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Immune Response Profile in Susceptibility and Protection in Visceral Leishmaniasis

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Abstract

Among the clinical forms of leishmaniasis, visceral leishmaniasis is the most debilitating clinical form because it compromises mainly liver and spleen in the host. The immune response against the parasite and immune factors relating to protection or susceptibility are not well established. Experimental models and natural infection in dogs also have different patterns to human disease. In the scientific community, many groups are developing different strategies to find an effective vaccine that could be used in dogs, and/or human in the future, with success. The vaccine to leishmaniasis, together with other sanitary strategies, could decrease the infection rates in endemic countries. Here, it is presented a review of the last studies focusing in a vaccine against visceral leishmaniasis.

Keywords: Leishmaniasis; Fever; Hematemesis

Introduction

Visceral leishmaniasis (VL), caused by the parasites *Leishmania donovani* and *L. infantum*, is endemic in 65 countries. The disease affects more than 200,000 people per year, with high case-fatality rates if not treated [1].

Human VL is characterized by long-term irregular fever, splenomegaly, hepatomegaly, lymphadenopathy, anemia, leukopenia, edema, epistaxis, hypergammaglobulinemia, hematemesis, weight loss and progressive weakness [2]. VL is considered to be the second most frequent cause of mortality and the fourth most frequent cause of morbidity in tropical diseases, with 20,000 to 40,000 deaths per year [1] and over 2 million years of Disability-Adjusted Life Years (DALYs) [3]. In addition, it is speculated that epidemiological data are underestimated, since clinical manifestation does not necessarily represent the absolute number of occurrences. For example, for each symptomatic case detected, there is estimation that there are, at least 6 to 20 asymptomatic infections [4]. In addition, the infection with *Leishmania* has gained attention as an opportunistic pathogen in HIV positive and other immunocompromised patients [5].

Among the factors that lead to the search for new alternatives to control VL are the development of resistant strains of the standard drug [6,7], toxicity of treatment and its inefficiency in patients co-infected with HIV virus [6,8] to the increased incidence in immunocompromised patients and the difficulties in control based on the elimination of seropositive dogs [9]. The gold standard would be a highly efficient vaccine against the parasite. However, no human vaccine is available at the moment. For veterinary use, there are three commercially available. One in Brazil based on recombinant A2 protein plus saponin-(Leishtec) [10]. The second licensed in Europe, composed of purified excreted-secreted proteins of *Leishmania infantum* (LiESP), using a highly purified fraction of the *Quillaja saponaria* saponin as adjuvant (Canileish) [11]. The third, also licensed in Europe, is composed of 4 proteins (3 ribossomal and 1 hypothetical), using no adjuvants (Letifend) [12].

The host immune response has an important role in protection/susceptibility in the course of *Leishmania* infection, besides the persistence and multiplication of the parasite. In the other hand, parasite exhibits different strategies to manipulate cell death and immune system to survive to establish infection [13]. The understanding of the mechanisms associated with immune evasion and disease progression is essential for the development of novel therapies and vaccine approaches. Here, we revise the immune response profile in natural and experimental infection by *Leishmania infantum*/*Leishmania donovani*, also after different vaccination approaches.

Immune Response in Experimental Infection

The events that occur after the inoculation of *Leishmania* sp. are essential to the course of the infection, and the involvement of the immune system is present by the complement system, polymorphonuclear neutrophils, natural killer cells (NK), macrophages, growth factors such as colony stimulating factor (GM-CSF) and (TGF-β), and [14,15]. In general, these elements are activated in an attempt to contain the infection, either by enhancing the innate immune response by chemotaxis of more cells, or by the activation of the adaptive immune response. Because it is an intracellular parasite, the adequate immune response to *Leishmania* involves the activation of the microbicidal mechanisms of infected cells, especially macrophages, resulting mainly in the production of molecules such as reactive oxygen (ROI) and nitrogen intermediates (RNI) [16,17] .

