

***Leishmania*, the phagosome, and host responses: the journey of a parasite**

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Abstract

Leishmania is the eukaryotic parasite responsible for leishmaniasis, a spectrum of diseases that puts at risk roughly 350 millions of people in 98 countries according to the Drugs for Neglected Diseases *initiative* (DNDⁱ). This parasite has a complex life cycle composed of two distinct stages, the promastigote form found in the female sand-fly vector and the amastigote form that replicates in the mammalian host [1]. To survive, the parasite interacts with its host immune system at multiple levels. In this review, we discuss the nature of those interactions, how they affect the host immune system, and how they affect parasite survival from the very beginning of the life cycle in the vector to its dissemination within the mammalian host.

1- Introduction

1.1.0 - Within the fly

The journey of *Leishmania* into its mammalian host begins in the midgut of female hematophagous sand flies, where *Leishmania* promastigotes proliferate and mature into infectious metacyclic forms. These infectious promastigotes migrate through the invertebrate vector oesophagus, causing its blockade, where they are finally ready to be inoculated into their mammalian host. Upon the following blood meal, sand flies regurgitate the parasites into the host bite wound. There, the parasites are rapidly taken up by various phagocytes populations recruited at the site of inoculation, including neutrophils, monocytes and macrophages [2, 3].

1.2.0 - A necessary sacrifice

During the establishment of infection within their mammalian hosts, not all *Leishmania* promastigotes are successful. Indeed, death of a sub-population of *L. major* is crucial for the infection process since injection of live parasite only leads to parasite clearance and to the development of host immunity [4]. This parasite mortality occurs naturally within the gut of infected sand flies and generates apoptotic-like *L. amazonensis* promastigotes that display phosphatidylserine (PS) at their surface, a lipid found on apoptotic cells, which are recognized by phagocytes [5]. Recognition of PS on apoptotic host cells is a mechanism that leads to a non-aggressive activation of phagocytes where inflammatory cytokines secretion is inhibited to avoid unnecessary immune reaction and where proinflammatory molecules such as TGF- β , PGE₂ and PAF are produced [6, 7]. Presence of apoptotic-like *L. major* promastigotes represents a crucial step that enables survival of the inoculated parasites, as they inhibit the immune response triggered by the

recognition of live parasites. Interestingly, this inhibition has been linked to an alternative internalization process, known as LC3-associated phagocytosis (LAP), and not to the parasite itself. Internalisation through LAP indeed causes an augmentation of IL-10 and TGF β , two important players of the tolerogenic pathway, and a reduction of proinflammatory cytokines IL-1 β and IL-6 secretion [8]. The importance for *L. amazonensis* to hijack the autophagic machinery has been highlighted through the use of PS targeting antibodies [5]. When PS are efficiently targeted, lesion size and parasite load are greatly diminished [5].

2.0.0-Before phagocytosis

2.1.0 - Complement and galectin

The zinc-metalloprotease GP63, or leishmaniolysine, is among the various pathogenicity factors expressed by *Leishmania* promastigotes [9, 10]. This major surface antigen exerts several functions before and after internalisation by phagocytic cells. It has been shown to degrade the extracellular matrix at a sub-cutaneous level, which is believed to facilitate the parasite movement under the skin following inoculation of promastigotes into the dermis [11]. GP63 is also able to inhibit complement activity by binding C3b and to increase the conversion of C3b into C3bi, an inactive form of C3, through direct cleavage, and thereby reducing fixation of the terminal C5-C9 membrane attack complex [12]. Under normal circumstances, complement-driven lysis is an important component of the host innate response that contributes to the elimination of pathogens. Inactivation of this host defense mechanism by *Leishmania* promastigotes thus represents an important step towards survival in mammals, and to entry inside

macrophages as it leads to enhanced phagocytosis through the complement receptors 3 and 1 [13, 14]. Consistent with the ability of GP63 to cleave C3, GP63-deficient *L. major* promastigotes were shown to be susceptible to complement-mediated lysis [15]. In addition to complement, Galectin-3, a mammalian soluble β -galactoside-binding lectin involved in host defense against *Leishmania* [16, 17], was shown to bind to the surface of *L. major* promastigotes through LPG [18]. Interestingly, bound Galectin-3 is cleaved by GP63 which prevents the formation of high orders of Galectin-3 lattices and may influence the immune response against *Leishmania* [18, 19].

