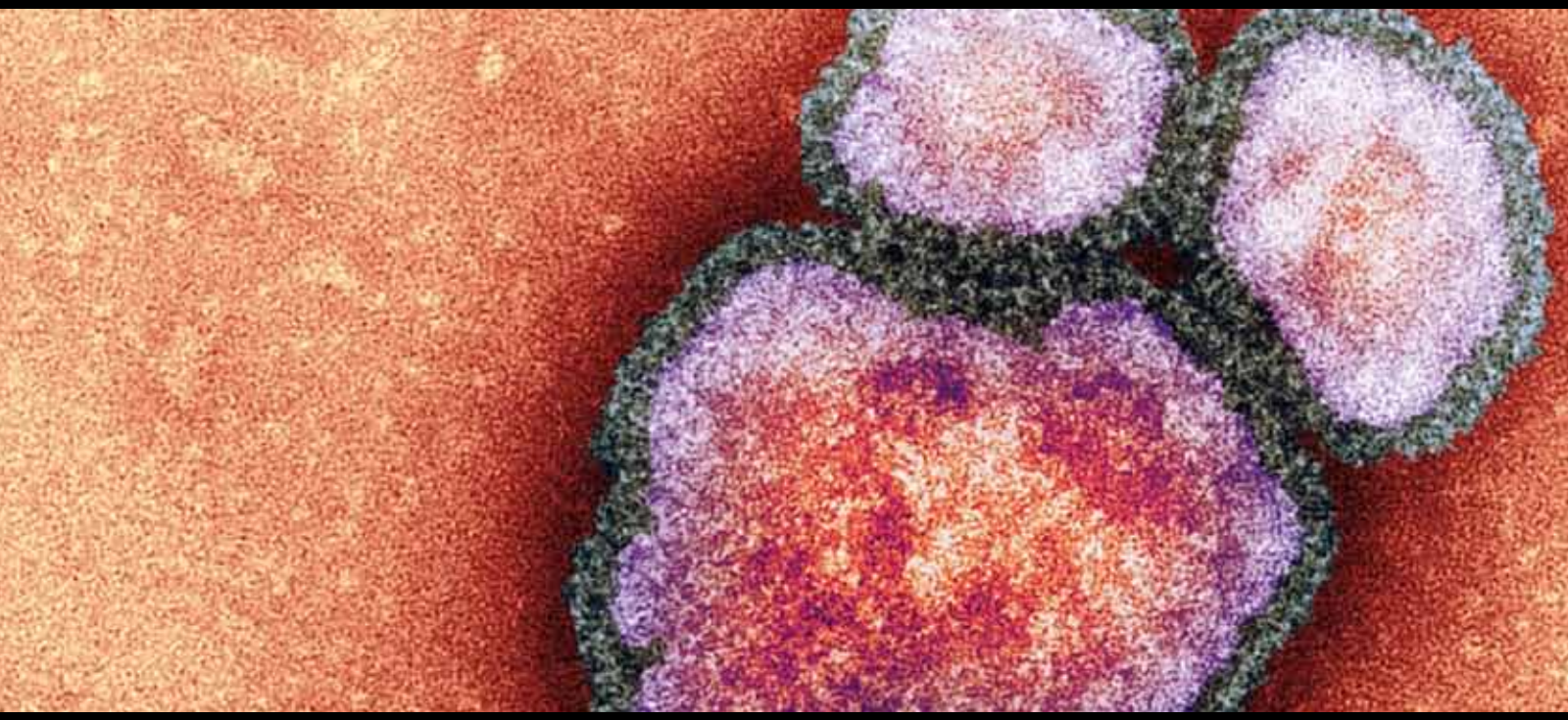
Journal of Tropical Medicine

Immunity to Visceral Leishmaniasis

Guest Editors: Nahid Ali, Asrat Hailu Mekuria, Jose M. Requena, and Christian Engwerda



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Editorial

Immunity to Visceral Leishmaniasis

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Leishmaniasis is a major vector-borne parasitic disease affecting 12 million people worldwide. With a broad range of clinical manifestations, ranging from self-healing skin ulcers to disfiguring mucosal lesions to life-threatening infections of visceral organs (liver and spleen), the disease has become a serious human health issue, particularly in developing countries. Among all of its forms, visceral leishmaniasis (VL, also known as kala-azar), caused by the *Leishmania donovani* complex (i.e., *L. donovani* and *L. infantum* in Old World and *L. chagasi* in New World), is often fatal in the absence of treatment. Although humans are the principal hosts for *L. donovani*, canine species are the main reservoirs of *L. infantum*. Canine VL affects millions of dogs and is associated with outbreaks of human VL and hence has become a major public health issue. The lack of vaccines to prevent and/or treat these infections, as well as the emergence of drug resistant parasites, is serious impediments to control leishmaniasis. Therefore, developing new prophylactic and therapeutic strategies against this disease is urgently required. However, for this to occur, a better understanding of the complex immune mechanisms generated in response to infection and defining those involved in resistance to infection is required. In this special issue on "*Immunity to visceral leishmaniasis*", several selected papers will discuss these issues.

The first paper in this special issue describes recent advances in vaccination strategies against VL. To date there is no vaccine available for any form of human leishmaniasis. The lack of knowledge relating to disease pathogenesis, the rise of coinfection with HIV, and cost of development are significant hindrances to vaccine development. Therefore, to reduce the burden of VL, particularly in resource poor nations, increased investment in VL vaccine development is required. The second paper in this issue focuses on the utility of live-attenuated parasites as vaccine candidates. Although subunit *Leishmania* vaccines have shown some efficacy in animal models, they have not yet been shown to have protective effects in humans. The recuperation of infected individuals and subsequent protection from reinfection indicates that infection with the parasite may be a prerequisite for the development of protective immunity. Thus, to achieve long lasting protection, genetically altered live-attenuated parasites could be a valuable tool. The third paper in this issue discusses recent advances made in cytokine and phenotypic cell profiles in different tissues and organs of dogs infected with *L. infantum*. Dogs represent an important reservoir of parasites in some regions, and infected dogs have been linked with human VL cases. This paper describes some of the complex immune responses developed by the dog in response to infection. The fourth paper in the issue describes evasion mechanisms employed by parasites that enable them to persist within the host. Parasites modulate the host immune response in the spleen and liver differently in mouse models, as indicated by a distinct pattern of organ-specific parasite growth during disease progression. While infection in the liver resolves within 6–8 weeks, a chronic infection becomes established in the spleen. Although the exact immune mechanisms responsible for parasite persistence in spleen are not known, it is now clear that the parasite

plays an important role in this process. The fifth paper in the issue describes novel immune evasion mechanisms employed by the *Leishmania* parasite, and in particularly, those used by virulent promastigote and amastigote forms that overexpress a glycoprotein enzyme (ectonucleotidase) on the parasite plasma membrane. The ectonculeotidase, with its catalytic sites facing extracellularly, hydrolyses extracellular nucleotides resulting in the production of adenosine. Increased levels of adenosine aid in the establishment of infection. Thus, the discovery of the immunomodulatory effects of adenosine and the characterisation of ectonucleotidases provide new insights into complex immune responses during leishmaniasis. The final paper in this issue discusses *Leishmania*-mediated manipulation of the signalling machinery of the host cell that enables parasite persistence. The identification of the key signalling mechanisms modulated by parasites will help to understand the strategies used by parasites to subvert the host immune system. Moreover, given that these signalling pathways could be manipulated pharmacologically, an improved understanding of the host-parasite interaction may allow the development of new therapies to control leishmaniasis.

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Review Article

Development of Vaccines against Visceral Leishmaniasis

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Leishmaniasis is a neglected disease resulting in a global morbidity of 2,090 thousand Disability-Adjusted Life Years and a mortality rate of approximately 60,000 per year. Among the three clinical forms of leishmaniasis (cutaneous, mucosal, and visceral), visceral leishmaniasis (VL) accounts for the majority of mortality, as if left untreated VL is almost always fatal. Caused by infection with *Leishmania donovani* or *L. infantum*, VL represents a serious public health problem in endemic regions and is rapidly emerging as an opportunistic infection in HIV patients. To date, no vaccine exists for VL or any other form of leishmaniasis. In endemic areas, the majority of those infected do not develop clinical symptoms and past infection leads to robust immunity against reinfection. Thus the development of vaccine for *Leishmania* is a realistic public health goal, and this paper summarizes advances in vaccination strategies against VL.

1. Introduction

There is currently no vaccine available for any form of leishmanias, including visceral leishmaniasis (VL), which if left untreated is almost always fatal. VL results from systemic infection with *L. infantum* (also known as *L. chagasi*) [1] which occurs in Europe, North Africa, South and Central America, and *L. donovani*, which is found throughout East Africa, India, and parts of the Middle East. Infection is initiated by the bite of an infected sand fly vector and parasites disseminate from the site of infection in the skin to reside and multiply within macrophages of the liver, spleen, and bone marrow [2]. VL, also known as kala-zar, is associated with fever, weight loss, enlargement of the spleen and liver, and anaemia. Leishmaniasis has strong links with poverty [3] and is considered one of the most neglected tropical diseases. Each year there are approximately 500,000 new cases of VL with over 90% of cases arising in India, Bangladesh, Nepal, Sudan, and Brazil. VL has become a frequent coinfection in HIV-positive individuals in endemic areas and is associated with enhanced onset of AIDS-related illness and increased VL treatment failure. Current VL therapy is based on the long-term parenteral administration of pentavalent antimonials, which, despite being expensive and highly toxic, has been the standard treatment for over 50 years.

Following *L. donovani* infection some individuals develop Post-kala-azar Dermal Leishmaniasis (PKDL), a complication that occurs either during or after treatment. During PKDL parasites reappear in the skin resulting in an array of small skin lesions, and patients are considered a significant infection reservoir because of the large number of parasites accessible to sand fly bites. Thus the treatment and control of PKDL are an important public health measure for controlling VL and must be considered in the development of VL vaccine strategies.

Host resistance to *Leishmania* infection is mediated by cellular immune responses leading to macrophage activation and parasite killing. Antileishmanial immunity is mediated by both innate and adaptive immune responses and requires effective activation of macrophages, dendritic cells (DCs), and antigen-specific $CD4^+$ and $CD8^+$ T cells [4]. Although strong humoral responses are induced by VL infection, antibodies play no role in protection and are often associated with disease exacerbation [5]. Effector $CD4^+$ T cells are responsible for the production of cytokines critical for the activation of macrophages and are required for optimal host response to infection [6]. Cytotoxic $CD8^+$ T cells also play

a host protective role, and are required for the effective clearance of parasites [7] and the generation of memory responses [8]. Interestingly, 80 to 90% of human infections are subclinical or asymptomatic, and this asymptomatic infection is associated with strong cell-mediated immunity. Only a small percentage of infected individuals develop severe disease, and patients who recover from VL display resistance to reinfection. This suggests the development of clinical immunity and provides a biological rationale for the development of VL vaccines that impart a strong cellular immunity.

Humans are the only known hosts for *L. donovani*; however *L. infantum* is primarily a zoonotic disease and canine species are the main animal reservoir. Canine visceral leishmaniaisis (CVL) affects millions of dogs in Europe, Asia, North Africa, and South America and has been associated with outbreaks of human VL [9]. Both symptomatic and asymptomatic *Leishmania*-infected dogs act as a source of parasites for VL transmission [10], and CVL represents a significant public health issue. A current approach to breaking the VL transmission cycle is the development of CVL vaccine, which may be crucial for controlling VL infection in human populations.

Vaccination against VL has received limited attention compared with cutaneous leishmaniasis (CL). Historically CL has been the focus of vaccination attempts, as it has been known for centuries that people who resolve a primary CL skin lesion are protected from further infections. It is generally acknowledged that human VL trials will follow on from any successful CL immunization programme. Ideally a vaccine would provide cross-protection against multiple *Leishmania* species. The recent comparative genomic analysis of three *Leishmania* species, which cause distinct disease pathologies, showed that *L. major*, *L. braziliensis*, and *L. infantum* genomes are highly conserved and have very few species-specific genes [11]. The level of amino acid conservation within coding regions is also high between species, suggesting that the major *Leishmania* antigens are conserved and that a pan species vaccine may be achievable. However there is a high degree of variability in the cross-protective immunity induced by infection with different *Leishmania* species [12, 13] and VL-specific vaccines may provide a more successful intervention.

Experimental infection models are used to screen and evaluate VL vaccines, and several animal species have been used including mice, hamsters, monkeys, and dogs [14]. However no single *in vivo* model accurately reflects all aspects of human VL disease, which has been a major limitation in the development of VL vaccines. The precise immune mechanisms underlying human VL are still not fully understood, and the responses necessary for protection by vaccination in experimental infection models may not reflect those required for efficacy in endemic areas [15].

The profile of an antileishmanial vaccine would need to incorporate several important features, such as safety, ease of production at a low cost in endemic countries, the induction of robust, long-term T cell responses, and both prophylactic and therapeutic efficacy. Ideally, such vaccine would offer cross-species effectiveness against CL and VL. As this might not be feasible, the development of a VL-specific vaccine remains an important global health priority.

2. First-Generation Vaccines

The only successful intervention against leishmaniasis is inoculation using virulent parasites, a process known as leishmanization (LZ). This ancient practise involves the administration of cutaneous *Leishmania* parasites to a discrete skin location, allowing a self-healing lesion to form. Initial immunological exposure then protects the individual from further infection and lesion development. LZ was traditionally practised by directly transferring infectious material from cutaneous lesions to uninfected individuals. However the establishment of an in vitro culture system in the early 20th century led to the large-scale production of promastigote forms of *Leishmania* for expanded clinical use. LZ induces a controlled, but full, infection and was successfully used as a prophylaxis throughout the Soviet Union, Asia, and the Middle East, with reported efficacy levels up to 100% [16, 17]. However LZ was largely abandoned due to safety issues associated with the use of live vaccines. Also, standardisation of the inoculum proved difficult as parasites used for LZ experience a dramatic loss of infectivity when subject to repeated subculturing [18]. Infection with live *Leishmania* also causes immunosuppression, which resulted in reduced immune responses to childhood vaccines and threatened the efficacy of immunization programmes [19, 20]. Currently only one country, Uzbekistan, employs the use of LZ, where a mixture of live and dead *L. major* is licensed as a vaccine for high-risk populations [21]. As LZ is the only vaccine strategy against *Leishmania* with proven efficacy in humans, efforts are being made to improve the safety of this practise. The inclusion of killed parasites in the inoculum and the use of adjuvants that promote rapid immune responses reduce the severity of primary lesions and accelerate wound healing during LZ [16, 22].

Research into first-generation vaccines based on whole-cell, killed *Leishmania* parasites dates back to the late 1930s, when pioneering work by Brazilian scientists demonstrated that killed parasites showed efficacy as both therapeutic and prophylactic vaccines [23]. Over the ensuing decades numerous preparations of killed parasites were tested, either alone or in combination with a variety of different adjuvants. Although displaying well-tolerated safety profiles, to date no first-generation vaccine using killed parasites has demonstrated sufficient efficacy as a prophylactic vaccine to be used in widespread control programmes [24]. Most vaccine studies focus on CL, and there have been no clinical trials of first-generation vaccines produced from VL *Leishmania* species. Due to the strongly conserved genomes of the *Leishmania* species, it is anticipated that human VL trials will follow any successful CL immunization program. Whether the same vaccine can show efficacy against both CL and VL remains to be determined. Interestingly, killed parasite vaccines using an alum-precipitated autoclaved *L. major* (ALM) given with a BCG adjuvant have shown promise as vaccines for VL and PKDL [25]. When given to patients with persistent PKDL in combination with antimonial therapy

this vaccine showed enhanced cure rates and lower incidence of relapse as compared to antimonial treatment alone [26]. Based on these initial studies, recommendations have been put forward for expanded trials to examine the prophylactic and therapeutic effects of the alum-ALM + BCG vaccine for PKDL and VL [27].

Evidence from experimental animal models supports the development of first-generation VL vaccines. Complete soluble antigen (CSA) from an attenuated *L. donovani* strain was effective as both a therapeutic and prophylactic vaccine in susceptible mice, without the use of an adjuvant [28]. Importantly, CSA immunization was effective against both pentavalent antimony sensitive and resistant strains of *L. donovani.* Vaccination with purified excreted-secreted antigens from *L. infantum* promastigotes (LiESAp) fully protected dogs from experimental challenge and induced a long-lasting cell-mediated immunity [29].

A major advantage of first-generation vaccines is that they are conceptually simple and relatively easy to produce in *Leishmania* endemic countries at low cost. However standardization of vaccines derived from cultured parasites is difficult, and this has hindered commercial development efforts. The route of administration, formulation, and adjuvant are also important considerations in the development of whole-parasite vaccines, and optimisation is essential for the induction of protective immune responses. The most recent clinical trials of first-generation vaccines have demonstrated a good safety profile but have not conferred significant levels of protection for use as prophylactic vaccines. However promising results from trials using therapeutic vaccination in combination with chemotherapy warrant further investigation.

3. Second-Generation Vaccines

The development of Second-generation vaccines for *Leishmania* has included recombinant proteins, polyproteins, DNA vaccines, liposomal formulation, and dendritic cell vaccine delivery systems. A variety of different molecules have been tested to date with varying degrees of efficacy (Table 1). In general VL vaccination studies have been hampered by the lack of a suitable animal model. The natural combination of dogs and *L. infantum* [30] and *L. donovani* in golden hamsters [31] reproduces many features of human VL. The canine model is particularly useful in evaluating vaccine candidates since successful vaccination of dogs might control the spread of disease to humans in endemic areas where the dog is the reservoir of the parasite [32]. However, both models suffer from lack of immunological reagents and assays needed for the characterisation of immune responses. Therefore, the mouse model of VL has been widely used to assess vaccine candidates. While experimental VL infection in mice does not fully reproduce the disease observed in humans, mice are competent hosts for both *L. donovani* and *L. infantum* and exhibit organ-specific pathology in the liver and spleen. Other major advantages of the mouse model are that it is amenable to genetic manipulation to create mutants with specific deficiencies in the immune system and a wide range of immunological reagents is available.

Only a small number of recombinant proteins have been tested against VL in murine models. Early studies showed that promastigote-derived membrane protein dp72 protected mice against *L. donovani* infection [59, 82], but there has been no further advance on the use of this antigen for the development of vaccines. The *L. donovani* amastigote LCR1 protein containing 67-amino-acid repeats homologous to repeats in a *Trypanosoma cruzi* flagellar polypeptide was tested for protection in mice. Recombinant protein led to partial protection against *L. infantum* challenge [44], while immunization with BCG-LCR1 elicited better protection [45]. Vaccine efficacy was influenced by the site of immunization with subcutaneous administration superior to intraperitoneal inoculation [45]. Recombinant hydrophilic acylated surface protein B1 (HASPB1), a member of a family of proteins expressed only in metacyclic and amastigote stages, has shown efficacy in an experimental mouse model of VL [8]. This vaccine did not require the use of adjuvant, and protection was associated with the induction of antigen-specific, IFN-γ producing $CD8^+$ T cells, a mechanism similar to DNA vaccination [8]. Immunization with the *L. donovani* A2 cysteine proteinase delivered as recombinant protein or as DNA also afforded protection against experimental challenge infection [60, 61]. Other antigens tested include amastigote cysteine proteases (CPs) [40], kinetoplastid membrane protein-11 (KMP-11) [65], amastigote LCR1 [45], leishmanial antigen ORFF [63], and NH36, a main component of the fucose-mannose ligand [47].

Apart from defined single molecules, multicomponent vaccines have been shown to protect against VL in experimental infection systems. Recombinant Q protein formed by fusion of antigenic determinants from four cytoplasmic proteins from *L. infantum* (Lip2a, Lip2b, P0, and histone H2A) coadministered with live BCG protected 90% of immunised dogs by enhancing parasite clearance [49]. To date, only one multicomponent vaccine, Leish-111f, has been assessed in clinical trials [83]. Leish-111f is a single polyprotein composed of three molecules fused in tandem: the *L. major* homologue of eukaryotic thiol-specific antioxidant (TSA), the *L. major* stress-inducible protein-1 (LmSTI1), and the *L. braziliensis* elongation and initiation factor (LeIF) [83]. There is some evidence that the Leish-111f vaccine can also induce partial protection against VL in animal models [56]; however, it failed to protect dogs against infection and did not prevent disease development in a Phase III vaccine trial in dogs [55]. An optimized version, known as Leish-110f, has recently demonstrated strong immunogenicity and some protective efficacy against *L. infantum* in mice [84]. The Leish-111f vaccine is moving forward into clinical trials as LeishF1 and is being trialled in combination with the MPL-SE adjuvant. This adjuvant consists of monophosphoryl lipid A, a potent TLR4 agonist, formulated with the antigen as a stable emulsion. A recent small-scale clinical trial in a *L. donovani* endemic area showed Leish-F1-MPL-SE was safe and well tolerated in people with and without prior VL exposure and induced strong antigen-specific T cell responses [85].

Table 1: Vaccines against visceral leishmaniasis.

