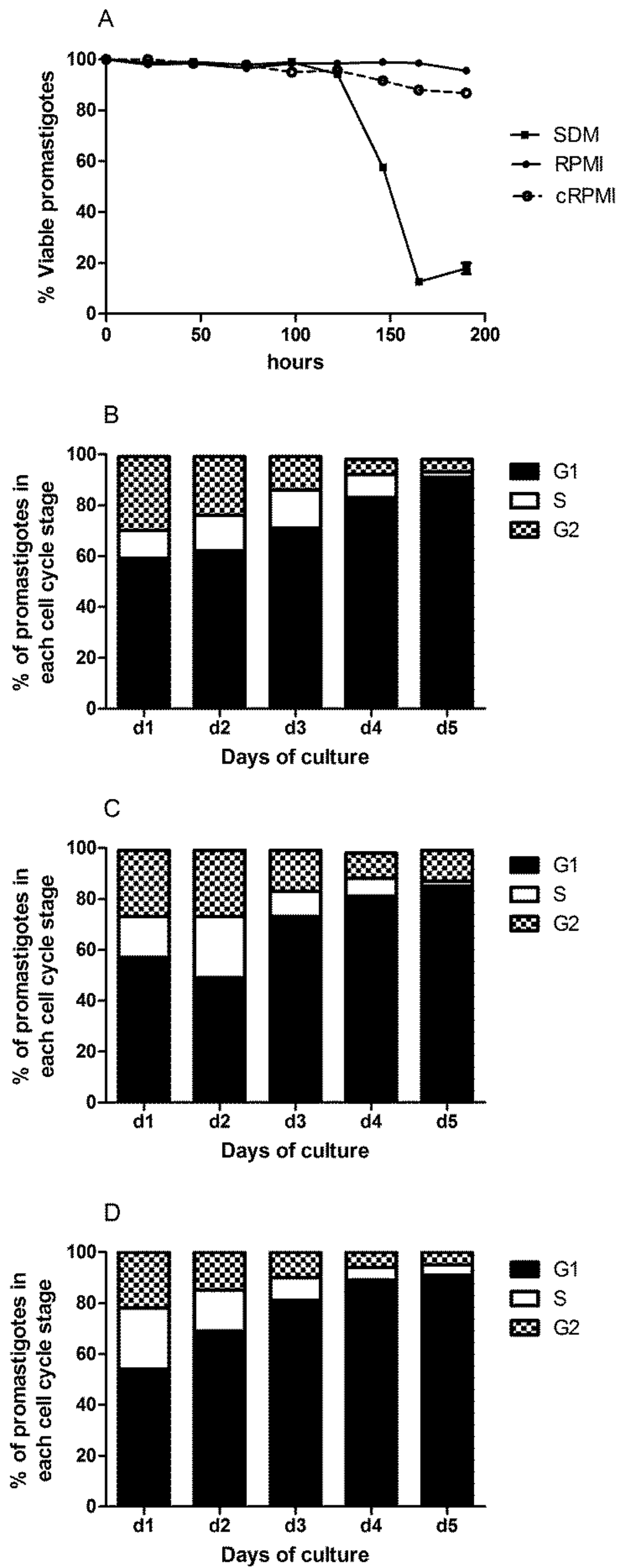


Figure 5

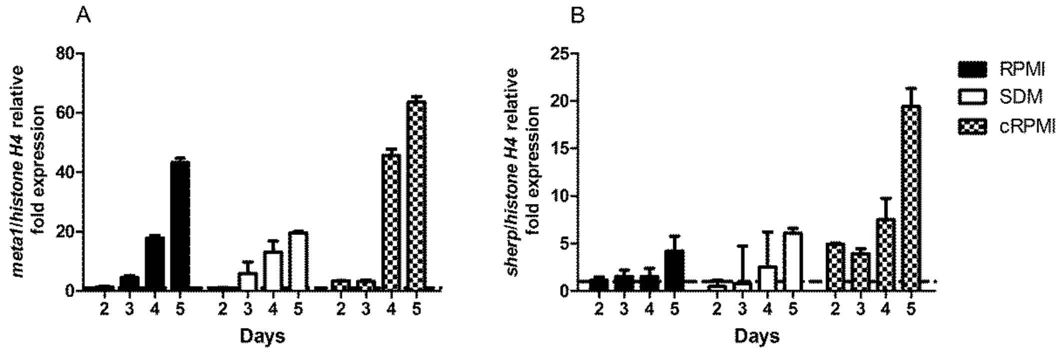
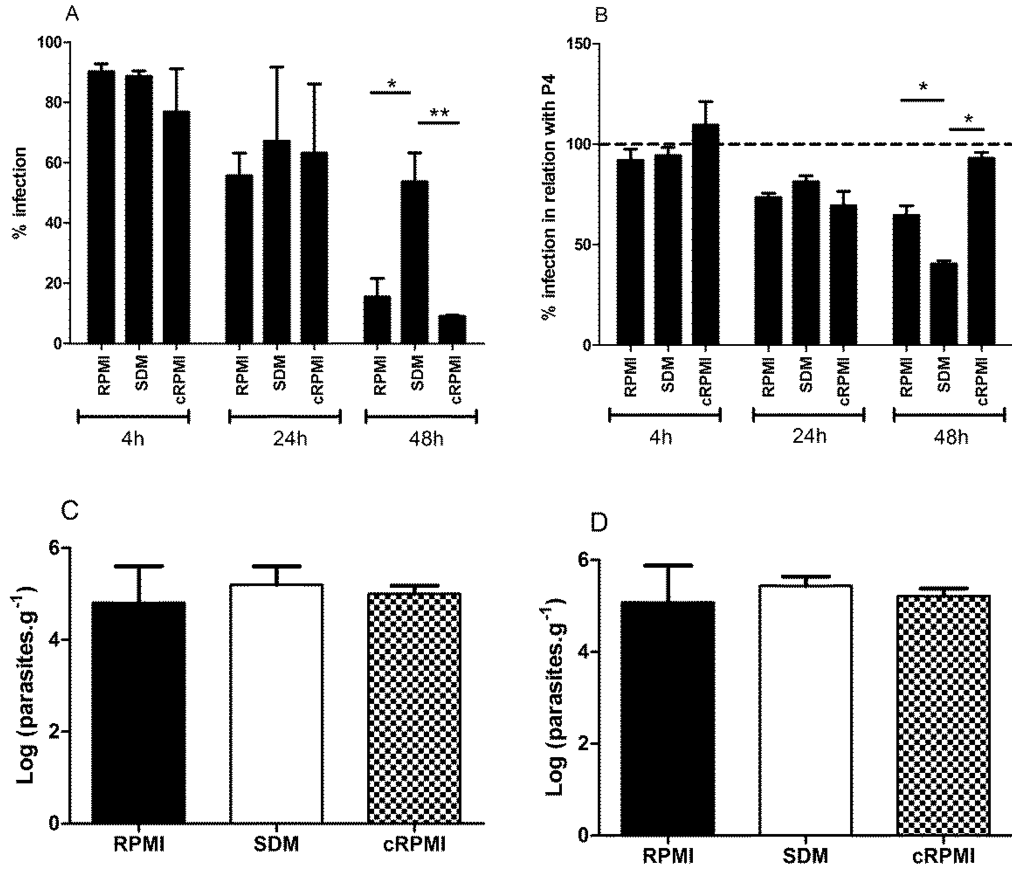
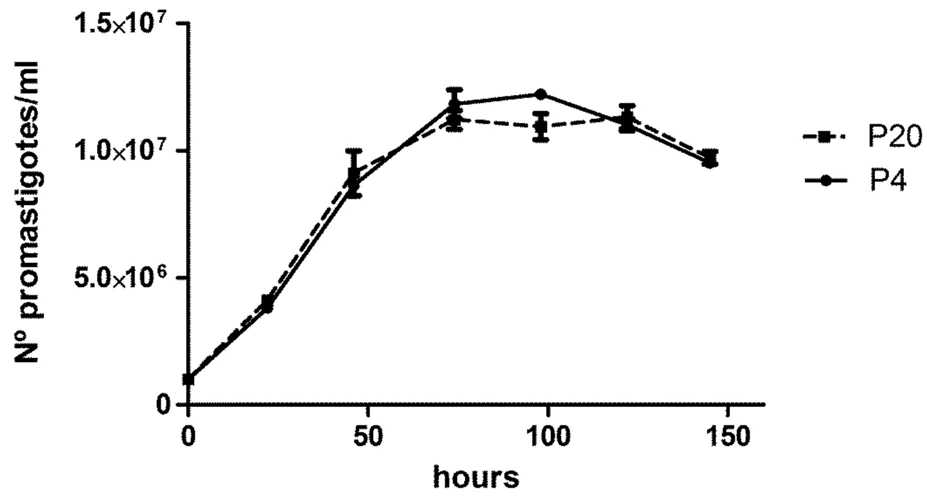


Figure 6



Supplementary figure 1



Supplementary table 1

	Price		Quantity/L		Cost (€)
Dulbecco's modified eagle medium powder	0,618	€/g	7	g	4,326
Medium 199	0,746849	€/g	2	g	1,493697
MEM amino acid	0,215	€/ml	8	ml	1,72
MEM nos ess. Amino acids	0,103	€/ml	6	ml	0,618
Glucose	0,189	€/g	1	g	0,189
Hepes	0,674	€/g	8	g	5,392
Mops	0,797	€/g	5	g	3,985
NaHCO3	0,0264	€/g	2	g	0,0528
L-Alanine	8,4	€/g	0,2	g	1,68
L-arginine	1,02	€/g	0,1	g	0,102
L-Methionine	1,84	€/g	0,07	g	0,1288
L-Phenylalanine	0,903	€/g	0,08	g	0,07224
L-Proline	0,884	€/g	0,6	g	0,5304
L-Serine	10,1	€/g	0,06	g	0,606
Taurine	1,09	€/g	0,16	g	0,1744
L-Threonine	11,5	€/g	0,351	g	4,0365
L-Tyrosine	0,518	€/g	0,1	g	0,0518
Adenosine	5,04	€/g	0,01	g	0,0504
Guanosine	33,9	€/g	0,01	g	0,339
D(+) Glucosamine	1,316	€/g	0,05	g	0,0658
Folic Acid	10,4	€/g	0,004	g	0,0416
p-Aminobenzoic acid	18,6	€/g	0,002	g	0,0372
d-biotin	152	€/g	0,0002	g	0,0304
Kanamycine	27	€/g	0,1	g	2,7
Ampicilie	6,04	€/g	0,05	g	0,302
NaCl	0,0616	€/g	0,05	g	0,00308
KH2PO4	0,168	€/g	0,012	g	0,002016
FCS	0,1993	€/ml	100	ml	19,93
Hemin	30,8	€/g	0,005	g	0,154
Stericup filtering device	11,38	€/unit	1	unit	11,38
			Total Cost		**60,19413**

Supplementary table 2

	Price		Quantity/L		Cost (€)
Glutamin	0,075	€/ml	5	ml	0,375
Hepes	0,16	€/ml	10	ml	1,6
Pen Strept	0,115	€/ml	5	ml	0,575
RPMI	0,015	€/ml	800	ml	12
FCS	0,1993	€/ml	100	ml	19,93
			Total Cost		**34,48**

Supplementary table 3

	Price		Quantity/L		Cost (€)
Dulbecco's modified eagle medium powder	0,618	€/g	7	g	0,7
Medium 199	0,746849	€/g	2	g	0,2
MEM amino acid	0,215	€/ml	8	ml	0,8
MEM nos ess. Amino acids	0,103	€/ml	6	ml	0,6
Glucose	0,189	€/g	1	g	0,1
Hepes	0,674	€/g	8	g	0,8
Mops	0,797	€/g	5	g	0,5
NaHCO3	0,0264	€/g	2	g	0,2
L-Alanine	8,4	€/g	0,2	g	0,02
L-arginine	1,02	€/g	0,1	g	0,01
L-Methionine	1,84	€/g	0,07	g	0,007
L-Phenylalanine	0,903	€/g	0,08	g	0,008
L-Proline	0,884	€/g	0,6	g	0,06
L-Serine	10,1	€/g	0,06	g	0,006
Taurine	1,09	€/g	0,16	g	0,016
L-Threonine	11,5	€/g	0,351	g	0,0351
L-Tyrosine	0,518	€/g	0,1	g	0,01
Adenosine	5,04	€/g	0,01	g	0,001
Guanosine	33,9	€/g	0,01	g	0,001
D(+) Glucosamine	1,316	€/g	0,05	g	0,005
Folic Acid	10,4	€/g	0,004	g	0,0004
p-Aminobenzoic acid	18,6	€/g	0,002	g	0,0002
d-biotin	152	€/g	0,0002	g	0,00002
Kanamycine	27	€/g	0,1	g	0,01
Ampicilie	6,04	€/g	0,05	g	0,005
NaCl	0,0616	€/g	0,05	g	0,005
KH2PO4	0,168	€/g	0,012	g	0,0012
Hemin	30,8	€/g	0,005	g	0,154
Glutamin	0,075	€/g	5	g	0,375
Sodium piruvate	0,075	€/g	5	g	0,375
Hepes	0,16	€/g	10	g	1,6
Pen Strept	0,115	€/g	5	g	0,575
RPMI	0,015	€/ml	800	ml	10,8
Stericup filtering device	11,38	€/unit	1	unit	11,38
			Total Cost		**29,4**

Supplementary table 4

	Cost/L (€)	Cost (€)/ 10^9 parasites
RPMI	34,5	1,41
SDM	60,2	0,43
cRPMI	29,4	2,44

4. Article name: Exoproteome dynamics in *L. infantum*

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In the present study we compared a continuous approach for exoproteome recovery with a discontinuous approach and used the continuous approach to compare the exoproteome components from stationary and logarithmic parasites.

Main Results:

-The continuous approach yielded a GP63-rich exoproteome that is distinct from the one obtained using a discontinuous approach.

-The recovered exoproteome is composed of vesicles and free proteins. Only 8% of the proteins in the exoproteome have a detectable secretion signal.

-The logarithmic parasites exoproteome is significantly enriched in proteins related to metabolism and gene information processing, hinting at an uncharacterized biological process of protein turnover associated to vesicle release.

-The stationary parasites exoproteome vesicle preparations contain proteins traditionally associated to apoptosis like histones or surface proteins.

-Vesicle release is a phenotype of parasite death.

Conclusions:

The exoproteome of *L. infantum* obtained from continuous cultivation is dominated by GP63. The composition of the exoproteome is stage-specific and dynamic hinting at specific phenomena like protein turnover and apoptosis.

Exoproteome dynamics in *L. infantum*

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Abstract

The extracellular proteins secreted/released by the protozoan parasite *Leishmania*, known as the exoproteome, are expected to be key mediators of the host-parasite interaction. To understand how the exoproteome changes during the promastigote life stage, we developed an approach enabling the recovery of the exoproteome of logarithmic and stationary parasites from *Leishmania infantum*. Significantly the exoproteome recovered from stationary phase parasites using continuous growth had GP63 as the most abundant protein and with the exception of 8% of the proteins it was mostly composed of proteins without any predicted conventional secretion signal. The exoproteome was separated into two fractions, vesicles and vesicl-depleted exoproteome. The composition of these fractions was distinct and dynamic during the transition from log phase parasites to stationary ones, reflecting the metabolic state of the parasites. The vesicle-derived exoproteome recovered from logarithmic parasites was enriched in ribosomal proteins, indicating a possible role for these vesicles in protein turnover. In stationary parasites there was an enrichment of GP63 both within and outside vesicles. A stage-specific enrichment of vesicles with properties related to apoptotic vesicles was noted in stationary phase parasites and evidence was obtained that the release of these vesicles was increased in response to a death stimuli. This study reports a new approach to explore the exoproteome and is providing new perspectives on *Leishmania* biology with the possibility of vesicles playing a major role in protein turnover and also in cell death.

Introduction

Digenetic protozoan parasites of the genus *Leishmania* are the causative agents of a group of tropical and subtropical infectious diseases collectively known as leishmaniasis [1]. The disease ranges from self-healing wounds, to a potentially fatal systemic disease known as visceral leishmaniasis. With 350 million people at risk, 2 million new cases each year and a yearly death toll of 70,000 people, human leishmaniasis is classified by the World Health Organization as an emerging and uncontrolled neglected topical disease [1]. The absence of vaccines, growing concerns related to HIV co-infection and the expansion of disease endemic areas, translates in a pressing need for a better understanding of the parasite biology to enable new control strategies for leishmaniasis [2,3]. *Leishmania* has a dimorphic life cycle existing either as the highly motile flagellated promastigotes or the non-motile amastigote form. The parasite life cycle starts when a female sand fly takes blood meal from an infected mammalian host. Ingested parasites differentiate into promastigotes, proliferate in the midgut of susceptible sandflies and eventually transform into infectious metacyclic promastigotes in a complex process that encompass migration towards the upper gut of the sandfly [4]. These metacyclic parasites are transferred to a mammalian host during subsequent blood meals. In a susceptible mammalian host, promastigotes are internalized by phagocytic cells, eventually transforming into amastigotes that proliferate in the normally inhospitable environment of the macrophage lysosome [5]. Remarkably, *Leishmania* and bacterium from the genus *Coxiella* are the only known organisms capable of thriving in mature phagolysosomes [6]. Much of the success of the infection is decided in the first moments of contact with the host. *Leishmania* is known to modulate the host immune response allowing the parasite to proliferate in the macrophage phagolysosome [7]. Most of the direct interactions with the host are expected to happen at the cell-parasite interface. In this process the parasite released proteins should play an active role in host-parasite interactions [8,9]. The collection of the extracellular proteins released from an organism under defined conditions is known as the exoproteome [9]. This includes the proteins released from the surface of the parasite or originated from cell lysis and also the secreted proteins actively released from the cell using either a classical or a nonclassical mechanism of secretion.

Surface associated molecules and few secreted proteins were considered to make up the bulk of *Leishmania* exoproteome [10]. In recent years the exoproteome of *Leishmania* and related trypanosomatids was studied resulting in a list of proteins that are found in the exoproteome from several species [11,12,13,14,15,16]. These studies demonstrated that the *Leishmania* exoproteome contains proteins with distinct cellular origins, most lacking a clear amino-terminal secretion signal [15]. Pathways such as exosomes release,

membrane translocation, secretory lysosomes and vesicle blebbing could explain the presence of proteins lacking signal peptides in the *Leishmania* exoproteome. Consistent with this proposal, recent proteomic approaches described the release of exosome like vesicles from the *Leishmania* surface [12]. The above mentioned exoproteome studies traditionally consisted in short term cultivation of the parasite in a serum free medium enabling the survival of the organism for enough time to recover the exoproteome, minimizing contamination derived from dead parasites or proteolytic activities. Most of the *Leishmania* exoproteome studies thus reflected short term response of the parasite to a defined environmental challenge such as temperature and pH [11,17], or medium change prior to exoproteome recovery. The use of a protein free medium sustaining the growth of the parasites should enable the study of the exoproteome dynamic during development of the promastigote stage.

The promastigote exoproteome of *L. infantum* was separated into free exoproteome proteins and vesicular components and this was done using two different approaches. The first approach involved a change in media prior to exoproteome recovery as done by most other investigators, while the second approach involved the continuous growth of parasites and recovery of the exoproteome. A GP63 dominated exoproteome was obtained from continuous growth that was distinct both in composition and relative abundance of proteins when compared to the exoproteome recovered upon media change. The continuous approach was then used to compare the exoproteomes from stationary and logarithmic parasites. These exoproteomes were shown to be enriched in specific set of proteins that were dependent on the promastigote stage of development. The exoproteome recovered from logarithmic and stationary parasites was found to be dynamic and associated with several specific biological processes.

Materials and methods

Parasites and cell culture

A cloned line of virulent *L. infantum* (MHOM/MA/67/ITMAP-263) freshly recovered from Balb/c mice was used for a total of ten passages after recovery. A miltefosine resistant strain (Ldi Mf 200.5) derived from the above parent strain was available [18]. Promastigote cells were maintained in RPMI 1640 medium (Lonza) supplemented with 10% Fetal Calf Serum (FCS) (Lonza) 2mM L-glutamine (Lonza), 100 U/ml penicillin (Lonza), 100 mg/ml streptomycin (Lonza) and 20 mM HEPES buffer (Lonza) (standard RPMI). Parasites used for exoproteome studies were maintained in cRPMI (RPMI base

complemented with 10% SDM-79 base [19] and 2,5 μg/ml of hemin), a media that we developed for exoproteome studies. All cultures were grown with a starting inoculum of $1x10^6$ parasites.

Growth curves

Promastigotes in cRPMI growing from a startup culture with an inoculum of $1x10^6$ were counted using a Neubauer chamber at defined time points .

Cell cycle analysis

At defined time points $2x10^6$ promastigotes were recovered from cultures, washed twice and resuspended in 1 ml of PBS 2% FCS. This was followed by the addition of 3 ml of cold absolute ethanol (Panreac) with continuous vortexing. Cells were fixed for at least 1 hour at 4ºC and then washed twice in PBS, centrifuged at 1200 g for 5 minutes. Before analysis, cells were resuspended in 50 μg/ml propidium iodide (Sigma) staining solution with 0.5 ng/ml RNase A (Sigma) and incubated 30 min at 4ºC. Data was collected in a BD FACScalibur cytometer (20.000 gated events) and analyzed by FlowJo software (Ashland).

Viability analysis

Promastigotes at a density of 10^5/ml were washed and suspended in Annexin V binding buffer (BD Pharmingen). Parasites were then incubated at room temperature for 15 minutes with AnnexinV-Cy5 (BD Pharmingen) and 7-AAD (Sigma). Parasites subjected to ultra violet light during 30 minutes and kept in culture for 4 hours were used as a positive control. Data were collected in a BD FACScalibur cytometer (20.000 gated events) and analyzed by FlowJo software.

Exoproteome preparation

Logarithmic promastigotes with a startup inoculum of $1x10^6$ in cRPMI were grown and the culture medium was recovered at 24h or 96h; the parasites were then removed by centrifugation followed by filtration through a 0.2 μm filter. Protease inhibitor phenylmethylsulfonyl fluoride (Sigma) was added to the spent medium at a concentration of 1 mM and then the exoproteome was centrifuged at 10000g for 10 minutes at 4ºC to remove cellular debris, and further centrifuged at 100000g for 3 hours. The vesicle depleted exoproteome (VDE) was recovered without disturbing the vesicle pellet, and then the final 1 ml of residual volume was decanted and discarded leaving only the pellet that was resuspended in 500 μl of PBS. The vesicles were stored at either 4ºC or -90ºC depending on further intended use. The recovered VDE was concentrated to a residual volume of 500 μl using centriprep Ultracel YM-3 filtering units. The samples were

dialyzed twice against PBS using the same YM-3 filtering devices. After the second dialysis step the VDE was again concentrated to a residual volume of 500 µl and stored at -90ºC. To evaluate the effect of change of media in the exoproteome the parasites were grown in standard RPMI for 3 days and then passed to cRPMI for 24 hours at parasite density of $1x10^7$/ml and the spent medium was then processed as described above for exoproteome recovery.