2.2.0 - Natural Killer cells

GP63 also counteracts various innate immune response actors such as natural killer (NK) cells through direct inactivation. This metalloprotease is indeed able to suppress the proliferation of IL-2-activated NK cells and affects expression of receptors at their surface [20, 21]. NK cells play an important role in the response to *L. major* through the production of IFN- γ which assists in driving an effective Th1 response [22]. In an experimental model of leishmaniasis, IL-12 was shown to drive a Th1 effective response associated with IFN- γ , IL-2 and TNF- α production while the Th2 susceptible response was associated to IL-4, IL-5, IL-10, IL-13 and TGF- β production [23, 24]. But even in resistant mice that establish a Th1 response with IFN- γ production throughout the infection, IL-10 production and accumulation in the entry wound after 2 weeks of infection was observed. This suggests that the recruitment of an higher amount of IFN- γ producing CD4+ T cells to the site of inoculation would be an effective way to obtain sterile cure [25].

2.3.0 - Phagocytes

Upon inoculation, species such as *L. chagasi* cause host cells migration to the wound in an attempt to repair damaged tissues. The first cells to arrive to the bite wound site are neutrophils followed by macrophages/monocytes/dendritic cells. This migration is favored by the saliva of the sand fly vector which was shown to enhance cell migration up to three-fold. An important diversity of cells is recruited to the inflammatory exudate but most of them surprisingly remain in the lining tissue surrounding the wound. *L. chagasi* also causes the influx of more neutrophils than macrophages which suggests a strategy to minimise the initial inflammatory response. An augmented flux of neutrophils to the wound is therefore quickly established to control the infection [26]. Neutrophils quickly undergo apoptosis and are taken up by macrophages and dendritic cells [27, 28]. Parasites taken up by neutrophils induce the expression of high levels of apoptotic markers. *Leishmania*-harboring apoptotic neutrophils are in turn internalized by dendritic cells, triggering an immunosuppressive response, which delays the development of acquired resistance [28]. This uptake of apoptotic neutrophils has been shown to allow *L. major* survival in the early infection steps, highlighting a mechanism by which *L. major* exploits the macrophage/dendritic cells-neutrophils interaction [29, 30]. Clearance of the infected apoptotic neutrophils by dendritic cells was also shown to lead to the inhibition of CD8+ T cells priming *in vitro* [31]. Consistent with this finding, depletion of neutrophils in susceptible BALB/c mice drove resistance to infection by inhibiting the Th2 response [32].

2.4.0 - Neutrophil Extracellular Traps

In addition to being internalized by neutrophils, promastigotes of various species including *L. amazonensis*, *L. donovani*, and *L. major*, can induce the rapid release of their DNA and a subset of their granule content, forming filamentous structures that capture and kill microorganisms named neutrophil extracellular traps (NETs) [33, 34]. Depending of the species, *Leishmania* are either killed or survive NETs [34]. Hence, *L. amazonensis* is killed by NETs, whereas in the case of *L. donovani*, NETs were proposed to immobilize promastigotes and facilitate their internalization by phagocytic cells such as monocytes. The ability to survive NETs is conferred by either LPG or the 3'-Nucleotidase/Nuclease, as parasites defective in LPG expression and parasites with diminished 3'-Nucleotidase/Nuclease activity were more susceptible to NETs killing [34, 35]. The 3'-Nucleotidase/Nuclease is a membrane anchored enzyme that was previously associated with nutrition since its activity is able to generate nucleotides and phosphate from nucleic acids [36-38].

3.0.0 -After phagocytosis

3.1.0 - Parasitophorous vacuole formation

Following internalisation via phagocytosis, promastigotes freshly established within newly formed phagosomes proceed to modify this compartment into a parasitophorous vacuole (PV), which can be either spacious and communal or tight and individual [39, 40]. These modifications are enabled by pathogenicity factors such as lipophosphoglycan (LPG) [41]. To influence PV formation and to promote parasite survival, LPG acts by disrupting phagosomal lipid microdomains. This disruption leads to the impairment of the NADPH oxidase assembly at the PV membrane and to the

exclusion of the vesicular proton-ATPase, thereby impairing vacuolar acidification [42-44]. One consequence of disrupting the integrity of the phagosomal lipid microdomains is a reduced phagosome fusogenicity towards endosomes and lysosomes [45]. This may be related to the fact that intact lipid microdomains are required for the phagosomal recruitment of synaptotagmin V, a regulator of membrane fusion [44]. LPG also induces the accumulation of periphagosomal F-actin, which may form a physical barrier that potentially prevents vesicular trafficking to the phagosome [46]. The role of LPG in the parasite survival is however restricted to the promastigote stage as this molecule is highly down-modulated in amastigotes [47]. Another pathogenicity factor, the cysteine proteinase B (CPB), contributes to *L. mexicana* virulence as knock-out parasite induced a protective Th1 response and poor lesion growth in BALB/c mice [48]. *L. mexicana* CPB was also shown to alter macrophages signaling and functions through the alteration of NF- κ B, STAT-1 α , and AP-1 which inhibits IFN- γ responses, production of nitric oxide, and lipopolysaccharide-induced macrophages IL-12 production [49, 50]. Recently, our group reported that *L. mexicana* CPB regulates virulence through the control of GP63 expression. Interestingly, expression of an exogenous GP63 into CPB knock-out parasites was sufficient to restore virulence in *L. mexicana* [51].