(a)

Vaccines tested against *L. infantum*					
Antigen	Source of antigen	Vaccine delivery	Animal model	Outcome	Reference
KMPII, TRYP, LACK, and GP63	*L. infantum*	DNA vaccine	Dog	No protection	Rodríguez-Cortés et al., 2007 [33]
H2A, H2B, H3, and H4	*L. infantum*	DNA vaccine	Mouse	No protection	Carrión et al., 2008 [34]
p36 LACK	*L. infantum*	DNA vaccine + protein expressed in vaccinia virus	Mouse	Protection	Dondji et al., 2005 [35]
p36 LACK	*L. infantum*	DNA vaccine	Mouse	No protection	Marques-da-Silva et al., 2005 [36]
p36 LACK	*L. infantum*	DNA vaccine	Mouse	Protection	Gomes et al., 2007 [37]
p36 LACK	*L. infantum*	DNA vaccine + protein expressed in vaccinia virus	Dog	Protection	Ramiro et al., 2003 [38]
p36 LACK	*L. infantum*	DNA vaccine + protein expressed in vaccinia virus	Dog	Protection	Ramos et al., 2008 [39]
CPA and CPB	*L. infantum*	DNA vaccine + recombinant protein	Mouse	Protection	Rafati et al., 2006 [40]
CPA and CPB	*L. infantum*	DNA vaccine + recombinant protein	Dog	Protection	Rafati et al., 2005 [41]
CTE of CPIII	*L. infantum*	DNA vaccine + recombinant protein	Mouse	No protection	Rafati et al., 2008 [42]
CPC	*L. infantum*	DNA vaccine + recombinant protein	Mouse	Protection	Khoshgoo et al., 2008 [43]
LCR1	*L. infantum*	Recombinant protein	Mouse	Partial protection	Wilson, et al., 1995 [44]
LCR1	*L. infantum*	Protein expressed in BCG	Mouse	Partial protection	Streit et al., 2000 [45]
PapLe22	*L. infantum*	DNA vaccine	Hamster	Partial protection	Fragaki et al., 2001 [46]
NH36	*L. donovani*	DNA vaccine	Mouse	Partial protection	Aguilar-Be et al., 2005 [47]
FML	*L. donovani*	Protein	Mouse	Partial protection	Aguilar-Be et al., 2005 [47]
FML	*L. donovani*	Protein	Mouse	Protection	Oliveira-Freitas et al., 2006 [48]
Q protein	*L. infantum*	Recombinant fusion protein of Lip2a, Lip2b, P0, and H2A + BCG	Dog	Protection	Molano et al., 2003 [49]
Q protein	*L. infantum*	Recombinant fusion protein of Lip2a, Lip2b, P0, and H2A + BCG	Mouse	Partial protection	Parody et al., 2004 [50]
A2	*L. donovani*	Recombinant protein	Dog	Partial protection	Fernandes et al., 2008 [51]
A2 and NH	*L. donovani*	DNA vaccine	Mouse	Partial protection	Zanin et al., 2007 [52]
LiESAp	*L. infantum*	Native protein	Dog	Protection	Lemesre et al., 2005 [53] and 2007 [54]
LiESAp	*L. infantum*	Native protein	Dog	Protection	Bourdoiseau et al., 2009 [29]
Leish-111f	*L. major*	Recombinant polyprotein of TSA, LmSTI1, and LeIF	Dog	No protection	Gradoni et al., 2005 [55]
Leish-111f	*L. major*	Recombinant polyprotein of TSA, LmSTI1, and LeIF formulated in MPL-SE	Dog	Protection	Coler et al., 2007 [56]
Leish-111f	*L. major*	Recombinant polyprotein of TSA, LmSTI1, and LeIF formulated in MPL-SE	Dog	Protection	Trigo et al., 2010 [57]

CP: cysteine proteinase; CTE: C-terminal extension; BCG: *Mycobacterium bovis* bacillus Calmette-Guerin; SLA: soluble leishmanial antigens; FML: fucose-mannose ligand; LiESAp: Leishmania infantum excreted-secreted antigen purified; LPG: lipophosphoglycan.

(b)

Vaccines tested against *L. donovani*					
Antigen	Source of antigen	Vaccine delivery	Animal model	Outcome	Reference
p36 LACK	Multiple species	DNA vaccine	Mouse	No protection	Melby et al., 2001 [58]
dp72	*L. donovani*	Native protein antigen	Mouse	Partial protection	Jaffe et al., 1990 [59]
A2	*L. donovani*	Recombinant protein	Mouse	Protection	Ghosh et al., 2001 [60]
A2	*L. donovani*	DNA vaccine	Mouse	Protection	Ghosh et al., 2001 [61]
HASPB1	*L. donovani*	Recombinant protein	Mouse	Protection	Stager et al., 2000 [8]
ORFF	*L. donovani*	Recombinant protein	Mouse	Partial protection	Tewary et al., 2004 [62]
ORFF	*L. donovani*	DNA vaccine + recombinant protein	Mouse	Partial protection	Tewary et al., 2005 [63]
ORFF	*L. donovani*	DNA vaccine	Mouse	Partial protection	Sukumaran et al., 2003 [64]
KMP-11	*L. donovani*	DNA vaccine	Hamster	Protection	Basu et al., 2005 [65]
KMP-11	*L. donovani*	DNA vaccine	Mouse	Partial protection	Bhaumik et al., 2009 [66]
gp36	*L. major*	Recombinant protein expressed in bacilli	Mouse	Partial protection	McSorely et al., 1997 [67]
gp36	*L. donovani*	Native protein in cationic liposomes	Mouse	Partial protection	Bhowmick et al., 2008 [68]
SLA	*L. donovani*	Native proteins in cationic liposomes	Mouse	Protection	Bhowmick et al., 2007 [69]
SLA	*L. donovani*	Native proteins	Mouse	Protection	Tewary et al., 2004 [70]
LD9, LD72, LD51, LD31	*L. donovani*	Native proteins in cationic liposomes	Mouse	Protection	Bhowmick and Ali, 2009 [71]
Leishmanial antigens	*L. donovani*	Native proteins in liposomes	Mouse	Protection	Mazumdar et al., 2004 [72]
F14	*L. donovani*	Recombinant protein	Hamster	Partial protection	Bhardwaj et al., 2009 [73]
γ-GCS	*L. donovani*	DNA vaccine	Mouse	Partial protection	Carter et al., 2007 [74]
FML	*L. donovani*	Native protein	Mouse	Partial protection	Palanik-de-Sousa et al., 1994 [75]
FML	*L. donovani*	Native protein	Mouse	Partial protection	Santos et al., 1999 [76]
FML	*L. donovani*	Formulation with QuilA saponin	Dog	Protection	Borja-Cabrera et al., 2002 [77]
Leishmune (FML)	*L. donovani*	Recombinant protein	Dog	Partial protection	Saraiva et al., 2006 [78]
Leishmune (FML)	*L. donovani*	Recombinant protein	Dog	Protection	Nogueira et al., 2005 [79]
H2A, H2B, H3, H4 and LACK	*L. donovani*	Multiunit DNA vaccine	Dog	Partial protection	Saldarriaga et al., 2006 [80]
LPG	*L. donovani*	Purified glycolipid + BCG	Hamster and mouse	No protection	Tonui et al., 2003 [81]

CP: cysteine proteinase; CTE: C-terminal extension; BCG: *Mycobacterium bovis* bacillus Calmette-Guerin; SLA: soluble leishmanial antigens; FML: fucose-mannose ligand; LiESAp: Leishmania infantum excreted-secreted antigen purified; LPG: lipophosphoglycan.

In addition to recombinant proteins, DNA has been extensively tested as means of vaccine delivery. The induction of Th1 responses leading to strong cytotoxic T cell immunity is a general property of DNA vaccines [86], and a growing body of evidence implicates $CD8^+$ T cells in protective antileishmanial responses [87]. The PapLe22 antigen, which is recognised by T cells from VL patients [88], was administered as a DNA vaccine and led to a marked decrease in parasite burden in immunised hamsters [46]. However stimulation of peripheral blood mononuclear cells from VL-infected individuals with recombinant PapLe22 induced IL-10 production [88], which is associated with VL pathogenesis in humans [89]. The *Leishmania* homologue for receptors of activated C kinase (LACK) is the most extensively studied DNA vaccine against both cutaneous and visceral leishmaniasis, but has shown inconsistent outcomes. DNA vaccination with a plasmid harbouring the LACK gene coadministered with, or without, IL-12 induced robust, long-lasting protection against *L. major* challenge in mice, which was dependent on $CD8^+$ T cells [90–92]. In a heterologous system, priming with *L. infantum* LACK followed by a vaccinia booster afforded protection against *L. major* infection [93]. The prime-boost regimen was also employed to immunise dogs against *L. infantum* infection and elicited protective responses in 60% of vaccinated animals [38], but this positive outcome has been overshadowed by studies

where immunisation with LACK offered no protection. In an experimental mouse model the LACK DNA vaccine induced strong Th1 responses, but failed to protect against *L. donovani* challenge [58]. Other studies in the *L. infantum* mouse model confirmed that LACK DNA vaccination does not confer protection against VL despite the presence of Th1 responses [36]; however, a strategy using a heterologous prime-boost vaccination using DNA and vaccinia viruses has shown some efficacy [35]. The heterologous DNA-prime protein-boost approach has also shown success for other VL vaccine antigens such as ORFF [63] and cysteine proteinases [94]. Heterologous prime-boost with gp63 antigen with CpG-ODN as adjuvant provided durable protection against *L. donovani* challenge in an experimental mouse model and was associated with robust cellular immune responses [95]. As gp63 is a major surface protein present on both amastigote and promastigote forms and shows a high homology between VL species [96] it is an attractive target for further vaccine development.

Insights into the role of the innate immune system, in particular dendritic cells (DCs), have provided the impetus for the use of DCs as a delivery system for *Leishmania* antigens [97–99]. DCs loaded with *L. donovani* soluble extract and expressing high levels of IL-12 induced protection in the mouse model of VL when used as a therapeutic vaccine [100]. Moreover, coadministration of DCs with antimonial therapy resulted in complete clearance of parasites from the liver and spleen, unlike DC immunisation alone which was not able to clear the infection from these organs [101].

Liposome formulations have been adopted as *Leishmania* drug delivery systems, and liposomal Amphotericin B is the current preferred drug treatment for VL in resource-rich settings [102]. Vesicle delivery systems are also being considered for VL vaccines and have been shown to adjuvant protein antigens and induce sustained Th1 immune responses [103]. These delivery systems have shown some protection against *L. donovani* infection in experimental mouse models [104] and provide a new approach to the development of VL vaccines.

Recently, Peters et al. [105] demonstrated that sand fly transmission of parasites abrogates vaccine-induced protective immunity. While mice vaccinated with killed parasites were refractory to a needle challenge, they were susceptible to the sand fly inoculum implying that the protective responses in vaccinated mice were either not generated or not maintained. These data provide a rationale for the inclusion of sand fly saliva components, which are specific to natural infection, in vaccine design. The sand fly injects Leishmania parasites in the presence of saliva, which contains a range of pharmacologically active molecules that can modulate host's immune and inflammatory responses and facilitate establishment of infection. For a number of years salivary gland antigens have been targeted as potential candidates for antileishmanial vaccine development, primarily against *L. major*. Nevertheless, it has been shown that children who underwent anti-*VL* delayed-type hypersensitivity (DTH) conversion also had increased titers of antibodies directed to sand fly saliva suggesting that mounting an effective antileishmanial response might be linked to neutralization of saliva components [106].

4. Development of Canine Vaccines

Eliminating animal reservoirs has been an essential public health tool for the control of many zoonotic diseases, such as rabies [107] and brucellosis [108]. Canines, particularly domestic dogs, are the main reservoir for VL species and are considered the main source of zoonotic transmission to humans. The development of an effective canine visceral leishmaniasis (CVLs) vaccine represents a cost-effective tool for interrupting the transmission cycle and controlling zoonotic VL infection in humans.

CVL is widespread throughout South America [9] and the Mediterranean [109] where *L.infantum* is the most significant causative agent of disease. *L. donovani* is considered to be zoonotic, but as yet there has been no clear identification of the reservoir host animal [110]. Asymptomatic infection is common in dogs, and as a large reservoir of parasites are present in the skin, asymptomatic animals are a major source of infection for vector transmission [10]. Human VL is an emerging disease in many areas of the world, including Northern Europe [111] and North America [112], and the spread of VL into nonendemic areas is often preceded by increased incidence of canine infection. There is concern that increased mobility of dogs and changes in vector habitat will result in increased transmission of human VL in previously nonendemic areas [113].

Treatment of CVL shows low efficacy with drugs successfully used for human VL chemotherapy, and drug treatment of dogs rarely results in cure [114]. Control programmes for CVL have a demonstrated capacity to reduce the prevalence of human VL disease following interventions that target dog populations in endemic regions [115]. However these public health campaigns are often complex and expensive to maintain, leading to varying degrees of efficacy. The use of insecticide-impregnated collars can reduce the risk of contracting CVL [116], but is costly and difficult to implement at a national level. The culling of seropositive dogs has long been recommended in Brazil; however this approach has not lead to a reduction in the number of human VL cases and may be of limited value [117]. Therefore the development of vaccines against CVL is an attractive approach to controlling infection in dogs, reducing the parasite reservoir and thus reducing the risk of transmission of VL to human populations.

Immunological characterisation of CVL reveals cellular and humoral immune responses comparable to human infection, including immune dysregulation and increased IL-10 which is associated with disease manifestation and progression [118]. Disease resistance is associated with strong Th1-type immune responses, including IFN-γ expression by antigen-specific T cells. Thus, analogous to a human VL vaccine, an effective CVL vaccine needs to induce strong and long-lasting cell-mediated immunity. Adjuvant choice must be carefully considered for CVL interventions, as live BCG is not appropriate for use in dogs and the identification of appropriate and effective adjuvants will be essential

for safe and effective CVL vaccines [119]. In addition, sand fly components are being considered for inclusion in CVL vaccine. Reactive antibodies to two sand fly saliva components (LuLo-D7 and LuLo YELLOW) were identified in infected dogs and proposed as possible vaccine candidates against CVL [120]. Evaluation of a killed *Leishmania* vaccine containing sand fly saliva extract indicated that the vaccine is highly immunogenic and provided support for further development of saliva components as candidates for anti-VL vaccine [121]. This is supported by vaccination studies using the hamster VL model, showing that salivary protein LJM19 was able to protect hamsters from fatal infection with *L. infantum* [122]. In addition, immunization with salivary proteins LJM17 and LJL143 induced strong cellular and humoral responses in dogs and might be an advantageous addition to anti-CVL vaccine [123].

Currently there are two commercially available CVL vaccines, Leishmune and Leishtec, and new vaccines under development include recombinant antigen vaccines and both live and killed whole-cell vaccines.

The Leishmune vaccine was the first commercially licensed vaccine for CVL, produced by Fort Dodge Animal Health and has been available in Brazil since 2004 [124]. This vaccine consists of the fucose mannose ligand (FML) isolated from *L. donovani* plus a saponin adjuvant. FML is a glycoprotein mixture, and the surface glycoconjugate GP36 is the major immunogen component [125]. This vaccine induced a significant and strong protective effect during phase III trials in dogs living in a VL-endemic area in Brazil with a vaccine efficacy as high as 80% [77, 126]. This protection lasted up to 3.5 years following vaccination, indicating induction of a long-lasting immunity [77]. As Leishmune-vaccinated dogs showed a complete absence of parasites, this renders them noninfectious and contributes to the breakdown of the zoonotic VL transmission cycle [79]. During phase III trials of Leishmune there was a concomitant reduction in human VL cases in districts where dogs were vaccinated [126] demonstrating that CVL vaccination interrupts the transmission of disease to humans. FML antigens are present on the surface of *Leishmania* parasites throughout the life cycle, and antibodies raised in vaccinated dogs prevented the binding of procyclic promastigotes to the sand fly midgut [78]. Thus Leishmune acts as a transmission blocking vaccine by clearing parasites from the animal reservoir and preventing survival of the parasite in the sand fly vector. Currently the Leishmune vaccine is used as a prophylactic and is recommended for asymptomatic noninfected dogs. However studies show that Leishmune is effective as a therapeutic vaccine for naturally infected dogs [127], particularly when given in combination with chemotherapy [128]. Emerging wide-scale field studies reveal that Leishmune decreases the incidence of both human and canine visceral leishmaniasis after dog vaccination with Leishmune [129].

A second vaccine, known as Leish-Tec, is being commercially developed by the Hertape Calier Saúde Animal and consists of adenovirus expressing the *L. donovani* A2 antigen. Whilst the results from phase-III trials of Leish-Tec are yet to be published it is known that immunization with a recombinant A2 protein elicits protection against the onset of clinical VL in experimental dog infections [51]. The recombinant adenovirus encoding the *A2* gene was capable of inducing strong Th1-type immune responses in vaccinated mice and reduced parasite burdens following challenge with VL parasites [130]. Together these studies indicate that A2 is an important candidate antigen for the development of CVL vaccines, and future studies should report on the impact of this intervention on both canine and human VL infection.

As many of the clinical and immunological features of CVL are similar to those observed in human VL, experimental challenge in dogs represents a useful system for evaluating the efficacy of vaccine candidates. The Leish-111f + MPL-SE vaccine is a leading vaccine candidate from human VL and has shown therapeutic efficacy in recent CVL trials [57]. Live attenuated parasites vaccines are also being explored in canine models, including a drug-attenuated line of *L. infantum* established by culturing promastigotes under gentamicin pressure. The attenuated *L. infantum* vaccine strain did not induce clinical symptoms of VL in dogs and provided protection from subsequent challenge with live virulent *L. infantum* [131].

The elimination of human VL will be difficult to achieve in the presence of persisting animal reservoirs, and veterinary intervention is an important tool for reducing the global burden of human VL disease. The identification of measurable and reliable biomarkers of immunogenicity and protection induced by CVL vaccines may also be informative for human VL vaccine efforts.

5. Live Attenuated Vaccines

Historically the most successful vaccines against intracellular pathogens have been based on live attenuated organisms. Vaccination strategies using live attenuated *Leishmania* parasites are attractive as they closely mimic the natural course of infection and may elicit clinically protective immune responses. A live attenuated vaccine strain would present a full complement of *Leishmania* antigens to the host immune system along with appropriate pattern-recognition molecules for the parasite. Live vaccines also deliver antigens to the correct cellular and tissue compartments for appropriate processing and presentation to the host immune system. Together, this enhances the capacity of live attenuated vaccines to promote antigen-specific effector and memory immune responses that confer long-lasting protective immunity.

The development of robust *in vitro* culture systems for growth and differentiation of *Leishmania* promastigote and amastigote life cycle stages has enabled the production of attenuated vaccine strains. It should be noted that most research in this area has utilized CL strains, such as *L. major*; however the attenuation techniques are broadly transferrable to VL causing species. It is has been known for some time that long-term *in vitro* culture of promastigote parasites leads to a loss of virulence *in vivo*. Studies in experimental mouse models of CL have shown that infection with cloned avirulent lines provides clear protection against

a virulent challenge infection [132]. Avirulent strains of the VL species *L. donovani* and *L. infantum* have been generated by repeated *in vitro* subculture of promastigotes in the presence of gentamicin [133]. These drug-attenuated promastigotes were able to invade macrophages but could not survive as intracellular amastigote forms [133]. Drug-attenuated *L. infantum* was avirulent in an experimental canine model, induced strong cellular immunity production and protection against challenge with live virulent *L. infantum* [131]. Early experiments showed that γ-irradiation rendered *Leishmania* parasites nonpathogenic and infection protected against challenge in a cutaneous *Leishmania* model. Protection depended on the presence of viable irradiated parasites, suggesting that transformation into amastigote forms is required for efficacy. Interestingly the underlying mechanism of protection may relate more to the induction of tolerization rather than immunization in this system [134]. Other approaches to the generation of attenuated parasites include chemical mutagenesis screens selecting for temperature sensitive CL strains [135] which are avirulent during infection and significantly protect against subsequent challenge.

The major concern regarding these approaches to attenuation is that the underlying genetic mechanisms are not defined. This creates safety concerns as the stability of parasite attenuation is uncertain and parasites could revert to a virulent form. Conversely, a progressive loss of virulence may occur, resulting in parasite lines that are incapable of establishing infection or inducing protective host responses. A loss of parasite virulence due to long-term *in vitro* culture has been demonstrated in both human patients undergoing leishmanization and experimental mouse models [19]. Thus in the absence of a clear genetic profile, nonspecific parasite attenuation is not acceptable for the development of a human VL vaccine.

Over the last few decades the development of a powerful "genetic toolkit" for *Leishmania* species has enabled research involving transgenic parasites [136]. *L. donovani* and *L. infantum* parasites can be stably transfected using integrating expression constructs that target genes for disruption by homologous recombination. As *Leishmania* organisms are diploid throughout their lifecycle, the production of null mutants requires each allele of a gene to be targeted individually with genetic constructs containing two different and independent selectable markers. The recent availability of *Leishmania* genome sequences has facilitated the identification and in-depth analysis of parasite genes crucial for infection and virulence. Comparative genomics studies of *Leishmania* species have shown a highly similar gene content and gene order and annotation studies have revealed only a few species-specific genes [11]. Increased knowledge of potential parasite virulence factors and a greater understanding of the antigens involved in the acquisition of immunity have generated much interest in the development of genetically attenuated parasite vaccines.

To date, there have been two general approaches to the genetic attenuation of *Leishmania* parasites. First, by deletion of genes encoding virulence factors or the enzymes responsible for their synthesis and second, by targeting genes essential for intracellular survival. Gene targeting aims to produce parasites that are capable of being produced and manipulated *in vitro*, usually in promastigote form, but incapable of sustaining virulent infection in the host, in amastigote form. The first genetically attenuated parasite vaccine was the *L. major* dihydrofolate reductase-thymidylate synthase (dhfr-ts) knockout, which targeted an essential metabolic gene [137]. This null mutant was able to establish a persistent infection in experimental mouse models, but remained avirulent with respect to disease. Importantly vaccination with dhfr-ts knockout parasites elicited substantial protective immunity, as mice were resistant to subsequent challenge with virulent *L. major*. Although further experiments in nonhuman primate models failed to show protection these initial studies provided proof of principle for the safety and immunogenicity of live attenuated *Leishmania* vaccines [138]. Drug-sensitive *Leishmania* mutants containing suicide genes [139–141] are also being developed for use during leishmanization, and inducible suicide mutants in *L. amazonensis* have shown protective efficacy in an experimental hamster model [142].

To date, only a small number of studies have focused on generating attenuated forms of the VL species *L. infantum* and *L. donovani* as a route to the production of an attenuated VL vaccine. One approach has targeted the transporters for the metabolic precursors of the folate pathway, as *Leishmania* parasites are auxotrophic for folate and pterin [143]. *L. donovani* parasites lacking the main biopterin transporter (BT1) showed a marked reduction in infectivity in an experimental mouse model, and this attenuated strain conferred protection to subsequent challenge with wild-type *L. donovani* [144]. Parasites incapable of intracellular reproduction were produced by targeting centrin, a calcium binding cytoskeletal protein. A loss of centrin from *L. donovani* parasites did not affect the growth of promastigote forms, but null mutants were unable to survive as axenic amastigotes or in human macrophages *in vitro* [145]. Immunization of mice and hamsters by infection with centrin deficient *L. donovani* protected against virulent homologous challenge. Importantly the centrin null vaccine strain elicited parasite-specific Th1 responses which strongly correlated with sustained protection and also induced a level of cross protection against *L. braziliensis* infection [146].

The genetic attenuation of *Leishmania* does not necessarily require the production of null mutants. Deletion of one allele of the *L. infantum* silent information regulatory 2 (SIR2) locus was sufficient to prevent amastigotes from undergoing intracellular replication in macrophages. Immunization with *L. infantum* lacking one SIR2 gene copy elicited strong parasite-specific T cell responses and conferred complete protection against virulent challenge in a VL mouse model [147].