Electron microscopy

Vesicles were placed onto formvar/carbon coated copper grids and stained with aqueous solution of 3% uranyl acetate for 10 minutes. Samples were viewed in a Jeol 1010 electron microscope.

In-gel protein digestion.

Gel plugs containing proteins of interest were either excised using a ProXcision robot (PerkinElmer) or cut manually from the gel with a scalpel using the guidance of the ProXcision robot and then identified by tandem mass spectrometry (Proteomics Platform of the Eastern Quebec Genomics Center). In-gel protein digestion was performed on a MassPrep liquid handling station (Waters) according to the manufacturer's specifications. Briefly, proteins were reduced with 10 mM DTT and alkylated with 55 mM iodoacetamide. Trypsin digestion was performed using 105 mM of modified porcine trypsin (Sequencing grade, Promega) at 58 °C for 1 h. Digestion products were extracted using 1% formic acid, 2% acetonitrile followed by 1% formic acid, 50% acetonitrile. Peptides were lyophilized in a speed vacuum and resuspended in 8 µl of 0.1% formic acid and 4 µl of this was used for mass spectrometry analysis.

Mass spectrometry

Peptide MS/MS spectra were obtained by capillary liquid chromatography coupled to an LTQ linear ion trap mass spectrometer equipped with a nanoelectrospray ion source (Thermo Electron). Peptides were loaded onto a reversed-phase column (PicoFrit 15-µm tip, BioBasic C18, 10 cm ×75 µm; New Objective) and eluted with a linear gradient from 2% to 50% acetonitrile in 0.1% formic acid at a flow rate of 200 nl/min. Mass spectra were acquired using a data-dependent acquisition mode (Xcalibur software, version 2.0) in which each full scan mass spectrum was followed by collision-induced dissociation of the seven most intense ions. The dynamic exclusion function was enabled (30 s exclusion), and the relative collisional fragmentation energy was set to 35%.

Interpretation of tandem mass spectra and protein

MS/MS spectra were analyzed using MASCOT (Matrix Science, London, U.K.; version 2.2.0) and searched against *Leishmania* in the GeneDB Leishpep database assuming a digestion with trypsin. A mass tolerance of 2.0 Da for peptides and 0.5 Da for fragments were tolerated, with 2 trypsin miss cleavages allowed. Carbamidomethylation of cysteine and partial oxidation of methionine were considered in the search. The Scaffold software (Proteome Software, Inc. version 3_0_05) was used to validate MS/MS-based peptide and protein identifications. Peptide identifications were accepted if they reached greater than 95% probability as specified by the Peptide Prophet algorithm [20]. Protein identifications were accepted if they reached greater than 90% probability and contained at least one unique peptide as specified by the Protein Prophet algorithm [21].

Functional classification and bioinformatics analysis

The classification was based on the gene ontology (GO) annotation for each gene on KEGG [22]. Relevant protein associated information like SignalP or transmembrane prediction was obtained from the annotation in TriTrypDB [23]. Other predictions were performed using WoLF PSORT [24] or SecretomeP [25].

GFP construction with LinJ26.2710 found in the exoproteome

The hypothetical gene LinJ26.2710 was amplified without the stop codon and with a 3' XbaI restriction site and 3' overlapping region to 5' GFP using primers 5'-TCTAGAATGGTGAGCAAGGGCGAGGA-3' and 5'-CACCACCCCGGTGAACAGCTCCTCGCCCTTGCTCACCATGAATACAGGTAGATAGATG-3', GFP was amplified with a 5' NdeI restriction site using 5'ATGGTGAGCAAGGGCGAGGA-3'and 5'CATATGTTACTTGTACAGCTCGTC CATGCCG- 3' both fragments were fused by PCR. The above mentioned PCR fusions were cloned in pGEM T-easy vector (Invitrogen). The resulting constructs were sequenced, digested with XbaI and NdeI and inserted into the *Leishmania* expression vector pSP72αNEOα [26] for overexpression studies. These constructs were used to transfect promastigotes as previously described [27].

Western blot analysis

Leishmania proteins, vesicles and VDE were run on 10% polyacrylamide gels and transferred onto nitrocellulose membranes as described previously [28]. The blots were blocked overnight in 5% skimmed milk in PBS. A polyclonal antibody against the GFP (MBL International) was diluted 1:1000 in PBS containing 0.1% Tween 20 (PBS/Tween) and incubated for 1 h with the membranes. The blots were washed three times for 5 min in PBS/Tween and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (antibodies-online, Aachen, Germany) for GFP diluted 1:10.000 in PBS/Tween. The blots

were washed as above, incubated with ECL Plus chemiluminescent substrate (Amersham Biosciences), and exposed using the Chemidoc XRS system (Biorad).

Fluorescence microscopy

L. infantum promastigotes episomally expressing GFP fusions with either serine hydroxymethyltransferase (SHMT) [29], hypothetical protein 26 (LinJ26.2710) or monomeric GFP [30] were recovered from cRPMI medium labeled with 1 µg/ml Hoechst dye 33342 (Sigma) during 1 hour in the dark at 27ºC. The parasites were mounted on a glass slide with 1% low melting agarose and covered with a cover slip. Fluorescence images were acquired on an AxioImager Z1 microscope (Carl Zeiss, Germany) using an Axiocam MR ver.3.0 (Carl Zeiss, Germany), and projected using ImageJ 1.3v software.

Induction of vesicle formation by parasite death assay

A starting inoculum of $1x10^7$ *L. infantum* wild-type or Ldi Mf 200.5 were treated with 10 µM of miltefosine or 4 µg/ml of G418 for 48 hours. Parasite viability was determined and vesicle recovery was performed as described above.

Statistical analysis

The data was analyzed with the Fisher exact test using GraphPad5 software.

Results

Growth properties in cRPMI

L. infantum grew to a maximum of $1,2x10^7$ parasites/ml in the serum free cRPMI medium (Fig 1A). Cell cycle analysis indicated that within 1 day of culture around 50% of the parasites were at S/G2 phase while on the fourth day only 6% of the parasites had an S/G2 profile (Fig 1A). Parasites grown in cRPMI for 24 hours and 96 hours were used for analysis of the exoproteome of logarithmic and stationary parasites respectively. At these time points parasite viability was higher than 94% (Fig 1A) and parasites had the morphology expected for procyclic and stationary parasites (Fig 1B) being

indistinguishable from parasites grown in standard serum supplemented RPMI medium (not shown).

Exoproteome preparation

The exoproteome is known to be composed of two distinct fractions: shed/secreted proteins and vesicles derived components [9]. Ultracentrifugation was used to maximize vesicle recovery from culture spent medium of both logarithmic (VL) and stationary cultures (VS) (Fig 2A). Electron microscopy was used to confirm the presence of vesicles in VL and VS (Fig 2B). The remaining vesicle depleted supernatant was concentrated to obtain the vesicle depleted exoproteome of logarithmic (VDEL) and stationary (VDES) parasites (Fig 2A). No vesicles were observed in VDEL and VDES (not shown). The SDS-PAGE protein patterns for the recovered fractions were different between vesicles and VDE, and also between stationary and logarithmic preparations (Fig 2C). The reproducibility of the exoproteome preparations was also confirmed with independent preparations (Fig 2C).

Traditionally, in exoproteome studies, parasites growing in standard media complemented with FCS are incubated for short time periods in serum free medium (discontinuous approach) [11,15,16]. In a similar discontinuous approach parasites were grown in standard RPMI based medium for 3 days and then passaged to cRPMI for 24 hours and then the exoproteome was recovered (Vesicles and VDE). This exoproteome was compared to the exoproteome of parasites growing continuously in cRPMI for 4 days. The 24 hour discontinuous approach enabled the detection of 215 proteins while 154 proteins were detected in the exoproteome of parasites grown continuously in cRPMI (Table S1). The relative abundance of the twenty most abundant proteins found either in vesicles or in vesicle depleted exoproteome samples prepared from cells either grown continuously in cRPMI or grown in a discontinuous approach are depicted in Figure 3. The most striking discrepancy is GP63 which is much more abundant in the continuous exoproteome in comparison to the discontinuous approach (Fig 3). The structural proteins alpha and beta tubulin (LinJ13.0330 and LinJ.1280) and the secreted protein histidine secretory acid phosphatase (LinJ36.6770) were over represented in the discontinuous approach (Fig 3). Only 36% (75/211) and 9% (14/159) of the protein identifications, in either vesicles or VDE respectively, were common to both approaches (Table S1).

Analysis of the exoproteome of *L. infantum* promastigotes

The samples derived from VS, VL, VDES and VDEL were loaded on gel normalized by parasite number and the proteins were identified as described in materials and methods.

The combined analyses provided a total of 494 protein identifications (Sup. Tables 1-2), distributed in the four fractions (Fig 4). VL had 108 detected proteins, 135 for VS, 141 for VDEL and 110 for VDES. Removing the redundant proteins detected in more than one fraction, the number of proteins in the comparative exoproteome study was 297. Out of these, 167 proteins (56% of the identified proteins) were only detected in one of the 4 study groups. The least unique group was VDES with only 19 unique proteins that accounted for 18% of the total VDES sample (19/110). We also found that 63, 47 and 38 proteins were uniquely identified in VDEL, VS, and VL respectively (Fig 4). The remaining 130 proteins were found in more than one fraction but only 17 were found in all fractions (Fig 4). The presence of common proteins in different fractions might be originating from cell lysis. Therefore to determine the most abundant intracellular proteins and verify the possibility of contamination from intracellular origin in the detected exoproteome, we performed MS/MS on the collected parasites during exoproteome recovery. We identified 285 proteins acting as a reference for the most prominent contaminants from intracellular origin. Among the exoproteome proteins 48% (142 identifications) were not among the 285 proteins identified in our control proteome (Table S1).

Proteins that were present in VDE and absent in vesicle fraction must have been enriched in the spent medium by vesicle independent pathways. To confirm vesicle independent enrichment we selected the hypothetical protein LinJ.26.2710 as a candidate for a vesicle independent secreted protein. This protein was only detected in the VDE and was predicted to be secreted by signalP and WoLF PSORT (Table S3). We created a GFP fusion of this hypothetical protein (MW, 68 KDa) and found it in the VDE but not in either the vesicle fraction or in intracellular parasites (Fig 5). Both the ubiquitous GFP monomer (27 kDa) and the cytosolic protein SHMT fused to GFP (78 kDa) [28] were found in both fractions as well as inside parasites. To verify if there was enrichment in secreted proteins in the VDE fraction, we used web based programs predicting protein secretion in eukaryotes (Table S2-3). Only 15% of the exoproteome (44 proteins) was predicted to have a secretion signal, as determined by SignalP and this value was reduced to 7% if proteins with transmembrane domains were removed. The cellular localization tool WoLF PSORT also only predicted that 7% of the exoproteome proteins (22 proteins) had extracellular localization. We also used SecretomeP to screen the exoproteome data for proteins secreted unconventionally and found 43% of the detected exoproteome predicted to be potentially unconventionally secreted (Table S2-3).

Exoproteome gene ontology and cellular localization analysis

A gene ontology (GO) analysis using the KEGG Ontology system [22] was performed on the exoproteome. Three different levels of analysis were done: general biological definition, specific biological process and defined biological function (Table 1 and Sup. Tables 4-6).

We first compared the GO annotation related to general biological definition between vesicles and VDE from either logarithmic or stationary parasites. Distinct profiles were observed between vesicles and VDE (Table 1). The best example is metabolism related gene ontology that is significantly overrepresented in VDEL and significantly underrepresented in VL (Table 1). Other examples of this inverse correlation are translation and ribosome related GO. Also we find different sets of proteins enriched between logarithmic and stationary parasites, as an example Gene Information Processing (GIP) is overrepresented in VL but not in VS, the reverse can be seen for proteins involved in oxidative phosphorylation (Table 1). There was a significant enrichment of presumed cytosolic proteins in VDEL and of exoproteome components in VS (Table 2). In contrast, there was a significant underrepresentation of nuclear proteins in VDEL (Sup table 7).

As result of the continuous approach for the exoproteome recovery the fractions from stationary parasites (VS and VDES) may also include the proteins that were released during growth in the logarithmic stage. To study individually the proteins that were enriched in the stationary phase we evaluated the unique proteins found in the stationary stage (VSU and VDESU). Also the VLU and VDELU proteins will be representative of proteins that were uniquely identified in the logarithmic stage. From 167 proteins unique to only one fraction 47 proteins were unique to VSU, 38 to VLU, 63 to VDELU and 19 to VDES fractions. The unique fractions from logarithmic parasites have even more homogenous specific GO assignments (Table S4-6). For example, GIP is overrepresented in VL accounting for 65% of all the protein identifications while in VLU this adds up to 80%. In contrast, unique fractions derived from stationary parasites have characteristics that make them distinct from the other fractions. Indeed, proteins involved in folding sorting and degradation and proteasome related gene ontology assignments are enriched in VDESU (Table 1). The same gene ontology assignments as VS are enriched in VSU and proteins involved in transport and catabolism, energy metabolism, replication and repair and chromosome were also found in VSU (Table 1). The VSU fraction is also underrepresented in GIP and ribosome related gene ontology assignments (Table 1). Interestingly, VSU also has the highest representation of plasma membrane proteins and proteins with transmembrane domains, (Table S4). Some characteristics of VSU, such as the presence of histones, seem reminiscent of apoptotic vesicles [31]. To confirm the capacity to recover vesicles as a direct result of *Leishmania* death we used miltefosine as

an apoptosis inducer [32]. We isolated vesicles from *L. infantum* strains treated with drugs. For an equal number of cells, the protein contents of vesicles were similar between the wild-type strain and the miltefosine resistant strain MF20.5 (Fig. 6, lanes 2, 5). However treatment with miltefosine of the wild type cell increased the number of vesicles as shown by the increase quantity of proteins detected (Fig. 6, compare lanes 2, 3). Interestingly the same drug treatment was not inducing an increase in vesicle formation in the resistant mutant MF20.5 (Fig. 6, lane 6). Treatment of either wild-type of MF20.5 cells with G418, however, increased the quantity of proteins detected, an indication of more vesicles produced (Fig. 6, lanes 4 and 7). This increase in vesicle production was confirmed by electron microscopy images were the size of the vesicles was bigger and the preparations were more densely packed with vesicles (Fig 2B).

Discussion

The ability to grow *L. infantum* in serum free medium (cRMPI) allowed the recovery of the exoproteome using continuous growth. This medium allowed a comparison of the exoproteome of procyclic and stationary promastigotes of *Leishmania*. The time point defined for the exoproteome recovery of actively multiplying parasites (40% S/G2) was 24 hours (Fig 1). The exoproteome of stationary parasites was recovered at 96 hours because the cell cycle phase S/G2 was at a minimum and culture viability was still high (Fig 1).

To define the best approach for exoproteome recovery we compared a discontinuous approach with a continuous approach using cRPMI. There were clear differences in relative abundance (Fig 3) and protein identifications (Table S1) when both approaches were compared. The most notable difference was GP63 that was the most abundant protein in the exoproteome collected from cells grown in cRPMI (Fig. 2C, Fig. 3). The dynamics of GP63 release are complex and known to be responsive to environmental stimuli [33]. The continuous approach seems to yield an exoproteome more related to the expected exoproteome *in vivo* where GP63 is actively released from the promastigote [34]. Indeed, GP63 of *L. major* can be found in the cytosol of macrophages as soon as 1 hour after infection [35]. This is consistent with the known abundance of GP63 in the promastigote stage representing up to 1% of total protein [36] and that pulse-chase experiments in *L. amazonensis* indicated that around 60% of newly synthesized GP63 was detected in the extracellular milieu after 12h of culture [37]. It is salient to point out that the use of standard discontinuous approaches failed to highlight GP63 as a main component of the exoproteome [11,12,15,16]. In fact 51% (77/151) of the proteins identified in our discontinuous approach were not detected in the continuous approach. The

continuous approach is probably better suited for the comparative studies because it minimizes the effect of the culture medium change.

For the comparative study, the exoproteome was separated in two distinct fractions, one containing vesicles and the other one containing the remainder of the exoproteome, the VDE. The protein content of vesicle fractions was distinct from the VDE (Fig 2C, 3 and 4). The distinct profiles were clearly demonstrated by the representation of ribosomal proteins that were significantly enriched in VL and significantly decreased in VDEL (Table 1). Vesicles in eukaryotes usually consist of shedding microvesicles, apoptotic bodies and exosomes [38]. Vesicles with exosome like characteristics were recovered in proteomic studies of *Leishmania* [12]. Proteins enriched in the VDE should be derived from secretion, cell lysis or vesicle breakdown [9]. To better evaluate the contribution of parasite lysis we used an internal control proteome from the parasites in the experiments. This enabled the evaluation of cell lysis as a contributor to the exoproteome. While 46% (131/285) of the control proteome was detected in the exoproteome (Table S1), 48% (142/297) of the exoproteome was not detected in the control proteome suggesting that several proteins were enriched in the exoproteome. The reduced overlap between the four samples is consistent with the fact that cell lysis was not an important contributor of the exoproteome composition. Indeed, only 5% (17/297) of the identified proteins were common to all exoproteome samples (Fig 4 and Sup Table 1). In fact, the proteins that were common to all exoproteome samples were also detected in other exoproteome studies that use shorter time of growth conditions minimizing parasite lysis [16]. The presence of proteins suspected to be of intracellular origin was not unexpected since proteins without a predicted secretion signal were already reported in the exoproteome of *Leishmania* [16]. Moreover, the GO analysis supports a distinct origin for proteins in each fraction (Table 1).

Apart from vesicle breakdown and cell lysis the physiological process for enrichment for proteins in VDE should be through protein secretion. Traditionally secreted proteins have only transient intracellular existence and are quickly processed through the Golgi and released in the flagellar pocket [10]. We used a GFP fusion of LinJ.26.2710, a protein predicted to be secreted, and showed that this protein was only detected in the VDE in a vesicle independent manner (Fig 5). In contrast cytosolic proteins such as SHMT could be found in both exoproteome components. No specific enrichment of putative secreted proteins was found in the two VDE fractions (Sup Table 3) and one other possibility could be an unconventional secretion as described for HASPB in *Leishmania* [39]. We thus used SecretomeP to screen the exoproteome data for proteins putatively secreted unconventionally and found 43% of the detected exoproteome predicted to be potentially

unconventionally secreted (Sup table 3). This value is similar to the 57% prediction found in the exoproteome of *L. braziliensis* [15].

Other processes of delivery of intracellular proteins outside the cell in a vesicle independent manner have been described [40]. A process similar to secretory lysomes could explain specific release of sets of cytoplasmic proteins [41]. The proteomic analysis of T-cell secretory lysosomes [42] revealed some proteins common to the VDE unique proteins (Sup Table 8), and these proteins were not found in any *Leishmania* vesicle preparations [12,17] nor are they commonly associated with exosomes [42]. An alternative delivery process of intracellular proteins consists in the fusion of amphisomes (resulting from the fusion of autophagosomes with endosomes or MVBs) with the plasma membrane as described for Acyl-coenzyme (Co)A binding protein [43]. This latter process connects nonconventional secretion with autophagy. Autophagy is a pivotal process in *Leishmania* development being essential for metacyclic differentiation [44]. The macroautophagy of intracellular structures like the glycosomes was described in *T. brucei* during its life cycle stage [45] and is also thought to occur in *Leishmania* [46]. Also microautophagy, a form of autophagy that involves the trapping and degradation of complete regions of the cytosol by the lysosomal system [47], in conjunction with the formation of amphisomes and their fusion with the membrane could explain the release of free cytosolic proteins into the VDE. While this may at first hand look wasteful, the release of cytosolic proteins can be seen as a preventive mechanism that guarantees the recycling of cellular components. This should be more prevalent in logarithmic parasites with their high metabolic rates associated with dividing parasites. Consistent with this proposal, putative cytosolic proteins were significantly increased in VDEL (Sup table 8). As result of this significant increase of cytosolic proteins in VDEL we find that metabolism related GO are significantly increased (Table 1).

The exoproteome was found to change with the parasite life cycle. In logarithmic parasites, its composition reflected the active state of multiplication of the parasites. The exoproteome of logarithmic parasites was enriched in GIP and metabolic related GO (Table 1). Interestingly this enrichment was fraction specific, vesicles were enriched in GIP while metabolic related GO was enriched in the VDE. The enrichment in GIP was mostly due to a significant enrichment in ribosomal proteins accounting for 35% GO annotations in VL, while in VS they account for only 16% (Sup table 6). The same trend can be seen in figure 3 where ribosomal proteins are more abundant in VL. The increase of ribosomal proteins during logarithmic growth was expected in actively multiplying organisms [48]. The extracellular accumulation of ribosomal proteins in a vesicle dependent manner may correspond to a physiological process of protein turnover. Possibly, non-functional

proteins are released from the parasites, instead of being recycled by traditional processes related to autophagy or proteasome degradation. This vesicle mediated protein turnover is probably not originating from the lysosomal compartment because ribosomal proteins in VL and identified by MS/MS were at the expected size in 2D gels (data not shown). Moreover, peptidases, the second most abundant GO designation found in the total exoproteome, were poorly represented in VL (Sup table 6), further suggesting that there was no enrichment for lysosomal content in these vesicles. The presence of ribosomal proteins in vesicles might be the result of a number of events that were described in other cell types. This could include ribophagy [49] or the selective degradation of parts of the endoplasmic reticulum by macroautophagy [50] or selective packaging into autophagosomes [51] or unspecific engulfment of ribosomal proteins by microautophagy [52]. The protein elongation factor 1 α (EF1-α) (LinJ17.0090) followed the trend of ribosomal enrichment in the vesicle fraction, being the most abundant protein in VL (Fig 3B). This protein is associated with translation, and its presence is consistent with the presence of ribosomal proteins and the metabolically active state of logarithmic parasites. The dominance of EF1-α in vesicles was observed in VL only. Indeed, GP63 and beta-fructofuranosidase (LinJ04.0300) were the most abundant protein in VS. GP63, was particularly abundant in the stationary exoproteome where 25% of all the spectra used for identification were derived from GP63 (Fig 3A). The importance of GP63 in the infection is well known and has been extensively studied [53]. High amounts of GP63 in the inoculum exoproteome will probably have a significant impact during the first contact with the host cells. Free or vesicle bound GP63 may contribute to immediate protection against complement mediated lysis [54] and might even enter the host cell cytoplasm through a lipid raft-dependent mechanism [55], to cleave the cytosolic host protein tyrosine phosphatases [55], contributing to macrophage energy [35]. The nucleoside diphosphate kinase b (LinJ32.3100) was enriched in VS (Fig 3B). Interestingly, in *L. amazonensis* this protein was shown to accumulate extracellularly in the stationary stage [56], and was shown to reduce nitric oxide microbicidal activity and to prevent ATP mediated cytolysis [56]. In another intracellular pathogen, *Mycobacterium tuberculosis*, nucleoside diphosphate kinase also exhibits virulence enhancing characteristics related to delayed phagosome maturation and cytotoxic activity [57,58].