Among the mechanisms involved in mammalian host protection against *Leishmania* infection, the development of Th1 immunity is crucial because triggers enhanced leishmanicidal activity by infected macrophages. In general, as demonstrated in murine model, protection against infection is attributed to the development of the cellular immune response triggered by $CD4^+$ Th1 T cells, characterized by the production of IL-12 and IFN-γ, which mediate the activation of

macrophages, and nitric oxide production and consequently elimination of the parasite [18-23].

Immunopathogenesis of VL has been extensively studied in murine models of systemic infection. Genetically susceptible animals, such as BALB/c and C57BL/6 mice, infected with *L. donovani* or *L. infantum*, intravenously or intraperitoneally, present visceral parasitism. In the liver, amastigotes multiply rapidly during the first four weeks, but are soon controlled by the formation of inflammatory granulomas. Infected Kupffer cells produce chemokines (CCL2, CCL3, CCL19, CCL21 and CXCL10) capable of recruiting neutrophils, monocytes and T cells that are indispensable in the formation of the granuloma. Neutrophils, in addition to eliminating the parasite, induce the polarization of the immune response to the Th1 pattern. $CD4^+$ T lymphocytes start to produce IL-12, which enhances the inflammatory response in granuloma among with production of IFN-γ, TNF-α and lymphotoxin α (LTα). These cytokines, in addition to increasing the recruitment of cells to structure the granuloma, activate the leishmanicidal mechanisms of infected cells producing reactive oxygen and nitrogen species and, in approximately eight weeks, liver infection is controlled [24,25].

In the spleen, multiplication of the parasite is late, but persistent compared to liver. Initially, IL-12 production is observed by dendritic cells migrating from the marginal zone to the periarteriolar zone in order to activate effector lymphocytes. However, this mechanism does not seem to be efficient in infection control, since the infection persists after several weeks and an enlargement of the spleen and changes in the splenic tissue are observed. This remodeling of the spleen is accompanied by an increase in the production of TNF-α which intensifies the depletion of macrophages and cells of the perateriolar zone, responsible for the release of CCL19 and CCL21, important in the migration of dendritic cells to the activation zone of effector T lymphocytes. In these models, IL-10 appears to be directly involved in parasite multiplication, since mice deficient in IL-10 production have infection controlled. It is suggested that IL-10 alters the expression of PD-1 (programmed death) and its ligand (PD-L1), inhibiting cytokine production and T-cell proliferation. IL-10 production can be induced by IL-27 and IL-21 *via* activation of Tr1 cells [24,25]. It was observed that mice deficient for the IL-27 receptor are resistant against infection by *L. donovani*, but develop severe liver lesions [26].

In different models, some studies have shown the hostile effect of the leishmaniasis, induced by B cells [27-30]. In experimental VL, disease exacerbation is contributed by B cells. Smelt and collaborators (2000) demonstrated that B cell-deficient mice are highly resistant to *L. donovani* infection. However, B cells contribution to infection is not well known. But it seems that *Leishmania* infection could induce a production of short-lived *Leishmania*-specific IgG, and it decreases during chronic phase, suggesting that most antibodies produced are not specific for the parasite [31].

Immune Response in Infected Patients

Although endemic, less than 10% of the individuals infected by *L. donovani* or *L. infantum* present the classic form of VL. The majority of people who become infected with visceralizing *Leishmania* spp. never develop disease. The two groups (asymptomatic and symptomatic) are targets of studies in an attempt to understand the immunological dynamics of the disease. Looking only at serological parameters, it has been observed the presence of several mediators of the immune response during the active form of the disease, the cytokine storm. Several studies report high serum levels of cytokines type 1 (IFN-γ and IL-12), type 2 (IL-4 and IL-13), Treg (IL-10 and IL-6), Th17 and TNF-α when compared with asymptomatic individuals [32-34]. After treatment, these levels of cytokines are similar to those of asymptomatic and healthy individuals. The clinical presentation of VL seems to be a consequence of the interaction parasite and the immune response developed by the host [35]. Thus, the outcome of infection would depend on the parasite's ability to evade non-specific defences of the host, to be recognized and phagocytosed, and survive within the phagocytes vacuole of macrophages [36]. During parasite-host interaction, complex signaling pathways are triggered by recognition of key parasite molecules [37]. Differences in virulence factors between species are responsible for the different clinical forms observed in leishmaniasis [38]. Protective immunity in leishmaniasis is mediated by the cellular immune response while the active disease is associated with a strong humoral response with absence of cellular response [38]. The control of infection is complex and the parasite is able to evade pro-oxidative responses and other macrophage effector mechanisms preventing the activation of a response [37,39].