Other approaches to developing live attenuated parasites as VL vaccines have utilised nonpathogenic *Leishmania* species, an approach comparable to the use of BCG as a vaccine against *Mycobacterium tuberculosis* infection. The lizard protozoan parasite *L. tarentolae* has never been found to be associated with any human leishmaniasis and is considered nonpathogenic. Whilst *L. tarentolae* is capable of infecting

mammalian cells and transforming into amastigotes, the parasite does not cause clinical symptoms of disease in either mouse or hamster models [148]. In experimental vaccine trials *L. tarentolae* elicited a strong Th1-driven protective immune response and conferred protection against infectious challenge with *L. donovani* in a susceptible mouse strain [149]. The use of *L. tarentolae* as a vaccine vector to deliver specific *Leishmania* antigens in the context of a live infection has also been explored. The *L. donovani* A2 antigen was expressed in *L. tarentolae*, which normally lacks this protein [150] and used as a vaccine strain in an experimental mouse model. Vaccination protected susceptible mice against *L. infantum* challenge and was associated with the production of high levels of IFN-γ production [151].

The use of live attenuated vaccines provides a promising vaccination strategy for VL; however safety issues regarding the use of genetically attenuated parasites as vaccines still need to be addressed. Many of the proposed live attenuated vaccines induce long-lasting immunity to reinfection by maintaining a low level asymptomatic infection. The establishment of subclinical infection is particularly valuable as the persistence of antigen is thought essential for the generation of effective memory responses to *Leishmania*. However reactivation of *Leishmania* has been observed in patients who are immunocompromised, such as following HIV infection, thus the safety of attenuated parasites that induce a subclinical infection will need to be carefully assessed.

Transgenic parasites provide an enticing lead for vaccine development. A continuing synergy between molecular and immunological approaches to the development of VL vaccines will accelerate development of the next generation of therapeutics. In addition, transgenic parasites are invaluable tools for understanding host-parasite interactions [152] and inform vaccine design by providing insight into immunity and pathogenesis during VL.

6. Concluding Remarks

Preventive vaccines are recognized as the best and most cost-effective protection measure against pathogens and save millions of lives across the globe each year. *Leishmania* vaccine development has proven to be a difficult and challenging task and is hampered by an inadequate knowledge of disease pathogenesis, the complexity of immune responses needed for protection, and the cost of vaccine development. The burden of VL is concentrated in resource poor nations, and a lack of political will and philanthropic investment further aggravates the situation. However, the rise of biotechnology industries in endemic countries, such as India, may provide an impetus for VL vaccine development and investment. A recent clinical trial in India assessed the safety and immunogenicity of the LEISH-F1+MPL-SE vaccine [85] which is the only Second-generation vaccine currently in clinical development for human VL. There are currently several new European-based VL vaccine efforts including a synthetic vaccine RAPSODI (http://www.fp7-rapsodi.eu/) [153], a DNA-based LEISHDNAVAX (http://www.leishdnavax.org/) [154], and an adenovirus vectored therapeutic vaccine (Paul Kaye, personal communication). New adjuvants are also being developed, and there are several clinical vaccine trials in progress and in planning [18]. Given the rapid progress in the fields of parasite immunology and genomics, a successful anti-*Leishmania* vaccine should be achievable sooner rather than later. There is a clear need for greater investment in research and development to move promising vaccine leads along the development pathway toward an effective, affordable VL vaccine.

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Review Article

Immunity to Visceral Leishmaniasis Using Genetically Defined Live-Attenuated Parasites

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Leishmaniasis is a protozoan parasitic disease endemic to the tropical and subtropical regions of the world, with three major clinical forms, self-healing cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL), and visceral leishmaniasis (VL). Drug treatments are expensive and often result in the development of drug resistance. No vaccine is available against leishmaniasis. Subunit *Leishmania* vaccine immunization in animal models has shown some efficacy but little or none in humans. However, individuals who recover from natural infection are protected from reinfection and develop life-long protection, suggesting that infection may be a prerequisite for immunological memory. Thus, genetically altered live-attenuated parasites with controlled infectivity could achieve such memory. In this paper, we discuss development and characteristics of genetically altered, live-attenuated *Leishmania donovani* parasites and their possible use as vaccine candidates against VL. In addition, we discuss the challenges and other considerations in the use of live-attenuated parasites.

1. Introduction

Leishmaniasis is caused by protozoan parasites of the genus *Leishmania* of the family *Trypanosomatidae* and is transmitted by the sand fly vector. It infects about 12 million individuals globally in tropical and subtropical regions, with ~2 million new clinical cases (0.5 million visceral leishmaniasis (VL) and 1.5 million cutaneous leishmaniasis (CL)) reported annually with an estimated death toll of ~50,000 persons/year [1]. The three major clinical forms of leishmaniasis, VL, CL, and MCL are the result of infection by different species of the parasite and the immune response of the host. VL, fatal if not treated, is caused by *L. donovani, L. infantum,* and *L. chagasi* [2, 3]. More than 90% of the visceral cases in the world are reported from Bangladesh, Brazil, India, and Sudan. Most affected patients (70%) are children under 15 years of age who already suffer from concurrent malnutrition and other secondary illnesses. The major clinical symptoms for VL are characterized by prolonged and irregular fever, splenomegaly, and hepatomegaly [3]. CL causes lesions that are self-healing and are caused by *L. major, L. tropica,* or *L. aethiopica* in the old world and by *L. mexicana* or *L. braziliensis* complex in the new world [4]. MCL is potentially life threatening and affects the mucosal region of infected individuals, typically seen in the Central and South America and caused by *L. braziliensis, L. amazonensis, L. panamensis, and L. guyanensis* [3].

In the *Leishmania* life cycle, motile promastigote forms that reside in the gut of the sand fly vector are transmitted to a mammalian host during a blood meal. Parasites are taken up by the neutrophils that are ingested by the host macrophages, differentiate into the nonmotile amastigote form, and reside and multiply in the phagolysosome compartment of the macrophages. These two major life stages have been adapted to *in vitro* culture for most *Leishmania* species allowing manipulation of the genome and assessment of the altered phenotypes *in vitro* and *in vivo*.

The most significant public health effects of leishmaniasis are concentrated in developing countries. Dogs represent an important reservoir for the parasite in Europe, the Middle East, as well as Latin America. In the USA, even though leishmaniasis is not endemic, infections can be observed in pockets of the country, mainly in the southwest [5, 6]. In addition, VL due to *L. infantum* was found in fox hounds in the northeastern part of the USA and transmission may involve exposure to an insect vector, direct transmission, or vertical transmission [7]. In addition, there have been several documented cases of parasite transmission by blood transfusion globally [8] and an outbreak of leishmaniasis among US troops led to deferral from donating blood for travel to Iraq in 2003 [9]. Studies in animal models, such as hamsters and dogs show that *Leishmania* not only survives blood-banking storage conditions, but also retains its infectivity [10, 11]. This retention of infectivity in blood raises the possibility of spread of disease from asymptomatic individuals through transfusion to healthy individuals in areas where the sand fly vector is absent.

Currently, the only treatment for leishmaniasis is drug treatment. However, either prolonged use or inefficient drug therapy has resulted in drug resistance. Currently, more than 60% of the clinical cases are resistant to the first line drug, antimony [12]. There is concern about the possibility of development of resistance to the new oral drug miltefosine in the near future as there are already reports of relapse among miltefosine treated cases [13]. In addition, about 50% of Sudanese and 5–10% of Indian kala azar patients within months after successful treatment develop post-kala-azar dermal leishmaniasis (PKDL) [14]. PKDL is a condition characterized by the appearance of diffuse lesions in the skin that harbor the visceral parasite and is considered a reservoir for transmission in these countries. There are no vaccines available at present. Attempts to develop vaccines for such parasitic agents such as heat killed, subunit, or DNA vaccines have not resulted in a successful vaccine candidate that could be applicable to humans [15–17]. However, past experience has revealed that individuals who recover from *Leishmania* infection develop a long lasting protection from future infections suggesting that a successful vaccine candidate needs to cause a controlled infection that evokes a protective immunity. Experience from other pathogens has suggested that a live-attenuated parasite vaccine could fulfill such requirements [18, 19]. Hence, in this paper we have focused on current knowledge about the (a) immunology of leishmaniasis, (b) need for and development of live-attenuated *Leishmania* parasites as vaccine candidates, (c) the safety challenges such parasites present, (d) the type of immune responses generated by such parasites that are protective, (e) experimental animal models for testing vaccines, and (f) routes of administration and mode of virulent challenge for vaccinated animals. Recently, various review articles have been published discussing the role of genetically altered *Leishmania* as vaccine candidates [3, 16, 17, 20–23]. In this paper we have limited our scope to genetically altered *L. donovani* parasites and their use as vaccine candidates.

2. Immunology of Leishmaniasis

Immunity to leishmaniasis is mediated by both arms of mammalian cellular immune system; innate (by neutrophils, macrophages, and dendritic cells) and adaptive (T cells) responses [20]. The sand fly bite causes minimal tissue damage that promotes recruitment of neutrophils to the site of injury as a primary immune defense mechanism of the host [24, 25]. However, regardless of *Leishmania* species, these neutrophils are the primary target of this parasite. Despite the harsh intracellular environment, parasites can survive for a time period, and eventually these parasitized neutrophils or viable parasites are engulfed by macrophages or dendritic cells. *Leishmania* can survive and replicate inside macrophages by modulating the normal antimicrobial machinery as well as increasing the host cell membrane fluidity and disrupting lipid rafts, which in turn affects the antigen presentation capability of host APCs [26]. These parasitized APCs then interact with T cells to stimulate cytokines, and in this whole process, several cytokines and chemokines are involved. In this way, *Leishmania* starts to hijack the whole immune system for its survival [27].

In leishmaniasis, host defense against intracellular *Leishmania* is cell mediated, which involves Th1 responses due to T-cells primed primarily by dendritic and macrophage cells producing IL-12 [28–31]. A clear dichotomy between Th1-mediated protection (mediated by major cytokines IFNγ, IL-2, TNF) and Th2-mediated disease progression (mediated by major cytokines IL-10, IL-4) has been demonstrated in mice against cutaneous leishmaniasis [21, 32]. However, this Th1/Th2 dichotomy is not as clear in visceral infection of mice and even less in human visceral leishmaniasis [33]. The immune response and pathology of visceral leishmaniasis are complex, involving a number of genetic and cellular factors in the process of susceptibility or resistance to parasites [34].

2.1. Nonhealing Response. Susceptibility to visceral leishmaniasis is correlated with the presence of a Th2 response [33]. *L. donovani* infections stimulate expression of Th2 associated cytokines (IL-10, IL-4, IL-5, and IL-13). Further, elevated levels of IL-10 in serum and enhanced levels of IL-10 mRNA expression in lesion tissue are a direct indication of severe visceral leishmaniasis in mice and humans [35–38]. IL-10 plays a crucial role in the establishment and maintenance of Th2-dependent immune responses by suppressing Th1-dependent cell-mediated immunity in CL [39, 40]. In the murine VL model, IL-10 directly inhibits the antimicrobial machinery of macrophages by modulating normal signal transduction mechanisms [41]. IL-10 inhibits killing of amastigotes by downregulating the production of TNF-α and nitric oxide by macrophages and dendritic cells and thereby strongly impairs their antimicrobial activity [41, 42]. IL-10 also inhibits the production of IFNγ, one of the major proinflammatory cytokines [43]. However, in visceral leishmaniasis, it has been suggested that the impaired function of cellular immunity that correlates with progression of active disease may be due to the inhibitory effects of IL-10 independent of the IFNγ level [44].

IL-10, in addition, is also a regulatory cytokine mainly secreted by T cells, B cells, macrophages, dendritic cells, and keratinocytes [39, 45]. In experimental cutaneous leishmaniasis in mice, natural T regulatory cells (CD4+CD25+FoxP3+) are a major source of IL-10, and these cells are crucial for maintenance of parasite persistence [46]. However, in experimental VL in mice [47] and in human visceral leishmaniasis [37], IL-10 comes from non-T reg cells (CD4+CD25−FoxP3−), indicating that therapeutic approaches require *Leishmania* species-specific understanding of the immune response. IL-4 has generally been considered a Th2 cytokine that helps in the proliferation of the Th2 cell population and consequently a significant downregulator of Th1 cell response [48, 49]. Its role in CL is well defined but not in VL [32]. For example, the parasite burden of *L. donovani* in infected IL-4−/− and IL-4+/+ BALB/c mice was similar [50]. However, IL-4 null mice are more susceptible to visceral leishmaniasis than their wild-type counter parts after drug therapy [50], suggesting that IL-4 is necessary for effective chemotherapy in visceral leishmaniasis [50].

2.2. Healing Response. In VL (both in murine and human), resolution of infection depends on the production of Th1 cytokines [21, 31, 38, 51–54]. Production of IL-12 by antigen presenting cells and IFNγ by T cells are crucial for controlling the parasite growth and development of host immunity [51, 55]. There seems to be a dichotomy in the role of IL-10 in VL. On one side, IL-10 suppresses host immunity and helps parasite survival, on the flip side, IL-10 also protects the host from tissue damage by exaggerated inflammation [56]. Recent findings suggest that some IFNγ producing cells are a crucial source of IL-10, which act as a negative feedback mechanism to control tissue damage [57, 58]. A study with VL patient splenic T cells showed elevated expression of both IFNγ and IL-10 [37] suggesting that at least some cells are producing both cytokines as part of a strong inflammatory response and limiting the tissue damage by a feedback control mechanism [33]. TNFα also shares a significant role in resistance because TNFα−/− mice readily succumb to infection with *Leishmania* species [59, 60]. In addition, TNFα was also shown to stimulate the action of IFNγ in the induction of nitric oxide production in macrophages to kill the parasite [61]. Chemokines also play an important role in killing parasites from host cells [51, 62–64]. Antileishmanial activity of chemokines has been demonstrated in both *in vitro* and *in vivo* infections in murine VL by induction of respiratory burst as well as by induction of Th1 cytokines [65, 66]. Another CD4+ T-cell lineage called Th17 cells was initially characterized as producing IL-17 family cytokines [67]. They are proinflammatory cytokines that stimulate the production of IL-6, TNFα, and chemokines. A recent study suggests that *L. donovani* strongly induced IL-17 and IL-22 in human PBMCs and enhanced secretion of these cytokines correlated with protection [68].

In addition to CD4+ T cells, the significant role of CD8+ cytotoxic T cells in the control of experimental CL and VL has been described [69]. In murine experimental VL, control of parasite multiplication and leishmaniacidal activity are both associated with development of a granuloma in the liver, which requires both CD4 and CD8 T cells as well as IL-12, IFNγ, and IL-2 [30, 55, 70–73]. On the other hand, B cell deficient animals showing resistance to *Leishmania* infection and increased levels of IgG in human and canine VL (with few recent reports showing exceptions in these hosts) suggest that antibodies are not necessary for protection in the absence of an appropriate cellular response [22]. Thus, in addition to the protective T-cell response, the role of other T-cell subsets, including regulatory T and Th17 cells in either susceptibility or resistance to experimental and human visceral leishmaniasis, has not been ruled out and requires detailed investigations to help in vaccine designs.

3. The Need for a Genetically Altered Live-Attenuated Vaccine against VL

Drug treatment requires long-term medication, which is expensive and highly toxic and often leads to resistance. Hence, vaccine strategies to combat leishmaniasis are very attractive. Though recombinant protein and DNA vaccines are under investigation, and in the past whole cell parasite lysates, heat killed parasites with or without adjuvants were used, a promising alternative is to develop live-attenuated parasite vaccines by genetic manipulation of genes that are necessary for virulence [22]. Moreover, live-attenuated pathogens have been used successfully as vaccine candidates against various viral and bacterial pathogens [19, 74]. In addition, the argument to use live-attenuated parasites as vaccine candidates becomes compelling based on the following facts: (a) complete *Leishmania* cDNA expression library injected into mice was more protective than any subpools of the library plasmids or a subunit, reinforcing the idea that the whole parasite makes the best vaccine [15]; (b) the cutaneous leishmanization practiced in the Middle East and Uzbekistan or the persistence of few parasites in the body even after cure protected individuals from reinfection [23]; (c) healing from natural or deliberate infection with *L. major* leads to the generation of more effective immunity against rechallenge in mice than vaccination with either killed parasites or defined leishmanial antigens that generally induce only short-term protection [75]; (d) in the past, researchers have developed attenuated strains by passaging through long-term culture, chemical mutagenesis, γ-irradiated, parasite culture under high drug pressure, and so forth [76–78]. Immunization with such attenuated strains showed a significant amount of protection upon virulent challenge in the mouse model, however, concerns about nonspecific attenuation that could result in reversion to infectivity of such strains does not make this a viable strategy. Further, nonspecific attenuation can lead to loss of protective immunity either by simply losing virulence or failure to establish subclinical infection. Therefore, the best alternate strategy would be to use "genetically defined live-attenuated" parasites. In the post genomic era with the help of *Leishmania* species-specific genomes, we can target a critical gene(s) that are responsible for survival inside macrophages, and deletion of such specific

gene(s) by homologous recombination allows the selection of parasites lacking those critical genes for virulence. Further, we expect that immunization with such attenuated parasites will render protection against virulent infection by eliciting parasite-specific immune response in the host just like after recovering from natural infection. Most importantly, the live-attenuated organisms will have the complete antigen spectrum like their wild-type counterparts, which may result in a robust immunity as compared to subunit vaccines that cannot provide the complete repertoire of antigens. Finally, by its nature, the attenuated parasite will persist in the host for a certain period of time providing the immune system persistent antigens that may allow the generation of antigen-specific memory cells that can be mobilized to provide a protective response immediately following subsequent infection. All of these features put together make a compelling argument that the genetically defined attenuated parasite could be an alternative approach for a vaccine against leishmaniasis, including for VL.

4. Development and Testing of Genetically Altered Live-Attenuated Vaccine Candidates against Leishmaniasis

Several targeted gene deletions have been carried out to develop *Leishmania*-attenuated vaccine strains. Among the vaccine candidates developed for CL, *L. major* dihydrofolate reductase thymidylate synthase (*dhfr-ts-*) knockout parasites protect mice [79] but not rhesus monkeys [80]. *L. major* deficient in surface and secreted phosphoglycans (*lpg2−*), although unable to survive in sand flies and macrophages, retained the ability to persist indefinitely in mice and conferred protection against virulent challenge, even in the absence of a strong Th1 response [81, 82]. However, *lpg2−/−* parasites, over time, unexpectedly, regained virulence [83]. *L. major* deleted for phosphomannomutase (PMM) protected mice, despite no increase in either effector or memory response [84]. Additionally, *L. mexicana* that also causes CL, deficient in cysteine proteinase genes (Δ*cpa* and Δ*cpb*), conferred protection in mice and hamsters against homologous challenge [85, 86]. Among the vaccination studies in VL (Table 1), mice immunized with a *L. donovani* strain deleted for biopterin transporter (*BT1*) were also similarly protected from virulent challenge [87]. Recent attempts using partial knockout parasites for the A2-A2rel gene cluster in *L. donovani* [88] and SIR2 gene in *L. infantum* [89] as immunogens induced protection against virulent challenge in BALB/c mice. However, such mutants cannot be used as vaccine candidates, because they still carry wild type allele/s and could cause disease. There were also efforts to enhance the vaccine safety by including drug sensitive *L. major* mutants with suicide genes for controlled infection [90, 91]. Hence, these experiments, despite limitations with most of the mutant strains, demonstrate the potential as well as the pitfalls of generating live-attenuated vaccines by targeted gene disruptions. Hence, it is critical to develop attenuated lines through complete gene knockouts that generate organisms either with controlled infectivity that persists for a long time or parasites that persists for a brief duration and are eventually eliminated by the host immune system, thereby inducing effective immunity without clinical disease or the risk of reactivation. Towards this concept, we have developed several *L. donovani* mutant parasites, which lack virulence-specific genes. We developed a *L. donovani* mutant (*LdCen*$^{-/-}$) deleted for the "centrin" gene. Centrin is a growth regulating gene in the protozoan parasites *Leishmania* [92], *Trypanosoma* [93] and *Plasmodium* [94], and higher eukaryotes [95]. *LdCen*$^{-/-}$ is specifically attenuated at the amastigote stage and not as the promastigote [96]. The mutant *Leishmania* amastigotes showed cytokinesis arrest in the cell cycle and persisted for a short duration in animals (mice and hamsters) or *ex vivo* in human macrophages and were eventually cleared [53]. In the animal studies, this attenuated parasite was found to be safe and protective in mice and hamsters against virulent challenge [53]. We also developed *L. donovani* mutant (Ldp27$^{-/-}$) deleted for the "*p27*" gene. *Leishmania donovani* 27 kDa mitochondrial inner membrane protein preferentially expressed in the amastigote stage is essential component of cytochrome c oxidase complex involved in oxidative phosphorylation [97]. The *Ldp27*$^{-/-}$ cell line is attenuated for infection of mice [97] and is currently being evaluated for its potential as a vaccine candidate. Taken together the examples of genetically altered parasites described above provide opportunities for live-attenuated vaccine candidates to be evaluated in preclinical and clinical conditions. However, there are various challenges to be addressed before such vaccine candidates become a reality.

5. Challenges for the Use of Genetically Altered Live-Attenuated Parasites as Vaccine Candidates

5.1. Monitoring of Attenuation in the Live-Attenuated Vaccines to Ensure Safety. A main concern with live-attenuated vaccines is the risk of reversion to a virulent parasite, since these parasites are expanded to production levels and used as vaccines to inoculate recipients. Hence, biochemical or molecular indicators are needed to assess the genetic and physiological traits of the organism to assess stability of the attenuated parasites. Such indicators must be measurable with an assay that is practical in the manufacturing setting [99]. Therefore, in addition to monitoring the obvious presence or absence of the interested deleted gene, other genes, that are uniquely altered in expression in such gene deleted parasites, provide as indicators of attenuation. To date, there are no known defined indicators of attenuation for genetically altered parasites. Towards that end, our laboratory has begun to identify such biochemical or molecular indicators. For example, to assess genes whose expression patterns are indicators of attenuation, the *LdCen*$^{-/-}$ line was compared to wild-type by gene expression microarray [100, 101]. Two genes, one coding for the mitochondrial inner membrane protein (27 kDa protein) and another coding for putative Argininosuccinate Synthase, that normally express a higher RNA level in the amastigote stage than in the promastigote

Table 1: Genetically altered live-attenuated vaccine candidates against visceral leishmaniasis.