The VDESU and VSU were enriched for GO assignments that differed from overall stationary fractions (Table 1). The VDESU was significantly enriched in proteins associated with folding and degradation. The secretion of proteasome components with specific proteolytic activities was already described for mammalian cells [59]. Selective secretion of specific proteasome proteins may regulate proteasome activity in parasite

preparing for apoptosis. The proteasome translocation from the nuclei to the cytoplasm is known to be an early event of apoptosis [60]. Apoptotic parasites were shown to be essential for *L. major* infection [61], therefore the existence of apoptotic components in the exoproteome of stationary parasites is not unexpected. Interestingly, we found components associated to apoptosis [44,61] such as histones (Sup Table 8) or nuclear and membrane proteins (Sup table 4 and 8) that were either unique or enriched in VSU. Moreover we observed vesicles of variable size (Fig 2B), some of them exceeding the predicted size for exosomes (40-100 nm) [62]. The existence of vesicles of variable size is common in shedding microvesicles and apoptotic bodies [38]. Also the presence of known/predicted surface proteins in vesicle preparations (Table S8) was inconsistent with traditional exosome release. Exosomes are accumulated in multivesicular bodies (MVBs) that can either fuse with lysosomes (degradative MVBs) or be released from the cell (exocytic MVBs) and traditionally do not have surface proteins [63]. The release of vesicles of variable sizes as a consequence of cell death was demonstrated using miltefosine, showing that formation of vesicles is linked to death inducing stimuli (Fig 2B and 6). The presence of surface proteins in vesicles is not restricted to apoptotic bodies, however. Shedding microvesicles were also shown to have surface proteins [38]. Since no apoptosis related components were enriched in the logarithmic exoproteome, it is possible that the vesicles isolated in these parasites originated from the shedding of microvesicles. Traditionally mammalian derived microvesicles are involved in major phenomena such as cell to cell communication [64] or protein turnover [65]. Therefore origin and composition of the vesicles recovered in the exoproteome warrants further investigation as they may have a significant impact in *Leishmania* biology.

Conclusion

The exoproteome recovery from continuous growth of the parasite enabled for the first time an insight into the dynamics of the exoproteome comparing log-phase with stationary parasites. We used a continuous approach that led to a GP63-rich exoproteome and allowed the comparison of exponential-phase and stationary parasites minimizing the effects of medium change. This comparison showed that the exoproteome is dynamic with protein contents dependent on the stage of recovery. The understanding of the exoproteome dynamics in *Leishmania* will advance our understanding of physiologically important processes and will enable the development of new therapeutic approaches.

Acknowledgements

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Figure 1. **Growth properties of *L. infantum* in cRPMI**

A. Promastigotes were cultured with an initial concentration of 1x10 ^{6} /ml parasites in cRMPI. The growth density was determined by counting the parasites at different time points in a hemocytometer. The growth curve is depicted in the left y axis. The percentage of viable parasites and of G2/S parasites is depicted related to the right y axis. Quantification of parasite viability was performed by Annexin V (Ann V) and 7-aminoactinomycin D (7-AAD) staining of in vitro culture. Percentage of viability represents the Ann V/7-AAD negative parasites at a given time point. Both cell cycle analysis and viability were done using FACSCalibur cytometer and analyzed by FlowJo software. One out of at least three independent experiments is shown.B. Fluorescence images of representative parasites in cRPMI after 24 (left panel) and 92 hours (right panel) of culture. Parasites were transfected with episomal GFP and stained with Hoechst dye 33342.

Figure 2. **Characterization of the recovered exoproteome fractions**

A. Scheme for exoproteome recovery B. Electron microscopy pictures of exoproteome fractions containing vesicles from logarithmic and stationary parasites stained with acetate uracil. Panel (I) vesicle preparation from logarithmic parasites, (II) detail on the relative size of a group of vesicles from a stationary parasite preparation (III) vesicle preparation from stationary parasites and (IV) vesicle preparation from WT parasites submitted to miltefosine treatment. C. SDS-PAGE with the equivalent of 8 x 10^{8} parasites, stained with Sypro Ruby. VS, vesicles recovered from stationary parasites, VL vesicles recovered from logarithmic parasites, VDES, secreted proteins depleted of vesicles recovered from stationary parasites, VDEL secreted proteins depleted of vesicles recovered from logarithmic parasites. The black arrows are for protein bands that are enriched in VDEL or VL. The white arrows are for protein bands that are enriched in either VL or VS. The numbers in parenthesis represents a biological replicate. MW are from New England BioLabs.

Figure 3. **Relative abundance of the 20 most represented proteins in vesicle depleated exoproteome and vesicle preparations**

A. The relative abundance of each protein in a defined sample was determined using the quantitative value of the Scaffold Software. The 20 most abundant proteins overall were compared to determine the relative amount of the protein in the sample. VDES, vesicle depleted preparations from stationary parasites; VDEL vesicle depleted preparations from logarithmic parasites; VDE(DA) vesicle depleted preparations from stationary parasites

grown for 24 hours in cRPMI. B. The relative abundance of each protein in a defined sample was determined using the quantitave value of the Scaffold Software. The 20 most abundant proteins overall were compared to determine the relative amount of the protein in the sample. VS, vesicles preparations from stationary parasites; VL, vesicles preparations from logarithmic parasites; V(DA), preparations from stationary parasites grown for 24 hours in cRPMI in a discontinuous approach.

Figure 4. **Distribution of the protein identifications in the different fractions.**

Venn diagram of the distribution of the protein identifications for the 4 different fractions studied. Vesicles from stationary parasites (VS); vesicle depleted exoproteome from stationary parasites (VDES); vesicle depleted exoproteome from logarithmic parasites (VDEL) and vesicles from logarithmic parasites (VL).

Figure 5. **Secretion of the Leishmania protein 26.0710**

Localization of GFP fusions with 26.2710 or SHMT (cytosolic) in the parasite and in the recovered exoproteome by confocal microscopy and western blot. Parasites expressing episomal GFP were used as a control. Upper and middle pannel, microscope phase contrast picture and fluorescent image respectively. Lower panel, detection of the fusion proteins by Western blot analysis using an anti-GFP antibody (V –Vesicle fraction; VDE – Vesicle depleted fraction). Molecular weights were determined using Rainbow™ Molecular Weight Markers (GE Healthcare).

Figure 6. **Vesicle production as a measure of cell death in *Leishmania***

Sypro staining of SDS-PAGE with volume equivalent of vesicles produced by $4x10^8$ parasites, treated with death inducer drugs. (1) Molecular weight marker (New England Biolabs), (2) *L. infantum* WT, (3) *L. infantum* WT treated with 10 µM of miltefosine, (4) *L. infantum* WT treated with 4 µg/ml of G418 , (5) *L. infantum* 200.5 miltefosine resistant mutant, (6) *L. infantum* 200.5 miltefosine resistant mutant treated with 10 µM of miltefosine, (7) *L. infantum* 200.5 miltefosine resistant mutant treated with 4 µg/ml of G418.

Tables:

Table 1 – **GO annotations significantly altered in the studied fractions of the exoproteome**

GO annotations associated with the different exoproteome fractions that are significantly different from the total proteome of the study with at least P < 0,05. Proteins from vesicles from stationary parasites, VS, vesicle depleted exoproteome from stationary parasites, VDES, vesicle depleted exoproteome from logarithmic parasites VDEL, vesicles from logarithmic parasites, VL, proteins that where unique to vesicles from stationary parasites, VSU and to vesicle depleted exoproteome from stationary parasites, VDESU. The symbol '↑' is associated with overrepresentation, '↓' underrepresentation and '--'.with no significant change.

Supplemental information:

Table S1 – **Protein identifications from the proteomic analysis**

Overall protein identifications derived from the proteomic analysis of this study.

Table S2 – **Bio informatic predictions for the exoproteome fractions**

WOLF pSORT, SecretomeP, SignalP and transmembranar predictions for the exoproteome fractions.

Table S3 – **Prediction of extracellular distribution or membrane localization**

Percentage of representation for each exoproteome fractions using the following parameters: presence of a secretion signal predicted by SignalP (SignalP); presence of a secretion signal predicted by SignalP and no predicted transmembrane domain (SignalP (-TM)); predicted to be secreted by WoLF PSORT (Sec (pSort); predicted to be the plasma membrane (Exo (pSort)); predicted to be potentially unconventionally secreted by SecreteP (SecreteP); presence of transmembranar domains (TM). Exoproteome fractions: Proteins from vesicles from stationary parasites, VS, vesicle depleted exoproteome from stationary parasites, VDES, vesicle depleted exoproteome from logarithmic parasites VDEL, vesicles from logarithmic parasites, VL, proteins that where unique to vesicles from stationary parasites, VSU and to vesicle depleted exoproteome from stationary parasites, VDESU, proteins that where unique to vesicles from logarithmic parasites, VLU and to vesicle depleted exoproteome from logarithmic parasites, VDELU. Values underlined in bold represent values that are significantly different from the total proteome of the study with at least P < 0,05.

Table S4 – **GO annotations in general biological process for the different exoproteome fractions**

Percentage of proteins from the defined exoproteome fractions containing the depicted GO annotations (general biological process). Exoproteome fractions: Proteins from vesicles from stationary parasites, VS; vesicle depleted exoproteome from stationary parasites, VDES; vesicle depleted exoproteome from logarithmic parasites VDEL; vesicles from logarithmic parasites, VL; proteins that where unique to vesicles from stationary parasites, VSU; and to vesicle depleted exoproteome from stationary parasites, VDESU; proteins that where unique to vesicles from logarithmic parasites, VLU; and to vesicle depleted exoproteome from logarithmic parasites, VDELU. Values underlined in bold represent values that are significantly different from the total proteome of the study with at least $P < 0,05$.

Table S5 – **GO annotations in specific biological process for the 10 most represented annotations**

Percentage of proteins from the defined exoproteome fractions containing the depicted GO annotations (defined biological process). Exoproteome fractions: Proteins from vesicles from stationary parasites, VS; vesicle depleted exoproteome from stationary parasites, VDES; vesicle depleted exoproteome from logarithmic parasites, VDEL; vesicles from logarithmic parasites, VL; proteins that where unique to vesicles from stationary parasites, VSU and to vesicle depleted exoproteome from stationary parasites, VDESU; proteins that where unique to vesicles from logarithmic parasites, VLU and to vesicle depleted exoproteome from logarithmic parasites, VDELU. Values underlined in bold represent values that are significantly different from the total proteome of the study with at least $P < 0,05$.

Table S6- **GO annotations in defined biological functions for the 15 most represented annotations**

Percentage of proteins from the defined exoproteome fractions containing the depicted GO annotations (defined biological function). Exoproteome fractions: Proteins from vesicles from stationary parasites, VS; vesicle depleted exoproteome from stationary parasites, VDES; vesicle depleted exoproteome from logarithmic parasites VDEL, vesicles from logarithmic parasites, VL; proteins that where unique to vesicles from stationary parasites, VSU and to vesicle depleted exoproteome from stationary parasites, VDESU; proteins that where unique to vesicles from logarithmic parasites, VLU and to vesicle depleted exoproteome from logarithmic parasites, VDELU. Values underlined in bold represent values that are significantly different from the total proteome of the study with at least $P < 0,05$.

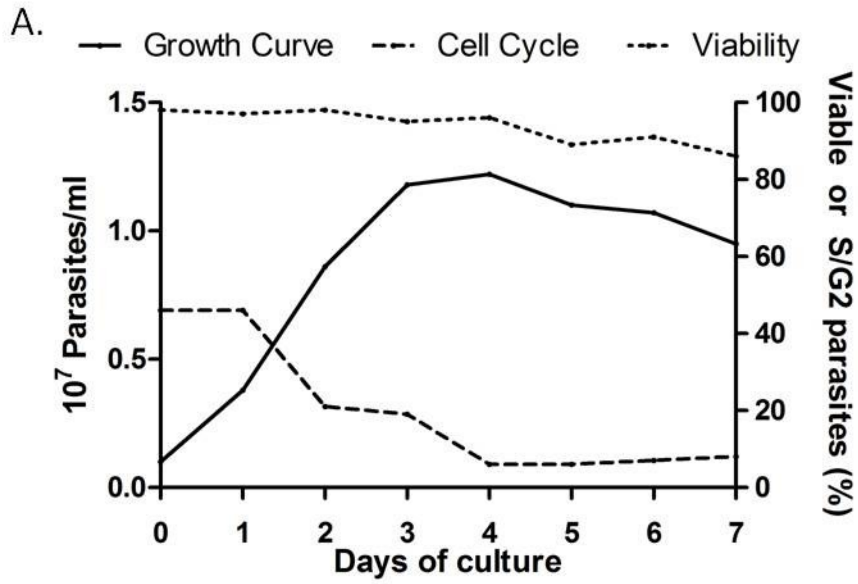
Table S7 – **Cellular localization prediction for the exoproteome fractions**

Percentage of proteins with a defined predicted cellular localization. Cyto, cytosolic; exo, plasma membrane and secreted; Mito, mithocondria; Nucl, nuclear; other, all other cellular localizations. Exoproteome fractions: Proteins from vesicles from stationary parasites, VS; vesicle depleted exoproteome from stationary parasites, VDES; vesicle depleted exoproteome from logarithmic parasites VDEL; vesicles from logarithmic parasites, VL; proteins that where unique to vesicles from stationary parasites, VSU and to vesicle depleted exoproteome from stationary parasites, VDESU; proteins that where unique to vesicles from logarithmic parasites, VLU and to vesicle depleted exoproteome from logarithmic parasites, VDELU. Values underlined in bold represent values that are significantly different from the total proteome of the study with at least $P < 0,05$.

Table S8 – **Groups of proteins specifically mentioned in this article**

Figure 1

A.



B.