Thus, the T cells and their producing cytokines play a central role in the immune response [40]. Parasitic macrophages, lymphocytes, dendritic cells and natural killers produce cytokines that are involved in both innate and adaptive responses to the parasite [22,41]. $CD4^+$ and $CD8^+$ T cells are important to restrain visceral disease and are involved with the production of IL-2, IFN-γ and IL-12 [42].

In contrast, if intense proliferation of B lymphocytes and the production of antibodies are abundant, the immune response is deleterious and non-protective for VL [43]. Therefore, the cellular immune response mediated by Th2 cells is associated with susceptibility, resulting in parasite persistence and infection progression (Kedzierski et al.). In VL, there is evidence of the predominance of Th2 cellular immune response during acute disease, leading to suppression of cellular reactivity against parasite antigens, absence of IL-2 and IFN-γ, predominance of IL-4 cytokine production and to polyclonal activation of B cells resulting in hypergammaglobulinemia [44-47]. Unlike the polarization observed in murine models, a distinct Th1 and Th2 response pattern is not detected in human VL; In this case, the mixed Th1/Th2 immune response is observed during the course of infection, indicated by the high simultaneous levels of IFN-γ and IL-10 in patients with the disease [48,49]. In VL, there is still no clear association between IL-4 production and active disease [50], but a direct correlation between IL-10 levels and disease progression has been described [46,51-56].

The cytokine IL-10 is indicated as the key factor of immunosuppression in VL. This fact is based on studies using murine model mainly, IL-10-deficient BALB/c and C57BL6 mice are highly resistant to *L. donovani* infection [53,57]. IL-10 has the ability to deactivate the leishmanicidal mechanisms of the macrophage and to down regulate the expression of MHC and co-stimulatory molecules [54,58,59]. It also decreases the production of IFN-γ by T cells and inhibits DC migration to T cell areas [60-63]. Therefore, the evolution of the disease may be induced by IL-10 and it can condition host macrophages to increase parasite survival and replication, since this cytokine renders macrophages non-responsive to activation signals and inhibits the killing of parasites, by down regulating the production of nitric oxide and TNF-α [54,64-66]. However, there are some evidence suggesting that IL-10 may be host protective; particularly, in regulating a detrimental inflammatory response in the liver. Indeed,

the extensive hepatic necrosis accompanying *L. donovani* infection in TNF-deficient mice may result from a concomitant defect in IL-10 induction [26,67]. Likewise, the severe hepatic pathology that follows *L. donovani* infection in IL27R$^{-/-}$ mice involves CD4^{+} T cells and may result from reduced IL-10 induction [26,68].

Studies in patients confirm the efficacy of IFN-γ and IL-12 mediated Th1 immune response to the elimination of *Leishmania*, to the detriment of Th2 IL-4 and IL-13 mediated immune response [41,45,69-71] . In classical VL, an absence of IFN-γ and IL-2 production in response to soluble *Leishmania* antigens is observed *in vitro* [72]. This suppression is restored when anti-IL-10 is added, suggesting the negative modulatory role of this cytokine [73]. Indeed, individuals with the active form of VL have elevated serum levels of various cytokines, called "cytokine storm", including IFN-γ and IL-10 [32,34,49,71,74-79]. Over time, other cytokines have been shown to regulate the immune response, as IL-6, IL-21 and IL-27 [74,80,81]. However, several aspects of this immune response in VL are still unclear.