Parasite	Characterization of attenuation	Animal model	Results of Immunization	References
L. donovani	Biopterin transporter gene deleted parasite ($BT1^{-/-}$)	BALB/c mice	Protective immunity, antigen-specific IFNγ secretion	[87]
L. tarentolae	Nonpathogenic strain expressing *L. donovani* A2 antigen	BALB/c mice	Protective immunity against *L. infantum* challenge, high IFNγ, low IL-5	[98]
L. donovani	Replication deficient centrin gene deleted ($Cen^{-/-}$)	BALB/c mice, Syrian hamster	Protective immunity against *L. donovani* and *L. braziliensis* challenge. Increased IFNγ, IL-2, and TNF producing cells and IFNγ/IL-10 ratio	[53]
L. infantum	Silent information regulatory 2 single allele deletion ($SIR2^{+/-}$)	BALB/c mice	Protective immunity, increased antigen-specific IFNγ/IL-10 ratio	[89]
L. donovani	Cytochrome c oxidase complex component *p27* gene deleted cell line ($Ldp27^{-/-}$)	BALB/c mice	12-week survival in host, initial evidence of protective immunity	[97] and Dey, unpublished

stage of wild type cells, were found downregulated in their RNA levels in $LdCen^{-/-}$ amastigote cells [99]. Northern blot analysis with these two genes showed that $LdCen^{-/-}$ parasites, recovered after five weeks of infection in mice, had the same expression pattern as they had prior to infection. Therefore, these two genes could be used as indicators of attenuation to monitor the safety of the $LdCen^{-/-}$ cell line as it is developed as a potential vaccine. However, further genetic evaluation to identify any compensatory mutations that could arise also will be an important aspect of any future such live vaccine candidate development.

5.2. Selection of Parasite Culture Conditions That Are Safe for Human Use. Live-attenuated parasite vaccines intended for injection into healthy individuals will need to be manufactured under strictly controlled conditions, that is, good manufacturing practices (cGMP) avoiding ingredients that could be hazardous for human use. One of the ingredients, bovine serum, that the parasite normally needs for its growth in culture, may not be suitable for human use. Even though in most cases the use of serum from BSE-negative animals is routine, there can be situations where it is not practical. Therefore, we have tested culture conditions for the genetically defined, attenuated parasites in serum free medium.

$LdCen^{-/-}$, $Ldp27^{-/-}$, and *Ld1S2D*, the parent wild-type strain of these genetically altered parasites, were adapted to growth in chemically defined medium (serum free media: SFM) [102]. SFM supported the growth of all tested *L. donovani* parasites at rates comparable with those obtained with serum-supplemented M199 medium (data not shown). Moreover, growth in such medium did not affect phenotypic characteristics of the parasites. We did not observe any difference in growth of mutant parasites compared to its wild type counterpart (data not shown). Parasite viability remained stable up to 6 days in culture (data not shown). Taken together, these results demonstrate the feasibility of cultivating *L. donovani* promastigotes in SFM and provide an alternative to use of serum for growing such parasites.

5.3. Vaccine Candidate Transmissibility by the Sand Fly. Another safety concern for genetically altered parasites to be used as vaccine candidates is whether such parasites, if taken up by sand flies, are able to survive in the sand fly gut and develop into infectious metacylic cells. The alterations made to date have been optimized for disrupting survival as amastigotes, yet allowing propagation as cultured promastigotes. Whether or not this will disrupt survival in sand flies cannot be predicted, but demonstration that the attenuated parasites do not survive or differentiate in the insect will be an important measure of safety blocking the potential for genetic recombination [103] or transmission of the vaccine strain. Preliminary studies from our laboratory suggest that genetically modified parasites may not be able to survive in the sand fly gut (Dey et al., unpublished data).

6. Immunoprotection against VL by Genetically Altered Live-Attenuated *Leishmania* Vaccines

In the literature, there are limited examples of genetically modified *L. donovani* parasites that have been tested against VL. The list of candidates is summarized in Table 1. Following are such examples. In our studies, mice immunized with $LdCen^{-/-}$ cells showed clearance of virulent challenge parasites in 10 weeks after challenge, with significantly reduced parasite burden in the spleen and no parasites in the liver. In contrast, high parasite loads were observed in the challenged mice previously immunized with heat-killed parasites or unimmunized [53]. Upon 10 week postvirulent challenge, the immunized mice displayed among the CD4+ T-cell population a significant increase of single and multiple Th1 type cytokine (IFNγ, IL-2, and TNF) producing T cells and increase in IFNγ/IL-10 ratio compared to nonimmunized naïve mice infected with wild type parasites. The naïve challenged mice displayed a reduced Th1 response and increased IL-10, a Th2 polarization accompanied by increased parasite burden in the organs. $LdCen^{-/-}$ immunized mice in addition showed increased IgG2a immunoglobulins and NO production compared to control [53]. These features indicated a

protective Th1 response. Protection was observed, even when challenged 16 weeks after immunization with *LdCen*$^{-/-}$ parasites and in some cases even longer periods (24 week), signifying a sustained immunity and suggesting generation of a memory response. Thus, the immunity may remain after the absolute clearance of attenuated parasites demanded of a safe vaccine candidate. Protection by immunization with *LdCen*$^{-/-}$ parasites was also seen in hamsters [53]. In addition, we found that immunization with these mutant cells also cross-protected mice against challenge with *L. braziliensis* that causes mucocutaneous leishmaniasis (MCL) [53] and against *L. mexicana* that causes cutaneous leishmaniasis (CL) (unpublished data), indicating *LdCen*$^{-/-}$ to be a safe and effective vaccine candidate against VL, MCL, and CL. We also have recently developed another live-attenuated *L. donovani* parasite that lacks a gene that is part of the cytochrome c oxidase complex and is necessary for oxidative phosphorylation [97]. Preliminary data suggest that this could also serve as a live-attenuated vaccine candidate similar to *LdCen*$^{-/-}$.

Th1 polarization towards protection against VL was also observed in animals after vaccination with other live-attenuated parasites (Table 1). Elevated IFNγ to IL-10 ratio with increased NO level and increase in both type 1 and 2 IgG antibodies correlating with parasite elimination was observed after *L. infantum* challenge in mice immunized with SIR2$^{+/-}$ *L. infantum* [89]. *BT1* null mutant *L. donovani* parasites upon immunization in mice produced significant amounts of IFNγ correlating with protection against virulent challenge [87]. Immunization of BALB/c mice with nonpathogenic strain of *Leishmania* (*L. tarentolae*) expressing *L. donovan*i A2 antigen resulted in protective Th1 type of protective immunity against *L. infantum* challenge [98]. Finally, safety and efficacy of the genetically altered *L. donovani* live-attenuated candidate vaccines need to be assessed in large animals as was done for other Leishmanias.

Generation and preservation of the immunological memory seems to be the most important but least studied aspect of antiparasitic vaccine development. In CL, the role of memory cells has been well studied and suggests generation of both effector (EM) and central memory (CM) T cells during infection [75, 104]. After antigenic stimulation, a few pathogen-specific EM T cells become CM T cells, which live for longer periods of time and stay in secondary lymphoid organs. Upon antigenic restimulation, CM cells become EM T cells and mediate protection [105]. Mice immunized with live-attenuated *L. major* parasites (DHFR deleted parasite) showed significant protection upon virulent parasite challenge, even after 25 weeks of immunization (in the absence of gene deleted parasites) and was due to the presence of CM cells [104]. Similarly, mice immunized with *LdCen*$^{-/-}$ vaccine candidate, that does not survive beyond 5 weeks after injection in mice, but shows protection when mice are challenged even 24 weeks after immunization [53] suggests *LdCen*$^{-/-}$ parasites could be generating a memory response that could be recalled upon challenge as was shown by Zaph et al. [104] for *L. major*. On the contrary, it is possible that protection by *LdCen*$^{-/-}$ parasites is achieved by establishing a low-grade persistent infection not detectable by current methods. This can be addressed by immunosuppressing immunized mice and monitor for parasite reemergence.

7. Other Considerations for Evaluation of Genetically Altered Live-Attenuated Vaccine Candidates

7.1. Experimental Models to Test Vaccines. The relevance of animal models is to understand the immunopathogenesis and to design, formulate, and test *Leishmania* vaccines/drugs. The models are expected to mimic the pathology and immunology observed in humans following inoculation with *Leishmania*. Criteria for establishment of infection in the animals are (i) susceptibility to infection, (ii) the age of the animal, and (iii) mode of infection. For *in vivo* testing of DNA, protein, or live parasite vaccines for VL, many animals have served as hosts, namely, BALB/c mice and Syrian golden hamster as primary screens, dogs as secondary screen, and squirrel, vervet, and Indian langur monkeys as tertiary screens [106–108]. Among such animals, the mouse may not be an ideal model for VL because in most situations the animals clear infection from both liver and spleen. However, the mouse model is used for VL studies in order to study the immune responses in the initial pathogenicity period since there are abundant immunological reagents available. Hence for VL, hamsters and nonhuman primates are more valuable models because of their similarity to human physiology and disease. The almost total lack of immunological tools for these animals is a disadvantage and it is not clear to what extent development or lack of immune responses in such animals are comparable to human. Among the nonhuman primates, African green monkeys were used as model to test infectivity of *Leishmania,* namely, *L. major* [109–111], *L. aethiopica* [112], *L. donovani* [113], and *L. infantum* [113], both langur monkey and squirrel monkey with *L. donovani* [114, 115], and owl monkey and marmoset with *L. braziliensis* [116, 117]. Few live-attenuated vaccines have been tested so far in mice/hamsters (Table 1). To our knowledge, no testing of live-attenuated vaccines against VL in non-human primates has been reported. However, a few vaccine studies conducted in monkeys against CL will provide insights in our understanding of vaccine studies against VL. The *dhfr-ts- L. major*, although protecting mice against CL, did not protect rhesus monkeys due to lack of protective immune response [79, 80]. However, in an another study, rhesus monkeys previously exposed to *L. major*, upon reinfection with *L. major* metacyclic promastigotes, showed reduced lesion compared to infection in naïve monkeys, signifying the immune response to live cutaneous parasites [118]. Limitations on the use of the dog or a monkey model are stipulated by the limited availability of such vaccine candidates in *Leishmania* endemic areas. Hence, it is important to forge collaborations with experts in nonendemic areas to address such questions.

Our recent studies [119] and studies by others [120–124], with PBMC from Indian patients of VL, indicated elevated serum levels of IFNγ, IL-10, and IL-6 during the

active disease, while TNF, IL-2, and IL-4 levels were minimal. Direct infection of healthy human PBMC *ex vivo* with live *L. donovani* parasites revealed elevated expression of IL-4 and IFNγ in the host cells [125]. Similarly, IFNγ upregulation was noticed in the PBMCs from nonexposed dogs after cocultured with *L. chagasi* promastigotes *ex vivo* [126]. Additionally, exposure of human PBMCs to peptides from a *Leishmania* surface antigen produced T cells that recognized *Leishmania*-infected autologous macrophages [127]. Since ethical reasons do not permit testing vaccine candidates directly in humans, as a preclinical evaluation of vaccines, the *ex vivo* grown human cells can be infected with live parasite vaccines (e.g., *LdCen*$^{-/-}$) for a brief period and immune responses (Th1) potentially correlating with protection can be analyzed.

7.2. Route of Administration and Mode of Virulent Challenge for Genetically Altered Live-Attenuated Vaccines. Studies to date with genetically targeted live-attenuated visceral *Leishmania* have primarily delivered the vaccine parasites intravenously [53]. If the vaccines are to be developed for human use, more acceptable routes of injection such as intramuscular, subcutaneous, or intradermal must be optimized. The intradermal infection route has been studied extensively for cutaneous *Leishmania* [128]; visceralization of *L. donovani* has been demonstrated after intradermal injection [129] and is being further investigated by our laboratory and others as a route of attenuated parasite vaccine administration. Subcutaneous injection of *L. chagasi*-attenuated parasites has been tested but failed to protect [130], though the nature of the vaccine strain, not the route of injection may be the cause of protection failure. As the attenuated parasite vaccine candidates are evaluated in larger animal models, these more favorable routes of administration will be tested.

The efficacy of a vaccine of any type is finally judged by its ability to protect recipients from natural infection. For leishmaniasis, this is a sand fly bite that delivers approximately 1,000 metacyclic promastigotes in the context of sand fly saliva, which is a potent modulator of the infection process [131, 132]. The traditional challenge models for visceral leishmaniasis are intravenous injection in the BALB/c mouse and intra cardiac injection of the hamster utilizing millions of stationary phase cultured virulent promastigotes- or spleen-derived amastigotes. The metacyclogenesis in culture is only partial; so stationary phase promastigotes is an imperfect model of the natural infection, but ficoll gradient separation of metacyclics improves the model [53]. The advantage of these challenge models is that they are well characterized, the disease outcome in a naïve animal is documented, and comparison of a current vaccine under evaluation can be made to earlier vaccine candidates. These models can be improved by dose modulation. A sufficiently virulent *L. donovani* strain that is maintained by continuous passage in hamsters will produce a robust infection with as little as 10^5 stationary promastigotes from the first culture passage after harvesting from a hamster spleen. Low dose needle injection intradermally in the ear is a challenge model much closer to the natural infection; however, the uncertainties of visceralization and the long delay to disease symptoms may not be worth overcoming, especially in the light of recent demonstration that vaccines that protect against needle challenge do not protect against infected sand fly bites [133]. Insectaries in which to perform *Leishmania* infected sand fly transmission to rodent models are not common and are challenging to maintain. However, the benefits of evaluating candidate vaccines for efficacy against the natural sand fly mode of transmission will vastly improve the chance of ultimate success of live-attenuated *Leishmania* vaccine candidates. Towards that end, we and others have initiated studies to develop visceralization animal models (mice and hamsters) upon challenge with infected sand flies, which could be evaluated for the efficacy of genetically altered live-attenuated *Leishmania* parasites.

8. Conclusions

Unlike most other pathogens, *Leishmania* is never fully cleared by the immune system, depriving researchers of any natural correlate of immunity to mimic in designing a preventive vaccine that seeks to achieve sterile immunity. With vaccine candidates being tested, despite having been made empirically, detailed understanding is needed about the mechanisms by which the vaccines stimulate protective immunity. A methodical understanding of protective immune responses and generation and maintenance of the immunological memory during *Leishmania* infection is needed for a sustained long-term protective response. While developing attenuated strains of *Leishmania*, focus is required towards obtaining attenuation selectively at the intracellular stage (amastigotes), while remaining nonattenuated as promastigotes to permit large scale cultivation for use as vaccines. In order to enhance the potential of live parasite vaccines, optimization of attenuation needs to be explored, probably via a combination of gene deletions by knocking out more than one gene in a single *Leishmania* strain. There is a need to develop broadly immunogenic vaccines that induce protection against a wide range of *Leishmania* strains that are causative agents of many different clinical forms of leishmaniasis. Lastly, safety of genetically altered parasites is of paramount importance if such vaccines are to be used in humans. Hence, studies are needed to develop indicators of safety for such vaccine candidates. Upon success, such approaches will be applicable to develop live-attenuated vaccine candidates for other intracellular pathogens like malaria and bacterial infections.

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Review Article

Cytokine and Phenotypic Cell Profiles of *Leishmania infantum* Infection in the Dog

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Leishmaniasis has reemerged in recent years showing a wider geographic distribution and increased global incidence of human and canine disease than previously known. Dogs are the main domestic/peridomestic reservoir hosts of zoonotic visceral leishmaniasis caused by *Leishmania infantum*. Since the evolution of leishmaniasis and clinical appearance is a consequence of complex interactions between the parasite and host immune response, a profound knowledge about the immune profile developed in dog's infection is crucial for vaccine and immunomodulatory therapy design. The main goal of this paper is to compile the recent advances made on cytokine and phenotypic cell profiles in different tissues and organs of dogs infected with *L. infantum*. This paper also stressed that the knowledge of the immune responses developed, namely, in liver, lymph node, and spleen is very limited. All data emphasizes that more research on canine leishmaniasis is necessary for the development of new and efficacious tools to control zoonotic leishmaniasis.

1. Introduction

Canine leishmaniasis (CanL) caused by *Leishmania infantum* (syn. *L. chagasi*, in Latin America), which is transmitted by the bite of phlebotomine sand flies, is endemic and affects millions of dogs in the Mediterranean basin, China, and Latin America and is an emergent disease in North America. Dogs are described as the best experimental animal model for visceral leishmaniasis caused by *L. infantum*, because many of the clinicopathological signs and immune responses observed in experimental CanL are similar to those observed on natural canine and human *Leishmania* infection [1, 2]. It has been claimed that dogs never achieve parasitological cure, and the widespread use of the available anti-*Leishmania* drugs for both canine and human treatment may contribute to parasite drug resistance. Therefore, an efficacious CanL vaccine able to block parasite transmission would be the best strategy to control the spread of the disease among other dogs and an essential part for the control of human zoonotic leishmaniasis [3]. The development of efficient immunoprophylactic molecules to maintain long-term immunity and to promote leishmaniases control relies on the identification and the characterization of the immune events associated with disease progression.

Clinical appearance and evolution of leishmaniasis is a consequence of complex interactions between the parasite and the genetic and immunological background of the host. It is widely accepted that in susceptible animals the progression of infection to active disease is characterized by a marked humoral response, a cellular immune depression against the parasite, and the appearance of a full array of clinical signs. On the other hand, resistant dogs lack clinical signs, develop low levels of anti-*Leishmania* antibodies and parasite load, and develop a strong *in vitro* lymphocyte proliferative response and a positive delayed-type hypersensitive response to leishmanial antigens in the skin [4–6]. Nevertheless, it is

important to keep in mind that animals considered resistant could be in an earlier stage of disease prior to developing signs of susceptibility [7].

Determining the role of T-helper-1 (Th1) and Th2 lymphocyte subpopulations in different tissues and organs of infected dogs is crucial to understand the immune mechanisms induced by infection. Previous studies described that cellular immune response in CanL was associated with activation of Th1 cells producing interferon-gamma (IFN-γ), interleukin-2 (IL-2), and tumour necrosis factor alpha (TNF-α) while cytokine pattern of active disease was characterized by a mixed Th1/Th2 response [5]. However, most of these works were made on peripheral blood (PB). Furthermore, other studies showed that the immune response to the parasite is not identical in whole host system but instead organ-specific [8]. In fact, a Th1, Th2, or mixed Th1/Th2 immune responses were observed in different organs of dogs infected with *L. infantum* and correlated with the presence or the absence of clinical signs and local parasite load [7, 9–12]. The organ-specific and mixed Th1/Th2 immune responses were also verified in leishmaniasis murine model [13, 14].

The challenges regarding local and systemic immune responses to the parasites need to be answered to achieve the development of efficacious strategies to control canine and human visceral leishmaniasis. The aim of this paper was to describe the recent studies about the cytokine and cell population profiles developed in different target organs/tissues, namely, bone marrow, lymph node, liver, peripheral blood, skin, and spleen developed by the dog to *L. infantum* infection.

2. Tissue and Organ Immune Responses in Canine Leishmaniasis

2.1. Peripheral Blood. IFN-γ expression or production by nonstimulated peripheral blood mononuclear cell (PBMC) lymphocytes or stimulated with soluble *Leishmania* antigen (SLA) from infected dogs has been correlated with disease resistance/asymptomatic status in both nonvaccinated and vaccinated animals [15, 16] as well as in dogs challenged with uninfected or infected colony reared *Lutzomyia longipalpis* [17]. Taking into account the results obtained in those works, the no expression of IFN-γ by PB (Table 1) could have been related with the persistence of infection in an experimental canine study performed in our laboratory [6]. On the other hand, Travi et al. [11] observed that the PMBC stimulated with SLA from 67% of symptomatic dogs experimentally infected with promastigotes isolated from the vector *Lutzomyia longipalpis* produced high levels of IFN-γ at the early stages of infection, and the proportion of individuals producing this cytokine increased over time, indicating that IFN-γ production and expression was not sufficient to prevent disease and, consequently, was not a good marker of resistance. On opposite, Carrillo et al. [16] found a depleted expression of IFN-γ in response to SLA in symptomatic experimentally infected dogs; according to Carrillo and Moreno [5], the low expression of this cytokine might have been associated with a diminished CD4+ T lymphocyte subset population. Several studies analysing lymphocyte subtypes by flow cytometry have pointed out that this PB population in sick dogs is decreased but returns to normal values after treatment [18–20], others observed an increased number of CD4+ cells in dogs with low parasitism [21]. Furthermore, others found out that the number of CD4+ T-cells in PB was similar in dogs with leishmaniasis and in healthy dogs and that there was no correlation between the clinical status or response to therapy and CD4+ counts [22, 23]. The contradictory results obtained highlights the complexity of the immune response mounted in response to *L. infantum* infection and that this lymphocyte subtype cannot be used alone as a prognosis marker. Reduction in CD3+ and increased CD5+ lymphocyte subpopulations in PB has been described in symptomatic dogs [18, 21, 23] while high CD8+ numbers were detected in animals with low parasitism [19, 21]. It was observed that a decreased CD21+ B cells, CD14+ monocytes, and class II molecules of the major histocompatibility complex (MHC-II) in symptomatic dogs were related with selective migration into lymphoid organs and lower ability for antigen presentation [21]. The contradictory results obtained were probably due to the comparison of the blood leukocyte subpopulations and parasite loads in different compartments. Moreover, the differences obtained between studies could also be related with the classification of the animals not only with the absence or presence of clinical signs but also with the development of specific anti-*Leishmania* antibodies or the detection of parasite since each methodology presents different sensitivity and specificity.

Regarding the expression and/or production of other cytokines in peripheral blood, results are also discrepant. While for some authors interleukin-6 (IL-6) [24] and IL-18 [25] are markers of active disease or asymptomatic infection; respectively, for others IL-6 [26] and IL-18 [15, 16] have no determinant role. Similar results were obtained for IL4 and IL10, where their expression was only observed in mitogen-stimulated PBMC from symptomatic dogs [27] while others detected it in animals presenting clinical signs or not [15, 16]. From these studies, IL-4 did not seem to contribute to canine susceptibility to infection. On the other hand, the increase of IL-10 production by PBMC stimulated with SLA along

Table 1: Frequencies of cytokine and iNOS expressions determined by reverse transcriptase PCR in 12 asymptomatic dogs (with high parasite load in viscera) infected with *L. infantum* amastigotes, six months postinfection.

Tissue/organ	INF-γ (%)	TGF-β (%)	TNF-α (%)	IL-10 (%)	iNOS (%)
Peripheral blood	0	91.66	66.66	0	0
Lymph node	0	36.36	54.54	81.81	36.36
Liver	9	18.16	0	18.19	18.19
spleen	11.11	11.11	0	33.33	33.33
Bone marrow	25	66.66	41.66	100	75

INF-γ: interferon-gamma; TGF-β: transforming growth factor-beta; TNF-α: tumour necrosis factor-alpha; IL-10: interleukin-10; iNOS: inducible nitric oxide synthetase.

with increase blood parasite burden has recently pointed out to be predictive of the evolution of canine infection [7]. Others have verified the association between IL-10 and active visceral leishmaniasis in humans [28]. Nonetheless, the spectrum of cytokines and the immunophenotypic of cells cannot be considered good markers to predict the evolution of infection since PB is not the tissue of election for parasite multiplication and persistence.