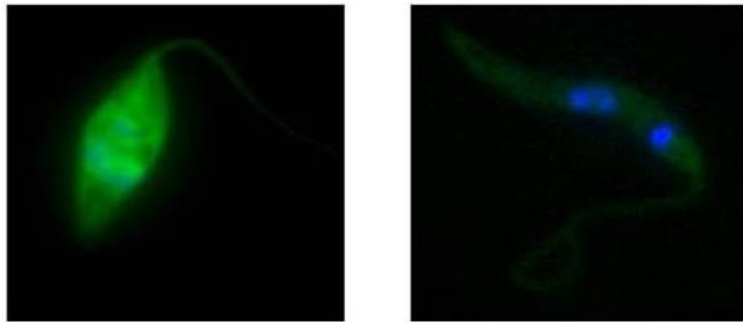


Figure 2

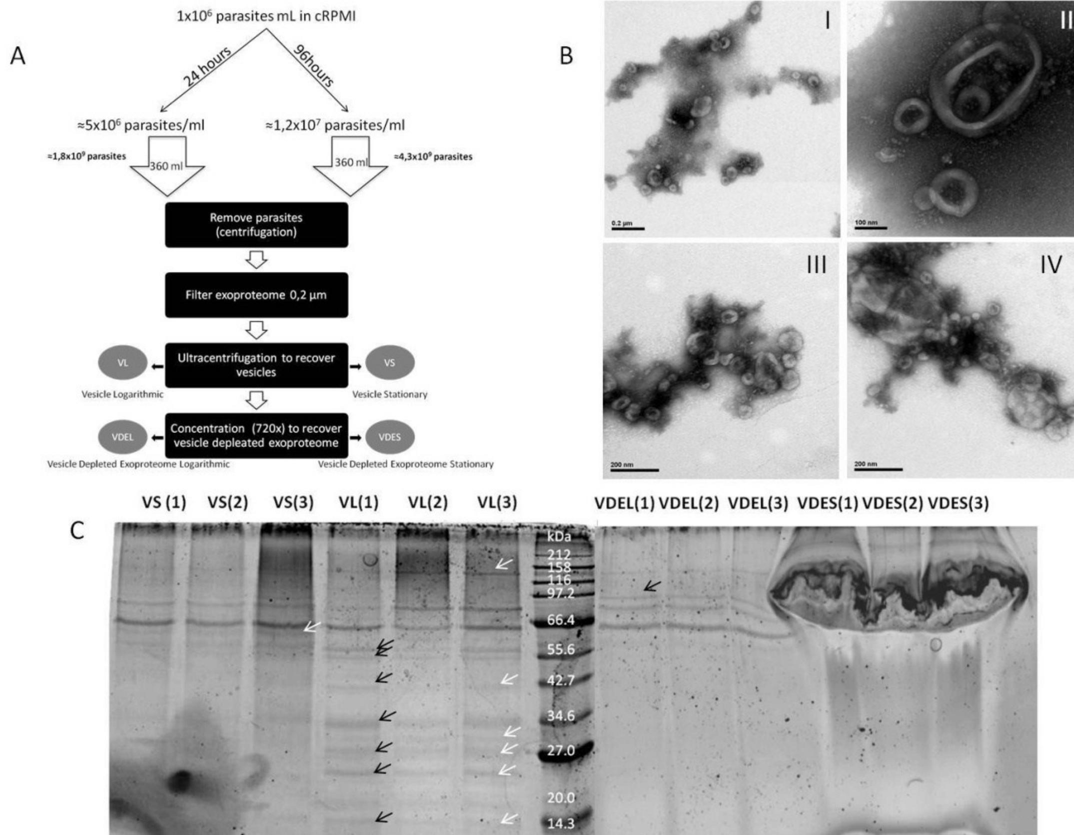


Figure 3

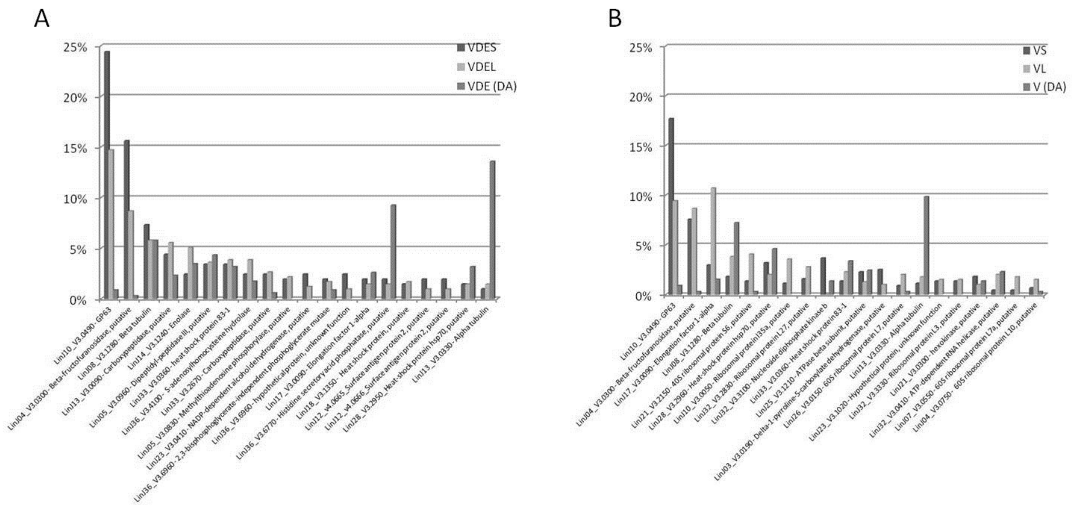


Figure 4

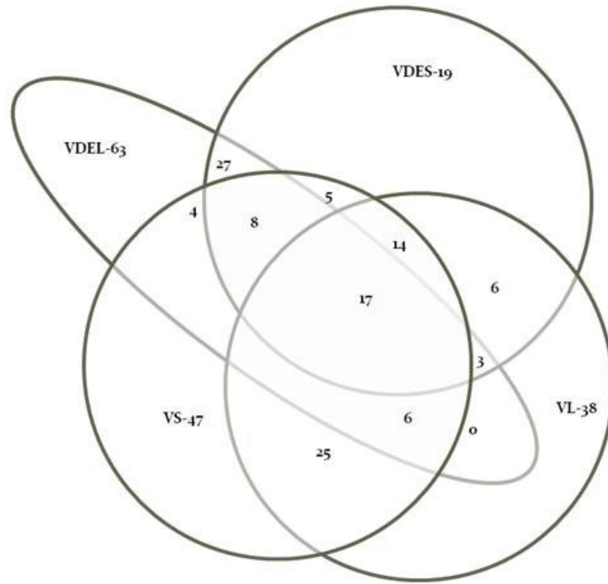


Figure 5

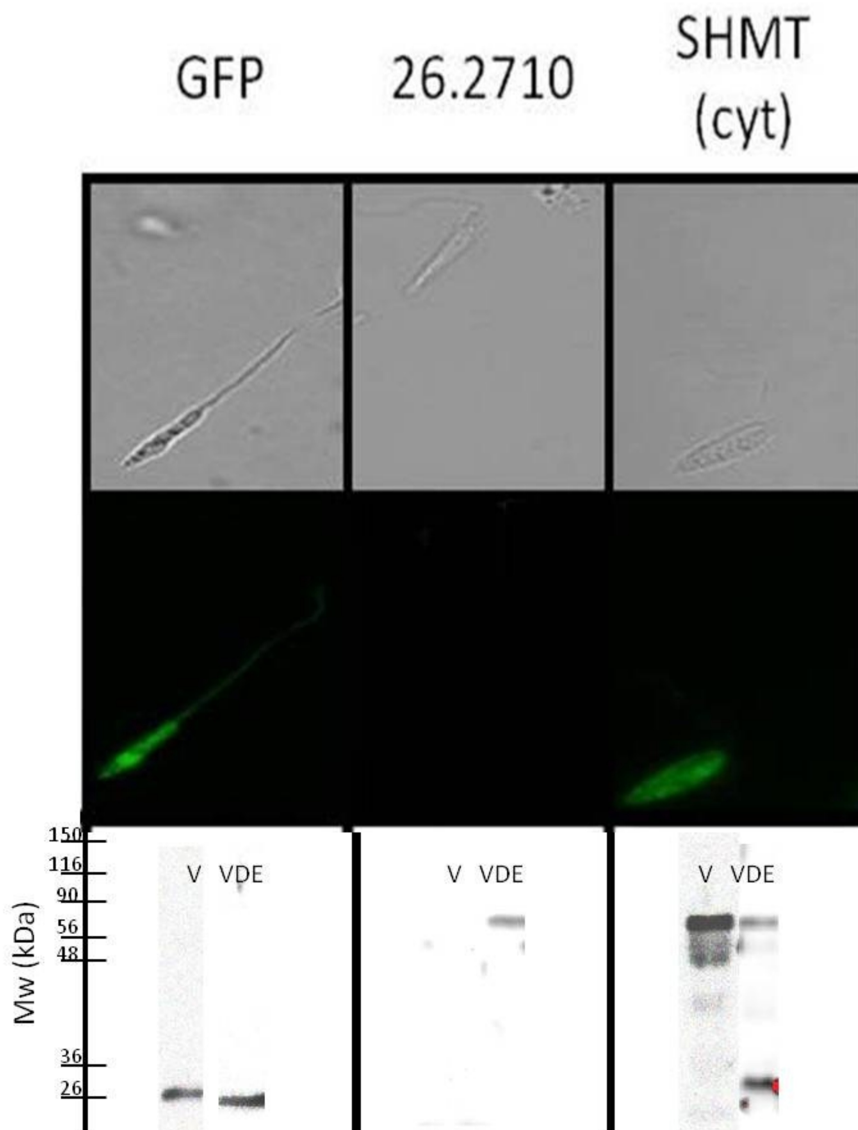


Figure 6

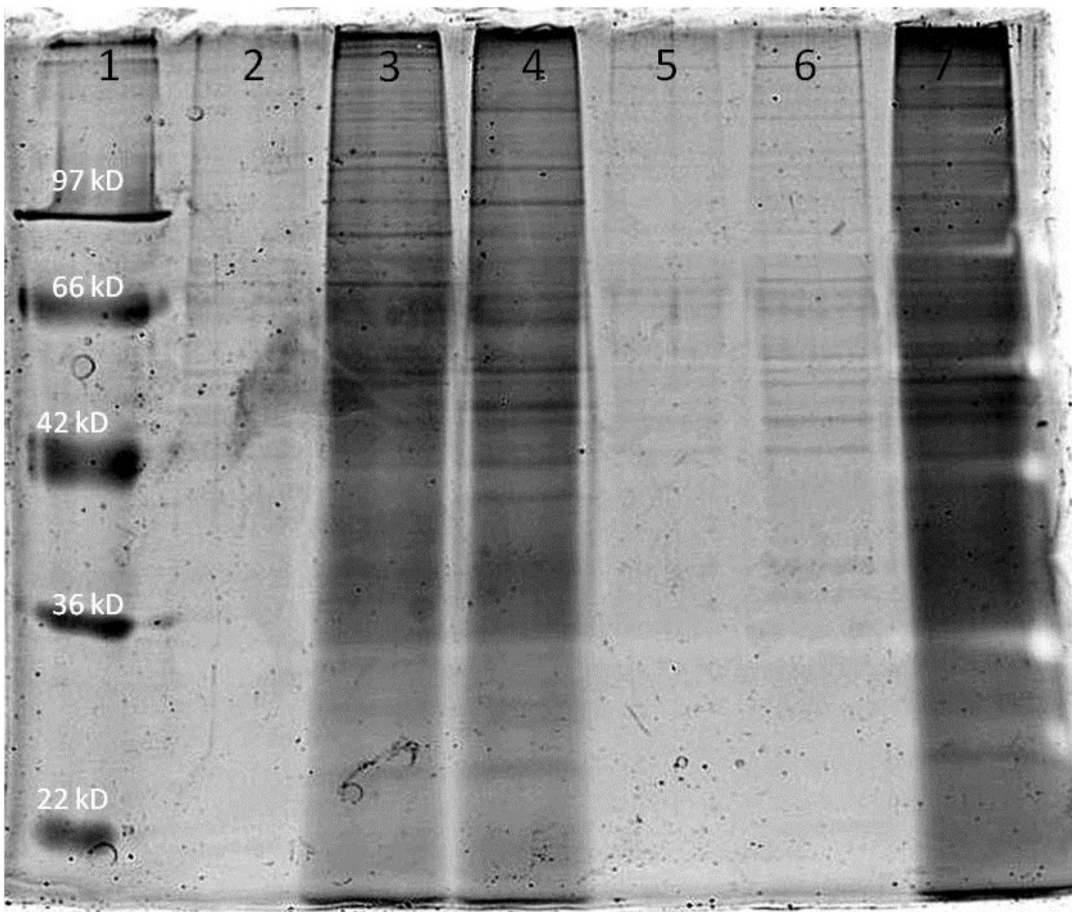


Table 1 – **GO annotations significantly altered in the studied fractions of the exoproteome**

Level of GO analysis	Specific GO assignment	Exoproteome fraction					
		VL	VS	VSU	VDEL	VDES	VDESU
General biological process	Metabolism	↓	--	--	↑	--	--
	Genetic information processing	↑	--	↓	↓	--	--
	Cellular process	--	↑	↑	--	--	--
Specific biological process	Translation	↑	--	--	↓	--	--
	Transport and catabolism	--	--	↑	--	--	--
	Folding sorting and degradation	--	--	--	--	--	↑
	Energy metabolism	--	--	↑	--	--	--
	Replication and repair	--	--	↑	--	--	--
	Carbohydrate metabolism	--	--	--	↑	--	--
	Aminoacid metabolism	--	--	--	↑	--	--
Defined biological functions	Ribosome	↑	--	↓	↓	--	--
	Oxydative phosphorylation	--	↑	↑	--	--	--
	RNA transport	↑	--	--	--	--	--
	Chromosome	--	--	↑	--	--	--
	Proteasome	--	--	--	--	--	↑
	Spliceosome	--	--	--	--	↑	↑

Table S1 – **Protein identifications from the proteomic analysis**

(See in the appendix section)

Table S2 – **Bio-informatics predictions for the exoproteome fractions**

(See in the appendix section)

Table S3 – **Prediction of extracellular distribution or membrane localization**

	SignalP	SignalP (-TM)	Extr (pSort)	Plas (pSort)	SecreteP	TM
Control Proteome	13%	8%	7%	3%	41%	7%
Total Exoproteome	15%	7%	8%	5%	42%	11%
VL	11%	4%	10%	3%	46%	7%
VLU	5%	5%	8%	0%	55%	**0%**
VS	18%	5%	8%	**10%**	42%	**17%**
VSU	23%	6%	9%	**22%**	43%	**30%**
VDEL	16%	9%	7%	3%	40%	9%
VDELU	14%	10%	2%	2%	43%	6%
VDES	15%	5%	12%	5%	36%	13%
VDESU	16%	5%	16%	11%	42%	21%

Table S4 – **GO annotations in general biological process for the different exoproteome fractions**

	Total Exo.	VL	VLU	VS	VSU	VDEL	VDELU	VDES	VDESU
Uncharacterized	22%	9%	5%	15%	23%	16%	15%	19%	28%
Metabolism	32%	**19%**	**10%**	31%	34%	**52%**	**54%**	35%	17%
Genetic information processing	36%	**65%**	**80%**	38%	**17%**	**24%**	**24%**	36%	44%
Celular process	7%	5%	0%	**12%**	**17%**	8%	6%	8%	6%
Environmental information processing	3%	2%	5%	4%	9%	1%	1%	2%	6%

Table S5 – **GO annotations in specific biological process for the 10 most represented annotations**

	Total Exo.	VL	VLU	VS	VSU	VDEL	VDELU	VDES	VDESU
Translation	25%	**50%**	**63%**	24%	5%	**12%**	**9%**	22%	14%
Carbohydrate metabolism	12%	9%	8%	8%	8%	**16%**	17%	12%	7%
Folding sorting and degradation	11%	12%	13%	10%	**5%**	8%	9%	14%	**43%**
Enzyme families	7%	4%	3%	7%	11%	10%	9%	8%	0%
Aminoacid metabolism	7%	5%	3%	6%	0%	**11%**	11%	7%	7%
Transport and catabolism	5%	4%	0%	9%	**13%**	5%	1%	7%	0%
Energy metabolism	4%	1%	0%	6%	**13%**	4%	4%	0%	0%
Replication and repair	3%	4%	8%	3%	**11%**	1%	3%	1%	0%
Nucleotide metabolism	3%	1%	0%	1%	3%	5%	4%	5%	0%
Metabolism of co-factors and vitamins	3%	1%	0%	1%	3%	3%	4%	4%	7%

Table S5 – **GO annotations in specific biological process for the 10 most represented annotations**

	Total Exo.	VL	VLU	VS	VSU	VDEL	VDELU	VDES	VDESU
Ribosome	14%	**35%**	**41%**	16%	**2%**	**3%**	**2%**	10%	6%
Peptidases	6%	2%	0%	6%	9%	7%	6%	7%	0%
Glycolysis / Gluconeogenesis	5%	5%	4%	5%	7%	7%	4%	6%	0%
Proteasome	4%	5%	7%	5%	4%	2%	3%	5%	**19%**
Pyruvate metabolism	3%	2%	4%	2%	2%	4%	6%	2%	0%
Citrate cycle	3%	4%	7%	1%	0%	2%	5%	1%	0%
Chromosome	3%	3%	**7%**	3%	**9%**	0%	1%	1%	0%
Purine metabolism	2%	1%	0%	1%	2%	3%	2%	4%	0%
Aminoacyl-tRNA biosynthesis	2%	2%	2%	0%	0%	3%	4%	2%	0%
Spliceosome	2%	3%	2%	3%	0%	1%	0%	**5%**	**13%**
Oxydative phosphorilation	2%	1%	0%	**4%**	**11%**	1%	0%	0%	0%
Cysteine and methionine metabolism	2%	1%	0%	2%	0%	3%	2%	3%	0%
Phagosome	2%	2%	0%	3%	4%	2%	0%	2%	0%
RNA transport	2%	**5%**	**9%**	1%	0%	1%	0%	2%	0%
Pentose phosphate pathway	2%	1%	0%	1%	2%	2%	2%	2%	0%

Table S6- **GO annotations in defined biological functions for the 15 most represented annotations**

	Total Exo.	VL	VLU	VS	VSU	VDEL	VDELU	VDES	VDESU
Ribosome	14%	**35%**	**41%**	16%	**2%**	**3%**	**2%**	10%	6%
Peptidases	6%	2%	0%	6%	9%	7%	6%	7%	0%
Glycolysis / Gluconeogenesis	5%	5%	4%	5%	7%	7%	4%	6%	0%
Proteasome	4%	5%	7%	5%	4%	2%	3%	5%	**19%**
Pyruvate metabolism	3%	2%	4%	2%	2%	4%	6%	2%	0%
Citrate cycle	3%	4%	7%	1%	0%	2%	5%	1%	0%
Chromosome	3%	3%	**7%**	3%	**9%**	0%	1%	1%	0%
Purine metabolism	2%	1%	0%	1%	2%	3%	2%	4%	0%
Aminoacyl-tRNA biosynthesis	2%	2%	2%	0%	0%	3%	4%	2%	0%
Spliceosome	2%	3%	2%	3%	0%	1%	0%	**5%**	**13%**
Oxydative phosphorilation	2%	1%	0%	**4%**	**11%**	1%	0%	0%	0%
Cysteine and methionine metabolism	2%	1%	0%	2%	0%	3%	2%	3%	0%
Phagosome	2%	2%	0%	3%	4%	2%	0%	2%	0%
RNA transport	2%	**5%**	**9%**	1%	0%	1%	0%	2%	0%
Pentose phosphate pathway	2%	1%	0%	1%	2%	2%	2%	2%	0%

Table S7 – **Cellular localization prediction for the exoproteome fractions**

	Cyto	EXO	Mito	Nucl	Other
Total Exo.	47%	13%	18%	18%	4%
VL	46%	10%	17%	23%	4%
VLU	58%	8%	13%	21%	0%
VS	40%	**18%**	17%	20%	5%
VSU	**22%**	**30%**	15%	26%	7%
VDEL	**57%**	10%	21%	**9%**	3%
VDELU	**60%**	**3%**	27%	**8%**	2%
VDES	55%	17%	12%	15%	2%
VDESU	53%	26%	5%	16%	0%

Table S8 – **Groups of proteins specifically mentioned during the article**

Protein identification	Fraction	Relevance
mitogen activated protein kinase (LinJ.33.1470)	VDEL	Proteins found in secretory lysomes of T-cells and in VDE and not in exosomes
adenylosuccinate lyase (LinJ.04.0440),	VDEL	
glucose-6-phosphate 1-dehydrogenase (LinJ.34.0080)	VDEL	
transketolase (LinJ.24.2150)	VDEL	
Surface Antigen 2 (LinJ.12.0665)	All	Proteins found in vesicles with predicted membrane localization by WoLF PSORT
Surface Antigen 2 (LinJ.12.0666)	All	
Surface Antigen Protein (LinJ.12.0671)	All	
Hypothetical Protein (LinJ.23.1020)	VL, VS	
GP63 (LinJ.10.0490)	All	
nucleobase transporter (LinJ.13.1110)	VS	
nucleoside transporter 1, putative (LinJ.15.1230)	VS	
P-type H+-ATPase, putative (LinJ.18.1500)	VS	
ADP,ATP carrier protein 1, mitochondrial precursor, putative,ADP/ATP translocase 1, putative (LinJ.19.0190)	VS	
hypothetical protein, conserved (LinJ.19.0560)	VS	
acetyl-CoA synthetase, putative (LinJ.23.0580)	VS	
hypothetical protein, unknown function (LinJ.23.1020)	VL, VS	
major surface protease gp63, putative,leishmanolysin, putative (LinJ.28.0600)	VS	
vacuolar-type proton translocating pyrophosphatase 1, putative (LinJ.31.1240)	VS	
3'-nucleotidase/nuclease precursor, putative (LinJ.31.2380)	VS	
nucleoside transporter 1, putative (LinJ.36.2040)	VS	
Histone 2B (LinJ.09.1410)	VS	Histones found in VS
histone H4 (LinJ.15.0010)	VS	
histone H3 (LinJ.16.0600)	VS	

5. Article name: Activation of bone marrow-derived dendritic cells by the exoproteome from *Leishmania infantum*

Mariana Resende[1], Nuno Santarém[1], Ricardo Silvestre[1] & Anabela Cordeiro-da-Silva[1,2]

To be submitted

In the present study we evaluated the immunological profile induced by the exoproteome of *L. infantum* on immature BmDCs

Main Results:

-The exoproteome induce the expression of BmDCs classical activation markers with the production of inflammatory cytokines.

-The activation is not dependent on the protein content of the exoproteome.

-Vesicles and vesicle-depleted exoproteome are also capable of activating the BmDCs

Conclusions:

The exoproteome is immunomodulatory, being capable of activating dendritic cells and inducing a pro-inflamatory profile.

Article title:

Activation of bone marrow-derived dendritic cells by the exoproteome from *Leishmania infantum*

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Keywords: Leishmania infantum; Bone marrow-derived dendritic cells; Toll Like receptors; Exoproteome; Vesicles

Abstract

Several evidences suggest that *Leishmania* exoproteome components can play a role in the initial steps of the infection. The immunological properties of the exoproteome were shown to be dependent on its protein composition. Therefore, we used a recently characterized continuous recovery of the exoproteome from stationary *L. infantum* to explore the role of the parasite-derived released components in the modulation of dendritic cell (DCs) function. Bone marrow DCs recovered from Balb/c mice were activated after an exoproteome stimulus, in a dose-dependent manner, inducing the up-regulation of costimulatory surface markers involved in antigen-presentation. This phenotype was maintained upon stimulation with either the vesicle fraction of the exoproteome or the vesicle depleated fraction. Moreover, the analysis of cytokine profile showed a dose dependent DC response towards exoproteome stimuli. Higher amounts of exoproteome components lead to an increased IL-10 release while lower quantities mainly induced TNF-α and IL12p40 secretion. The activation profile was independent of protein content suggesting that other structures rather than proteins are involved in this process. Protein independent activation is associated with Toll like receptors (TLR). TLR2 or TLR9 were not responsible for the activation profile. The early activation of DCs by *Leishmania* derived exoproteome components might contribute to an early inflammatory state in the site of infection leading to the recruitement of neutrophils and monocytes enabling the quick acces of the parasites to these susceptible cells.

Introduction

Leishmania spp. are protozoan parasites with a digenetic life cycle. The extracellular promastigotes proliferate within the digestive tract of hematophagous sandflies until a subsequent blood meal enables the transmission of the parasite to a mammalian host. In this host the protozoan differentiates into the intracellular amastigotes that thrive in the phagolysosome of macrophages (Bates 2007; Mougneau, Bihl et al. 2011). More than 20 *Leishmania* spp. are ethological agents for a group of vector borne neglected tropical diseases collectively known as leishmaniasis. The disease distribution endangers a population of 350 million distributed by 88 countries. It is characterized by different clinical manifestations that can range from self healing cutaneous presentations to fatal visceralizing complications (Kedzierski 2010). No effective human vaccine exists and the treatment is often toxic (Kedzierski 2010). In consequence there is a growing need of better understanding of these protozoa to enable the development of better and affordable therapeutical approaches. The dendritic cells (DCs) are among those cells involved in the initial steps of infection. They act as sentinels for intruding pathogens playing central role in the initiation and regulation of the immune response to invading microorganisms such as *Leishmania* spp. (Antoine, Prina et al. 2004; De Trez, Magez et al. 2009). It is known that uptake of *Leishmania major* leads to DC maturation and interleukin 12 (IL-12) release (Konecny, Stagg et al. 1999) while in *Leishmania donovani* these effects are not clear with reports often contradictory (McDowell, Marovich et al. 2002). Therefore it is clear that the effect of *Leishmania* spp. infection on DCs maturation and cytokine production is not only dependent on the infecting species but also on DCs subtype and experimental culture conditions (McDowell, Marovich et al. 2002; Ghosh and Bandyopadhyay 2004). The secretome of these parasites is expected to be an active agent in the interplay with the DCs (Revest, Donaghy et al. 2008; Neves, Silvestre et al. 2010; Silverman, Clos et al. 2010). Much like the study of parasites parasite interaction with DCs, the study of the properties of the exoproteome present the same limitations (Revest, Donaghy et al. 2008) with another major variable related to exoproteome recovery (Santarem et al 2011, submitted). Recent developments in the exoproteome recovery lead to the characterization of the exoproteome *Leishmania infantum* as a highly enriched in GP63 (Santarem et al 2011, submitted). The experimental approach that enabled the recovery of this proteome aims at reproducing the exoproteome present in the early stages of infection (Santarem et al 2011, submitted). Therefore we evaluated the immunomodulatory effects of the *L. infantum* stationary promastigotes exoproteome on mouse bone marrow-derived DCs (BmDCs) to better understand the contribution of the parasite-derived exoproteome in the onset of infection.

Materials and Methods

Animals and Parasites

Ten to twelve-week-old female from BALB/c, C57BL/6, TLR2-KO (C57BL/6 background) mice were obtained from Instituto de Biologia Molecular e Celular (IBMC; Porto, Portugal) animal facilities. Under laboratory conditions, the animals were maintained in sterile cabinets and allowed food and water *ad libitum*. Animal care and procedures were in accordance with institutional guidelines. All conducted experiments were done in accordance with the IBMC.INEB Animal Ethics Committee and the Portuguese Veterinary Director General guidelines. RS has an accreditation for animal research given from Portuguese Veterinary Direction (Ministerial Directive 1005/92). A cloned line of virulent *L. infantum* (MHOM/MA/67/ITMAP-263) was grown at 26°C in RPMI 1640 medium (Lonza, Swtzerland) supplemented with 10% heat-inactivated Fetal Bovine Serum - FBS (Lonza, Switzerland), 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin and 20 mM HEPES buffer.

Purification of exoproteome components from stationary *L. infantum* promastigotes

Logarithmic promastigotes with a startup inoculum of $1x10^6$ in cRPMI were grown and the culture medium was recovered at 96h; the parasites were then removed by centrifugation followed by filtration through a 0.2 µm filter. The recovered culture medium containing the exoproteome components was concentrated to a residual volume of 500 µl using centriprep Ultracel YM-3 filtering units (Millipore). The samples were dialyzed twice against PBS using the same YM-3 filtering devices. After the second dialysis step the exoproteome was again concentrated to a residual volume of 500 µl, sterile filtered through a 0,2 µm filter and stored at -90ºC. For MVs the culture medium was centrifuged at 10000g for 10 minutes at 4ºC to remove cellular debris, and further centrifuged at 100000g for 3 hours. The vesicle depleted exoproteome (VDE) was recovered without disturbing the vesicle pellet, and then the final 1 ml of residual volume was decanted and discarded leaving only the pellet that was resuspended in 500 µl of PBS. The vesicles were stored at either 4ºC or -90ºC depending on further intended use. The recovered VDE was concentrated to a residual volume of 500 µl using centriprep Ultracel YM-3 filtering units. The samples were dialyzed twice against PBS using the same YM-3 filtering devices. After the second dialysis step the VDE was again concentrated to a residual volume of 500 µl and stored at -90ºC.

As a control for the recovery process cRPMI was passed through the same recovery procedure and used as a negative control. Quantification of endotoxins in the exoproteome samples was made by a semi-quantitative assay using the E-TOXATE kit from SIGMA.

Bone Marrow-Derived Dendritic Cells

Bone marrow-dendritic cells (BM-DCs) were derived from precursors in bone marrow from femurs and tibiae of 10- to 12-week-old BALB/c mice were flushed with RPMI 1640, using syringes and 25-gauge needles. The tissue was resuspended, and BM-DCs were obtained by seeding $5x10^6$ bone marrow cells in 25 ml RPMI 1640 medium (Lonza) supplemented with 10% FCS, 2mM L-glutamine (Lonza), 100 U/ml penicillin (Lonza), 100 mg/ml streptomycin (Lonza), 20 mM HEPES buffer (Lonza), 50 µmol/L 2-mercaptoethanol (Sigma Chemical Co.) and 200 U/ml of granulocyte macrophage–colony-stimulating factor (GM-CSF) (PeproTech, Rocky Hill, NJ) – DC medium. Cells were cultured at 37°C and 5% CO2 for 3 days, after which the same amount of DC medium was added to each flask. At days 6 and 8, half of the culture supernatant was collected and centrifuged, and the cell pellet was resuspended in the same amount of fresh DC medium and put back into the original flasks. At day 10, the same procedure was performed but with use of only 100 U/ml of GM-CSF. BM-DCs obtained after 12 days of culture displayed a phenotype highly enriched in $CD11c^+$ cells (~95%).

In Vitro Stimulation of bone marrow derived dendritic cells

For *in vitro* infection, 12-day nonadherent BmDCs were seeded at $1x10^6$ cells/ml of RPMIc containing 3 U/ml of GM-CSF in 24-well plates (for flow cytometry and enzyme-linked immunosorbent assay [ELISA] assays),. After an overnight incubation period, BmDCs were stimulated for 24h with the theoretical volume of exoproteome produced by $1x10^6$ parasites or $1x10^7$ parasites. MVs and VDE also used using the same ratio. As positive control cells were also stimulated with LPS (1 µg/ml), FSL-1 (5µg/ml) or INF-γ (100ng/ml). Cells were maintained for 24 hours with the respective stimuli and then recovered.

Flow cytometry determination of surface markers

For the analysis of surface costimulatory markers, $2x10^5$ BMDCs were incubated for 20 minutes with saturating concentrations of fluorescein isothiocyanate-conjugated monoclonal antibodies to either CD40 (clone 3/23), CD80 (clone 16-10A1), CD86 (clone GL1), or anti CD11c-PE (clone HL3). Mouse isotype controls were used when necessary. All of the antibodies were obtained from BioLegend (San Diego, CA), except for anti-CD11c-PE antibody, which was obtained from BD Pharmingen (San Diego, CA). After two washing steps with PBS/2% fetal bovine serum, cells were acquired in a FACSCalibur Cytometer. Data was analyzed with FlowJo software, cells were selected on the basis of

forward scatter/side scatter values and BM-DCs were gated on $CD11c^+$ population and dead cells were excluded from all samples by propidium iodide labeling.