The factors that influence susceptibility to VL remain an area of intense interest but are still largely a matter of speculation. As mentioned, many of the cytokines measured at elevated levels in VL patients indicate that the immune system responds appropriately but that other factors render these responses inadequate to contain the infection effectively. In addition, VL mostly affects populations in poor countries, where malnutrition or/and co-infection with helminthes or other organisms might be common, especially in rural area. It is demonstrated that low nutritional status can impair both innate and adaptive immune response [82,83]. In addition, co-infection with helminthes alters the Th1/Th2 balance, supporting *Leishmania* infection [84,85]. Also, it is demonstrated that testosterone induces parasite replication, resulting in more infected men with VL than women. This could suggest that hormone could induce or interfere in the susceptibility in this gender [54,86-88].

Immune Response in Infected Dogs

In Canine visceral leishmaniasis (CVL), cell-mediated immunity is able to control infection, and correlates with the absence of symptomatology [89-91], while the lack of this response has been associated with the presence of an exacerbated humoral response allowing the progression of the disease [92,93].

Illustratively, the positive skin test (late hypersensitivity reaction) for *Leishmania* spp. in dogs is a good indicator of resistance to infection, and can be used as a prognostic factor [94,95].

The mechanisms of cellular immune response in the natural infection canine leishmaniasis are different from those found in models where the classical Th1/Th2 response paradigm is associated with resistance or susceptibility [96]. Although not following the classic paradigm, some studies associate asymptomatic infection in dogs with the activation of Th1 cells that produce IFNγ, IL-2 and TNFα [89,91]. Furthermore, the cytokine profile in blood cell culture (PBMC) of infected asymptomatic dogs experimentally with *L. infantum*, shows predominantly Th1 response, mediated by expression of IL-2, IFNγ and IL-18, but absence of IL-4 expression [35]. In this way, the parasites are killed by macrophages activated by lymphocytes producing IFNγ through a mechanism dependent on nitric oxide production [97]. However, there is evidence that IFN-γ increases in the early stages of infection but is not sufficient to prevent disease and that levels increase as a consequence of infection, regardless of clinical symptomatology [97,98].

Both asymptomatic and symptomatic dogs are able to produce cytokines from Th1 and Th2 profiles, and these profiles can coexist in CVL [99]. Thus, studies on the expression of cytokines in CVL have conflicting results that may be related to the compartmentalization of the response in the different tissues evaluated. Alves et al. [100] demonstrated the gene expression of IFN-γ and TNF-α in asymptomatic dogs and IL-10 and TGF-β in symptomatic dogs. The association of parasite load, clinical evolution, gene expression and synthesis of certain cytokines such as IFNγ, IL-2, IL-6, IL-10 and IL-12 has already been demonstrated [91,101-104]. Another study showed that increased IL-4 gene expression in peripheral blood mononuclear cells after six months of infection was quantitatively similar in both asymptomatic and symptomatic dogs [105]. In symptomatic dogs infected with *L. infantum*, no correlation was found between serum TNF-α level and active disease [102]. Unlike human and murine leishmaniasis, in CVL no difference was observed between levels of IL-10 in the tissues and the expression of IL-4 in the bone marrow of naturally infected dogs, especially those most severely affected, suggests an association between IL-4 and disease [97]. However, when analyzing the relationship between skin parasite density and the expression of mRNA for IL-10 in skin, it is observed a positive correlation between high burden and IL-10 expression [106]. In summary, the immunological mechanisms responsible for the resistance or progression of CVL and the detection of cytokine synthesis and its association with *L. infantum* infection are still controversial.

The understanding of the role of Th1 and Th2 subpopulations in the different tissues is crucial to comprehend the profile of immune response induced by the infection. In fact, the immune response to the parasite is not identical in any host system, but organ-specific [107]. The liver is the site of resolution of the acute infection, exhibits minimal tissue damage and resistance to reinfection, whereas the spleen becomes a site of persistence of the parasite as evidenced in a murine model of infection [67].