2.2. Skin. Skin is essential for the transmission of *Leishmania* since it is the tissue where an infected sand fly inoculates parasites into the vertebrate and the first barrier of the immune system. In the study performed by Solano-Gallego et al. [29], muzzle skin of asymptomatic dogs had no histological demonstration of lesions neither amastigotes, but parasitesDNA was detected. According to these authors, asymptomatic dogs found positive to *Leishmania* parasites by PCR do not play a significant role in the infection of phlebotomine sand flies. However, in a recent work performed by Madeira et al. [30], *L. chagasi* was isolated from intact skin from different body regions of 292 out of 394 seropositive dogs despite that only 21.9% of them were recognised as symptomatic. Furthermore, Guarga et al. [31] through xenodiagnosis concluded that asymptomatic dogs were infectious to the sand fly vectors. All these data suggest that parasites have large distribution in the skin of infected dogs in spite of remaining asymptomatic for prolonged time and for that reason not submitted to any control measures. The presence of parasites in these animals highlights their importance as reservoir hosts. Thus, from an epidemiological point of view, it would be important to correlate the local immune response of skin to the presence of the parasite as well as with the infectiousness to competent vectors and therefore, allow the development of tools for blocking transmission.

A variety of cells, such as intraepithelial T lymphocytes and Langerhans cells, are present in the skin and are capable to generate local immune reactions. Branchelente et al. [9] demonstrated that the local immune response in lesional skin of naturally infected dogs included IFN-γ, TNF-α, and IL-4 expression. The increased expression of IL-4 was associated with severe clinical signs and a high parasite burden in the skin biopsies. More recently, Menezes-Souza et al. [12] analysed the expression of proinflammatory, anti-inflamatory, and immunoregulatory cytokines as well as the levels of transcription factors T-bet (associated with Th1 immune response), GATA-3 (associated with Th2 immune response), and FOXP3 (involved in the regulation of cytokine gene transcription) in the skin without lesion of dogs naturally infected. A mixed Th1/Th2 cytokine profile was observed in asymptomatic dogs. Additionally, low levels of transcription factors GATA-3 and FOXP3 were also correlated with the absence of clinical signs. The data obtained indicate that in asymptomatic infection or in cases with lower skin parasitism, a mixed inflammatory and regulatory immune response profile may be of major relevance for both the maintenance of the clinical status of the dogs as well as for parasite replication at low levels.

Fondevila et al. [32] and Papadogiannakis et al. [33] investigated the cellular immunophenotyping and the number of amastigotes in the epidermis and dermis in dogs with patent leishmaniasis. For these authors, alopecic and sebaceous adenitis were associated with an effective local immune response characterized by the activation of epidermal Langerhans cells, upregulation of MHC-II molecules on keratinocytes, dermal infiltration by CD8+ and a lower number of CD4+ cells, presence of CD21+ cells, and a relatively low parasite burden. On the other hand, dogs with generalized nodular disease mounted an impaired immune response characterized by a low epidermal expression of MHC-II molecules, small numbers of T cells in the dermal infiltrate, and a high parasite burden [32]. Taking into account data obtained in several studies, it would be important to determine the immunophenotype associated with the absence of parasites in the skin.

2.3. Lymph Nodes. Lymph nodes (LN) are widely accepted to be the first relevant lymphoid tissues affected after dissemination of the parasite from skin macrophages; thus, the evaluation of their immune response to *Leishmania* might help in determining the infection outcome. However, the cellular immune responses developed in LN to *L. infantum* are scarce. Giunchetti et al. [34] analysed the immunophenotypic profile in popliteal LN from naturally infected dogs and its relation with parasite burden in this organ and skin. A significant increased number of T lymphocytes, particularly CD8+ cells, in addition to decreased levels of CD21+ B cells and upregulation of MHC-II molecules were the major LN immunophenotypic changes observed. Interestingly, the highest number of CD8+ T cells was observed in animals harbouring the highest skin parasitism. According to the authors, LN CD8+ T cells may present a distinct activation status during CanL, probably associated with immunomodulatory or suppressor cell activity. In fact, the immunomodulatory effect of these cells was recently observed by Alexandre-Pires et al. [23] where that CD8+ subpopulation in LN from treated dogs was significantly lower than in asymptomatic dogs. Moreover, CD4+ T-cell subset in LN from both asymptomatic and treated dogs was significantly higher than that in noninfected dogs. Together, these findings suggested that lymphocyte activation in the LNs with the expansion of CD4+ subpopulation may favour the control of *Leishmania* infection through a local reduction of parasite replication and/or parasite clearance while an increase of the number of CD8+ cells seem to be related with parasite persistence and immunomodulatory cell activity.

Regarding cytokine profile, Alves et al. [35] evaluated its relation with parasite burden in prescapular LN from naturally infected dogs and observed that the balance of expression of IFN-γ, TNF-α, IL-10, and transforming growth factor-beta (TGF-β) determines parasite load and clinical expression. LN from asymptomatic dogs had higher expression of proinflamatory cytokines and lower number of parasites indicating that IFN-γ and TNF-α could play a role in protection against disease while LN from symptomatic dogs expressed more anti-inflamatory cytokines suggesting

a role for IL-10 and TGF-β in disease progression. This event is in agreement with the cytokine profile observed by us in popliteal LN from dogs experimentally infected where the balance between the percentage of IL-10 and TNF-α expression (Table 1) could have been the responsible not only for a lower parasite load in this tissue than in others (skin, hepatic, and splenic), but also for the absence of lymphadenomegaly or other clinical signs at the end of the study in most of the infected dogs (only one dog had popliteal adenomegaly).

All these data highlight that more studies focused on specific immunological events in LN, namely, on cytokine profile and CD8+ T and CD4+ subpopulations, should be performed in order to determine if triggering an effective immune response in this lymphoid tissue could avoid an intense multiplication and consequent dissemination of the parasite to other organs [35].

2.4. Liver. One of the most relevant organs involved in the parasite-host interface during *L. infantum* infection is the hepatic compartment. However, and as far as we are aware, only one study quantified the cytokine production by the liver of dogs infected and observed that the production of IFN-γ, IL-10, and TGF-β1 was higher in those with no clinical signs [36]. Our results on experimental CanL correlated these findings as liver cells of the infected animals also expressed those cytokines as well as iNOS (Table 1). On the other hand, there was no expression of TNF-α and IL-4. The presence of interlobular granulomas of variable severity in infected dogs could be a reaction of the organism to parasitism in an attempt to control the multiplication of the parasites [37]. The presence of hepatic granulomas was also correlated with subclinical human VL [38]. In addition, Stanley and Engwerda [39] suggested that apart from IFN-γ responsible for the generation of leishmanicidal mechanisms, TNF-α is also involved in hepatic granuloma formation and contributes to the resolution of local infection in the murine model. Thus, the no expression of TNF-α by the hepatic cells of our experimentally infected dogs could have been associated with the high parasitism observed [2].

As mentioned above, data regarding cytokine profile are also quite limited highlighting the necessity to perform more studies in order to improve our knowledge concerning the immune response developed in liver during visceral *Leishmania* infection.

2.5. Spleen. CanL is associated with splenic architecture disruption, which is characterized by disorganization of normal lymphoid tissue, loss of normal spleen leukocyte diversity via replacement of leukocytes by plasma cells, and eventual atrophy of the lymphoid tissue [40]. Thus, whilst the spleen is responsible for the major immune response in leishmaniasis, the present knowledge of the cytokines and leukocytes that participate in its immune response is very limited since few studies were performed. RNA expression levels of a wide range of cytokines (IFN-γ, TNF-α, IL-4, IL-5, IL-10, IL-12, IL-18, and TGF-β), transcription factors (T-bet and GATA3), and chemokines (IP-10, RANTES, MIP-1α, MCP-1) were evaluated in the spleen from naturally and experimentally infected dogs [10, 41]. A positive correlation between the expression of IL-10 by splenocytes with both increased parasite load and progression of the disease was observed in naturally infected dogs [41]. According to Santana et al. [42], the production of IL-10 within splenic granulomas may provide immunological conditions for the survival and growth of the parasite. On the opposite, Strauss-Ayali et al. [10] did not find any change in the expression of IL-10 by splenocytes throughout experimental infection with 8.6×10^8 *L. infantum* amastigotes, even in animals with a high parasite load. Similar result was obtained by Corrêa et al. [36] where no differences were found in the production of IL-10 by spleen extracts between symptomatic and asymptomatic naturally infected dogs. On the other hand, Strauss-Ayali et al. [10] suggested that the early increase of IL-4 might have a role in the persistence of parasites in the presence of high IFN-γ expression. An association between high levels of IFN-γ and chemokines expression and splenic parasitism with a worst disease prognostic was also observed [10, 36, 41]. Interestingly, in our experimental infected dogs, IFN-γ was only expressed by the tissues with high parasite load, namely, spleen, bone marrow, and liver (Table 1), suggesting that the presence of this cytokine is not synonymous of parasite clearance. One possible explanation for the association between IFN-γ presence and the high parasite load could be that new parasite generations are constantly being seeded from other infected tissues stimulating its expression [10]. Furthermore, the levels of IFN-γ and chemokines expression significantly decreased after treatment, reflecting a reduced recruitment of immune cells into the spleen due to the minimal amount of parasites remaining in the organ. It is important to mention that at this moment the source of such IFN-γ is not known, and probably it is not produced by T cells, since symptomatic dogs show T-cell depletion in the spleen and a specific immunosuppression against the parasite.

The elevated levels of the tested chemokines observed by Strauss-Ayali et al. [10] were suggestive of an accumulation of infiltrating monocytes attracted by MIP1-α and MCP-1, as well as of CD4+Th1 and CD8+ cells which could have been recruited by IP-10. In agreement with these data, Guerra et al. [19] observed an increased frequency of CD8+ T cells in the spleen with low parasite load.

Nevertheless, in the few studies performed up to now, none of the Th2 [36], Th1/Treg [41], or Th1/Th2 [10] cytokine immune responses neither the chemokine nor phenotypic cell profiles obtained were able to eliminate the parasite locally.

2.6. Bone Marrow. Progression of *Leishmania* infection has been related with a granulomatous inflammation in bone marrow accompanied by an increased percentage of lymphocytes and plasma cells, erythroid and megakaryocytic hypoplasia, and/or dysplasia and erythrophagocytosis [4]. According to Manzillo et al. [43], megakaryocytic and erythroid dysplasia were probably related to an increased number of bone marrow macrophages producing high levels of TNF-α and IFN-γ. In agreement with this hypothesis,

Quinnell et al. [44] observed an increased accumulation of these cytokines in bone marrow of naturally infected dogs with and without clinical signs. These authors also detected a significant positive correlation between disease severity and IL-4. However, this cytokine was not expressed by the bone marrow cells of dogs experimentally infected six months after infection (Table 1). Instead, iNOS and a mixed pattern of proinflamatory (TNF-α) and regulatory (TGF-β and IL-10) cytokines were detected in those asymptomatic dogs. Thus, one of the reasons for the asymptomatic evolution of the infection observed in those dogs despite the high parasite load in bone marrow, liver, and spleen could have been related with the no expression IL-4 by these organs.

In a study on bone marrow leukocyte subpopulations in naturally infected dogs with and without clinical signs of CanL and after being treated for leishmaniasis, Alexandre-Pires et al. [23] observed that symptomatic and asymptomatic animals exhibited a significant increase of MHC-II expression in bone marrow lymphocytes probably reflecting the presentation of *Leishmania* antigens. Moreover, treated animals also showed increased expression of MHC-II monocytes pointing out to elevated levels of antigenic presentation activity, possibly due to the availability of parasite antigens as a consequence of treatment. No differences in CD8+ and CD4+ T-cell populations were observed between the three studied groups allowing hypothesizing that the control of infection in the bone marrow is not related with the expansion of these cells.

3. Conclusions

The cytokines and phenotypic cell profiles that participate in immune responses in different compartments where the parasite replicates seem to have variable effects on local parasite control, highlighting the complexity of the cellular immune response developed by the dog to *L. infantum* infection. Moreover, these studies have disclosed interesting facets of the immune response, even contradicting some dogmas such as the role of IFN-γ in parasite clearance. Furthermore, this paper also stressed that the knowledge of the immune responses in some lymphoid compartments (liver, lymph node, and spleen) is very limited.

Since the presence and intensity of parasites in blood in CanL are normally low and transient, the generation of immunological data in this tissue might not be an accurate reflection of immune responses that occur in the body compartments where a high parasitism is normally observed. On the other hand, could the immune response in PB be the result of the immune responses developed in the different organs and thus be used as a prognosis marker? Or could local immune response developed in spleen, the major lymphoid organ involved in parasite-host interaction, be used to predict the evolution of infection? In fact, here, the invasive method of sampling must be considered.

Future integrated studies are needed in order to clarify the association between the trafficking of various cell subpopulations, cytokine, and chemokine gradients in association with the immune responses and parasite loads in the different visceral and peripheral tissues developed at the same time and in the same animal in order to improve the knowledge of local and systemic immune responses in CanL. The effective immune response able to control the parasite must be evaluated in animals with no clinical signs and with very low parasite burden since only these animals can be considered immune competent/resistant to clinical disease. In addition, the identification of specific cell subpopulations that are involved in disease control in different organs will allow control strategies namely, the development of efficacious therapeutic and prophylactic tools.

Conflict of Interests

All authors declare that they have no conflict of interests concerning the work reported in this paper.

Ethical Guidelines

The study on *Leishmania infantum* experimental infected dogs followed the International Guiding Principles for Biomedical Research Involving Animals and the guidelines of the Portuguese Legislation (Lei n°92/95, 12.9) and was approved by the Ethics Committee of the Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa.

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Review Article

Differential Regulation of the Immune Response in the Spleen and Liver of Mice Infected with *Leishmania donovani*

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Immunity to pathogens requires generation of effective innate and adaptive immune responses. *Leishmania donovani* evades these host defense mechanisms to survive and persist in the host. A better understanding and identification of mechanisms that *L. donovani* employs for its survival is critical for developing novel therapeutic interventions that specifically target the parasite. This paper will highlight some of the mechanisms that the parasite utilizes for its persistence and also discuss how the immune response is regulated.

1. Introduction

Visceral leishmaniasis (VL) is caused by the intracellular parasites *Leishmania donovani* and/or *Leishmania infantum/chagasi*. In the mouse model of visceral leishmaniasis, there is a distinct organ-specific pattern of parasite growth during the disease progression. Infection in the liver is characterized by a rapid increase in the parasite burden in the first 4 weeks of infection followed by clearance of the parasite within 6–8 weeks. This self-curing mechanism in the liver is attributed to the development of a Th1 dominated granulamatous response [1] characterized by high IFNγ production by CD4 and CD8 T cells. In contrast to liver, infection in the spleen has serious consequences demonstrated by increased parasite burden, disruption of splenic microarchitecture and impaired immune responses resulting in the establishment of parasite persistence [2]. Although the exact mechanism by which the parasite establishes chronic infections in the spleen still remains elusive, it is now becoming evident that the parasite targets and alters the functions of host immune system for evasion. Some of the mechanisms that are altered include suppression of host protective Th1 responses, generation of defective CD8 T cells and inhibition of dendritic cell (DC) functions [2–4]. In addition to modifying DC and T-cell function, the parasite also modulates B-cell function for its survival. Furthermore, by directly interacting with different cellular subsets, the parasite also generates an immunosuppressive environment by inducing IL-10 production and thus favoring its survival in the host. In the first part of this paper we will discuss the above mentioned mechanisms utilized by the parasite to evade host immune response and establish chronic infection in the spleen. The second part of the paper will focus on the *L. donovani* infection in the liver and the regulation of the inflammatory response in this organ.

2. Infection in the Spleen

In the experimental model of VL, the spleen is a site of chronic inflammation, characterized by parasite persistence. Chronic infection of the spleen is associated with splenomegaly and changes in the splenic microarchitecture [2, 5, 6], which have consequences on the generation of the immune response to the parasite [5–8]. Typically, following encounter with antigen, dendritic cells (DCs) migrate from the marginal zone (MZ) of the spleen to the T-cell-rich area known as periarteriolar lymphoid sheaths (PALS). In

the PALS, the DCs interact with the T cells, and this interaction results in the induction of an antigen-specific T cell response [9]. During chronic *L. donovani* infection, increased production of TNF by macrophages results in the disruption of the splenic MZ [5]. As a consequence of this disruption, DCs [2] and naïve T-cells [5] fail to migrate to the PALS, which may results in diminished priming of T-cells. DCs not only show an impaired migratory capacity, but they also down regulate important costimulatory molecules and increasingly express inhibitory molecules [3]. Hence, it appears that DC functions are defective at later stages of infection. This hypothesis is supported by the fact that adoptive transfer of LPS-activated bone marrow-derived DCs into mice at d21 p.i. results in a significant reduction of the splenic parasite burden [2, 10] and possibly in the induction of protective T cell responses. Splenomegaly is also associated with destruction of the follicular DC network [11] and the $gp38^+$ fibroblastic reticular cell network [2]. This disruption is particularly critical since treating or blocking it increases the frequency of protective IFNγ-producing CD4 T cells [12]. Conventional $CD11c^{hi}$ splenic DCs not only show impaired migratory capacity during chronic VL, but also increasingly upregulate the inhibitory molecule B7-H1 during chronic infection [3]. B7-H1 is constitutively expressed on subsets of macrophages, thymocytes, and B cells [13], but its expression can also be induced on DCs, epithelial, and endothelial cells [13, 14]. B7-H1 binds to its receptor PD-1 and inhibits T cell proliferation and cytokine production [15]. Furthermore, B7-H1 can also induce programmed cell death of effector T cells by ligation to a yet unknown receptor [16]. During chronic VL, antigen-specific CD8 T cells gradually upregulate PD-1 and display signs of exhaustion characterized by the loss of IL-2, TNF, and finally IFNγ production. These functionally exhausted cells eventually die [3]. Exhausted CD8 T cells have also been recently observed in patients with diffuse cutaneous leishmaniasis [17]. The functional exhaustion and deletion of antigen-specific CD8 T cells is in part induced by the upregulation of B7-H1 on splenic DCs. Indeed, in vivo blockade of B7-H1 during chronic *L. donovani* infection results in increased survival of antigen-specific CD8 T cells and also partially restores the functional capacity of exhausted CD8 T cells [3]. The fact that CD8 T-cell functions are not completely restored by B7-H1 blockade suggests the involvement of additional pathways in the suppression of cytokine production by these cells. Other inhibitory molecules, such as LAG3 [18–20] and CTLA4 [21, 22], may also be involved in this suppression. Nevertheless, the partial recovery of CD8 T cell functions is sufficient to reduce the splenic parasite burden, suggesting that B7-H1 blockade and the reactivation of CD8 T-cells may be exploited as a therapeutic intervention.

Other costimulatory and inhibitory molecules were also shown to play a suppressive role during acute experimental VL. Indeed, blocking of either B7-2 [23], OX40, or CTLA-4 [24, 25] results in improved T-cell responses in *L. donovani* infections. Taken together, these results highlight the crucial role of DC during VL and imply that manipulation of the DC response to the parasite may be beneficial for future therapeutic interventions.

Another major factor contributing to progression of disease in VL is IL-10 [10, 26–28]. The role of IL-10 in augmenting disease has been demonstrated by studies that show that IL-10 receptor blockade aids in abrogation of the disease [26]. Additionally, $Il10^{-/-}$ mice are highly resistant to VL [27]. During chronic infection in mice, IL-10 is mainly expressed by $CD25^-FoxP3^-CD4^+$ T cells [10], which also produce IFNγ. These cells have also been identified in human patients and their presence correlates with disease progression [10]. Although there is evidence linking IL-10 and susceptibility to *L. donovani* infection in humans, inter-individual differences in IL-10 production might contribute to variation in susceptibility to *L. donovani* [29]. Using transgenic mice expressing human IL-10 ($Il10^{-/-}$/hIL10BAC), we could investigate the role of cellular-specific IL-10 production on susceptibility to infection. Interestingly, in these transgenic mice IL-10 was properly regulated in the myeloid compartment and resulted in the rescue of $Il10^{-/-}$ mice from LPS toxicity. However, transgenic IL-10 was weakly expressed in T cells resulting in resistance to *L. donovani* infection, indicating that T-cell-derived IL-10 inhibits the control of parasite burden in the spleen and liver. Thus, T-cell-derived IL-10 plays an essential role in the establishment of chronic infection [30].

IL-10 also plays an important suppressive role during the early stages of infection. We have recently demonstrated that IL-10 contributes to suppression of CD8 T-cell expansion during the early stages of infection (Bankoti et al., submitted). IL-10 blockade also resulted in increased effector functions in CD8 and CD4 T cells. Several cell populations have been identified that upregulate IL-10 production during *L. donovani* infection. These include CD4 T cells [10], NK cells [31], macrophage [26], and DCs [32], and potentially one of these cell populations could be responsible for suppression of CD8 T-cell expansion and CD8 and CD4 T-cell function. However, data from our laboratory suggest that there is probably no major source of IL-10 responsible for the suppression of T-cell function, but several IL-10 sources contribute to it (Bankoti and Stäger, unpublished).

Several studies indicate that B cells have an adverse effect on disease outcome in various models of leishmaniasis [33–36]. In experimental VL, B cells also contribute to disease exacerbation. Indeed, in the absence of B cells, mice are resistant to *L. donovani* infection [35]. The mechanism by which B cells contribute to parasite persistence has not yet been completely defined. One of the possible mechanisms by which B cells exacerbate disease is by secretion of antibodies. The role of antibodies in progression of disease was demonstrated by using J_H mice that produce no antibodies. Infection of J_H mice with *L. major* resulted in smaller lesions and limitation of the parasite burden. However, reconstituting J_H mice with anti-*L. major* sera at d21 postinfection resulted in exacerbation of the disease [37, 38]. This increased susceptibility to infection could be explained by the fact that host IgG on the amastigote surface ligates the macrophage FcγRs and induces IL-10 production by macrophages [37]. One of the consequences of this increased IL-10 production is the inactivation of macrophages which further contributes to parasite persistence. In addition to IgG, increased IgM

production and polyclonal B-cell activation has also been recently demonstrated as a cause of disease exacerbation in *L. infantum* infected mice during the early stages of infection [34].