Quantification of cytokines by ELISA

The levels of IL-10, TGF-β, TNF-α, IL-12p40 and IL-12p70 were measured in the culture supernatants by ELISA after 24h of culture. All cytokine quantification was done according to the manufacturer's recommendations (BD Pharmingen for IL-10 and IL-12p70, BioLegend for IL-12p40, and TNF- α, and R&D system for TGF- β. Samples were assayed in triplicate, and the data expressed as the average of each cytokine assayed.

Exoproteome inactivation

In order to test if DCs activation was due to proteins present in the exoproteome, 250 µL exoproteome were digested for two hours at 55ºC with 750 µg or 1000 µg of proteinase K, followed by 10 minutes at 100ºC.

SDS-PAGE and Silver Nitrate Staining

SDS-PAGE was performed as previously described (Laemmli 1970). A 12% or 10% resolving gel and 5% stacking gel were used to separate the proteins of interest. To define a molecular weight of loaded proteins, the molecular weight (Sigma) marker was loaded and separated in parallel. The protein concentration in the samples was adjusted by dilution of more concentrated samples in PBS. Equal volumes of protein samples and 5x 2-mercaptoethanol PAGE-loading buffer were mixed, submitted to heating at 95°C for 5 min, and loaded in the gel. Proteins were separated using the Mini- PROTEAN system (Bio-RAD).Visualization of the protein bands form the gels were made through silver nitrate staining. Briefly, proteins were fixed trough an overnight incubation of the gel in a 30% ethanol, 10% glacial acetic acid, 60% water solution. After a 1h of ethanol incubation, the gel was washed several times with deionized water, incubated with a 0.1% solution of silver nitrate for 30 min, and gently washed with deionized water. To develop the gel it was used an aqueous solution of 2.5% sodium carbonate, 0.02% formaldehyde. In few minutes stained bands of proteins started to appear, and the reaction was quenched with a 1% acetic acid solution.

Statistical analysis

The results are presented as means ± SD, and the statistical difference between two groups was determined by the two-sided unpaired Student's *t*-test. The tests were performed

using GraphPad Prism (version 5.02; GraphPad Software, San Diego, CA). Statistically significant values are as follows: *p<0.05, **p< 0.01, and ***p<0.001.

Results

Recovery of *Leishmania* exoproteome components

To analyze the immunomodulatory effects of *L. infantum e*xoproteome on BmDCs we stimulated the cells directly with the recovered exoproteome. The exoproteome components we recovered using a serum free medium, cRPMI. This medium was used to recover the exoproteome from stationary parasites as previously described by our group (Santarem at al submited). As a quality control we confirmed the protein profiles in SDS PAGE (data not shown).

The exoproteome molecules induce an activation profile in BmDCs

As a preliminary approach, we stimulated BmDCs with a total exoproteome volume corresponding to the theoretical volume produced by parasites in a ratio of 1:1 and 10:1 (parasite:cell). As controls cells were either stimulated with cRPMI concentrated through the same recovery process as the exoproteome or LPS. After 24 hours of incubation, the surface expression markers CD40, CD86, CD80 and MHCII were up-regulated when stimulated with total exoproteome in a dose-dependent manner (Fig 1). To further characterize the effect of the exoproteome on BmDCs, we determined the levels of secreted cytokines upon 24 hour stimulation. The total exoproteome induced a statistically significant increase in the secretion of the pro-inflammatory TNF-α and IL-12 (p40) and the anti-inflammatory IL-10 cytokines (Fig 2). A negative dose-response effect was observed upon stimulation with total exoproteome for the pro-inflamatory cytokines, that decrease upon higher concentrations of exoproteome. The contrary was seen with the anti-inflammatory IL-10 that increased in a dose dependent manner (Fig 2). The negative control with cRPMI medium concentrated trough the same recovery procedure induced no alteration on co-stimulatory surface markers nor induced secretion of any of the cytokines (Data not shown).

Induced BmDCs activation profile by the exoproteome is via TLR

To verify if the activation of BmDCs involved proteins we used proteinase k to exclude the protein contribution (Fig 3). Non protein activation is associated with TLRs. These are known to interact with conserved motifs of evolutionarily conserved structures on pathogens, pathogen-associated molecular patterns (PAMPs). To define if a single the toll

like receptor was involved we performed similar experiments recurring to BmDC recovered from TLR2-KO or TLR9-KO mice. DCs stimulation with total exoproteome induced an up-regulation of the costimulatory molecules CD40, CD80 and CD86 (Fig 4). As a positive control, FSL-1, a synthetic lipopeptide that is a classical TLR-2 agonist or ODC a TLR9 agonist (Kiura, Kataoka et al. 2006) were used as positive controls. Since TLR2-KO and TLR9-KO mice were backcrossed with C57BL/6 strain, all experiments were also performed with BmDCs from C57BL6 mice showing a similar behavior as Balb/c mice (Fig 4).

Leishmania infantum microvesicles and vesicle depleated exoproteome induce BmDCs surface markers up-regulation

The *Leishmania* spp. exoproteome is composed of distinct components (Corrales, Sereno et al. 2010) that are enriched in specific proteins (Nuno et al 2011 submited). To evaluate the fraction responsible for the DCs activation we recovered microvesicles (MVs) and sesicle depleated exoproteome (VDE) (Nuno et al 2011 submited). We were able to isolate a fraction enriched in MVs (data not shown). Both MVs and VDE were able to up-regulated BmDCs (Fig 5).

Discussion

Over the years, numerous exoproteome molecules were implicated in the host-pathogen interaction have been identified (Chenik, Lakhal et al. 2006; Ellis and Kuehn 2010; Silverman and Reiner 2011). Also it is known that the exoproteome components from other intracellular pathogens, such as *Mycobacterium tuberculosis* or *Toxoplasma gondii*, contain highly immunogenic antigens (Prigione, Facchetti et al. 2000; Chenik, Lakhal et al. 2006). Several molecules found in the exoproteome of these intracellular organisms were shown to be targeted by host humoral and/or cellular responses, proving to be protective in several vaccine models (Prigione, Facchetti et al. 2000; Mustafa 2002; Daryani, Hosseini et al. 2003; Pym, Brodin et al. 2003; Shams, Klucar et al. 2004). While it was already published that exoproteome antigens from *Leishmania* spp. can induce protection (Bourdoiseau, Hugnet et al. 2009; Kaur, Sobti et al. 2011), the specific effect of exoproteome components in activation or maturation of antigen presenting cells remains elusive. The study of the effect of *Leishmania* spp. infection on DCs maturation and cytokine release is hampered by the lack of uniformity relating to parasite species and strain, DCs origin, maturation stage and culture conditions (McDowell, Marovich et al. 2002; Brandonisio, Spinelli et al. 2004; Soong 2008). In fact a strong dependency on the state of maturation of DCs was also verified in studies developed in our group (Neves,

Silvestre et al. 2010). Therefore there we opted to work with immature DCs (data not shown). The exoproteome of *L. infantum* stationary promastigotes induced a clear DCs activation profile, with the increase in expression of classic activation markers (Fig 1). This activation did not follow the trend of other exoproteome works that attribute to the exoproteome components immunosuppressive properties (Revest, Donaghy et al. 2008; Silverman, Clos et al. 2010). It was already shown that the immunological properties of recovered exoproteomes were dependent on the composition of the exoproteome, as was shown for vesicle preparations from HSP100 KO in *L. major* (Silverman, Clos et al. 2010). The vesicles recovered from the *L. major* exoproteome have anti inflammatory properties leading to the deactivation of BmDCs (Silverman, Clos et al. 2010). Remarkably vesicles from *L. major* HSP100 KO, whose composition is distinct from the *L. major* vesicles, have pro-inflammatory properties (Silverman, Clos et al. 2010) in a clear indication of an immunological effect dependent on the composition of the exoproteome. The four day continuous recovery procedure originated an exoproteome that was distinct in composition from the traditional short term exoproteomes (Santarem et al 2011, submitted). In consequence, this difference in composition might be responsible for the distinct profile of activation registered (Fig 1). Interestingly the activation of BmDCs was also reported in *L. braziliensis* infection (Carvalho, Pearce et al. 2008). The cells responsible for this activation were the bystander cells (cells that were in contact with the parasite but never infected). Using a transwell assay the authors separate DCs from parasites and showed that the activation of bystander DCs was mediated by soluble parasite product (Carvalho, Pearce et al. 2008). Therefore this is compelling evidence that the continuous exoproteome retained the properties of the naturally released parasite components. These bystander DCs have CD40, CD86 and MHCII markers up-regulated and produced significant amounts of IL-12 and TNF-α (Carvalho, Pearce et al. 2008). The cytokine profile of the BmDCs upon exoproteome stimulation induced a significant increase of the IL-12 and TNF- α supporting once again the profile seen for *L. braziliensis* (Fig 2 C and D). The IL-12 production is associated with the shift towards a TH1, and was shown to be associated with protection in cutaneous species (Sypek, Chung et al. 1993). The general inflammatory environment associated with the exoproteome might also contribute to the recruitment of neutrophils (Lukacs, Strieter et al. 1995) important hosts in the first steps of infection (Peters, Egen et al. 2008). The significant diminution of inflammatory cytokines in a concentration dependent manner (translated by a decrease of IL-12p40 and TNF-α and an increase in IL-10) is indicative of a possible dual effect of the exoproteome (Fig 2). This might be indicative of a two stage interaction, the first mediated by direct contact with the surface of the DC and the second mediated by released components inside the cell. It was already shown that GP63, the dominant component of

the recovered exoproteome (Santarem et al, 2011), can interact with intracellular machinery leading to deactivation of macrophages by directly cleaving intracellular components of the pro-inflammatory transcriptor factors AP-1 (Contreras, Gomez et al. 2010) or mTOR (mammalian/mechanistic target of rapamycin) (Jaramillo, Gomez et al. 2011). Interestingly it was reported that antigen presenting cells (APCs) could uptake vesicles associated to the exoproteome (Silverman, Clos et al. 2010). Consequently it is possible that the content of these vesicles might be released in the cytosol leading to the effects above described. It is also possible that these exoproteome components can be processed and presented to CD8 by MHCI leading to the selective elimination of these DCs by CD8 cells. In consequence the truly infected DCs parasites will not be affected because of the general surface marker down regulation associated with the infection (Carvalho, Pearce et al. 2008) leading to an impaired response to the parasite. This results in the selective elimination of DCs, leading to an impaired immune response. The most likely mechanism of interaction with DCs was the activation of specific surface receptors and the delivery of MVs cargo into the cytosol/endosomes and subsequent interaction with intracellular components of the DCs. It is known that DCs interaction with antigens can occur through different groups of receptors families, such as Fc receptors for antigen-antibody complexes, C-type lectin receptors for glycoproteins and pattern-recognition receptors such as TLRs. This myriad of receptors allows DCs to recognize a broad range of microbial stimuli (Brandonisio, Spinelli et al. 2004). We first concluded that a TLR pathway should be involved on exoproteome because the pathways are known to recognize a broad spectrum of pathogen-associated molecular patterns (PAMPs) that include lipids, DNA, RNA, carbohydrates and proteins (Akira 2006). To try and discover the nature of the agent responsible for the activation we used proteinase k digestion to eliminate proteins in the exoproteome (Fig 3A). The presence of intact proteins was not essential to the activation profile (Fig 3B). A non protein candidate responsible for the activation profile seen was LPG. This lypophosphoglycan can activate murine mDCs and natural killer (NK) cells through TLR2 (Brandonisio, Spinelli et al. 2004). Hence, we hypothesized that LPG could be among the responsible molecules for the DCs activation profiles induced by the exoproteome could be via the TLR-2 pathway. Free LPG is expected to exist because the molecular weight of the LPG exceeds the cut-off of the membrane used in the concentration of the exoproteome. Therefore as an abundant element in the surface of the parasite and being constitutively shed is possible that could be the major contributor to the activation profile. Nevertheless this hypothesis turned out to be untruth, since TLR2-KO BmDCs were also activated by exoproteome components indicating that LPG was not the single element involved in activation (Fig 4A). Still we cannot exclude the hypothesis of LPG playing a role in the exoproteome induced DCs activation however it was not the

sole responsible molecule for the activation. However, it was possible that glycosylated fractions of proteins might be able to activate DCs through other receptors, such as TLR-4 pathway, associated with polysaccharide recognition. Other protozoan PAMPs known to interact with TLRs include glycosylphosphatidylinositol (GPI) anchors (Almeida and Gazzinelli 2001; Debierre-Grockiego, Campos et al. 2007), which activate TLR2 and TLR4, and also the TLR9 activating unmethylated DNA (Gazzinelli and Denkers 2006). To exclude the later we used TLR9 KO mice to evaluate the activation with BmDCs. It was clear that the activation was not determined only dependent on DNA content (Fig 4B). One of the most prominent proteins found in the exoproteome was GP63 (Santarém et al). This protein can be found in the exoproteome by conventional secretion or by the release for the parasite surface where is anchored by a GPI anchor (McGwire, O'Connell et al. 2002; Jaffe and Dwyer 2003). Therefore the high abundance of GP63 in the exoproteome reported by Santarem et al might explain the activation seen in this report because of the GPI anchor also other GPI anchored proteins might be responsible for this activation. Therefore TLR2 and 4 seemed to be the most likely candidates acting in perhaps in synergy. If TLR-4 is the receptor involved glycosylated proteins might contribute to the observed modulatory effects on BmDCs. To better understand if MVs or soluble proteins were involved in the activation of DCs we separated the total exoproteome into two distinct fractions, VDE and MVs. Both components were able to up-regulate BmDCs co-stimulatory (Fig 5). Therefore as both fractions induced a similar activation profile, it is possible that common elements should be responsible for this activation. Prominent surface proteins like GP63 were found in both fractions, in fact it was reported a 25% overlap in protein identifications in proteomic study between VDE and MVs from stationary parasites (Santarem et al, submitted). Also LPG is expected to be in both fractions because it is likely to be associated to surface vesicles and is also known to be shed. Continuing work with TLR antagonists and also TLR4 and MyD88 KO might help unravel the mechanism of activation. The activation profile induced by the exoproteome of *L. infantum* indicates that it is a source of highly immunogenic components that might be involved in the Immunopathogenesis by induction of precocious transient inflammatory state that will lead to the recruitment of neutrophils and other cells that might work as safe heavens for the promastigote parasites in the first hours of the infection.

Conclusion:

The exoproteome of *L. infantum* leads to the activation of BmDCs and to the production of pro-inflammatory cytokines. These effects are independent of the protein composition of the exoproteome indicating that TLR signaling might be involved. The TLR2 and TLR9 are not involved in the activation profile. LPG and GPI anchors might be involved in the

activation through TLR4 and TLR2. Also VDE and vesicles from the exoproteome are also capable of activating the BmDCs.

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Figures

Figure 1. Effect of exoproteome molecules on BmDC maturation. BmDCs ($1X10^6$) were incubated for 24h in RPMI medium with, LPS (1µg/ml), or volume equivalents to the exoproteome produced by $1x10^6$ parasites (1:1); $1x10^7$ parasites (1:10) or $4x10^7$ parasites (1:40). DCs were harvested and the surface expression levels of the indicated markers were measured on $CD11c^+$ gated cells by flow cytometry. Black dashed profiles (Ctrl) correspond to cells labeled with the isotype-matched control Ab. Data are representative of at least three independent experiments

Figure 2. Cytokine production by BmDcs upon stimuli with the exoproteome
Cytokine secretion was measured by ELISA on the supernatants after 24 h of culture. BmDCs ($1X10^6$) were incubated for 24h in RPMI medium (Cells), LPS (1µg/ml), volume equivalent to the exoproteome produced by $1x10^6$ parasites (1:1) or $1x10^7$ parasites (1:10). Results are expressed as mean ± SD of three independent experiments. Statistically significant differences were determined comparing with unstimulated control (cells) or between the indicated conditions (*$p<0.05$, **$p<0.01$ and ***$p<0.001$).

Figure 3. BmDC activation profile induced by proteolytic processed exoproteome.
A. Exoproteome treated or not with proteinase K was migrated in 12% SDS-PAGE gel and stained with silver nitrate. Lane 1, marker; lane 2, untreated exoproteome; Lane 3, exoproteome sample treated with 750 µg/ml, Lane 4, exoproteome sample treated with 1000 µg/ml of proteinase K. Lane 5: proteinase K control.

B. BmDCs ($1X10^6$) were incubated for 24h, with LPS (1µg/ml); Total exo, volume equivalent to the exoproteome produced by $1x10^7$ parasites; ExoK, volume equivalent to the exoproteome produced by $1x10^7$ parasites proteolytic processed by proteinase K; and Pki proteinase K inactivated. Balb/c cells were harvested and surface expression levels of the indicated markers were measured on CD11c+ cells by flow cytometry. Black dashed profiles (Ctrl) correspond to cells labeled with the isotype-matched control Ab. Data are representative of two independent experiments

Figure 4. Exoproteome activation profile on TLR2 and TLR 9 KO BmDCs
BmDCs ($1X10^6$) derived from TLR2 **(A)** or TLR9 KO mice **(B)** were incubated for 24h in RPMI medium with, LPS (1µg/ml), or volume equivalents to the exoproteome produced by $1x10^7$ parasites (Total exo). DCs were harvested and the surface expression levels of the indicated markers were measured on $CD11c^+$ gated cells by flow cytometry. Black dashed profiles (Ctrl) correspond to cells labeled with the isotype-matched control Ab. Data are representative of at least three independent experiments

Figure 5. Effect of exoproteome components on BmDC maturation. BmDCs ($1X10^6$) were incubated for 24h in RPMI medium with, LPS (1µg/ml); Total exo, volume equivalents to the total exoproteome produced by $1x10^7$ parasites; MVs volume equivalents to vesicles produced by $1x10^7$ parasites; VDE; volume equivalents to the vesicle depleted exoproteome produced by $1x10^7$ parasites. DCs were harvested and the surface expression levels of the indicated markers were measured on $CD11c^+$ gated cells by flow cytometry. Black dashed profiles (Ctrl) correspond to cells labeled with the isotype-matched control antibody. Data is representative of three independent experiments.