The spleen is among a major organ affected in CVL and is largely responsible for the immune response to infection, but the knowledge of the immunopathological mechanisms involved is limited, with most studies focused on the murine model of experimental infection. It is an important secondary large-volume lymphoid organ with primary function of responding to systemic pathogens [108]. The lymphoid tissue is highly organized and the disorganization of the splenic architecture in dogs with VL has been associated with the progression of the disease and the reduction of the expression of several chemokines and their receptors [109]. The mRNA levels of a wide variety of cytokines (IFNγ, TNFα, IL-4, IL-5, IL-10, IL-12, IL-18, TGFβ), transcription factors (Tbet and GATA3), and chemokines (IP-10, RANTES, MIP-1α, MCP-1) were evaluated in the spleen of naturally and experimentally infected dogs [99,110]. It has been suggested that during *L. infantum* infection, a Th1/Th2 mixed response occurs in the spleen of symptomatic and oligosymptomatic dogs, insufficient to control the parasite locally. The authors showed that the high expression of IFN-γ in the spleen did not eliminate the parasites completely, since a new parasite load from other sites, where IFN-γ is not elevated, is constantly released into the spleen [111].

One of the most relevant organs involved in the parasite-host interface during *L. infantum* infection is the hepatic compartment. Few studies have quantified the production of cytokines and

chemokines in the liver of infected dogs. The mRNA levels of CCL1, CCL17, CCL26, CCR3, CCR4, CCR5, CCR6 and CCR8 were higher in asymptomatic dogs as well as the synthesis of IFN-γ, IL-10 and TGF-β1 [99,112]. On the other hand, it seems that the infection alone causes an increase in the synthesis of IL-4 and IL-10 in the liver, independent of symptomatology, with increased TNF-α level in symptomatic dogs, which was not observed in the spleen. In addition, the parasite burden of the liver is lower than in the spleen [113]. In the liver, the presence of granulomas, in different degrees of maturation in infected dogs, is a reaction of this organ to try to control the proliferation of parasites, and in the murine model TNFα is involved in the formation of hepatic granulomas [67,114]. The presence of granulomas is also observed in human VL and CVL [115].

Immune Response Profile after Different Vaccination Approaches

The control of LV is dependent of early diagnosis, treatment of infected individuals, and control of vectors and outbreaks in domestic reservoirs [2]. These measures together could eliminate or drastically reduce transmission [116,117] when used over a long period of time [118]. However, the number of available drugs is limited, most overpriced, have toxic side effects or prove ineffective due to co-infection with HIV or induce emergence of resistant strains. In addition, the vector control methods available are not so efficient. Therefore, vaccination is the most effective method for the control of zoonotic LV. The application of an effective vaccine in human/dogs can also drastically reduce the transmission of *L. infantum* [119]. The development of vaccines against human and canine infection has been the target of many research groups [50,61,120,121].

It has been demonstrated that the treatment of *Leishmania* infection confers a strong immunity against reinfection in cutaneous species [47,122], the partial protection observed in animal models after immunization with different recombinant or crude antigens [50,123,124] and protection against infection resulting from the leishmanization process [125]. However, despite the extensive search for vaccine development against leishmaniasis in recent decades, no vaccine against human leishmaniasis, whether cutaneous or visceral, is available for prophylactic use.

Between the different methodologies, the vaccination approaches include killed or live attenuated *Leishmania* parasite (first generation), recombinant *Leishmania* proteins (second generation), DNA encoding *Leishmania* proteins (third generation), and immunomodulators [61,126-128] Here is tabled the available immunization studies according to the animal model and the protection observed (Table 1).

Immunization	Animal model	Profile of immune response	Protection	Reference
L. major antigens+BCG	Clinical trial	-	Partial protection	[129]
L. major antigens+BCG	Dogs	-	Partial protection	[130]
L. braziliensis+BCG	Dogs	Cell proliferation and IFN-γ production	Partial protection	[131]
Leish 111 (LeIF+TSA+LmSTI1)+MPL	Dogs	IgG1 and IgG2 production	-	[132]
MML	Dogs	-	5/8 protected dogs	[133]
H1, HASPB1, H1+HASPB1	Dogs	Antibody response	Partial protection	[133]
Protein Q chimera+BCG	Dogs	DTH response	Protection	[134]
Multicomponent LBSap vaccine	Mice	Humoral and cellular response	Partial protection	[135]
LBSapSal	Dogs	NO and Th1 cytokines response	Protection	[136]
LBSap	Dogs	Cell proliferation, IFN-γ production	Protection	[137,138]
pCI-neo-LACK+MVA or M65	Human PBMC, Hamsters	Th1-type response	Protection	[139]
rA2+Alum+CPG	Mice	High IgG, IgG1, IgG2, IFN-g and IL-10	82% decrease spleen	[140]
CL-14 (Trypanosoma cruzi CL-14 non-virulent strain)+A2				
Th1 responses with enhanced IFN-γ, IL-12, nitric oxide,	Mice	Th1 responses with enhanced IFN-, IL-12, nitric oxide,	Protection	[141,142]
IgG2a/IgG1 ratio and reduced IL-4 and IL-10		IgG2a/IgG1 ratio and reduced IL-4 and IL-10		
responses		responses		
Th1 responses with enhanced IFN-γ, IL-12, nitric oxide,		High IFN-g, IL-12, NO, IgG2/IgG1 ratio		
IgG2a/IgG1 ratio and reduced IL-4 and IL-10		Low IL-10, IL-4		