Our recent findings suggest that, in addition to inducing IL-10 production by macrophages via antibody secretion [37], B cells directly interact with *L. donovani* and this interaction results in the suppression of protective T-cell responses in an antibody-independent manner. Following i.v. injection, amastigotes move to the spleen where they initially come in contact with cells of the marginal zone (MZ). The amastigotes are primarily internalized by marginal zone macrophages (MZM) and metallophilic macrophages (MM) present in the MZ of the spleen [4, 9]. The marginal zone also consists of the marginal zone B cells (MZBs) [39] that are located strategically to encounter the parasite early on during infection. Marginal zone B cells are noncirculating B cells and initiate T-independent responses in response to blood borne pathogens [40, 41]. MZBs also have the capacity to rapidly differentiate into short lived IgM producing antibody forming plasma cells [42]. We recently observed that *L. donovani* closely interacts with B cells in vivo, inducing activation and cluster formation (Bankoti et al., submitted). We also observed an association between amastigotes and cells that displayed an MZB phenotype in vivo, 24hr after infection, suggesting that MZBs capture parasites during the very early stages of disease. Although, the exact mechanism of parasite capture remains unclear, it is possible that MZB cells recognize amastigotes through surface IgM and/or through the complement receptor 2 (CD21). This interaction of the parasite and MZBs resulted in the upregulation of the costimulatory molecules CD80 and CD86 and the production of IL-10 by two B-cell subpopulations: marginal zone B-cell-like cells and regulatory B-cells-like cells (Bankoti et al., submitted). Moreover, in agreement with previous report [34], B cells upon interacting with *L. donovani* increased surface IgM expression and secrete IgM (Bankoti et al. submitted). In addition to inducing IL-10 production by MZB cells, cross-linking of CD21 also induces the migration of MZBs to the white pulp [41, 43, 44]. Hence, it is possible that the MZB cells capture the parasite and migrate to the white pulp where they can then transfer the antigen to the follicular DCs [45–47]. Follicular DCs can then retain the antigen for a long duration of time and initiate CD4 and CD8 T-cell responses. Follicular DCs can also interact with the follicular B cells and result in polyclonal B-cell activation [48], which is typically observed during human VL [49–52]. Activation of MZBs by *L. donovani* is detrimental to the course of infection, since depletion of MZB results in significantly lower splenic parasite burdens and in stronger CD8 and CD4 T-cell responses. This suggests that marginal zone B cells suppress protective T-cell responses during early stages of *L. donovani* infection and contribute to the establishment of chronic disease. Although MZBs secrete IL-10, this suppressive effect is only partially mediated by MZB-derived IL-10. Thus, it is possible that MZB inhibit APC functions and/or induce IL-10 production by other cell populations, such as macrophages or T cells. Further investigations are needed to test this hypothesis.

IL-10 production by B cells was also shown to induce Th2 responses in a model of cutaneous leishmaniasis [53]. B cells also produce IL-10 upon encounter with *L. infantum* [34]; however, this B-cell-derived IL-10 does not seem to affect the parasite burden at least at d28 p.i. [34].

3. Infection in Liver

The liver is another main target organ in the experimental model of VL. In contrast to the spleen that stays chronically infected, infection in the liver is self-resolving within 6–8 weeks. Resolution of disease in the liver is associated to the development of granuloma formation which is one of the key features of hepatic resistance [54]. The resolution of the infection in the liver is attributed to the development of a Th1-dominated granulomatous response, characterized by high IFNγ production by CD4 T cells. The Th1 response in turn is initiated by IL-12 secreted from the DCs [9, 55]. IL-12 is an essential cytokine in the development of protective immunity to *L. donovani,* since blocking of IL-12 reduced both the IFNγ production and granuloma formation in the liver of infected mice [56]. Furthermore, supplementing exogenous IL-12 to *L. donovani*-infected mice early during infection decreased the liver parasite burden [57]. CD8 T cells also have an essential role in the clearance of the parasite in the liver. Indeed, depletion of CD8 T cells inhibits the development of the granulomatous response and results in disease exacerbation [1]. Moreover, CD8 T cells have been shown to be mediators of resistance to rechallenge [58]. In addition to the adaptive T-cell response, TNF production and expression of inducible nitric oxide synthase (iNOS or NOS2) by macrophages aid in clearing the infection. Hepatic resistance in VL is also attributed to the generation of reactive nitrogen and oxygen intermediates, both of which have been shown to play a role in containing parasite growth during the early stages of infection [59]. These might in turn be related to the T-cell-dependent recruitment of monocytes at these early time points [60]. At the later stage of infection, iNOS gene regulation appears to play an important role and generation of NO indicates T-cell-dependent macrophage activation [60].

Initiation of the immune response requires the recognition of the parasite by the immune system. The presence of certain molecules on the pathogens, known as pathogen-associated molecular patterns (PAMPs) allows the immune system to recognize the pathogen and elicit an immune reaction. These PAMPs are recognized by Toll-like receptors (TLRs) present on the antigen presenting cells. Recognition of a pathogen by TLRs initiates a signaling cascade that ultimately results in the induction of cytokines. The interferon regulatory factors (IRFs) are important parts of this signaling chain. One of the IRFs, IRF5, is of particular interest and has previously been demonstrated to induce proinflammatory cytokines such as IL-12, TNF, and IL-6 in response to viruses [61, 62]. This was further supported by the fact that in *Irf5*$^{-/-}$ mice there is attenuation of type I IFNs, TNF and IL-6 production in response to viral infection [63–65]. We have recently demonstrated that *L. donovani*

elicits a defective Th1 responses in the liver of *Irf5*$^{-/-}$ mice and results in fewer liver granuloma and exacerbation of the hepatic infection [66]. Additionally, the granulomas in *Irf5*$^{-/-}$ mice were also considerably smaller in size suggesting a defective recruitment of inflammatory cells into the liver of *Irf5*$^{-/-}$ mice. These results emphasize the importance of IRF5 as an essential transcription factor to initiate the inflammatory response following *L. donovani* infection. Recently, IRF5 was shown to promote inflammatory macrophage polarization [67]. M1 macrophages, also known as the classically activated macrophages, are induced by IFNγ to produce proinflammatory cytokines, for example, TNF, IL-6, and IL-23. In addition to producing pro-inflammatory cytokines, M1 macrophages also exhibit enhanced microbicidal activity. M2 macrophages on the other hand are known as alternatively activated macrophages, which produce anti-inflammatory cytokine and enhance tissue repair [68, 69]. Interestingly, IRF5 influences the polarization of macrophages. M1 macrophages have high IRF5 expression and therefore activate genes for IL-12p40, IL-12p35, and IL-23p19 and repress IL-10 gene [67]. Classically activated macrophages upon triggering of interferon and TLR pathway also increase expression of iNOS that results in production of NO [70–72]. Hence, it is not surprising that iNOS protein is not produced in the granulomas of *Irf5*$^{-/-}$ mice and that *Irf5*$^{-/-}$ mice are more susceptible to VL. The exact pathway triggered by *L. donovani* that results in the induction of IRF5 and the mechanism by which IRF5 induces the inflammatory response during *L. donovani* infection are still undefined. IRF5 is downstream of TLR7 and 9, but can also be directly induced by type I IFN [73]. Thus, one could speculate that *L. donovani* may trigger TLR7 and/or TLR9 and induce IRF5 expression. It would be interesting to examine whether IFR5 is induced by *L. donovani* in *Tlr9*$^{-/-}$*Tlr7*$^{-/-}$ double-knockout mice and whether TLR7 and TLR9 have any overlapping functions. IRF5 upregulation appears to be required by different cells at different time of infection. For instance, the absence of IRF5 does not seem to affect the early development of Th1 responses. In contrast, this transcription factor is essential for the maintenance of Th1 responses and is increasingly expressed in T cells during chronic infection. Conversely, IRF5 deficiency severely impaired the infiltration of inflammatory cells in the liver already at earlier stages of infection. How this timely expression of IRF5 regulates the immune response and the role of IRF5 in T cells are still some of the questions that remain unanswered and need further investigations. Several studies have demonstrated the importance of other IRFs during *L. donovani* infection. *L. donovani* has been shown to induce IRF-binding activity in macrophages *in vivo* [74]. Furthermore, IRF7 was shown to have a role in regulating the killing of parasites by splenic marginal zone macrophage [75]. Additionally, *L. donovani* amastigotes disrupt the interaction between STAT1α and importin-α5 (a nuclear transport adaptor protein) resulting in the inhibition of IFNγ-induced expression of IRF1 in macrophages [76]. Although these studies reveal the importance of IRFs as an important regulator of signaling events and modulating the outcome of disease, further investigations are required to completely understand the exact mechanisms of IRF-induced pathways to use it for therapeutic purposes in *L. donovani* infections.

4. Conclusion

Leishmania donovani utilizes several mechanisms to evade the host immune system depending on the cell that it interacts with during the course of infection. Although these events might not be chronologically linked, clearly the parasite encounters different cells of the immune system and efficiently evades its clearance. Upon intravenous infection with *L. donovani* in mice, the cells that first come in contact with the parasite in the spleen are DCs, macrophages, and the MZ B cells. The parasite evades these immune cells by either interfering with proper DC function or by inducing IL-10 production by macrophages, B and T cells, resulting in an immunosuppressive environment. The effect of these altered functions early during the disease is reflected by generation of defective CD8 T and CD4 T-cell responses. Clearly there is a dearth of knowledge regarding the complex mechanism that the parasite utilizes to evade the immune system, and a better understanding of the complex interaction of various cell types with the parasite is needed to aid in development of novel therapeutics interventions.

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Review Article

Adenosine and Immune Imbalance in Visceral Leishmaniasis: The Possible Role of Ectonucleotidases

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Visceral leishmaniasis (VL) is the most severe form of leishmaniasis and is responsible for most *Leishmania*-associated deaths. VL represents a serious public health problem that affects many countries. The immune response in leishmaniasis is very complex and is poorly understood. The Th1 versus Th2 paradigm does not appear to be so clear in visceral leishmaniasis, suggesting that other immunosuppressive or immune-evasion mechanisms contribute to the pathogenesis of VL. It has been demonstrated that generation of adenosine, a potent endogenous immunosuppressant, by extracellular enzymes capable to hydrolyze adenosine trinucleotide (ATP) at the site of infection, can lead to immune impairment and contribute to leishmaniasis progression. In this regard, this paper discusses the unique features in VL immunopathogenesis, including a possible role for ectonucleotidases in leishmaniasis.

1. Introduction

Parasites that belong to the genus *Leishmania* are among the most diverse human pathogens, both in terms of geographical distribution and in the variety of infection-induced clinical manifestations they generate. Of the 88 countries affected by leishmaniasis, 72 are classified as developing countries, including 13 of the least developed countries. Despite the widespread distribution of leishmaniasis, 90% of VL cases occur in just five countries: Bangladesh, India, Nepal, Sudan, and Brazil [1]. *Leishmania* are obligate intracellular protozoan parasites transmitted by phlebotomine sand flies. Flagellated promastigotes injected by sand fly bites replicate as intracellular amastigote stages inside mononuclear phagocytic cells of mammalian hosts [2, 3]. The epidemiology of leishmaniasis is diverse, with 20 *Leishmania* species that are pathogenic in humans and 30 sand fly species that have been identified as vectors [1]. This range of species variability culminates in different symptomatology and clinical manifestations that can be classified into two broad disease presentations: cutaneous leishmaniasis and VL [4].

Visceral leishmaniasis (VL) is caused by *Leishmania donovani* (*L. donovani*) in the Indian subcontinent, Asia, and Africa and by *Leishmania infantum* (*L. infantum*) or *Leishmania chagasi* (*L. chagasi*) in the Mediterranean region, southwest and central Asia, and South America; however, other species, such as *Leishmania amazonensis* (*L. amazonensis*) in South America, are occasionally viscerotropic [5, 6]. VL is the most severe form of leishmaniasis and is responsible for most deaths caused by leishmaniasis. The disease commonly affects the liver, spleen, and lymph nodes. The pathophysiology of the disease is characterized by the onset of symptoms of a persistent systemic infection, including loss of appetite, weight loss, intermittent fever, fatigue, and exhaustion. The incubation period usually ranges from two to six months. Parasite dissemination occurs via the vascular, lymphatic, and mononuclear phagocyte systems

and results in bone marrow infiltration, hepatosplenomegaly, and lymphadenopathy [7, 8].

In VL, the immune response is closely related to disease progression and the development of clinical manifestations. The syndromes and causative mediators are typical of a slowly developing systemic inflammatory response syndrome with multiorgan failure. The absence of parasite actions or products that would harm the host cells or tissues is a further indication that the systemic pathogenicity of VL is dependent on the host response [9].

2. Immune Response in Visceral Leishmaniasis: The Th1 versus Th2 Paradigm

Cytokine production and the subsequent stimulation or inactivation of certain immune cells in the early stages of an infection essentially determines the type of immune response [10]. The complexity of the immunological events triggered during active VL and the relevance of the segregation of human immune responses during VL into type 1 and type 2 still remain unclear. Several studies have identified that the major immunologic dysfunction observed in VL is the inability of T cells to produce IL-2 and IFN-γ upon stimulation with *Leishmania* antigens and to activate macrophages and kill *Leishmania* parasites. Analysis of Th1 and Th2 cell-associated cytokines in peripheral blood samples from patients with *L. donovani*-induced VL showed reduced IFN-γ levels and increased IL-4 levels in comparison to healthy patients in the control group [11].

Recent data have challenged the simplicity of Th1 versus Th2 model and revealed further complexities in cytokine regulation and the mechanisms of acquired resistance and immune escape [3]. For some cases of VL, it appears that the immune response is a mixed Th1 and Th2 type. In patients with VL, although the production of type 1 cytokines was not depressed, cells appeared to be unresponsive to stimulation with type 1 cytokines [10]. Increased production of multiple cytokines and chemokines in VL patients was also observed; the response was predominately proinflammatory, as indicated by the elevated plasma levels of IL-1, IL-6, IL-8, IL-12, IL-15, IFN-γ-inducible protein-10 (IP-10), monokine induced by IFN-γ (MIG), IFN-γ, and tumor necrosis factor (TNF)-α [2, 12]. Gene expression analysis in an *in vitro* model, using human monocyte-derived macrophages (MDMs) challenged with *L. chagasi* promastigotes that were subsequently cocultured with or without *Leishmania*-naïve autologous T-cells, found that the initial encounter between *L. chagasi* and cells of the innate and adaptive immune system primarily stimulated type 1 immune cytokine responses. Type 1 cytokine responses were produced despite a lack of classical macrophage activation, suggesting that the local microenvironment at the site of parasite inoculation may determine the initial course of T-cell differentiation [13].

Investigations into the mechanisms underlying the immunosuppression observed during acute VL have demonstrated defective antigen-specific proliferation and IFN-γ responses [14], which suggest that the parasites suppress macrophage microbicidal responses, and IFN-γ signaling pathways [15] at the earliest stages of infection. Also supporting the absence of a clear Th1 versus Th2 dichotomy in VL is the observation that inhibition of Th1 cytokines in a murine model of *L. chagasi*-induced VL results in parasite clearance independent of Th2 cytokines [16]. Studies, not only in mice, but also in humans, suggested that cure was independent of the differential production of Th1 and Th2 cytokines, and both IFN-γ and IL-4 producing T cells have been isolated from asymptomatic and cured patients [17]. These immunosuppressive mechanisms could differ depending upon etiologic agent, T-cell subpopulation predominance at site of infection, stage of infection, and target organ. Such mechanisms could be even more complex because the course *Leishmania* infection may vary widely depending on the species and strain of parasite.

3. Immunomodulatory Effects of Adenosine and Adenosine Triphosphate (ATP) Nucleotidases

In studying the complexity of immune system regulation, especially at the site of injury, several groups have recently begun to demonstrate a possible role for the nucleoside adenosine and adenosine nucleotidases in regulating *Leishmania*-specific immune responses. It was proposed that ecto-ATP diphosphohydrolases and ectonucleotidases, which are membrane-associated enzymes with their catalytic sites turned extracellular, would act to dephosphorylate adenosine nucleotides into free adenosine [18, 19]. Before discussing the role of ectonucleotidases in leishmaniasis, concepts and comments regarding the immunomodulatory roles of adenosine nucleotides and nucleosides must be stressed.

Extracellular nucleotides are involved in a variety of physiological functions by participating in extracellular signaling through the activation of cell surface metabotropic purinergic (G protein coupled) and ionotropic (ion channel coupled) receptors. The major classes of purinergic receptors include the type P2 receptors (P2X-P2Y) and ionotropic and metabotropic receptors [20–22]. The activation of these receptors by ATP leads to proinflammatory effects by initiating a response characterized by the secretion of IFN-γ, IL-12, and TNF-α [23].

During acute inflammation, the ATP released by the leakage of cellular contents can be sequentially dephosphorylated by the action of ectonucleotidases, causing the concentration of extracellular adenosine to increase markedly [22, 24]. Adenosine exerts distinct effects on the immune system compared to ATP. The immunosuppressive actions of adenosine are triggered by activation of four receptor subtypes (A_1, A_{2A}, A_{2B}, and A_3) belonging to the family of purinergic receptors known as P1 or A. These receptor subtypes are members of a superfamily of receptors composed of seven transmembrane regions that are coupled to G proteins [22, 25] and expressed in neutrophils, macrophages, dendritic cells [26], and T cells [27]. These four adenosine receptor subtypes are expressed concomitantly in various immune cells [28, 29].

Purinergic signaling depends on several factors, including receptor expression, receptor sensitivity, and the levels of extracellular nucleotides and nucleosides. The concentration of extracellular adenosine can determine the activation of a given receptor due to the difference in A receptors

affinity [28]. In physiological adenosine concentrations (0.2–0.5 μM), the A_1 and A_3 receptors are preferentially activated, while at higher adenosine concentrations (16,2–64,1 μM), such as those seen in inflammation, A_{2B} receptors exert their effects [30].

Activation of A_{2A} and A_{2B} receptors leads to increased levels of intracellular cAMP by activating adenylyl cyclase, which inhibits immune cell function [31]. This regulatory effect is caused mainly by inhibiting the production of IFN-γ, IL-12, and TNF-α coupled with an increased production of IL-10 [32] as summarized in Figure 1. Inhibition of monocyte maturation and the suppression of macrophage phagocytic function are also related to the activation of A_2 receptors [33]. The signaling pathway that couples A_{2A} and A_{2B} adenosine receptors with G proteins is balanced by the signaling that results from the association of A_1 and A_3 receptors with Gi protein, which inhibits adenylyl cyclase. Adenylyl cyclase inhibition then leads to decreased levels of cAMP, which limits the premature inhibition of immune cells by A_2 receptors [34].

4. Adenosine and the Establishment of *Leishmania* Infection: The Possible Role of Ectonucleotidases in Immune Impairment

Ectonucleotidases are glycoprotein enzymes present in the plasma membrane with their catalytic sites facing extracellularly [18, 19], which are capable of hydrolyzing extracellular nucleotides. Fundamentally important in maintaining the homeostasis of extracellular nucleotides, these enzymes are also regulatory (termination of signaling events triggered by ATP) and metabolic (generation of nutrients) in function [35]. The hydrolysis of extracellular nucleotides occurs in a sequential manner by the action of ecto-ATPase, ecto-ADPase, ecto-ATPDase, and ecto-5′-nucleotidase [36]. The classification of ectonucleotidases in different families takes into account kinetic aspects such as enzyme specificity and substrate affinity and molecular aspects of protein structure [37].

Since Gottlieb and Dwyer [38] provided information in 1981 about the biochemistry of surface membranes of *Leishmania*, a large number of authors have observed nucleotidases located on the outer surface of the plasma membrane of the parasite. For example, ecto-ATPases, ecto-5′-nucleotidase, and ecto-3′-nucleotidase have all been described in *Leishmania* sp. [18, 19, 39, 40].

In *Leishmania* parasites, it was proposed that an increase in ectonucleotidase activity would increase the production of adenosine, which would consequently aid in the establishment of infection through its immunosuppressive mechanisms. Infective *L. amazonensis* promastigotes exhibit higher ATPase activity compared to nonvirulent promastigotes [39]. Furthermore, amastigotes, which are responsible for maintenance of leishmaniasis in the vertebrate host, are capable of hydrolyzing ATP at higher rates than promastigotes [40]. *L. amazonensis* strains that possess higher ectonucleotidase activity are more effective in establishing infection in murine models [41]. Comparison of ectonucleotidase activities between *L. amazonensis*, *Leishmania braziliensis* (*L. braziliensis*), and *L. major* showed that the more virulent parasite causing nonhealing lesions in C57BL/6 mice (e.g., *L. amazonensis*) hydrolyzes higher amounts of ATP, ADP, and 5′AMP [42]. Furthermore, adenosine treatment at the time of *L. braziliensis* inoculation delays lesion resolution and induces increased parasite burdens. Consistent with this, inhibition of adenosine receptor A_{2B} led to decreased lesion size and lower parasite burden [43]. In clinical practice, results that are consistent with the idea that ecto-ATPase activity is involved in virulence were also observed, whereby *L. amazonensis* strain isolated from a human case of VL possess higher ecto-ATPase activity than strains isolated from CL cases [44].

In addition to these ectonucleotidases, *Leishmania* parasites also express a bifunctional enzyme called 3′-nucleotidase/nuclease (3′NT/NU) in the plasma membrane with a high capacity to hydrolyze 3′ribonucleotides and nucleic acids [43, 45]. Although first identified in *L. donovani* [43, 45], it was later found in *L. chagasi* [46], *L. major* [47], *L. mexicana* [48], and *L. amazonensis*. Although the 3′-nucleotidase enzyme is found solely in some trypanosomatids, 3′-nucleotides are available through nucleic acid hydrolysis in several mammalian tissues [49], especially the spleen, an organ commonly targeted by *Leishmania* parasites. Recently, our group has characterized the 3′-nucleotidase activity of *L. chagasi* and demonstrated that such activity could be related to aspects of parasite virulence [46]. Interestingly, the viscerotropic *L. chagasi* and *L. donovani* had higher 3′-nucleotidase activity compared to the New World and Old World dermatotropic species (e.g., *L. amazonensis*, *L. major*, *L. tropica*, and *L. braziliensis*). *L. chagasi* metacyclics (infective promastigote stage) had higher 3′-nucleotidase activity compared to *L. chagasi* nonmetacyclics (noninfective promastigote stage) [46]. Similar results were also observed with *L. amazonensis*, where infective promastigotes possessed twofold higher 3′-nucleotidase activity compared to nonvirulent promastigotes [50].

In addition to the role of ectonucleotidase in the establishment of *Leishmania* infection by ectonucleotidases, some interesting data have further demonstrated that high expression/activity of such enzymes on immune cells contributes to immune imbalance. $CD4^+CD25^+$ regulatory T cells (Tregs) from mutant mice deficient in CD39 have impaired regulatory function manifesting as a 50% decrease in the ability of CD39-null Tregs to modulate effector T-cell function *in vitro* and *in vivo*. These results indicate that CD39 expressed by Treg is the major and rate-limiting ectonucleotidase responsible for the generation of adenosine and suggest that a putative CD39/CD73-adenosinergic axis may contribute to the immunoregulatory function of Treg [51, 52].