Fig 1

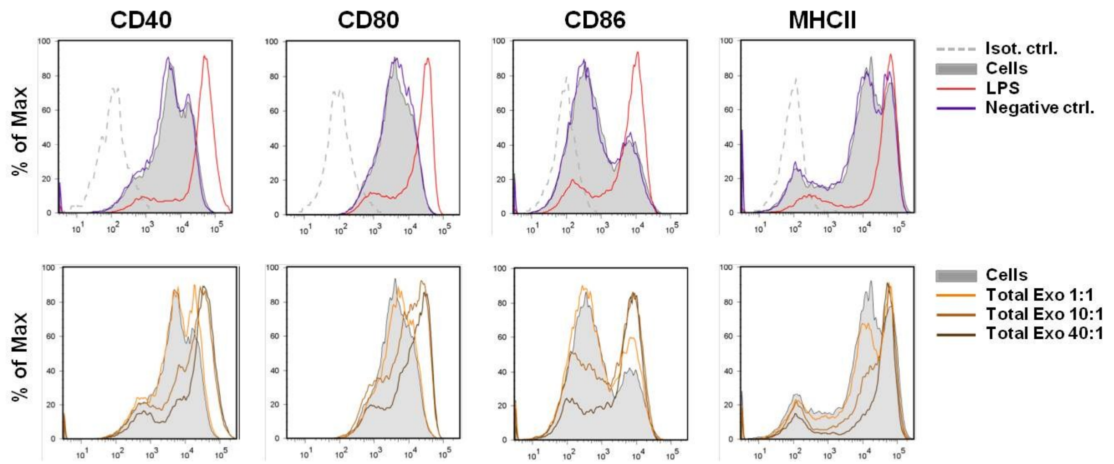


Fig 2

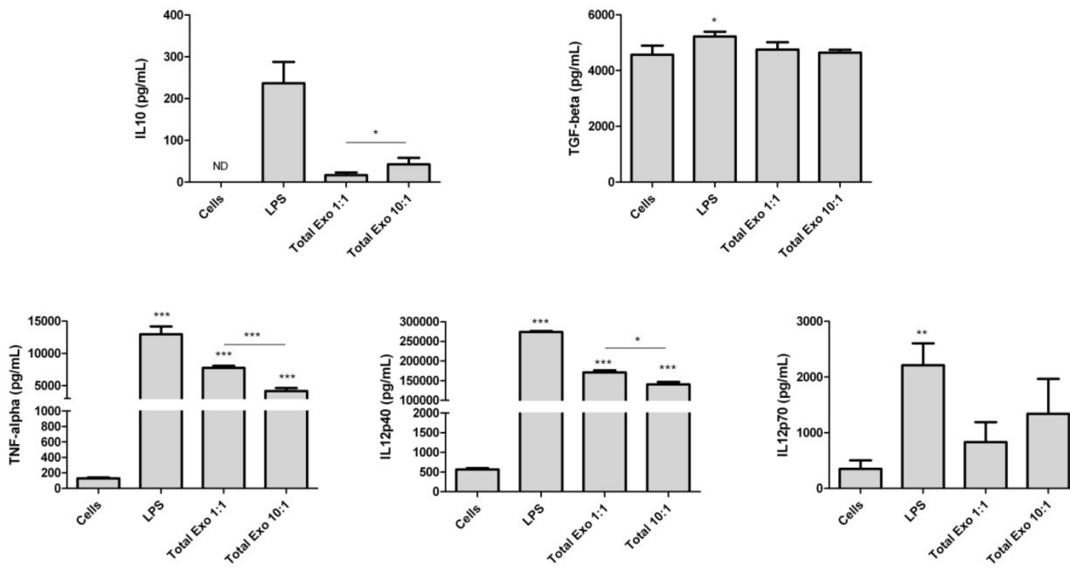


Fig 3A

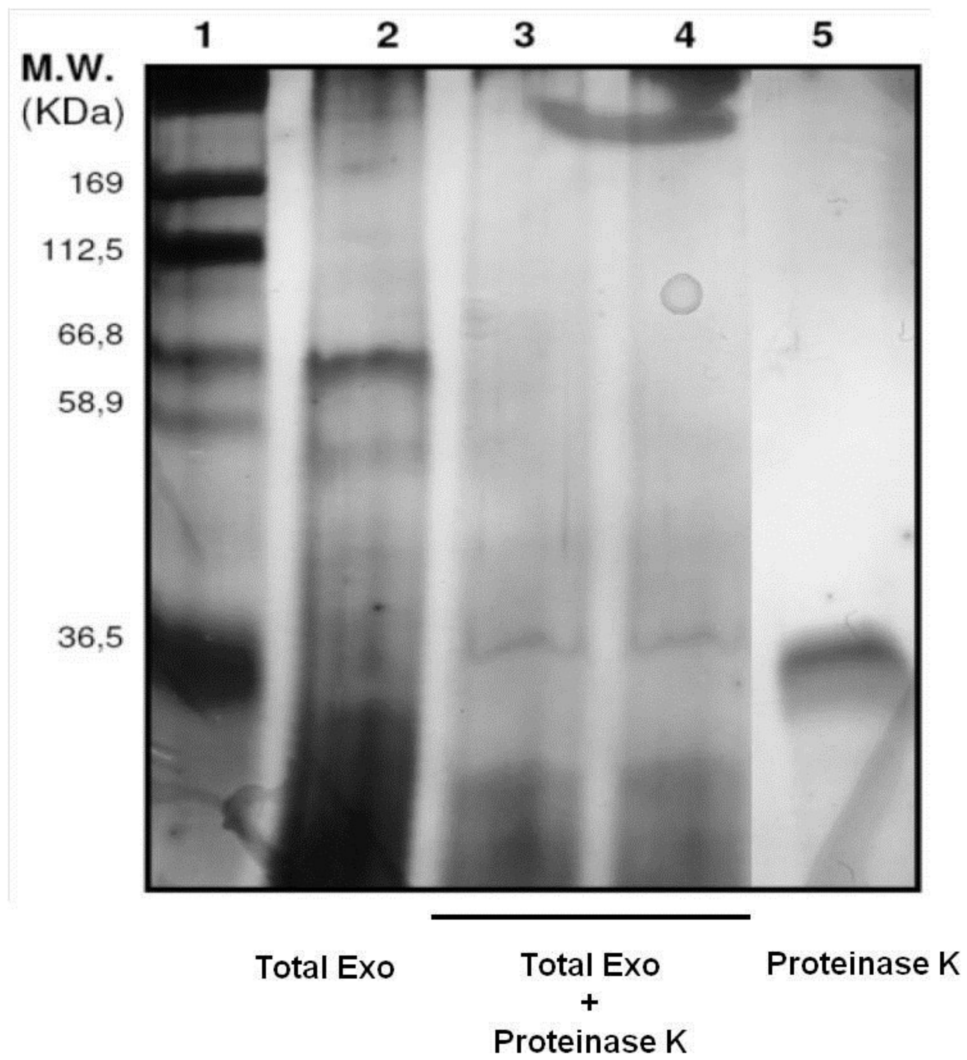


Fig 3B

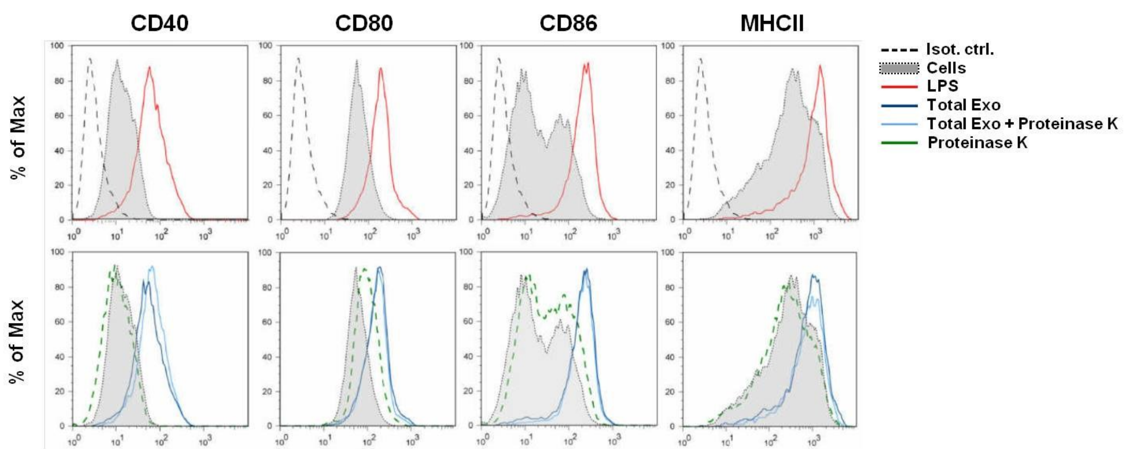


Fig 4A

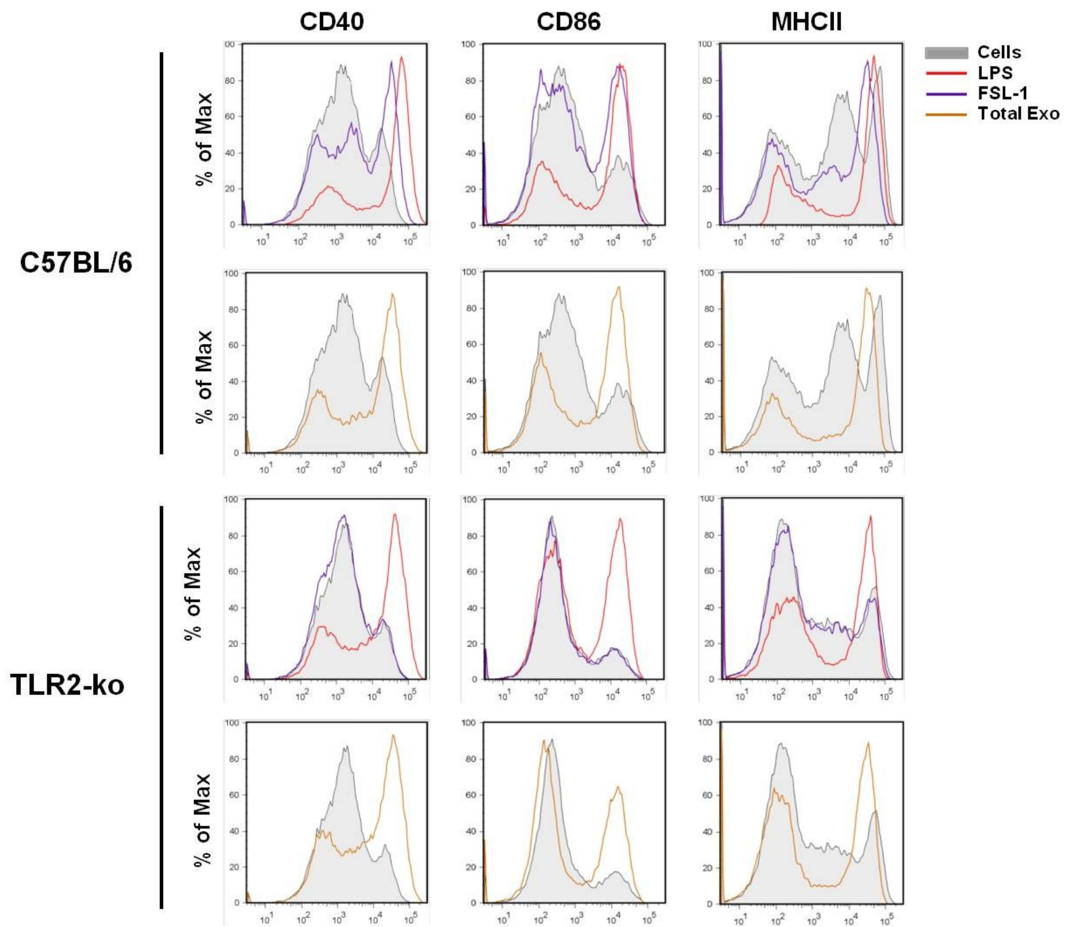


Fig 4 B

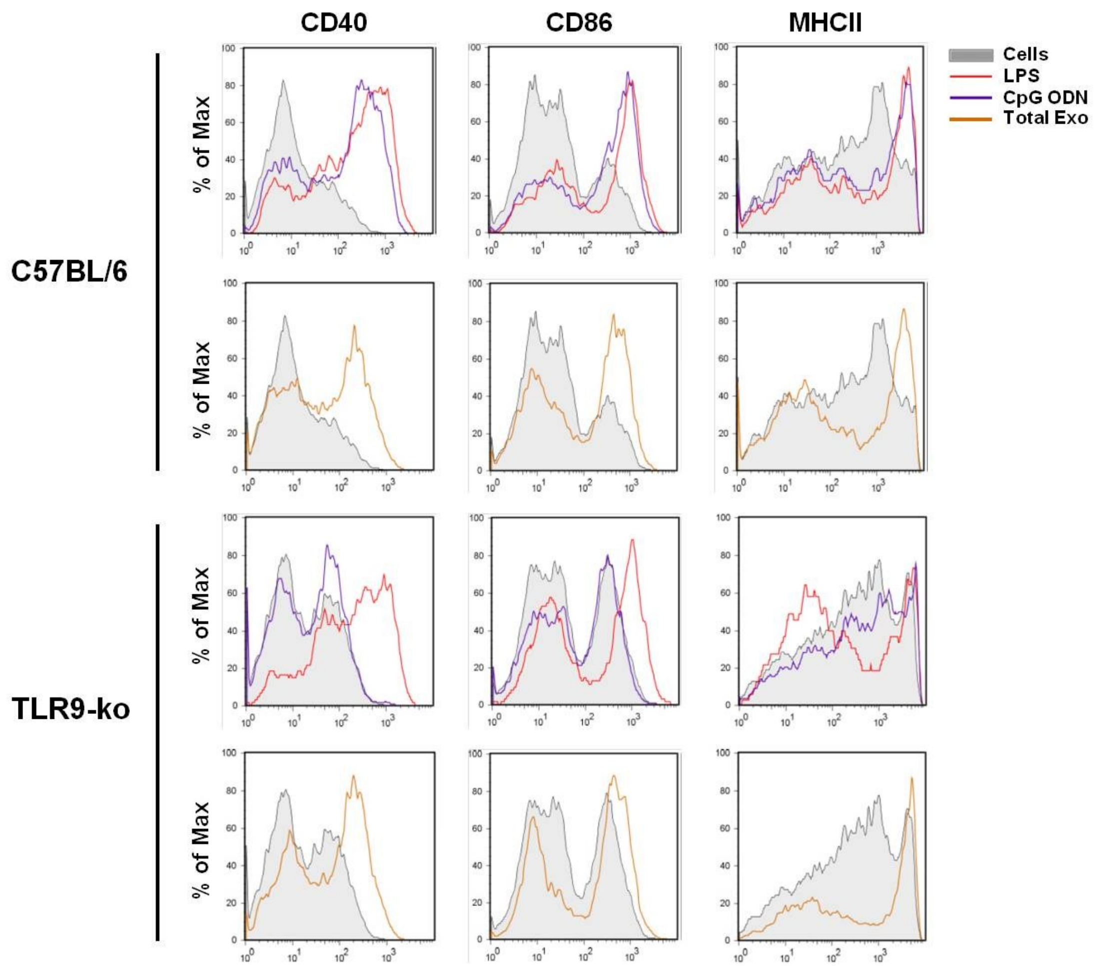
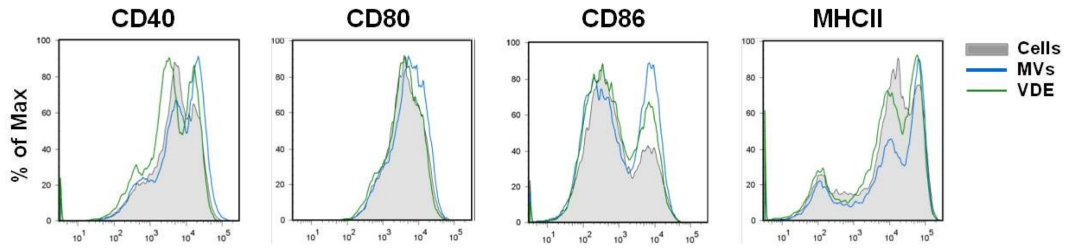


Fig 5



Chapter III

Discussion and perspectives

R III

1. Discussion

When the project was defined in 2007, fewer than 10 secreted proteins were known (Santarem et al., 2007). During the subsequent years, the characterization of the *Leishmania* spp. secretome attracted significant attention resulting in several proteomic studies that present the *Leishmania* spp. secretome as highly complex with more than 300 proteins identified as part of the secretome (Cuervo et al., 2009; Hassani et al., 2011; Silverman et al., 2008; Silverman et al., 2010a). The current state of knowledge indicates that the secretome is mostly composed of proteins with no predicted secretion signal (Silverman et al., 2008). The identification of exosome like vesicles in the secretome was a major breakthrough in the understanding of the dynamics of proteins release by these parasites (Silverman et al., 2010a). Our observations that confirmed these conclusions were described in Chapter II section 4. All the studies concerning the systematic identification of secretome proteins do not take into consideration the stress induced by the experimental approach, therefore the real value of these approaches in the context of *in vivo* or *in vivo* infections is debatable. Nonetheless these exoproteomes were used in several immunological approaches (Silverman et al., 2010a; Silverman et al., 2010b). Traditionally parasites are grown in standard medium supplemented with FCS and then cultivated in a serum free minimal medium for the time necessary for the recovery of the exoproteome. The time of recovery is defined as the minimum time of culture that enables the release of a detectable amount of secreted proteins in order to minimize the contribution of dead parasites in the minimal medium used (Corrales et al., 2010). We considered this method for exoproteome recovery as a discontinuous approach because it implies the growth of the parasites in standard medium supplemented with FCS and then changing the parasites to a minimum recovery medium without FCS. To our knowledge no study involving virulence of *Leishmania* spp. uses this discontinuous approach because the medium change is a stress inducing factor. Therefore we tried and implement a continuous approach for our secretome studies to recover proteins that would relate more to the naturally released proteins and not stress proteins associated with medium change. To accomplish this we first clarified the mechanism of virulence loss upon subculture (Chapter II, section 2). Working with virulent parasites was a pivotal point in our approach. Therefore we were interested in determining the span of time that we could work with parasites without detectable loss of virulence. It is known that parasites lose virulence with consecutive sub passages *in vitro* (Mitchell et al., 1984). To define the number of generations that would allow to work with parasites fully virulent we performed a study using parasites grown for a defined number of passages in standard RMPI medium (Chapter II, section 2). We defined that less than 10 passages (fourty generations) enabled

the use of parasites without loss of virulence (Chapter II, section 2). A loss of virulence was observed after 10 passages and was related to an incapacity to differentiate into amastigotes (Chapter II, section 2). This data was also supported by the *in vitro* infections with different media where we once again showed virulence loss dependent on the number of divisions (Chapter II, section 3). After defining the culture condition that retained virulence we developed an *"in house"* medium that enabled the maintenance of fully virulent parasites (Chapter II, section 3). We first evaluated the importance of the FCS complementation to two standard media to verify if this supplementation could be eliminated. Studies using completely defined medium already showed that the *Leishmania* spp. could be grown in the absence of FCS using a completely defined medium (Merlen et al., 1999). Using two distinct media, RPMI1640 and SDM79, that are complemented with 10% FCS we showed that serum complementation, although increasing the parasites yield in culture (probably by the presence of proteins), was not essential for basic multiplication and infectivity (Chapter II, section 3). Therefore, using a combination of both bases we developed a medium, cRPMI, which was able to support the growth of the parasites and retain virulence both *in vitro* and *in vivo* (Chapter II, section 3). This media was used in the subsequent exoproteome studies.

In the first of these studies we started by evaluating two different approaches for the recovery of the exoproteome (Chapter II, section 4). An exoproteome dominated by GP63 was obtained from the continuous approach, while the discontinuous approach presented a distinct profile were GP63 was not dominant, suggesting that different methods can lead to different results. GP63 in the context of the infection (Gomez et al., 2009), had been already extensively studied (Santos et al., 2006). The presence of GP63 in the continuous exoproteome was, in fact, determinant in the choice of this approach for the exoproteome studies with *L. infantum* (Chapter II, section 4). The recovered exoproteome was separated into distinct fractions, vesicles and Vesicle Depleated Exoproteome (VDE), which were characterized by the dominant presence of proteins without predictable signal for secretion. This observation was consistent with the actual dogma of the *Leishmania* spp. secretome being dominated by proteins without secretion signal. Other studies in *L. donovani* (Silverman et al., 2008), *L. braziliensis* (Cuervo et al., 2009), *L. mexicana* (Hassani et al., 2011) support this atypical secretion. The presence of these proteins of intracellular origins presented a real limitation for the exoproteome studies because it was difficult to evaluate the contribution of cell lysis to the actual exoproteome. The strategy used to evaluate the actual contribution of these components was to use a control proteome of the parasites used to produce the exoproteome. The presence of proteins enriched in the exoproteome that were not detected in the control proteome was clear

(Chapter II, section 4), indicating a specific enrichment of specific sets of proteins. This approach was similar to the first study of the secretome in *L. donovani* which showed a specific enrichment of putative intracellular proteins that was not compatible to passive release of intracellular proteins upon parasite death (Silverman et al., 2008). In fact, the presence in the exoproteome of proteins derived from dead parasites is also expected to happen *in vivo*, therefore we accepted these as an expected component of the exoproteome (Chapter II, section 4). Still we did not consider it to be a dominant fraction because the recovered proteins from the exoproteome presented distinct profiles when comparing to the recovered proteome. We were also able to isolate vesicles in both logarithmic and stationary promastigotes confirming that these are produced during two promastigote stages (Chapter II, section 4). The presence of these vesicles confirmed observations made in *L. donovani*, *L. major* and *L. mexicana* using discontinuous approaches (Hassani et al., 2011; Silverman et al., 2010a; Silverman et al., 2010b). For the first time the content of vesicles of logarithmic parasites was compared to stationary parasites, indicating specific enrichment in different subsets of proteins (Chapter II, section 4). This specific enrichment suggests a still uncharacterized phenomena concerning vesicle trafficking related to cell homeostasis and autophagy (Chapter II, section 4). In the logarithmic stage, proteins related to gene information processing, like ribosomes, where significantly enriched in the vesicle fractions. This specific targeting of ribosomes to the vesicles might be part of a protein turnover process. The vesicles from stationary parasites lead to the observation that vesicle release was a mechanism associated with parasite death (Chapter II, section 4). Death in *Leishmania spp.* is associated to a controlled process similar to apoptosis in higher eukaryotes (Wanderley and Barcinski, 2010). In fact, traditional apoptosis markers like histones were found in proteins unique to the vesicles of stationary parasites, while being absent from the corresponding VDE or from any logarithmic fraction (Chapter II, section 4). The presence of apoptotic vesicles in *Leishmania* spp. was already suggested by others (Silverman et al., 2010a), but the results presented in Chapter II, section 4 are currently the best evidence for apoptotic vesicles in *Leishmania* spp. The characterization of these vesicles as related to apoptosis and cell death was supported by several observations, like the enrichment of proteasome components in the VDE, and the presence of surface proteins in the vesicles (Chapter II, section 4). Apoptotic bodies are known to have surface proteins because they originate from the surface of the cell by blebbing of the membrane (Cocucci et al., 2009). The presence of surface proteins in the vesicles was inconsistent with exosome release. One possibility is that the vesicle population in *Leishmania* is composed of distinct vesicles from distinct origin that might include, exosome shedding microvesicles and apoptotic bodies. The existence of these vesicles is interesting because the presence of

apoptotic Leishmania parasites in the infectious inoculum is a prequisite for successful infection *in vivo* (van Zandbergen et al., 2006). One of the characteristics of the MVs of higher organisms is their long half life and the possibility of functioning as signaling platforms (Cocucci et al., 2009). Taking into consideration the development of the parasite inside the sandfly it is expected that vesicles (and also free proteins from the exoproteome) released during the later stages of the promastigote development will probably remain imbedded in the PSG. Therefore during the blood meal of the infected sandflies, vesicles will be released, in conjunction with the PSG and parasites, into the feeding pool. Consequently the exoproteome will be among the first components of the parasite to contact the immune system. As was already mentioned, some reports combining proteomic and immunological approaches already evaluated the capacity of the recovered exoproteome to induce an immune response (Hassani et al., 2011; Silverman et al., 2010a; Silverman et al., 2010b). These reports aimed at mimicking the exoproteome during differentiation. Therefore, they collected the exoproteome upon temperature and pH change to reproduce the conditions during the invasion of the mammalian host (Hassani et al., 2011; Silverman et al., 2010a). These reports attributed anti-inflammatory properties to the recovered exoproteomes. Interestingly it was shown that the properties were dependent on the vesicle content. Vesicles recovered from *L. major* HSP100 null mutants presented distinct cargo from the ones recovered from WT parasites (Silverman et al., 2010b). Remarkably these changes in cargo had a distinct effect on DCs; vesicles derived from wild type were anti-inflammatory, while the ones derived from the HSP100 null mutant presented pro-inflammatory properties. These cargo dependent properties increased the interest in studying vesicles recovered from stationary parasite as our exoproteome recovered was distinct from the published exoproteomes. Therefore we could expect a distinct immunological profile. Furthermore we were not interested in the exoproteome obtained from parasites that are differentiating or subjected to temperature or pH stress, we were mostly interested in recovering an exoproteome with minimum stress. The proteins recovered in stress conditions can only be representative of the proteome of the parasites differentiating because there is no positive control from exoproteome of differentiating parasites into true amastigotes (or axenic amastigotes). Furthermore, the release of these components in the extracellular environment is not likely to represent the events during infection because the parasites are quickly removed from circulation by neutrophils and resident APCs. In fact, exoproteome components will most likely be released during differentiation inside the phagosome. This is an obvious limitation of the exoproteome studies involving recovered MVs in *Leishmania* spp in conditions mimicking differentiation into amastigotes. The interactions between the exoproteome during differentiation are not expected to happen at the surface of the APC.

These are more likely to happen inside the parasitophorous vacuole, where the differentiation actually takes place. Therefore, the exogenous stimulation of cells with the exoproteome is not the most appropriate approach for these studies. Taking this into consideration the exoproteome that we choosed to study does not represent the exoproteome upon differentiation or upon arrival to a new host but represents mostly the exoproteome mixed with the PSG in the early stages of infection. We aimed to evaluate the impact of the exoproteome in the early stages of infection using immature DCs as a model. The delivery of these exoproteome components during the blood meal enables the direct interaction with host cells. These interactions are expected to be mediated by the recognition of parasite derived material through specific receptors at the surface of the host cells. This interaction is not exclusive to the cell surface because we cannot eliminate the possibility of exoproteome components entering into the cell and interfering with the cellular machinery. Some reports using vesicles derived from GFP parasites feed exogenously to macrophages showed that the cells were able to internalize the vesicles (Silverman et al., 2010a). Although the fate of the vesicle upon entry into the cells is not clear the results suggest an accumulation in the lysosomal/endosomal compartment (Silverman et al., 2010a). Recent data seem to indicate that the exoproteome is capable of inducing cleavage of tyrosine phosphatases in the macrophage cytosol suggesting that there can be some release of proteins inside the cytosol (Hassani et al., 2011). Still it is not clear if this uptake is mediated by vesicles, or through the uptake of individual proteins. In fact free GP63 is thought to enter the cells through the lipid rafts of the membranes (Gomez et al., 2009).