3.2.0 - Cytokines modulation

3.2.1 - By the promastigote

Once inside the macrophage, *L. major* proceeds to modulate the phagocytic cell cytokines secretion pattern to promote its phagocytosis and its survival. To accomplish this feat, the parasite uses a variety of mechanisms. Studies from the group of Olivier

revealed that GP63 causes the activation of protein tyrosine phosphatases such as SHP-1, PTP1B, and TCPTP that act along the JAK, MAPK and IRAK-1 kinase pathways [52-55]. GP63 also selectively downregulates host cell protein synthesis through alterations of the mTORC1-dependent signaling [56]. GP63 inactivates various transcription factors such as AP-1 and NF- κ B through specific cleavage and degradation of different subunits [57, 58]. More recently, GP63 was shown to target the nuclear envelope, where it degrades nucleoporins of the nuclear pore complexes [59]. These signaling and transcription modifications were proposed to inhibit host responses associated to the control of infection, such as the release of TNF, IL-12, and NO, which will contribute to the survival of the parasite and facilitate its replication [50, 55, 60]. In contrast, our group recently described a role for GP63 in inducing cytokine release, whereby GP63 cleaves synaptotagmin XI, a SNARE present on recycling endosomes and lysosomes and that negatively regulates the secretion of preformed stored TNF and IL-6 [61]. We showed that both of these pro-inflammatory cytokines are responsible for the augmentation of neutrophils and inflammatory monocytes influx to the parasite inoculation site. This augmented influx then indirectly contributes to the spread and maintenance of infection [62].

3.2.2 - By the amastigote

Between 24 to 48 h post-internalization, promastigotes transform into the non-flagellated amastigote forms. The signals that trigger the differentiation process are not fully understood and are believed to involve a temperature shift and acidification of the parasitophorous vacuole [63]. The effective differentiation into amastigotes has been

shown to significantly up-regulate the Th2-associated cytokines while avirulent parasites failed to differentiate into amastigotes and to induce such an immune response [64]. Another *Leishmania* pathogenicity factor, the kinetoplastid membrane protein-11 (KMP-11), whose expression is increased during metacyclogenesis and in *L. amazonensis* amastigote stage, was shown to exacerbate BALB/c mice peritoneal macrophages infection by increasing IL-10 secretion [65, 66]. KMP-11 was also shown to increase the macrophage arginase activity while reducing the nitric oxide production, thus promoting parasite survival [65]. KMP-11 is currently regarded as a potential vaccine target [67]. *Leishmania* amastigotes mimic apoptotic cells by exposing PS on their surface, and this contributes to the ability of *Leishmania* to evade the immune response. The levels of PS exposed at the parasite surface were positively linked to parasite infectivity, since parasites recovered from infected susceptible BALB/c exposed more PS than parasites isolated from resistant C57/BL6 mice, and were found to be more infectious [68].

4.0.0 Modulation of immune responses

4.1.0 Antigen presentation

Once established within its host cell, the parasite begins to modulate the host adaptive immune response. Hence, in addition to the suppressive effects of GP63 in macrophages, this protease targets specific proteins in other cell types [20, 52, 69, 70]. Indeed, GP63 alters the expression of surface receptors in NK cells and thereby inhibits their proliferation [20]. In T lymphocytes, GP63 cleaves the surface co-receptor CD4 [69]. Since CD4 is required for T lymphocyte activation, its cleavage by GP63 is likely to reduce T lymphocyte responses to antigen presenting cells. In line with these observations, *L.*

donovani and *L. major* inhibits cross-presentation (i.e. presentation of exogenous microbial antigens in the context of MHC class I to CD8⁺ T lymphocytes) by dendritic cells and macrophages, an event that is dependent on the cleavage of VAMP8 by GP63. As a result, activation of CD8⁺ T lymphocytes is reduced [70]. The importance of cross-presentation in *L. major* immunity was demonstrated in Balf3 null mice lacking the IL-12 producing and cross-presenting CD8a⁺ and CD103 DC subset [71]. These mice developed larger lesions with higher parasite burden linked to an impaired IFN- γ production and increased Th2 and Th17 cytokines with *Leishmania* specific immunoglobulin 1 (IgG1). IFN- γ was also shown to be mainly produced by the cross-presenting DC subset compared to the CD103 DC subset. Furthermore, Balf3 deletion and cross-presenting DC depletion during infection led to the similar increases in wound size and parasite load [72].