In *L. infantum*-infected BALB/c mice, Tregs are present. The high levels of Foxp3 gene expression and surface expression of Eb7 integrin (CD103) suggest a predisposition for Treg retention within sites of *L. infantum* infection, as is the case of the spleen and draining lymph nodes, consequently influencing local immune response [53]. It was attributing to $CD4^+CD25^+FOXP3^+$ regulatory T cells an important role in

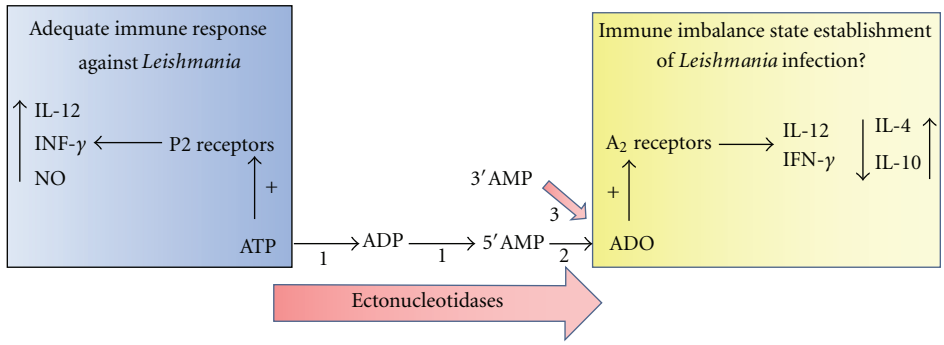

Figure 1: Partial reactions catalyzed by ecto-nucleoidases: (1) ecto-ATPase, ectonucleoside triphosphate diphosphohydrolase; (2) ecto-5′-nucleotidase; (3) ecto-3′-nucleotidase. The sequential hydrolysis of extracellular ATP by *Leishmania* ectonucleotidases triggers the host inflammatory and immune response following P2 receptor activation by extracellular ATP (blue square) and P1 (A_2) receptors activation by extracellular adenosine nucleoside (yellow square). ADO: Adenosine.

immune suppression because in those cells, CD39 and CD73 are overexpressed. Inhibitors of ectonucleotidase activities and antagonists of the A_{2A} receptor blocked Treg-mediated immunosuppression [54]. Consistent with this view, it was demonstrated that patients with VL possess high levels of adenosine, which was related to ectonucleotidase activities and disease progression [55].

This paper about the immune modulatory effects of adenosine and the production of such nucleoside by parasite and immune cells provide new perspectives for the understanding of the complex immune response in leishmaniasis. Further studies are needed, especially using visceral leishmaniasis models, to clearly delineate the role of ectonucleotidases in the establishment of infection and *Leishmania*-induced immunomodulation.

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Review Article

Host Cell Signalling and *Leishmania* Mechanisms of Evasion

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Leishmania parasites are able to secure their survival and propagation within their host by altering signalling pathways involved in the ability of macrophages to kill pathogens or to engage adaptive immune system. An important step in this immune evasion process is the activation of host protein tyrosine phosphatase SHP-1 by *Leishmania*. SHP-1 has been shown to directly inactivate JAK2 and Erk1/2 and to play a role in the negative regulation of several transcription factors involved in macrophage activation. These signalling alterations contribute to the inactivation of critical macrophage functions (e.g., Nitric oxide, IL-12, and TNF-α). Additionally, to interfere with IFN-γ receptor signalling, *Leishmania* also alters several LPS-mediated responses. Recent findings from our laboratory revealed a pivotal role for SHP-1 in the inhibition of TLR-induced macrophage activation through binding to and inactivating IL-1-receptor-associated kinase 1 (IRAK-1). Furthermore, we identified the binding site as an evolutionarily conserved ITIM-like motif, which we named kinase tyrosine-based inhibitory motif (KTIM). Collectively, a better understanding of the evasion mechanisms utilized by *Leishmania* parasite could help to develop more efficient antileishmanial therapies in the near future.

1. Background

Apart from the impact of *Leishmania* on world health, Leishmaniasis represents an elegant infection model that can teach us a lot about host-parasite interactions and immune evasion. This parasite has the ability to enter host macrophages (M∅s) safely and replicate inside the very same phagocytes that were recruited to destroy it. The inability of M∅s to kill the parasite and activate cells of the adaptive immune system is a product of the parasite's long-reported capacity to alter several key signalling pathways in the host. Many signalling alterations are seen early in the course of infection suggesting they start upon the initial contact between the parasite and the M∅. These rapid alterations of signalling pathways serve at least two main functions: firstly, inhibition of M∅ killing mechanisms that are triggered upon phagocytosis of foreign particles (e.g., production of reactive oxygen species), and secondly, inhibition of leishmanicidal functions that can be triggered in response to M∅ activation in infected tissues in response to stimuli such as lipopolysaccharides (LPS) or interferon-γ (IFN-γ) (e.g., nitric oxide production). In this review, we will discuss the roles of *Leishmania* in disease establishment, focusing on the signalling pathways that they interfere with and the M∅ functions that are affected by the alteration of these pathways.

2. Alteration of Macrophage Signalling Molecules by *Leishmania*

Several pathogens (i.e., *Acanthocheilonema viteae* [1], African trypanosomes [2], and *Toxoplasma gondii* [3]) are able to alter the signalling of their target cells to their own advantage and *Leishmania* is no exception. *Leishmania* achieves this by either employing strategies to inhibit proteins that play a positive role in immune cell activation or by activating molecules known to play key roles in the negative regulation of immune cell signalling and function [4]. We will discuss below the main signalling molecules altered by *Leishmania* in an effort of the parasite to survive inside host M∅s.

Firstly, protein kinase C (PKC), a protein family comprising 10 serine/threonine kinases, initially characterized as Ca^{+2} and phospholipid dependent [5, 6], is classified into three subfamilies: the conventional (PKC-α, -βI, -βII, and -γ), novel (PKC-δ, -ε, -η, and -τ), and atypical (PKC-ζ and -λ) isoforms [7]. PKC signalling is known to play a key role in the regulation of M$\varnothing$ functions activating, for instance, cytokines such as IFN-γ and TNF-α [8, 9], both having important roles in driving several M$\varnothing$ functions including NO production [8] and oxidative burst [10]. Promastigote LPG has been described to be able to block PKC activity [11–13]. This inhibition is achieved through the binding of LPG to the regulatory domain of PKC which contains the DAG, Ca^{+2}, and phospholipid binding sites [14]. It is interesting to observe that amastigotes, which lack LPG, are also able to inhibit PKC activity in monocytes [15], suggesting that factors other than LPG can also mediate this inhibitory effect. Indeed, *Leishmania*-induced ceramide generation [16] and GIPLs [12] have been shown to be able to do so, providing a possible mechanism whereby amastigotes can inhibit PKC activity.

Later on, Janus kinase 2 (JAK2) as one of four members of the Janus family of tyrosine kinases (JAK1, JAK2, JAK3, and TYK2), has been identified to be importantly affected by *Leishmania* infection. JAK activation plays an important role in cell proliferation, differentiation, migration, apoptosis, and immune activation [17]. The JAK signalling pathway is initiated when a cytokine or a growth factor binds to its receptor inducing receptor multimerization followed by JAKs transphosphorylation and activation, ultimately leading to the phosphorylation of signal transducer and activator of transcription (STAT), a transcription factor (TF), that will then dimerize and proceed to nucleus by translocation and to bind target regulatory sequences to activate or repress transcription [17, 18].

Importantly, the iNOS gene promoter responsible for NO production has binding sites for several TFs including STAT-1 [19, 20]. *Leishmania* has the ability to block the JAK/STAT signalling pathway in response to IFN-γ stimulation, therefore avoiding the induction of NO. Indeed, it has been reported that infection with *L. donovani* amastigotes was able to block IFN-γ-induced JAK1, JAK2, and STAT-1 phosphorylation in PMA-differentiated U-937 promonocytic cells and human monocytes [21]. However, we went further in studying the effect of *Leishmania* on JAK2 phosphorylation by reporting that *L. donovani* promastigotes were rapidly activating host SHP-1 leading to the subsequent inhibition of IFN-λ-induced JAK2 phosphorylation [22], see Figure 1. Others have suggested that IFN-γ unresponsiveness to stimulation can be due to the inhibition of the IFN-γ receptor (IFN-γR) complex formation [23], however they did not provide any clues on how the parasite could do so. Another complication with this report is that the authors infected cells for 24 hours to see an appreciable effect on receptor expression and phosphorylation, which cannot explain the rapid dephosphorylation of JAK2 seen when BMDMs are infected with *Leishmania* promastigotes [22], supporting the notion that early JAK/STAT inhibition must depend on parasite-induced alterations of existing signalling molecules of the host and not on alterations at the transcriptional level.

In the same line of ideas, several members of the mitogen-activated protein kinases (MAPKs) family (e.g., extracellular signal-regulated kinase1/2 (Erk1/2), proline for Jun N-terminal kinase (JNK), and glycine for p38), known to play critical role in the activation of several TFs [24], have been found to be exploited by *Leishmania* parasite (Figure 1). Indeed, as was the case with the JAK family, it is remarkable, though not unexpected, that the *Leishmania* parasite developed tactics to render several MAPK members inactive in response to parasite entry to M$\varnothing$s or to activating stimuli that follow infection. For instance, it was reported that the phagocytosis of *L. donovani* promastigotes by naive M$\varnothing$s does not lead to the activation of any of the three MAPKs (Erk1/2, JNK, p38) [25]. Furthermore, activation of several MAPKs in response to LPS has been shown to be inhibited in infected cells. For instance, *L. amazonensis* amastigotes are able to block LPS-mediated Erk1 phosphorylation in infected M$\varnothing$s [26], and *L. donovani* amastigotes can block PMA-induced Erk1/2 phosphorylation in RAW264 M$\varnothing$s leading to the inhibition of Elk-1 and c-fos expression [27]. The authors of the latter study suggested a role for host PTPs in Erk1/2 inactivation, a hypothesis supported and more deeply explored by our laboratory where we provided evidence that PTP-SHP-1 is able to dephosphorylate and inactivate Erk1/2 through demonstrating that this MAPK was still able to be activated in *Leishmania*-infected SHP-1-deficient M$\varnothing$s in response to IFN-γ stimulation [28]. Alternatively, it has been suggested that ceramide production by *L. donovani*-infected M$\varnothing$s can lead to reduce Erk1/2 phosphorylation [29]. Interestingly, amastigotes of *L. mexicana* were also reported to inhibit Erk1/2 signalling not by inhibiting their phosphorylation, but rather by degrading them using the parasite's cysteine proteinases. Similar cysteine proteinase-dependent degradation was observed for JNK [30].

In regard to p38, it has been shown that this MAPK is nonresponsive when M$\varnothing$s infected with *L. major* are stimulated with a CD40 antibody to mimic the M$\varnothing$-T cell interaction. p38 inactivation correlated with impaired iNOS2 expression and NO production and therefore impaired leishmanicidal functions [31]. In fact, this inactivation makes sense in the light of experiments showing the importance of p38 activation in the control of *Leishmania* infection. The use of anisomycin, a p38 activator, enhanced parasite killing in M$\varnothing$s by triggering p38-dependent antileishmanial effects [31, 32].

In order to inhibit gene expression of proinflammatory cytokines and microbicidal molecules, *Leishmania* developed several strategies to interfere with TFs that bind to the promoters of those genes. Several TFs are involved in this process including NF-κB, STAT-1α, and AP-1, all of which known to be modulated by the parasite. In fact, several groups have reported different strategies employed by *Leishmania* to alter, for instance, the TF NF-κB. *Leishmania*-induced ceramide generation by M$\varnothing$s was shown to play a role in NF-κB inhibition [29]. One study provided evidence that *L. major* amastigotes blocked the nuclear translocation of the p65/p50 complex selectively favouring the c-Rel/p50

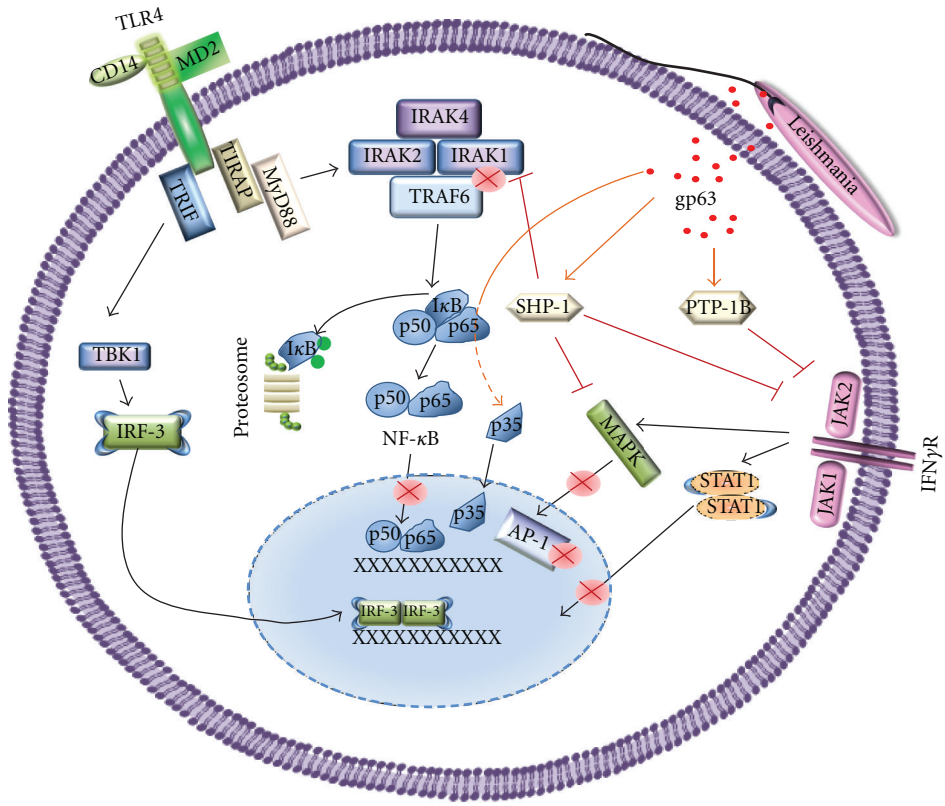

Figure 1: *Downregulation of macrophages signalling by Leishmania infection. Leishmania* infection modulates phosphatases (SHP-1 and PTP-1B) activity by mechanism involving the metalloprotease gp63. SHP-1 was found to interact with IRAK-1, a key kinase involved on TLR-triggered signaling pathway. Whereas, both SHP-1 and PTP-1B are involved in the downregulation of IFNγ-induced pathway (JAK/STAT1) as well as MAPK activation. In addition, transcription factor such as NF-κB and AP-1 are cleaved/degraded in part by *Leishmania* gp63. Orange arrows indicate gp63 involved modulation; black arrows indicate activation; red abrogated lines indicate downregulation.

complex that, they proposed, plays a role in the gene expression of immunosuppressive cytokines in M$\varnothing$s such as IL-10 [33]. Another study reported that cysteine proteinases of *L. mexicana* mediated NF-κB degradation and caused its inability to bind its DNA consensus sequence, thus partially explaining how the parasite can inhibit LPS-mediated IL-12 production [30]. Work from our laboratory showed that promastigotes of several pathogenic *Leishmania* species were able to cleave the p65 RelA subunit to generate a p35 RelA fragment translocating to the nucleus and bind DNA. This p35 fragment was suggested to be involved in the parasite's ability to drive NF-κB-mediated chemokine gene expression in infected M$\varnothing$s [34].

In addition to NF-κB and the fact we have previously described *Leishmania*'s ability to inhibit the JAK/STAT pathway [22], our laboratory has also reported that the parasite is able to repress IFN-γ-mediated signalling in M$\varnothing$s by interfering with STATs. We showed that *L. donovani* promastigotes were able to cause proteasome-mediated STAT-1 degradation in infected M$\varnothing$s. However, whereas STAT-1 degradation was reversed using proteasome inhibitors [35], its capacity to respond to IFN-γ was still altered due to JAK2 inactivation (unpublished data).

AP-1 is a structurally complex TF formed by dimmers from Jun and Fos protein family [36] that can be activated by many kinds of stimuli such as growth factors, cytokines, hormones, and pathogens, which do so using several signalling molecules. Consequently, the previously mentioned tactics employed by *Leishmania* to interfere with PKC, Erk1/2, JNK, and p38 activities have a direct impact on the ability of the parasite to block AP-1 signalling in M$\varnothing$s. Importantly, work from our group demonstrated a role for SHP-1 in AP-1 inhibition [28, 37] and, more recently, that the parasite's surface protease gp63 is responsible for the cleavage and degradation of key AP-1 subunits [38]. The latter finding provides the first demonstration that a parasite-derived molecule can directly interfere with AP-1 in host M$\varnothing$s in order to block its downstream functions.

3. Negative Regulation by Protein Tyrosine Phosphatases

Protein tyrosine phosphatases (PTPs) are proteins that have the ability to dephosphorylate substrates and are divided into receptor-like and nonreceptor PTPs. Nonreceptor PTPs can either dephosphorylate tyrosines only or can possess dual specificity dephosphorylating tyrosines as well as serines/threonines [39]. One common feature of PTPs is the presence of a PTPs catalytic domain in which a critical cysteine is found within a conserved signature motif,

(I/V)HCxxGxxR(S/T), and mediates the hydrolysis via the formation of a thiophosphate intermediate [40]. Receptor-like PTPs include RPTP-α, CD45, and CD148, and the function of some like CD45 in immune cell signalling are well known [41]. However, herein we will focus on a selected group of soluble PTPs that have been shown to play a role in *Leishmania* host evasion mechanisms, namely, PTP-1B, TC-PTP, PTP-PEST, and most importantly SHP-1. PTP-1B and TC (T cell)-PTP: they are two ubiquitously expressed PTPs that have more than 73% identity in their catalytic domain [42]. PTP-1B is known to play important regulatory functions in metabolism, as demonstrated by the insulin hypersensitivity of PTP-1B$^{-/-}$ mice and their resistance to high-fat-diet-induced obesity [43, 44]. This insulin hypersensitivity was shown to be due to the ability of PTP-1B to dephosphorylate the insulin receptor [45]. PTP-1B also seems to play a role in the regulation of cytokine signalling through its ability to interact with and dephosphorylate members of the JAK family, namely, JAK2 and TYK2 [46]. In addition to PTP-1B's role in the regulation of JAK/STAT signalling, a role for this phosphatase in the regulation of TLR4 signalling was proposed. PTP-1B$^{-/-}$ M$\varnothing$s had increased LPS-induced iNOS expression and NO production compared to WT M$\varnothing$s and were more susceptible to endotoxic shock following low-dose LPS injection [41]. As PTP-1B was found to be an important negative regulator of M$\varnothing$s signaling, its role in Leishmaniasis could be critical. In fact, we recently showed that *Leishmania* gp63 was able to enhance PTP-1B activation by cleaving it. PTP-1B activity seems to inhibit M$\varnothing$ activation and help in parasite survival as seen in the delayed onset of footpad swelling and reduced parasite burden in PTP-1B$^{-/-}$ mice infected with *L. major* [47]. In addition, we found that TC-PTP that also plays important roles in the negative regulation of JAK1, JAK3 [48], and nuclear STAT-1 [49] was modulated by gp63 in *Leishmania*-infected M$\varnothing$s [47]. This gp63-mediated TC-PTP cleavage along with the cleavage of PTP-PEST was recently reported by our group and M.L. Tremblay's group to enhance the catalytic activity of the PTPs in question and/or allow them to access additional substrates that might help the parasite establish itself [50].

Another PTP modulated by *Leishmania* gp63 is SHP-1. This PTP contains two N-terminal SH2 domains (N-SH2, C-SH2), followed by a PTP domain responsible for dephosphorylating substrates, and a C-terminal tail [41]. This phosphatase is mostly expressed in hematopoietic cells [51, 52], but is also expressed at lower levels in epithelial [52], endothelial [53, 54], and central nervous system cells [55]. The SH2 domains have two main functions: firstly, the N-SH2 domain plays an important autoinhibitory role by interacting intramolecularly with the PTP domain, keeping the PTP in the inactive state. Secondly, both SH2 domains have the ability to bind to phosphotyrosine (p-Y) residues usually found within immunoreceptor tyrosine-based inhibitory motifs (ITIMs) whose consensus sequence is (I/V/L/S)xYxx(L/V) [36]. This second feature of SH2-domains is thought to play a role in the detachment of the N-SH2 from the PTP domain once the C-SH2 domain binds to a target p-Y, therefore opening up and activating the PTP [41].

At the signalling level, our laboratory have clearly demonstrated that *Leishmania* was able to rapidly activate host SHP-1 causing SHP-1-mediated JAK2 inactivation in M$\varnothing$s [22]. Additionally, we and others have implicated SHP-1 in the negative regulation of Erk1/2 activity [27, 28] and in the regulation of the downstream TFs NF-κB and AP-1 [28] during *Leishmania* infection. At the functional level, our laboratory showed that the injection of PTP inhibitors (bpV-phen, a bis-peroxovanadium compounds) to mice infected with *L. major* or *L. donovani* helped control the infection [56] in a manner dependent on iNOS expression and NO production [57]. Furthermore, we demonstrated that SHP-1-deficient viable moth-eaten mice infected with *L. major* did not develop footpad swelling and had significantly reduced parasitic loads [58]. This decreased pathology was associated with increase activated neutrophil recruitment to the footpad and more iNOS mRNA expression [58].

As to how *Leishmania* is able to activate SHP-1, it has been proposed that *Leishmania*'s elongation factor-1α (EF-1α) is responsible for the activation of host SHP-1 seen 16 hours after infection [59]. This report cannot explain, however, how SHP-1 is activated in earlier infection times nor does it explain how EF-1α of the parasite can shuttle from the phagolysosome where the parasite is to the cytosol where SHP-1 is found. A more plausible mechanism has been recently suggested by our group, where SHP-1 was shown to be activated via cleavage by the parasite's protease gp63, which gains access to the cytosol by going through the lipid raft of host M$\varnothing$s [47].

Collectively, it appears that the rapid activation of SHP-1 by *Leishmania* is a key host evasion step whereby the parasite is able to utilize this phosphatase to negatively regulate several key M$\varnothing$ pathways and render them unresponsive to activating stimuli such as IFN-γ and LPS. By doing so, the parasite is able to block several M$\varnothing$ functions such as NO production and the synthesis of many proinflammatory cytokines that can be deadly to the parasite if allowed to be produced.