The immunogenicity of the exoproteome from stationary parasites was evaluated in Chapter II, section 5 of this report. Unlike most of the described exoproteomes we showed that the recovered exoproteome can indeed induce a pro-inflammatory response with the activation of DCs (Chapter II, section 5). Furthermore this activation did not seem to be dependent of protein interactions with receptors (Chapter II, section 5). Interactions with non-protein components are traditionally associated to TLRs. This activation was also evident with either vesicles or VDE fractions (Chapter II, section 5). Among the possible activators of TLRs described for trypanosomatid protozoa we find the GPI anchors as possible candidates, due to its TL4 activating capacity. Moreover the GP63 abundance in the exoproteome might contribute to the unusual abundance of GPI anchors when compared to other exoproteomes. In fact no other exoproteome presented this level of GP63 (Chapter II, section 4).The LPG also can be responsible for this activation and can explain the vesicle mediated activation, because surface vesicles will contain LPG. This activation might be helpful for the establishment of the infection because it will contribute

to create an early pro-inflammatory environment enabling the recruitment of neutrophils and monocytes. These recruited cells might become infected but eventually will be unable to kill the parasite due to its capacity to down regulate the cell to respond to stimuli upon successful infection (Chapter II, section 2) (Lapara and Kelly, 2010). Interestingly the cytokine profile of DCs upon stimulation was indicative of a diminishing pro-inflammatory response when higher quantities of exoproteome where present (Chapter II, section 5). This might correlate with a secondary effect upon interaction related to the deactivation of the cell by free or vesicle associated GP63. The DCs that were activated by the vesicles and are not infected will present antigens that are likely to induce a non productive response to the parasite inducing a cytotoxic response against the cells that present the parasite proteins. On the other hand cells that are infected will not present them because *L. amazonensis* infection can induce down regulation of several surface markers including proper presentation of MHC II (Antoine et al., 1999). This is reminiscent of the theory of panantigens in association with *Leishmania* spp. infection. In fact the exoproteome will act as a smoke screen enabling the infection by inducing a non productive response against antigens presented by these exoproteome components.

In conclusion the validation of this novel exoproteome approach using continuous cultivation unraveled a dynamic exoproteome. This dynamic profile of the exoproteome seams to be related to physiological processes like protein turnover and parasite death. The exoproteome is also immunologically active inducing activation of DCs. Biologically this activation might be relevant in the precocious stages of the infection inducing the recruitment of neutrophils and other APCs.

2. Perspectives

While the work presented here largely answered the goals of the project, inevitably we are left with more questions than answers.

The knowhow acquired over the last few years will be exploited to evaluate the exoproteomes from different species, and also to evaluate the immunogenicity of individual components, by using liquid chromatography to separate and evaluate the different components of the exoproteome. This might provide a source of immunomodulatory molecules with potential for medical use. Furthermore the importance of microvesicles in parasite-parasite communication and parasite-host interaction is an area with great potential. A growing number of reports point to the capacity of these organisms to modulate the functions of macrophages and DCs by direct interaction with the host cell machinery. To evaluate the importance of microvesicles in the interaction with host cells it is important to characterize the different vesicle populations sorting them into exosomes, apoptotic bodies and shedding microvesicles. Also the mechanisms of the fusion process with the host cell must be studied. Murine macrophages and dendritic cells can be used to determine the fate of these vesicles using electron and confocal microscopy. A particularly enticing prospect is the possibility of communication mediated through protein or RNA transfer. *Leishmania* spp. exosomes may serve as a source of shuttle RNA's. Exosomes from human mast cells contained bioactive shuttle RNA's -both mRNAs and microRNAs- that could be transferred between cell types changing the biological characteristics of the recipient cells (Valadi et al., 2007).

Ultimately the main purpose of the work carried out in this thesis was to advance in fundamental aspects of *Leishmania* cell biology, and its interaction with the host cells. The new knowledge has valued in its own right, but it is also possible that this knowledge will form the basis of new and exciting implications for the biology of *Leishmania* spp. and of other eukaryotic pathogens that invade host cells.

This thesis will contribute to a better understanding of *Leishmania* interactions with the host and will contribute to mitigate the effects of a disease that disfigures, disables, and kill hundreds of thousands of individuals every year.

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Annexed files

Supplementary figures 1 and 2 from chapter 2, section 4.

Supplemental table 1 – Protein identifications from the proteomic analysis

VL - Vesicules fraction from the exoproteome of logarithmic parasites
VS - Vesicules fraction from the exoproteome of stationary parasites
VDEL - Vesicules depleated exoproteome from logarithmic parasites
VDES - Vesicules depleated exoproteome from stationary parasites
Ext - Detected proteome from parasites growing in cRPMI (Control Proteome)
V (NC) - Vesicules fraction from the non continuos exoproteome
VDE (NC)- Vesicules depleated exoproteome from the discontinuos recovery

Fraction									
VL	VS	VDEL	VDES	Ext	V (NC)	VDE (NC)	Name	Acess Number	MW
X				X	X		ribosomal protein S7, putative	LinJ01.0430	24 kDa
		X	X	X	X		hypothetical protein, conserved	LinJ01.0500	39 kDa
X		X	X	X	X		eukaryotic initiation factor 4a, putative	LinJ01.0790	45 kDa
	X						phosphoglycan beta 1,3 galactosyltransferase	LinJ02.0140	90 kDa
X							proteasome regulatory non-ATPase subunit 6, putative	LinJ02.0340	59 kDa
	X			X			hypothetical protein, conserved	LinJ02.0430	32 kDa
				X			LinJ02.0660 peptidyl-dipeptidase, putative	LinJ02.0660	77 kDa
				X			long chain fatty Acyl CoA synthetase, putative	LinJ03.0080	79 kDa
X	X	X	X	X	X	X	delta-1-pyrroline-5-carboxylate dehydrogenase, putative	LinJ03.0190	62 kDa
				X			hypothetical protein, conserved	LinJ04.0060	59 kDa
X	X	X	X		X	X	beta-fructofuranosidase, putative	LinJ04.0300	72 kDa
		X					adenylosuccinate lyase, putative	LinJ04.0440	54 kDa
				X			60S ribosomal protein L11 (L5, L16)	LinJ04.0470	22 kDa
X	X		X	X	X		60S ribosomal protein L10, putative	LinJ04.0750	25 kDa
				X			nascent polypeptide associated complex subunit-like protein	LinJ04.0770	29 kDa
					X		fructose-1,6-bisphosphatase, cytosolic,	LinJ04.1180	39 kDa
X	X	X		X	X		actin	LinJ04.1250	42 kDa
					X		phosphoprotein phosphatase, putative	LinJ05.0100	72 kDa
	X						protein tyrosine phosphatase, putative	LinJ05.0280	25 kDa
		X	X	X		X	trypanothione reductase	LinJ05.0350	53 kDa
				X		X	microtubule-associated protein, putative	LinJ05.0380	165 kDa
	X	X		X	X		ATPase alpha subunit	LinJ05.0500	63 kDa
		X	X	X			methylthioadenosine phosphorylase, putative	LinJ05.0830	33 kDa
	X	X	X		X	X	dipeptidyl-peptidase III, putative,metallo-peptidase, Clan M-, Family M49	LinJ05.0960	76 kDa
		X					mercaptopyruvate sulfurtransferase	LinJ05.0970	40 kDa
	X						vacuolar ATPase subunit-like protein	LinJ05.1140	42 kDa
					X	X	histone h4	LinJ06.0010	11 kDa
				X			CYP cyclophilin	LinJ06.0120	20 kDa
					X		proteasome beta 6 subunit, putative	LinJ06.0140	28 kDa
X	X			X			60S ribosomal protein L19, putative	LinJ06.0410	28 kDa
X				X			60S ribosomal protein L23a, putative	LinJ06.0590	16 kDa
			X				hypothetical protein, unknown function	LinJ06.0840	381 kDa
			X				coproporphyrinogen III oxidase	LinJ06.1330	35 kDa
	X			X			cytochrome c1, heme protein, mitochondrial precursor, putative	LinJ07.0210	30 kDa
				X			acetylornithine deacetylase-like protein	LinJ07.0250	43 kDa
				X			maoc family dehydratase-like protein	LinJ07.0460	17 kDa
X	X		X	X			60S ribosomal protein L7a, putative	LinJ07.0550	39 kDa
X	X		X	X			40S ribosomal protein S9, putative	LinJ07.0760	22 kDa
					X		splicing factor ptsr1-like protein	LinJ07.0940	42 kDa
		X	X	X		X	iron superoxide dismutase	LinJ08.0300	26 kDa
				X			hypothetical protein, conserved	LinJ.08.1010	42 kDa
X				X			stress-induced protein sti1	LinJ08.1020	62 kDa
X	X	X	X	X	X	X	beta tubulin	LinJ08.1280	50 kDa
X							kinesin, putative	LinJ09.0250	133 kDa
				X			prefoldin subunit 2, putative	LinJ09.0720	15 kDa
		X		X	X		oligopeptidase b,serine peptidase, clan SC, family S9A-like protein	LinJ09.0820	83 kDa
		X					hypothetical protein, conserved	LinJ09.0890	43 kDa
X	X		X				polyubiquitin	LinJ09.0950	98 kDa
				X		X	CALA2 calmodulin, putative	LinJ09.0970	17 kDa
		X	X	X	X		elongation factor-1 gamma	LinJ09.1020	52 kDa
		X	X		X		hypothetical protein, conserved	LinJ09.1070	67 kDa
	X				X	X	histone H2B	LinJ09.1410	12 kDa
X	X			X			ribosomal protein l35a, putative	LinJ10.0050	16 kDa
X	X	X	X	X	X	X	GP63, leishmanolysin,metallo-peptidase, Clan MA(M), Family M8	LinJ10.0490	64 kDa
X	X						GP63, leishmanolysin,metallo-peptidase, Clan MA(M), Family M8	LinJ10.0530	64 kDa
				X	X	X	isocitrate dehydrogenase [NADP], mitochondrial precursor	LinJ10.0610	48 kDa
		X					nuclear transport factor 2, putative,ntf2-like	LinJ10.0900	14 kDa
		X					FKBP-type peptidyl-prolyl cis-trans isomerase, putative	LinJ10.0940	17 kDa
					X		histone h3	LinJ10.1210	15 kDa
	X						rab1 small GTP-binding protein, putative	LinJ10.1250	25 kDa
				X			small GTP-binding protein Rab11	LinJ10.1250	23 kDa
		X		X			seryl-tRNA synthetase, putative	LinJ11.0100	53 kDa
		X					proteasome alpha 7 subunit, putative	LinJ11.0240	28 kDa
		X		X	X		14-3-3 protein, putative	LinJ11.0350	29 kDa
X	X	X		X	X		aminopeptidase, putative,metallo-peptidase, Clan MF, Family M17	LinJ11.0640	57 kDa
X							40S ribosomal protein S21, putative	LinJ11.0770	17 kDa
X							hypothetical protein, conserved	LinJ11.0820	37 kDa
				X			ribosomal protein S5	LinJ11.0960	21 kDa
				X			pyruvate phosphate dikinase, putative	LinJ11.1000	100 kDa
				X			hypothetical protein, conserved	LinJ11.1030	28 kDa
X	X			X			60S ribosomal protein L28, putative	LinJ11.1110	16 kDa
X	X	X	X				40S ribosomal protein S15A, putative	LinJ11.1180	15 kDa
					X		HGPRT,hypoxanthine-guanine phosphoribosynthesis protein	LinJ12.0120	24 kDa
X							proteasome regulatory ATPase subunittcc1l8.3, putative	LinJ12.0190	46 kDa
		X	X	X	X	X	glucose-6-phosphate isomerase	LinJ12.0490	67 kDa
	X				X		cytochrome c oxidase subunit iv	LinJ12.0620	37 kDa
		X					NADH:flavin oxidoreductase/NADH oxidase, putative	LinJ12.0730	39 kDa
		X					proteasome beta-1 subunit, putative	LinJ12_v4.0030	30 kDa
		X					MAR1 ribonuclease, putative	LinJ12_v4.0060	22 kDa
X	X	X	X				surface antigen protein 2, putative	LinJ12_v4.0665	49 kDa
X	X	X	X				surface antigen protein 2, putative	LinJ12_v4.0666	44 kDa
X	X	X	X				surface antigen protein, putative	LinJ12_v4.0671	60 kDa
		X	X	X	X	X	carboxypeptidase, putative,metallo-peptidase, Clan MA(E), family 32	LinJ13.0090	57 kDa
				X			LinJ13.0160 protein kinase A regulatory subunit	LinJ13.0160	56 kDa
X	X	X	X	X	X	X	alpha tubulin	LinJ13.0330	50 kDa
					X	X	hypothetical protein, conserved	LinJ13.0320	13 kDa
				X			cysteinyl-tRNA synthetase, putative	LinJ13.0350	88 kDa
		X		X			40S ribosomal protein S12, putative	LinJ13.0460	16 kDa
				X		X	60S ribosomal protein L18, putative	LinJ13.0490	22 kDa
				X	X		mitochondrial processing peptidase alpha subunit, putative	LinJ13.0800	58 kDa
				X			hypothetical protein, conserved	LinJ13.0810	76 kDa
				X			NADH-cytochrome B5 reductase, putative	LinJ13.0990	35 kDa
				X			leucyl-tRNA synthetase, putative	LinJ13.1030	122 kDa
	X						nucleobase transporter	LinJ13.1110	55 kDa
				X			adenylosuccinate synthetase, putative	LinJ13.1120	78 kDa
X	X			X	X		40S ribosomal protein S4, putative	LinJ13.1120	31 kDa
				X			programmed cell death 6 protein-like protein	LinJ13.1390	26 kDa
			X				pyrroline-5-carboxylate reductase	LinJ13.1420	29 kDa
		X					nucleoside hydrolase-like protein	LinJ14.0130	39 kDa
		X					carboxypeptidase, putative,metallo-peptidase, Clan MA(E), Family M32	LinJ14.0180	57 kDa
		X		X			hypothetical protein, conserved	LinJ14.0190	22 kDa

		X	X				hypothetical protein, conserved	LinJ14.0240	15 kDa
				X			hypothetical protein, conserved	LinJ14.0460	36 kDa
	X		X	X	X	X	calpain-like cysteine peptidase, putative,cysteine peptidase, Clan CA, family C2, putative	LinJ14.0910	13 kDa
X	X	X	X	X	X	X	enolase	LinJ14.1240	46 kDa
		X					myo-inositol-1-phosphate synthase	LinJ14.1450	46 kDa
	X				X	X	histone H4	LinJ15.0010	11 kDa
		X					hypothetical protein, conserved	LinJ15.0040	38 kDa
X	X		X	X			60S ribosomal protein L13a, putative	LinJ15.0220	25 kDa
				X	X		ribonucleoprotein p18, mitochondrial precursor	LinJ15.0320	21 kDa
	X	X	X	X	X	X	40S ribosomal protein S3, putative	LinJ15.1010	24 kDa
X	X			X			60S ribosomal protein L6, putative	LinJ15.1060	21 kDa
		X			X		glutamate dehydrogenase	LinJ15.1070	115 kDa
X	X	X	X	X	X	X	tryparedoxin peroxidase	LinJ15.1100	22 kDa
	X						nucleoside transporter 1, putative	LinJ15.1230	54 kDa
X							glutaminyl-tRNA synthetase, putative	LinJ15.1490	66 kDa
		X					proliferative cell nuclear antigen (PCNA), putative	LinJ15.1500	32 kDa
X	X			X			60S ribosomal protein L21, putative	LinJ160470	18 kDa
		X	X	X			dihydroorotate dehydrogenase, putative	LinJ160540	34 kDa
		X		X			aspartate carbamoyltransferase, putative	LinJ160550	35 kDa
	X						histone H3, putative	LinJ160600	15 kDa
		X	X	X			transaldolase, putative	LinJ160760	37 kDa
			X				hypothetical protein, unknown function	LinJ161030	99 kDa
					X		paraflagellar rod protein 2C	LinJ16.1500	69 kDa
		X	X				kinesin, putative	LinJ161550	305 kDa
X	X			X			hypothetical protein, conserved	LinJ17.0010	88 kDa
X	X	X	X	X	X	X	elongation factor 1-alpha	LinJ17.0090	49 kDa
				X			hypothetical protein, conserved	LinJ17.0710	11 kDa
	X						hypothetical protein, conserved	LinJ17.0970	48 kDa
	X		X				hypothetical protein, conserved	LinJ17.0990	12 kDa
					X		META1 hypothetical protein, conserved	LinJ17.1020	12 kDa
					X		hypothetical protein, conserved	LinJ17.1400	8 kDa
			X				hypothetical protein, conserved	LinJ17.1400	22 kDa
		X					diphosphomevalonate decarboxylase, putative	LinJ18.0020	42 kDa
		X					hypothetical protein, conserved	LinJ18.0280	76 kDa
		X		X	X	X	aconitase, putative	LinJ18.0510	97 kDa
X				X	X		60S ribosomal protein L10a, putative	LinJ18.0630	25 kDa
	X						hypothetical protein, conserved	LinJ18.0660	137 kDa
				X	X	X	citrate synthase, putative	LinJ18.0690	52 kDa
		X	X				prolyl-tRNA synthetase, putative,bifunctional aminoacyl-tRNA synthetase, putative	LinJ18.1220	90 kDa
	X	X	X	X	X		heat shock protein, putative	LinJ18.1350	94 kDa
X	X		X				pyruvate dehydrogenase E1 component alpha subunit, putative	LinJ18.1360	43 kDa
X				X			60S ribosomal protein L34, putative	LinJ18.1380	19 kDa
	X						P-type H+-ATPase, putative	LinJ18.1500	107 kDa
		X	X	X			nonspecific nucleoside hydrolase	LinJ18.1570	34 kDa
				X	X		hypothetical protein, conserved	LinJ18.1650	13 kDa
X	X			X			40S ribosomal protein S2	LinJ19.0050	29 kDa
		X	X				aminopeptidase, putative,metallo-peptidase, Clan MG, Family M24	LinJ19.0150	43 kDa
	X			X	X	X	ADP,ATP carrier protein 1, mitochondrial precursor, putative,ADP/ATP translocase 1, putative	LinJ19.0190	35 kDa
X	X						C-terminal motor kinesin, putative	LinJ19.0250	94 kDa
X							nucleosome assembly protein, putative	LinJ19.0440	40 kDa
				X			glycosomal malate dehydrogenase	LinJ19.0540	34 kDa
	X						hypothetical protein, conserved	LinJ19.0560	58 kDa
X							proteasome regulatory non-ATP-ase subunit, putative	LinJ19.1100	47 kDa
	X				X		hypothetical protein, conserved	LinJ19.1150	41 kDa
	X	X	X	X	X		cysteine peptidase A (CBA)	LinJ19.1460	39 kDa
					X		peptidylprolyl isomerase-like protein	LinJ19.1440	48 kDa
	X						inosine-5'-monophosphate dehydrogenase	LinJ19.1590	56 kDa
				X	X		PGKC phosphoglycerate kinase C, glycosomal	LinJ20.0110	52 kDa
		X	X				hypothetical protein, conserved	LinJ20.0980	39 kDa
X	X		X	X			calpain-like cysteine peptidase, putative,cysteine peptidase, Clan CA, family C2, putative	LinJ20.1210	112 kDa
	X	X					calpain-like cysteine peptidase, putative,cysteine peptidase, Clan CA, family C2, putative	LinJ20.1220	92 kDa
				X	X		calpain-like cysteine peptidase, putative	LinJ20.1320	17 kDa
X	X			X	X	X	calpain-like cysteine peptidase, putative,calpain-like cysteine peptidase, Clan CA, family C2	LinJ20.1350	15 kDa
				X			phosphatase-like protein	LinJ20.1560	23 kDa
X	X			X			ribosomal protein s11 homolog	LinJ20.1620	16 kDa
					X		aminoacylase, putative	LinJ20.1670	43 kDa
X	X	X		X	X	X	hexokinase, putative	LinJ21.0300	52 kDa
					X		hypothetical protein, conserved	LinJ21.0330	47 kDa
				X			dihydrolipoamide acetyltransferase precursorlike protein	LinJ21.0450	40 kDa
		X		X			la RNA binding protein, putative	LinJ21.0600	37 kDa
				X			hypothetical protein, conserved	LinJ21.0720	249 kDa
X				X		X	60S Ribosomal protein L36, putative	LinJ21.0800	12 kDa
X	X						proteasome regulatory non-ATP-ase subunit 5, putative,19S proteasome regulatory subunit	LinJ21.0840	54 kDa
		X					methionyl-tRNA synthetase, putative	LinJ21.0890	84 kDa
				X			adenylate kinase, putative	LinJ21.0920	30 kDa
	X						histone H2A	LinJ21.1160	14 kDa
					X		hypothetical protein, conserved	LinJ21.1270	47 kDa
X							60S ribosomal protein L9, putative	LinJ21.1290	22 kDa
X	X		X	X			40S ribosomal protein S23, putative	LinJ21.1300	16 kDa
				X	X		ATP synthase F1 subunit gamma protein, putative	LinJ21.1530	34 kDa
			X		X		RNA helicase, putative	LinJ21.1820	59 kDa
	X						hypothetical protein, conserved	LinJ21.1830	47 kDa
X	X		X	X			proteasome alpha 2 subunit, putative	LinJ21.2070	25 kDa
X							60S ribosomal protein L32	LinJ21.2090	15 kDa
X	X	X	X	X	X		40S ribosomal protein S6, putative	LinJ21.2150	28 kDa
X							60S ribosomal protein L37a, putative | location=Lin.chr21:742926-743204(+) | length=92	LinJ21.2190	10 kDa
X	X		X	X			proteasome alpha 5 subunit, putative,20S proteasome subunit alpha 5, putative	LinJ21.2200	27 kDa
				X			hypothetical protein, conserved	LinJ22.0170	28 kDa
				X			LinJ22.1230 centrin, putative	LinJ22.1230	16 kDa
				X			LinJ22.1280 i/6 autoantigen-like protein	LinJ22.1280	23 kDa
X				X			40S ribosomal protein L14, putative	LinJ22.1370	26 kDa
				X			CCR4 associated factor, putative	LinJ22.1450	38 kDa
				X	X		peroxidoxin	LinJ23.0050	25 kDa
			X				mannose-1-phosphate guanyltransferase	LinJ23.0120	42 kDa
	X	X	X	X	X	X	endoribonuclease L-PSP (pb5), putative	LinJ23.0220	17 kDa
	X		X				pteridine reductase 1	LinJ23.0310	30 kDa
				X			(H+)-ATPase G subunit, putative	LinJ23.0380	12 kDa
	X	X	X	X	X		NADP-dependent alcohol dehydrogenase, putative	LinJ23.0400	38 kDa
					X		hypothetical protein, conserved	LinJ23.0410	14 kDa
		X					aldose 1-epimerase-like protein	LinJ23.0470	44 kDa
	X						acetyl-CoA synthetase, putative	LinJ23.0580	78 kDa
	X						kinesin, putative	LinJ23.0720	75 kDa
		X		X	X		3-ketoacyl-coa thiolase-like protein	LinJ23.0860	47 kDa
		X		X			acetyl-CoA synthetase, putative	LinJ23.0880	77 kDa
X	X						hypothetical protein, unknown function	LinJ23.1020	57 kDa
	X						hypothetical protein, unknown function	LinJ23.1150	34 kDa
	X						hypothetical protein, conserved	LinJ23.1160	80 kDa
	X			X			hypothetical protein, unknown function	LinJ23.1190	15 kDa
				X	X		t-complex protein 1, gamma subunit, putative	LinJ23.1420	60 kDa
		X					hypothetical protein, conserved	LinJ23.1880	27 kDa
	X		X		X		hypothetical protein, conserved	LinJ23.1980	17 kDa
X	X			X			60S ribosomal protein L17, putative	LinJ24.0040	19 kDa
		X					malic enzyme	LinJ24.0780	63 kDa
		X	X	X		X	triosephosphate isomerase	LinJ24.0870	27 kDa