responses				
Th1 responses with enhanced IFN-γ, IL-12, nitric oxide,				
IgG2a/IgG1 ratio and reduced IL-4 and IL-10				
responses				
gp63				
gp63+HSP70 DNA	Mice	High IFN-g	-	[143]
Proteophosphoglycan	Hamsters	NO and pro inflammatory cytokines	80% of protection	[144]
KMP-11	Mice/Hamsters	$CD4^+$ and $CD8^+$ T cells producing IFN-g, IL-12, TNF-a	Protection	[145-148]
Amastigote gene	Mice	IgG, pro inflammatory cytokines	-	[149]
KMP-11+Ljm19	Hamsters	High IFN-γ/IL-10	Protection	[147]
Centrin deleted live parasite *L. donovani*	Mice, hamsters, dogs	High IFN-g, IL-12, TNF-a, IgG, IgG1, IgG2, T cell proliferation	Protection	[150-154]
Ascorbic acid-deficient live mutants of *L. donovani*	Mice	T cell proliferation, pro inflammatory cytokines	Protection	[155]
p27 gene knockout *L. Donovani* parasites	Mice	T cell proliferation, pro inflammatory cytokines, NO	Protection	[156]
L. major (ALM)+BCG	Mice	Antibody production, DTH response	Protection	[125]
KBMA *L. infantum* and *L. chagasi*	Mice	Th1 cytokine pattern	Partial protection	[157]
Leishmune (FML)	Mice, dogs	High lymph proliferative response and IgG2 production	Partial protection	[158]
Leish F1	Clinical trial	Th1 response	Safe	[159]
Leish 111f+MPL	Clinical trial	Th1 response	Protection	[160]
HASPB1	Mice	Th1 response	Protection	[161]
rSMT	Mice	Th1 response	Protection	[162]
A2	Mice	High IFN-γ production	Protection	[163]
LD91+LD72+ LD51+LD31	Mice	High pro inflammatory cytokines production	Protection	[164]
TRYP	Mice	IgG and IgG2 production	Partial protection	[165]
ORFF	Hamsters	IFN-γ and IL-12 production	Partial protection	[166]
LPG 3	Mice	Mixed Th1/Th2 response	Partial protection	[167]
LEISHDNAVAX	Mice	IgG production	Partial protection	[168]

Table 1: Immunization studies to VL according to animal model, immune response profile and protection

Conclusions

The pattern of immune response is well studied in murine and human, whose Th1/Th2 paradigm is well established. In the murine model of leishmaniasis, Th1 cells secrete IL-2, IFN-γ and TNF-α and are associated with resistance and infection control, whereas Th2 cells secrete IL-4 and IL-10, and are responsible for the susceptibility and progression of the disease In human VL, the immune response Th2 is represented by the low synthesis of IFNγ and by the increase of IL-4 levels. In addition, IL-10 has been indicated as one of the major cytokines suppressors of the protective immune response, both in murine models and in human VL. Although some evidence demonstrates the feasibility of vaccination against leishmaniasis, the differences among the host species and the organs affected by the disease creates difficulties to develop a broad vaccine [169-177]. Those particularities still need more studies and a better understanding to develop a well-established vaccine to VL.

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