4. Macrophage Functions Altered by *Leishmania*

Modulation of signalling pathways by *Leishmania* is intended to alter critical M$\varnothing$ functions to the advantage of the parasite. Upon the initial contact of *Leishmania* with the M$\varnothing$, certain functions such as the production of chemokines and chemokine receptors are induced, whereas others are inhibited. Among the functions inhibited by the parasite are those related to M$\varnothing$ activation and to their ability to present Ag and communicate with cells of the adaptive immune system. Hereby, we will discuss the main functions that *Leishmania* can interfere with initial interaction (0–6 h) or chronic infection (>6 h) of host M$\varnothing$s.

One of the important early challenges confronted by *Leishmania* is the ability to preferentially recruit cells of the immune system to the site of inoculation in order to infect them and establish disease in the host without getting killed. One key mechanism by which the parasite is able to do so is the induction of chemokine expression and production

by host immune cells. One study showed that infection of mice with *L. major* upregulated the gene expression of several chemokines (RANTES/CCL5, MIP-1α/CCL3, IP-10/CXCL10, and MCP-1/CCL2) in cells collected from the footpad and their draining lymph nodes [60]. Additionally, we have shown that *L. major* infection caused an upregulation in the expression of several chemokines (RANTES, MIP-1α, MIP-1β/CCL4, IP-10, MCP-1, and MIP-2/CXCL1) in cells being rapidly recruited at the site of inoculation [61]. It is interesting to see that most of these chemokines are monocyte chemoattractants, recruiting M$\varnothing$s to infected tissues and helping the parasite get installed. It is equally interesting to see that none of these chemokines, with the exception of MIP-2, attract neutrophils. This is in accordance with our previous finding that exacerbated neutrophil recruitment to infection sites is associated with parasite killing in SHP-1 deficient viable moth-eaten mice [58].

So far, we have considered chemokine upregulation as beneficial to the parasite, yet it is important to bear in mind that secreted chemokines during Leishmaniasis can act as a double-edged sword. Whereas selective activation of chemotactic factors can help the parasite to recruit M$\varnothing$s and neutrophils that they can infect and/or utilize during the initial step of infection, treatment of susceptible BALB/c with recombinant IP-10 in the early course of *L. major* infection has been shown to increase NK cell cytotoxic activity in the draining lymph nodes and to drive a healing IFN-γ-mediated Th1 response [62]. In chronic infections, chemokine types, amounts, and duration of chemotactic effect have been implicated in parasite clearance or persistence. For instance, in visceral Leishmaniasis, clearance of parasites from the liver is strongly associated with increased late phase IP-10 production and the Th1 effects associated with its presence [62]. Parasite persistence in the spleen, on the other hand, has been correlated with sustained MCP-1, but not IP-10 levels [63].

Whereas *Leishmania* can modulate selected chemokines, importantly the inhibition of key microbicidal functions is crucial for its initial survival. For instance, one of the dangers that *Leishmania* encounters recruiting and entering M$\varnothing$s is the ability of these cells to produce deadly free radicals such as NO [64] and reactive oxygen intermediates (ROIs) [65]. NO is produced by NOS which converts one of the terminal nitrogens of the guanidino group of L-arginine to NO producing L-citrulline [66, 67]. The importance of this free radical in Leishmaniasis was demonstrated by several groups. An early study showed the ability of activated M$\varnothing$s to kill *L. major* amastigotes by an L-arginine-dependent mechanism [68]. Later confirmed by the observation that L-N-monomethyl arginine (L-NMMA), an L-arginine analogue and inhibitor of the NO pathway was able to inhibit the leishmanicidal effect of M$\varnothing$s activated *in vitro* with IFN-γ or LPS. They also showed the ability of NO in cell-free suspensions to kill the parasite. Importantly, the same group demonstrated the importance of NO *in vivo* by rendering resistant CBA mice susceptible to *L. major* infection upon local administration of L-NMMA [64].

However, parasite is able to block its synthesis in response to stimuli such as IFN-γ [69], but how can *Leishmania* achieve this inhibition? A critical role for host SHP-1 has been proposed. As previously stated, *Leishmania* has the ability to rapidly activate SHP-1 in infected M$\varnothing$s and by doing so can interfere with several molecules involved in NO production including JAK2, Erk1/2, and the TFs NF-κB and AP-1. Indeed, SHP-1 deficient M$\varnothing$s infected with *L. donovani* are still able to produce NO in response to IFN-γ stimulation, unlike infected WT M$\varnothing$s which are refractory to a similar stimulation [28]. As expected, the IFN-γ-mediated NO production in infected SHP-1 deficient M$\varnothing$s correlated with successful JAK2 and Erk1/2 phosphorylation and the activation of NF-κB and AP-1. These findings further elucidate the role of SHP-1 activation in parasite survival and propagation through its ability to contribute to NO inhibition [28]. Another mechanism involved in the downregulation of NO production is by conversion of arginine to ornithine and urea via the arginase pathway [70]. Supporting this mechanism, recently, it has been shown that arginase as well as polyamines gene expression are upregulated by *L. amazonensis* amastigote [71]. Whereas those latter mechanisms of evasion are to be of general use by all, *Leishmania* parasite will need further investigation.

In addition to NO, ROIs represent another source of danger to *Leishmania*. These intermediates include the superoxide radical and hydrogen peroxide produced by cells of the immune system such as neutrophils and M$\varnothing$s in response to various agonists. Although important in parasite killing, the activity of the respiratory burst in mice was shown to have an early and transient effect only. This conclusion is based on the delayed granuloma formation and resolution of infection seen in respiratory burst-deficient X-CGD mice infected with *L. donovani* compared to WT [72]. Despite the critical role that NO seems to play in *Leishmania* killing [72], ROIs do contribute to parasite clearance and are therefore a target to be inhibited by the parasite. Indeed, *L. donovani* has been shown to inhibit the oxidative burst in infected M$\varnothing$s [15, 73, 74], and this inhibition was in part mediated by the parasite surface molecules LPG and gp63 [14, 75] involving PKC inactivation [15]. Interestingly, it was later shown that LPG of *L. donovani* promastigotes is able to block NADPH oxidase assembly at the phagosome membrane without interfering with p47(phox) phosphorylation and its ability to form complexes with p67(phox) [76]. *L. donovani* amastigotes, on the other hand, were shown to effectively block superoxide release through inhibiting the phosphorylation of the NADPH oxidase component p47(phox), leading to defective recruitment of p47(phox) and p67(phox) to the phagosome [77]. The inhibition of p47(phox) phosphorylation could be a result of the previously reported ability of *Leishmania* amastigotes to inhibit PKC activity [15], which is reported to be required for p47(phox) phosphorylation [78]. Downregulation of ROIs seems also to be modulated by ERK as inhibitor of ERK decreased ROIs production, increasing the killing of *L. amazonensis* amastigote, however this mechanism is not applied to all species as *L. major* still survives [79].

In addition to those nitrous and oxygen derivatives, IL-1 and TNF-α have been correlated with antimicrobial activities against bacteria and parasites *in vitro* and *in vivo* [80–83],

and IL-12 is well known for its ability to promote Th1 differentiation and to activate NK cells [84]. In regard to IL-1 and TNF-α, it has been shown that these molecules are not produced upon a 12 h *in vitro* infection of human monocytes with *L. donovani* amastigotes [85]. Interestingly, preinfection of those cells diminished LPS-mediated IL-1 production, but not IL-1 m-RNA, suggesting inhibition at the translational level [85]. Another study showed that preincubation of human monocytes with purified LPG was able to cause inhibition of LPS-mediated IL-1β secretion [86]. The role of LPG in IL-1β inhibition was later shown to involve LPG ability to inhibit IL-1β gene transcription in a manner dependent on the nucleotide region -310 to -57 of the promoter region [87]. This inhibitory effect of LPG on IL-1β gene transcription was suggested to involve an inhibition of the binding of an activation factor or an induction of an unknown transcription repressor [87]. Interestingly, a study by our laboratory revealed that SHP-1 deficient mice infected with *L. major* produced significantly higher amounts of IL-1 and TNF-α compared to their littermates [61], suggesting that *Leishmania*-induced SHP-1 activity could play a pivotal role in the attenuation of the inflammatory response repressing the proinflammatory cytokines production.

IL-12 is another key cytokine inhibited by *Leishmania*. This inhibitory effect is necessary for parasite survival given the established role of this molecule in driving Th1 differentiation and production of IFN-γ by T cells and NK cells, which in turn can activate M$\varnothing$s to kill the parasite. It has been reported that infection of BMDMs with promastigotes of *L. major* or *L. donovani* fails to induce IL-12 production, both following infection alone and upon subsequent LPS or heat-killed bacterial stimulation of M$\varnothing$s [88]. Similar observations were seen when murine M$\varnothing$s were infected with amastigotes of *L. major* and *L. mexicana* [89]. Furthermore, incubation of activated murine M$\varnothing$s with LPG led to the inhibition of IL-12 production by these cells, with the inhibition occurring at the transcriptional level [90]. The mechanism by which IL-12 is inhibited by *Leishmania* remains not fully understood. Roles for the M$\varnothing$ CR3 [91] and Fc-γR [92] have been proposed. Recently, we have reported a very interesting mechanism whereby *Leishmania* can inhibit LPS-mediated proinflammatory functions such as IL-12 and TNF-α production. We showed that *Leishmania*-induced SHP-1 is able to bind to an evolutionarily conserved immunoreceptor tyrosine-based inhibitory motif (ITIM)-like motif (which we renamed kinase tyrosine-based inhibitory motif (KTIM)) found in the kinase domain of IL-1 receptor-associated kinase 1 (IRAK-1), causing its inactivation. SHP-1-bound IRAK-1 is no longer able to detach from Myeloid differentiation factor 88 (MyD88) to bind TNF receptor-associated factor 6 (TRAF6) and activate downstream signalling pathways, therefore explaining in part how the parasite is able to block LPS-mediated MyD88-dependent proinflammatory functions in host macrophages [93].

One remarkable tactic the parasite utilizes to subvert the immune response is its ability to inhibit IFN-γ-induced MHC class II expression in infected M$\varnothing$s. Indeed, *L. chagasi* and *L. donovani* were both shown to inhibit MHC II expression in response to IFN-γ stimulation [94–96]. Surprisingly, M$\varnothing$s infected with *L. major* or *L. amazonensis* showed normal phagocytosis, Ag processing, and MHC II production, yet these cells failed to present parasitic Ags to T-cell hybridomas [97, 98]. Authors of both studies concluded that the failure to present Ags to T cells is due to the parasite's ability to interfere with the loading of Ags onto MHC II molecules. Another interesting mechanism to control Ag presentation is shown by amastigotes of *L. amazonensis* being able to internalize MHC II molecules and to degrade them using their cysteine proteinases [99].

Activation of CD4+ T cells involves a "two-signal model" whereby two signals are required to activate the T helper cell. The first signal is triggered by the binding of the T-cell receptor (TCR) to the MHC II-Ag complex on the APC, and the second is provided by the binding of CD28 or CD40L on T cells to costimulatory molecules of APCs such as those of the B7 family or CD40. Interestingly, apart from interfering with the first signal by inhibiting MHC II presentation, *Leishmania* has been demonstrated to interfere with M$\varnothing$ costimulatory signals. *L. donovani* infection was reported to block LPS-mediated B7-1 expression in infected M$\varnothing$s [100], a mechanism that seems to be mediated by prostaglandins [101]. Furthermore, *L. major* was reported to interfere with CD40 signalling in infected M$\varnothing$s in a p38-dependent manner [31]. This result is very interesting, especially because previous studies have established a protective role for CD40 in *Leishmania major* infections [102, 103], while others have reported that the disruption of CD40/CD40L ligation results in increased susceptibility to *L. amazonensis* infection [104]. The increased susceptibility caused by the disruption of CD40/CD40L ligation was in part due to the inhibition of iNOS expression [102, 104] and IL-12 production [105] by infected M$\varnothing$s.

So far, we have discussed several mechanisms by which *Leishmania* can interfere with key signalling pathways involved in M$\varnothing$ activation such as the JAK/STAT pathway. We also discussed alterations that occur to signalling molecules involved in TLR signalling such as MAPKs and the TFs NF-κB and AP-1. However, this does not give justice to TLR signalling, given its extremely important role in the activation of APCs to kill invading pathogens and/or activate cells of the adaptive immune system. Equally important are the strategies developed by pathogens to block TLR signalling pathways that can lead to undesirable activation of immune functions. Therefore, the last portion of this review will discuss TLR signalling and how *Leishmania* parasite deals with this important group of pathogen sensors.

5. Modulation of Toll-Like Receptor Signalling by *Leishmania*

TLR family members are known for their critical role in bridging the innate immune response to the adaptive one through recognizing pathogen-associated molecular patterns (PAMPs). In the light of the ongoing host-pathogen arms race, the detection of parasite PAMPs by TLRs has two main implications: first, the ability of cells of the immune system

to detect parasites and eliminate them when favourable conditions are present. Second, the ability of parasites to counteract TLR detection by interfering with TLR signalling keeping immune cells in an inactive state and rendering them refractory to subsequent TLR stimulation.

One of the main parasite-derived molecules involved in TLR binding and activation is GPI-anchored proteins. *Trypanosoma cruzi*-derived GPI-anchors were shown to be detected by TLR2/TLR6 and CD14 and to activate NF-κB [106, 107], while GIPLs of *T. cruzi* activated Chinese hamster ovary (CHO) cells in a TLR4/CD14-dependent manner [108]. It has been also shown that GPI-mucin of *T. cruzi* is able to activate TLR signalling on first exposure and induce tolerance to secondary TLR stimulation [109]. This was later shown to be mediated by the ability of GPI-mucin to induce the expression and activation of the serine/threonine phosphatase PP2A that acts on cellular IRAK-1, MAPKs, and IκB causing their inhibition and leading to tolerance [110]. The induction of PP2A was shown to require p38 and NF-κB, the very same molecules PP2A is induced to inhibit, therefore giving rise to an autoregulatory loop [110]. LPG of *Leishmania* is another GPI-anchored protein detected by TLRs. It has been shown that LPG of *L. major* directly binds to TLR2 of M$\varnothing$s and NK cells [111, 112] and that LPG of *L. donovani* is also detected by TLR2 of activated M$\varnothing$s [113]. Interestingly, GPI-anchors derived from *Plasmodium falciparum* merozoites can induce TNF production in human monocytes and mouse M$\varnothing$s through interacting with TLR1/TLR2 and to a lesser extent TLR4 [114, 115]. Moreover, GPI- anchors of *Toxoplasma gondii* are detected by TLR2 and TLR4, which can thus play an important role in host defense against *T. gondii* infections [116].

Although less numerous than GPI-anchored ligands, non-GPI-related ligands represent an important group of parasite-related molecules detected by TLRs. An example is the *T. cruzi*-derived protein Tc52, which is able to induce proinflammatory cytokine production in DCs in a TLR2-dependent manner [117]. Other important non-GPI ligands include the DNA of *T. cruzi*, *T. brucei*, and *Babesia bovis*, which are able to activate M$\varnothing$s and DCs [118, 119], possibly through unmethylated CpG motifs [120] detected by TLR9 [121, 122]. TLR3 was recently shown to be upregulated in IFN-γ-primed M$\varnothing$s and to play a role in their leishmanicidal activity. The silencing of TLR3 led to impaired NO and TNF-α production in IFN-γ-primed M$\varnothing$s in response to *L. donovani* infection and increased parasite survival [113]. Given that the only known ligand of TLR3 is dsRNA, the parasite component that activates TLR3 remains unclear. The authors ruled out the presence of dsRNA *Leishmania* virus infection in their parasite strain and also failed to detect natural *Leishmania*-derived double-stranded RNA structures such as rRNA or tRNA [113]. As far as apicomplexans are concerned, *Plasmodium*-derived hemozoin crystals were shown to induce proinflammatory cytokines in M$\varnothing$s [123, 124]. Initially, TLR9 was proposed as the binding receptor of hemozoin [125], this remains controversial as it has been later shown that TLR9 activation by hemozoin is mediated by malaria DNA attached to the crystal and that the activation of TLR9 by hemozoin was abolished upon treatment with nucleases [126]. In fact, recent data from our laboratory show that the proinflammatory cytokine IL-1β is induced by hemozoin through the Nod-like receptor family, pyrin domain containing 3 protein (NLRP3), and the adaptor protein Asc, which lead to caspase 1 activation [127]. Concerning *Toxoplasma*, a profilin-like protein from *T. gondii* (PFTG) activates TLR11 in mouse cells [128], and heat shock proteins and partially purified preparations isolated from tachyzoites activate TLR4 and TLR2, respectively [129, 130].

The many parasite-related molecules that are detected by TLRs suggest an important role for TLR-related signalling molecules in the resistance to parasitic infections [131]. Given the fact that Th1-driving proinflammatory responses are beneficial to the host in several types of parasitic infections, it is not surprising that the activation of the MyD88-dependent pathway is crucial in the resistance to many protozoan diseases. Indeed, MyD88-deficient mice are highly susceptible to *T. cruzi* [132], *T. brucei* [122], *L. major* [133], and *T. gondii* [134] infections due to the decreased inflammatory response and the impaired production of Th1-associated cytokines such as IL-12 and IFN-γ in these mice. It is important to mention that MyD88-driven proinflammatory events are not always favourable to the host in the fight against protozoans. The decreased inflammatory and Th1 responses in MyD88-deficient mice were seen to improve pathology and outcome of *P. berghei* infection in mice. This suggests that *Plasmodium*, in this case, utilizes the MyD88-dependent pathway to cause tissue injury and worsen disease symptoms [135].

It is quite remarkable that the amount of susceptibility to several protozoan infections conferred by the absence of MyD88 is significantly higher than that observed when mice lacking a single TLR are used. This strongly suggests that several TLRs are simultaneously involved in the recognition of parasites, thus explaining why the loss of MyD88 can have a bigger impact on susceptibility compared to the loss of a single TLR [131]. Nevertheless, deficiency of relevant TLRs increases susceptibility to certain infections. For example, TLR9-deficient mice have higher parasitemia and mortality when infected with *T. cruzi* [121] or *T. brucei* [122]. TLR4-deficient mice are more susceptible to *L. major* infection with bigger lesion size and parasite loads compared to WT mice [136, 137], and TLR11-deficient mice are more susceptible to *T. gondii* infection manifesting increased cyst formation in the central nervous system and decreased IL-12 and IFN-γ production compared to WT mice [128].

The ability of TLRs to detect parasite PAMPs put together with the fact that many successful infections are associated with silent entry to target cells suggests that parasites must have evasion tactics to block TLR signalling and functions. Some of these mechanisms have been already described, while others are still to be discovered. We will hereby discuss some evasion strategies employed by *Leishmania*, *Plasmodium*, and *Toxoplasma*.

The ability of *Leishmania* to interfere with TLR signalling components has been already discussed in this chapter under the "signalling pathways altered by *Leishmania*" section. These evasion mechanisms include the previously discussed ability of the parasite to interfere with the activation of all

three MAPKs (Erk1/2, JNK, and p38) (see MAPK section) and its ability to interfere with IκB, NF-κB, and AP-1 (see TF section). There is also evidence that signalling through CR1 and CR3, which *Leishmania* is known to bind to, can inhibit LPS- and IFN-γ-induced IL-12 production through impaired STAT-1 phosphorylation [91]. A similar role for Fc-γR ligation has been proposed [92, 138]. Nevertheless, very little is known about how the parasite can interfere with critical upstream proteins unique to IL-1/TLR signalling such as members of the IRAK family. Our laboratory has been interested for many years in exploring mechanisms utilized by *Leishmania* to block TLR signalling in M$\varnothing$s, and to evaluate the role of host SHP-1 in this process (Figure 1).

Of utmost interest, we have recently established SHP-1 as a central regulator of TLR signalling which can be exploited by *Leishmania* to inhibit IRAK-1 leading to the inability of M$\varnothing$s to respond to a wide range of TLR ligand stimulation including LPS, favoring parasite survival [93].

Other pathogen evasion tactics include the ability of *P. falciparum* to cause infected erythrocytes to express *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) which was shown to interact with the scavenger receptor CD36 on the surface of DCs [139] making the cells that phagocytose these infected erythrocytes become unresponsive to LPS stimulation, ultimately leading to defects in T-cell activation [140–142].

T. gondii is yet another parasite able to block LPS-mediated IL-12 and TNF-α production, the upregulation of costimulatory molecules, and the activation of T cells [143–146]. One way the parasite is able to do so is by activating STAT3 in IL-10-dependent and -independent manners [147, 148]. Although this *T. gondii*-induced inhibition of subsequent LPS stimulation might somehow resemble LPS tolerance in that it inhibits MAPKs like p38 [149], important differences between infection and LPS tolerance exist. Unlike LPS tolerance, *T. gondii* infection followed by LPS stimulation resulted in the activation of MKK3 and MKK6 (upstream activators of p38) and in the degradation of IκB [149]. This suggests that the inactivation of p38 observed when LPS stimulation is preceded by *Toxoplasma* infection is either due to the inhibition of another p38-activating kinase such as MKK4 or is mediated by a *T. gondii*-induced MAPK phosphatase that prevents the phosphorylation-dependent activation of p38 [131]. It is interesting to note that although *T. gondii* infection followed by LPS stimulation causes IκB activation, the liberated NF-κB fails to translocate to the nucleus [150, 151]. Later studies suggested that the lack of NF-κB translocation might actually be due to increased nuclear export of this TF rather than inhibition of nuclear import [152].

Collectively, it is clear that TLRs play a crucial role in mounting innate and adaptive immunity against invading pathogens. Alteration of TLR signalling by pathogens or by clinical drugs can play a key role in the outcome of infections. We have discussed in good detail strategies used by pathogens or by the clinic to alter TLR signalling. The activation of MyD88-dependent signalling and Th1 responses can turn out very useful in the elimination of many pathogens including *Leishmania*. However, these efforts must always be perceived with caution as exaggerated activation of inflammation can cause edema, pain, and tissue injury and in severe conditions could be deadly. In addition, certain infectious models like malaria seem to benefit from MyD88-dependent signalling and inflammation in their pathology, and thus a completely different approach should be used when trying to fight *Plasmodium*. As opposed to using TLR ligands which can worsen the disease, TLR agonists could prove clinically effective in treating malaria. Nevertheless, the effects of blocking TLR-TLR-L interactions on the ability of the immune system to fight off other pathogens that can be/become present have to be taken into serious consideration.

6. Concluding Remarks

Over the last 15 years, research stemming from our laboratory and others has provided strong evidence that parasites of the *Leishmania* genus are able to establish themselves and to propagate within the mammalian host due to its ability to alter key signalling pathways, therefore interfering with the induction of critical M$\varnothing$ functions that can otherwise threaten parasite survival. Importantly, we have identified one key way *Leishmania* can do so, and this is by exploiting host negative regulatory mechanisms such as the modulation of M$\varnothing$ PTPs.

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