4.2.0 - T cells activation

Inhibition of cross-presentation is only one of the many ways devised by *Leishmania* to evade the adaptative immune response. Hence, *L. donovani* possesses the capacity to downregulate CD80 and ICAM-1 expression in infected cells which leads to the failure to optimally activate T cells while other species such as *L. major* efficiently modulate the expression of CD40, anergising T cells and favoring the development of Treg in the context of a lower expression of CD40. These Treg will in turn counteract the proinflammatory Th and exacerbate the infection [73-76]. The importance of CD40 in *L. major* infection has been shown through the use of CD40 knockout mice where a

significantly lower amount of IL-12 was produced leading to an ineffective Th2-response [74, 75].

4.3.0 - Dissemination

Subversion of the host immune response may represent an important step to allow dissemination of the parasites. Depending on the *Leishmania* species, dissemination may or may not occur. Species such as *L. donovani* will develop in the spleen and liver causing potentially fatal visceral leishmaniasis, whereas other species such as *L. major* will remain cutaneous and other such as *L. braziliensis* may disseminate and become mucosal [77-79].

4.3.1 - Matrix metalloproteinases (MMP)

The inflammation marker matrix metalloproteinase 9 (MMP-9) plays various roles including immune cell migration, macrophage recruitment, and effective granuloma formation. It has been shown to be associated with the dissemination of *Mycobacterium tuberculosis* and to have an augmented secretion and activation during *L. braziliensis* infection *in vitro* [79]. MMP-9 and MMP-2 contribute to cell migration through the degradation of the type IV collagen in the basal lamina [80]. Furthermore, macrophages from treated mucosal leishmaniasis, which results from *L. braziliensis* dissemination, displayed increased MMP-9 activity compared to macrophages from treated cutaneous leishmaniasis [80]. Recently, the soluble secretory serine protease (pSP) of *L. donovani* has been used as a new vaccine target adjuvanted with IL-12 to drive a protective Th1 response. The presence of antibodies against pSP led to reduced parasite internalization

by macrophages and to protection against the parasite. Groups receiving this vaccine had an increased Th1 associated cytokines such as IFN- γ and TNF while their Th2 associated cytokines such as IL-4 and IL-10 were decreased. More specifically, this protection is linked to the modification of the cytokine-mediated MMP-9 expression through TNF-induced MMP-9 expression down regulation by IFN- γ [81, 82]. Release of MMP-9 was increased by over 40% and an augmented secretion in co-culture of hepatocytes and infected macrophages leading to hepatocyte damage [83]. A similar phenomenon was observed in dogs suffering from visceral leishmaniasis where the level of active MMP-9 was much higher than in control dogs. Higher levels of proMMP-9 and proMMP-2 were also noted although no augmentation of active MMP-2 was found [84]. In cutaneous leishmaniasis patients receiving antimonial treatments, an important gelatinase activity was detected in the wounds of poorly responding patients, linked with an augmented presence of IFN- γ , TGF- β and IL-10 producing cells with a prevalence of IFN- γ while an augmentation of MMP-2 was detected in the wound of good responding patients with a prevalence of IL-10 [85]. MMP-2 was previously shown to be important for skin re-epithelialization[86].

4.3.2 - Effective cure markers

Although the increase in MMP-9 could be used by the parasite to migrate through the host body, its drastic augmentation combined with an augmentation of the soluble CD40L during antimonial treatment of visceral leishmaniasis was negatively correlated with parasite load and spleen size, identifying MMP-9 and CD40L as potential markers for an effective cure [87].

5.0.0 Conclusions and perspectives

Leishmania is a very complex and specialised parasite that possesses the ability to survive and thrive within its human host. To survive, *Leishmania* uses an arsenal of pathogenicity factors that includes LPG and GP63 to modify the phagosome into a parasitophorous vacuole, modulate the expression and secretion of cytokines, modulate the immune cells flux to the infection site and modulate the antigen presentation favoring a Th2 inefficient response. A lot is still unknown as to how *Leishmania* modifies the phagosome to ensure its survival, modulates the immune response, and migrates throughout the host body. A better understanding of this complex parasitic relationship will also require to take into consideration host genetic factors as well as the diversity of parasite species and strains.

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