				X			hypothetical protein	LinJ24.1360	30 kDa
				X			60S ribosomal protein L26, putative	LinJ24.1440	16 kDa
				X	X	X	hypothetical protein, conserved	LinJ24.1500	55 kDa
		X	X	X	X		IgE-dependent histamine-releasing factor, putative	LinJ24.1560	19 kDa
		X		X			transketolase	LinJ24.2150	72 kDa
X				X	X	X	40S ribosomal protein S8, putative	LinJ24.2160	25 kDa
			X	X			RNA-binding protein, putative, UPB1	LinJ25.0500	27 kDa
			X	X			eukaryotic initiation factor 5a, putative	LinJ25.0740	18 kDa
	X	X	X	X	X	X	cyclophilin a	LinJ25.0940	19 kDa
					X		dynein heavy chain, putative	LinJ25.1010	536 kDa
	X	X		X		X	aldehyde dehydrogenase, mitochondrial precursor	LinJ25.1160	54 kDa
				X	X		cytochrome c oxidase VII, putative	LinJ25.1170	19 kDa
X	X	X		X	X		ATPase beta subunit, putative	LinJ25.1210	56 kDa
				X			GTP-binding protein, putative	LinJ25.1470	24 kDa
X				X			hypothetical protein, conserved	LinJ25.1670	37 kDa
X				X			pyruvate dehydrogenase E1 beta subunit, putative	LinJ25.1790	38 kDa
		X		X	X		hypothetical protein, conserved	LinJ25.2090	30 kDa
				X			hypothetical protein, conserved	LinJ25.2110	33 kDa
				X			succinyl-CoA synthetase alpha subunit, putative	LinJ25.2230	31 kDa
				X			serine/threonine-protein kinase, putative	LinJ25.2460	44 kDa
				X			APRTASE adenine phosphoribosyltransferase	LinJ26.0120	26 kDa
X	X			X	X		60S ribosomal protein L7, putative	LinJ26.0150	31 kDa
		X					C-1-tetrahydrofolate synthase, cytoplasmic, putative	LinJ26.0310	32 kDa
				X			CPN10 10 kDa heat shock protein, putative	LinJ26.0590	11 kDa
				X			protein disulfide isomerase, putative	LinJ26.0630	41 kDa
				X			glutathione peroxidase-like protein, putative	LinJ26.0780	19 kDa
				X	X		ribosomal protein S16, putative	LinJ26.0840	17 kDa
	X		X	X			heat shock protein 70-related protein	LinJ26.1220	70 kDa
				X			prefoldin-like protein	LinJ26.1340	22 kDa
		X	X	X			thimet oligopeptidase, putative,metallo-peptidase, Clan MA(E), Family M3	LinJ26.1550	77 kDa
				X	X		cytochrome c oxidase subunit V, putative	LinJ26.1720	22 kDa
	X						mitotubule-associated protein Gb4, putative	LinJ26.1950	287 kDa
				X			hypothetical protein, conserved	LinJ26.1970	39 kDa
		X					nitrilase, putative	LinJ26.2290	31 kDa
X							60S ribosomal protein L35, putative	LinJ26.2350	15 kDa
		X	X				hypothetical protein, unknown function	LinJ26.2710	41 kDa
				X			calpain-like cysteine peptidase, putative	LinJ27.0420	69 kDa
				X			hypothetical protein, conserved	LinJ27.0840	35 kDa
				X	X		T-complex protein 1, beta subunit, putative	LinJ27.0850	58 kDa
				X			hypothetical protein, conserved	LinJ27.0920	60 kDa
				X			60S acidic ribosomal subunit protein, putative	LinJ27.1000	35 kDa
		X		X			arginyl-tRNA synthetase, putative	LinJ27.1230	78 kDa
				X	X		glycosomal phosphoenolpyruvate carboxykinase, putative	LinJ27.1470	58 kDa
X	X						hypothetical protein, conserved	LinJ27.1630	38 kDa
		X		X			hypothetical protein, conserved	LinJ27.1920	22 kDa
					X		heat shock protein DnaJ, putative	LinJ27.2030	44 kDa
	X			X			proteasome beta 3 subunit, putative	LinJ28.0110	22 kDa
X				X			ribosomal protein s26, putative	LinJ28.0570	13 kDa
	X						major surface protease gp63, putative,leishmanolysin, putative	LinJ28.0600	61 kDa
				X			ribonucleoside-diphosphate reductase large chain, putative	LinJ28.0930	91 kDa
					X		hypothetical protein, conserved	LinJ28.0970	46 kDa
				X			hypothetical protein, conserved	LinJ28.1080	44 kDa
		X			X		ribosomal protein s20, putative	LinJ28.1100	13 kDa
				X			electron-transfer-flavoprotein, alpha polypeptide, putative	LinJ28.1170	34 kDa
	X			X	X	X	glucose-regulated protein 78, putative	LinJ28.1310	72 kDa
	X			X			glycosomal membrane protein, putative	LinJ28.2430	24 kDa
X				X			eukaryotic translation initiation factor, putative	LinJ28.2480	46 kDa
					X		vacuolar ATP synthase subunit B, putative	LinJ28.2540	56 kDa
X	X						2-oxoglutarate dehydrogenase, E2 component, dihydrolipoamide succinyltransferase, putative	LinJ28.2600	42 kDa
				X	X	X	40S ribosomal protein S17, putative	LinJ28.2680	16 kDa
		X	X	X			activated protein kinase c receptor (LACK)	LinJ28.2940	34 kDa
	X	X					heat-shock protein hsp70, putative	LinJ28.2950	68 kDa
X	X	X	X	X	X	X	heat-shock protein hsp70, putative	LinJ28.2960	71 kDa
		X		X			cytosolic malate dehydrogenase	LinJ28.3090	34 kDa
	X	X					glutamate dehydrogenase, putative	LinJ28.3140	49 kDa
				X			proteasome regulatory non-ATPase subunit, putative	LinJ29.0120	99 kDa
				X	X		40S ribosomal protein S2	LinJ29.0310	29 kDa
					X	X	ABC transporter, putative	LinJ29.0770	207 kDa
				X			hypothetical protein, conserved	LinJ29.0470	37 kDa
				X			cofilin-like protein	LinJ29.0660	16 kDa
		X	X				guanine deaminase, putative	LinJ29.0920	49 kDa
				X			LPG3 lipophosphoglycan biosynthetic protein, putative	LinJ29.0920	87 kDa
				X	X		cysteine peptidase C (CPC)	LinJ29.0990	37 kDa
					X		hypothetical protein, conserved	LinJ29.1070	53 kDa
				X			ADP ribosylation factor 3, putative	LinJ29.1080	20 kDa
X	X				X		ribosomal protein L1a, putative	LinJ29.1160	41 kDa
		X					hypothetical protein, unknown function	LinJ29.1330	79 kDa
				X	X		tryparedoxin	LinJ29.1400	17 kDa
					X		paraflagellar rod protein 1D, putative	LinJ29.2030	69 kDa
		X					fumarate hydratase, putative	LinJ29.2080	63 kDa
X				X			GTP-binding protein, putative	LinJ29.2310	78 kDa
X	X			X	X		60S ribosomal protein L13, putative	LinJ29.2570	25 kDa
	X				X		ATP-dependent phosphofructokinase	LinJ29.2620	54 kDa
	X			X	X		40S ribosomal protein S19-like protein	LinJ29.2970	18 kDa
				X			inosine-adenosine-guanosine-nucleoside hydrolase, putative	LinJ29.3130	37 kDa
		X					2-hydroxy-3-oxopropionate reductase, putative	LinJ30.0170	31 kDa
		X		X			aspartyl-tRNA synthetase, putative	LinJ30.0470	63 kDa
					X		heat shock protein, putative	LinJ30.0610	85 kDa
				X			kinesin, putative	LinJ30.0670	68 kDa
		X					hypothetical protein, conserved	LinJ30.0680	80 kDa
				X			hypothetical protein, conserved	LinJ30.1210	17 kDa
				X			adenosine kinase, putative	LinJ30.1250	37 kDa
		X					Pyridoxal kinase, putative	LinJ30.1310	33 kDa
				X			p22 protein precursor, putative	LinJ30.1340	23 kDa
		X		X	X	X	heat shock 70-related protein 1, mitochondrial precursor, putative	LinJ30.2470	69 kDa
		X		X			heat shock 70-related protein 1, mitochondrial precursor, putative	LinJ30.2480	72 kDa
					X	X	PAPLE22 reticulon domain protein, 22 kDa potentially aggravating protein	LinJ30.2910	22 kDa
	X			X	X	X	glyceraldehyde 3-phosphate dehydrogenase, glycosomal	LinJ30.2990	39 kDa
X				X			eukaryotic translation initiation factor 3 subunit 7-like protein	LinJ30.3080	61 kDa
				X			hypothetical protein, conserved	LinJ30.3220	13 kDa
				X			hypothetical protein, conserved	LinJ30.3480	36 kDa
	X	X	X	X	X	X	S-adenosylmethionine synthetase	LinJ30.3560	43 kDa
				X			LIP 60S acidic ribosomal protein P2, putative	LinJ30.3570	10 kDa
				X			HMGR 3-hydroxy-3-methylglutaryl-CoA reductase, putative	LinJ30.3600	46 kDa
X	X			X			ribosomal protein L15, putative	LinJ30.3710	24 kDa
					X	X	60S ribosomal protein L9, putative	LinJ30.3760	21 kDa
				X			ATP synthase, epsilon chain, putative	LinJ30.4090	20 kDa
				X			hypothetical protein, conserved	LinJ31.0080	26 kDa
	X				X		cytoskeleton-associated protein CAP5.5, putative,cysteine peptidase, Clan CA, family C2, putative	LinJ31.0450	80 kDa
		X	X	X			biotin/lipoate protein ligase-like protein	LinJ31.1070	29 kDa
					X		small GTP-binding protein Rab1	LinJ31.0990	22 kDa
	X			X	X		vacuolar-type proton translocating pyrophosphatase 1, putative	LinJ31.1240	83 kDa
				X			hypothetical protein, unknown function	LinJ31.1400	39 kDa

				X			hypothetical protein, unknown function	LinJ31.1400	39 kDa
X				X			nucleosome assembly protein-like protein	LinJ31.1770	45 kDa
	X						GP63-like protein, leishmanolysin-like protein,metallo-peptidase, Clan MA(M), Family M8	LinJ31.2040	67 kDa
	X	X	X	X	X		prostaglandin f2-alpha synthase	LinJ31.2210	32 kDa
	X						3'-nucleotidase/nuclease precursor, putative	LinJ31.2380	42 kDa
		X					phosphoglycan beta 1,3 galactosyltransferase 5	LinJ31.3210	124 kDa
				X			adp-ribosylation factor, putative	LinJ31.3500	20 kDa
X	X		X	X	X	X	ATP-dependent RNA helicase, putative	LinJ32.0410	67 kDa
				X			hypothetical protein, conserved	LinJ32.0730	38 kDa
			X	X	X		RNA binding protein, putative	LinJ32.0790	25 kDa
				X			hypothetical protein, conserved	LinJ32.0920	21 kDa
X	X			X	X		60S ribosomal protein L18a, putative	LinJ32.0930	21 kDa
				X			hypothetical protein, conserved	LinJ32.1240	56 kDa
				X			chaperonin containing t-complex protein, putative	LinJ32.1410	59 kDa
		X	X	X	X	X	iron superoxide dismutase, putative	LinJ32.1910	22 kDa
X				X			hypothetical protein, conserved	LinJ32.2330	26 kDa
X	X			X			ribosomal protein L27, putative	LinJ32.2830	15 kDa
X	X	X	X	X	X	X	nucleoside diphosphate kinase b	LinJ32.3100	17 kDa
	X				X		hypothetical protein, conserved,leucine rich repeat protein, putative	LinJ32.3200	96 kDa
X	X			X		X	ribosomal protein L3, putative	LinJ32.3330	48 kDa
		X		X			glucosamine-6-phosphate isomerase, putative	LinJ32.3460	31 kDa
		X		X		X	dihydrolipoamide dehydrogenase, putative	LinJ32.3510	51 kDa
				X	X		chaperonin alpha subunit, putative	LinJ32.3830	59 kDa
X				X			60S ribosomal protein L2, putative	LinJ32.4050	28 kDa
		X			X		thiol-dependent reductase 1	LinJ33.0260	50 kDa
X	X		X	X	X	X	heat shock protein 83-1	LinJ33.0350	79 kDa
X	X	X					heat shock protein 83-1	LinJ33.0360	81 kDa
	X			X			hypothetical protein, conserved	LinJ33.1090	19 kDa
		X					mitogen activated protein kinase, putative,map kinase, putative	LinJ33.1470	47 kDa
				X			hypothetical protein, conserved	LinJ33.1470	45 kDa
				X			peptidase M20/M25/M40, putative	LinJ33.1710	52 kDa
				X			cyclophilin, putative	LinJ33.1730	29 kDa
	X						hypothetical protein, conserved	LinJ33.2570	56 kDa
					X		hypothetical protein, conserved	LinJ33.2600	17 kDa
				X			D-alanyl-glycyl endopeptidase-like protein	LinJ33.2650	44 kDa
		X	X	X		X	carboxypeptidase, putative,metallo-peptidase, Clan MA(E), Family M32	LinJ33.2670	57 kDa
		X					aminopeptidase, putative,metallo-peptidase, Clan MF, Family M17	LinJ33.2700	56 kDa
				X			hypothetical protein, conserved	LinJ34.0010	33 kDa
		X					glucose-6-phosphate 1-dehydrogenase, putative	LinJ34.0080	63 kDa
		X		X	X		malate dehydrogenase	LinJ34.0150	33 kDa
				X			eEF1B beta translation elongation factor 1-beta	LinJ34.0730	26 kDa
	X		X	X			hypothetical protein, conserved	LinJ34.1620	17 kDa
	X						hypothetical protein, conserved	LinJ34.1630	15 kDa
				X			acyl-coa binding protein	LinJ34.2210	10 kDa
X			X	X	X	X	hypothetical protein, conserved	LinJ34.2410	22 kDa
						X	regulatory subunit of protein kinase a-like protein	LinJ34.2460	72 kDa
X	X						40S ribosomal protein S19 protein, putative	LinJ34.2620	20 kDa
				X			hypothetical protein, conserved	LinJ34.2880	24 kDa
X							60S ribosomal protein L21, putative	LinJ34.3440	18 kDa
				X			hypothetical protein, conserved	LinJ34.3860	89 kDa
				X			ubiquitin-like protein	LinJ34.4120	9 kDa
X	X			X	X		20s proteasome beta 7 subunit, putative	LinJ34.4390	25 kDa
		X	X	X	X		pyruvate kinase	LinJ35.0030	50 kDa
X			X				60S ribosomal protein L30	LinJ35.0240	11 kDa
					X		hypothetical protein, conserved	LinJ35.0240	14 kDa
					X		ATP-dependent DEAD-box RNA helicase, putative	LinJ35.0470	46 kDa
X	X		X	X	X		40S ribosomal protein S3A, putative	LinJ35.0400	30 kDa
		X	X				hypothetical protein, conserved	LinJ35.0450	98 kDa
			X				beta-fructofuranosidase-like protein	LinJ35.0640	40 kDa
X	X		X				beta-fructofuranosidase-like protein	LinJ35.0650	78 kDa
	X			X			proteasome activator protein pa26, putative	LinJ35.0770	24 kDa
		X	X				aspartate aminotransferase, putative	LinJ35.0840	46 kDa
X				X			casein kinase, putative	LinJ35.1030	40 kDa
				X			aldose 1-epimerase-like protein	LinJ35.1080	42 kDa
		X					short chain dehydrogenase, putative	LinJ35.1240	28 kDa
	X			X	X		mitochondrial processing peptidase, beta subunit, putative,metallo-peptidase, Clan ME, Family M16	LinJ35.1390	55 kDa
				X			arginase, putative	LinJ35.1640	36 kDa
X					X	X	60S ribosomal protein L5, putative	LinJ35.1870	34 kDa
							60S ribosomal protein L32	LinJ35.2200	15 kDa
		X	X	X		X	kinetoplastid membrane protein-11	LinJ35.2250	11 kDa
		X	X			X	aminopeptidase P, putative,metallo-peptidase, Clan MG, Family M24	LinJ35.2400	54 kDa
					X		ATP-dependent RNA helicase, putative	LinJ35.3200	101 kDa
	X	X		X	X		cystathione gamma lyase, putative	LinJ35.3280	45 kDa
					X		6-phosphogluconate dehydrogenase, decarboxylating, putative	LinJ35.3440	52 kDa
X				X			60S ribosomal subunit protein L31, putative	LinJ35.3330	27 kDa
				X	X		Gim5A protein, putative	LinJ35.3800	25 kDa
X				X			60S ribosomal protein L27A/L29, putative	LinJ35.3810	16 kDa
X	X			X			60S ribosomal protein L23, putative	LinJ35.3840	15 kDa
		X					proteasome beta 2 subunit, putative	LinJ35.3880	28 kDa
				X			LinJ35.3950 t-complex protein 1, eta subunit, putative	LinJ35.3950	62 kDa
X				X			poly(a) binding protein, putative	LinJ35.4200	65 kDa
			X	X			hypothetical protein, conserved	LinJ35.4540	22 kDa
			X				peptidyl-prolyl cis-trans isomerase (cyclophilin- 40), putative	LinJ35.4830	39 kDa
	X	X	X		X		proteasome alpha 1 subunit, putative	LinJ35.4910	27 kDa
		X	X				pyruvate kinase	LinJ35.5450	55 kDa
				X			chaperone protein DNAJ, putative	LinJ36.0110	38 kDa
X		X	X	X	X		elongation factor 2	LinJ36.0190	94 kDa
			X	X			proteasome beta 2 subunit, putative	LinJ36.0340	23 kDa
				X	X		hypothetical protein, conserved	LinJ36.0430	29 kDa
				X			chaperonin, putative	LinJ36.0590	58 kDa
				X			PDI protein disulfide isomerase	LinJ36.0630	52 kDa
				X	X		eukaryotic translation initiation factor 3 subunit 8, putative	LinJ36.0670	82 kDa
			X				40S ribosomal protein S18, putative	LinJ36.0990	19 kDa
			X	X			ribosomal protein L24, putative	LinJ36.1130	15 kDa
X	X	X		X	X	X	fructose-1,6-bisphosphate aldolase	LinJ36.1320	41 kDa
		X		X			translation elongation factor 1-beta, putative	LinJ36.1490	23 kDa
				X			40S ribosomal protein S10, putative	LinJ36.1640	19 kDa
			X	X			proteasome alpha 1 subunit, putative	LinJ36.1670	30 kDa
				X	X	X	40S ribosomal protein S18, putative	LinJ36.1690	17 kDa
			X	X			proteasome beta 5 subunit, putative	LinJ36.1730	34 kDa
				X			Eukaryotic translation initiation factor 6 (eIF-6), putative	LinJ36.1740	27 kDa
	X						nucleoside transporter 1, putative	LinJ36.2040	54 kDa
		X	X	X			phosphomannomutase, putative	LinJ36.2070	28 kDa
				X			ubiquitin-like protein, putative	LinJ36.2100	33 kDa
			X	X		X	chaperonin Hsp60, mitochondrial precursor	LinJ36.2130	61 kDa
				X			hypothetical protein, conserved	LinJ36.2180	26 kDa
					X		EIF3-interacting protein-like protein	LinJ36.2410	63 kDa
		X					dipeptidyl-peptidase 8-like serine peptidase, putative,serine peptidase, Clan SC, Family S9B	LinJ36.2550	94 kDa
				X			stress-inducible protein STI1 homolog	LinJ36.2590	29 kDa
	X						membrane-bound acid phosphatase 2, putative	LinJ36.2720	63 kDa
				X			hs1vu complex proteolytic subunit-like	LinJ36.2760	24 kDa
X							dihydrolipoamide acetyltransferase precursor, putative	LinJ36.2790	49 kDa
	X		X	X			40S ribosomal protein S24e	LinJ36.3010	16 kDa
		X		X			succinyl-CoA ligase [GDP-forming] beta-chain, putative	LinJ36.3100	44 kDa

			X		X	X	X	14-3-3 protein-like protein	LinJ36.3360	30 kDa
			X		X			cysteine synthase	LinJ36.3750	34 kDa
	X							40S ribosomal protein S27-1, putative	LinJ36.3940	10 kDa
			X	X		X		glycyl tRNA synthetase, putative	LinJ36.4030	70 kDa
	X					X		eukaryotic translation initiation factor 3 subunit, putative	LinJ36.4070	39 kDa
	X	X	X	X	X	X	X	S-adenosylhomocysteine hydrolase	LinJ36.4100	48 kDa
					X			UMSBP1 universal minicircle sequence binding protein, putative	LinJ36.4120	13 kDa
					X	X		clathrin heavy chain, putative	LinJ36.4140	191 kDa
			X	X	X	X		oxidoreductase, putative	LinJ36.4380	36 kDa
					X	X		chaperonin Hsp60, mitochondrial precursor	LinJ36.4560	59 kDa
					X		X	glyceraldehyde 3-phosphate dehydrogenase, cytosolic, putative	LinJ36.4900	35 kDa
					X			sterol 24-c-methyltransferase, putative	LinJ36.4930	40 kDa
	X		X	X	X	X		40S ribosomal protein SA, putative	LinJ36.5240	28 kDa
		X				X		hypothetical protein, conserved	LinJ36.5380	71 kDa
						X		hypothetical protein, conserved	LinJ36.5660	103 kDa
	X			X		X		isoleucyl-tRNA synthetase, putative	LinJ36.5870	126 kDa
					X			hypothetical protein, conserved	LinJ36.6350	58 kDa
	X	X		X				tartrate-sensitive acid phosphatase acp-3.2, putative	LinJ36.6740	35 kDa
			X	X		X	X	histidine secretory acid phosphatase, putative	LinJ36.6770	71 kDa
			X	X				2,3-bisphosphoglycerate-independent phosphoglycerate mutase	LinJ36.6960	61 kDa
			X					prolyl oligopeptidase, putative,serine peptidase clan SC, family S9A, putative	LinJ36.7060	78 kDa
Number identifications	108	135	141	110	285	151	64			

Total number of unique identifications in all groups = 994
Total number of unique proteins = 446