Immunopathogenesis of infection with the visceralizing Leishmania species

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Abstract

Human leishmaniasis is a spectral disease that includes asymptomatic self-resolving infection, localized skin lesions, and progressive visceral leishmaniasis. With some overlap, visceral and cutaneous leishmaniasis are usually caused by different species of Leishmania.

This review focuses on host responses to infection with the species that cause visceral leishmaniasis, as they contrast with species causing localized cutaneous leishmaniasis. Data from experimental models document significant differences between host responses to organisms causing these diverse syndromes. The visceralizing Leishmania spp. cause localized organ-specific immune responses that are important determinants of disease outcome. Both the Leishmania species causing cutaneous and those causing visceral leishmaniasis require a Type 1 immune response to undergo cure in mouse models. However, during progressive murine infection with the visceralizing Leishmania sp., the Type 1 response is suppressed at least in part by TGF-β and IL-10 without type 2 cytokine production. This contrasts with the cutaneous species L. major, in which a Type 2 response suppresses type 1 cytokines and leads to murine disease progression. Population and family studies are beginning to elucidate human genetic determinants predisposing to different outcomes of Leishmania infection. These studies should eventually result in a better understanding of the immunopathogenesis and the spectrum of human leishmaniasis.

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1. Introduction

The Leishmania spp. are a diverse group of organisms belonging to the order Kinetoplastida and the family Trypanosomatidae. The genus can be divided into two sub-genera, Leishmania (Leishmania) spp., and Leishmania (Viannia) spp. (Table 1). The visceralizing Leishmania spp. belong to the L. (Leishmania) sub-genus, and include L. donovani and L. infantum in the Old World and L. chagasi in the New World. Although different in name and geographic origin, molecular data are suggesting that L. infantum and L. chagasi may actually be one and the same species [1]. Leishmania donovani and L. infantum/chagasi usually cause disseminated disease in humans, and in turn most cases of visceral leishmaniasis can be attributed to them. However, there is considerable overlap in clinical manifestations caused by the various Leishmania spp., and there are reported cases of cutaneous lesions due to L. chagasi/infantum and L. donovani and cases of visceral disease due to L. amazonensis [2] and L. tropica [3–5].

Infection with the visceralizing Leishmania spp. leads to variable manifestations in humans and in experimental rodent hosts. The extent to which the variation in manifestations of disease is caused by genetically defined host versus parasite factors is not settled, but studies have made it clear that there are contributions from both. This review will focus on host responses to infection with L. donovani and L. infantum/chagasi, and how these differ from responses to species of Leishmania that typically cause human cutaneous leishmaniasis (e.g. L. major).
Finally, much of the literature on experimental cutaneous leishmaniasis focuses on *L. major*, and it is becoming apparent that other cutaneous species, such as *L. amazonensis*, elicit yet distinct host responses [6,7].

2. The Th1/Th2 paradigm: *L. major* models of cutaneous leishmaniasis in mice

Studies of murine models have underscored biological differences between *L. major*, a species that typically causes cutaneous leishmaniasis in humans, and the *Leishmania* spp. that cause visceralizing leishmaniasis. Work of Mossman and Coffman in the 1980s documented distinct subsets of CD4+ T cells including Th1 cells that lead to macrophage activation and B cell secretion of immunoglobulin subtypes (e.g. IgG2a) able to fix complement and neutralize viruses. In contrast, Th2 cells help antibody production of IgG1, IgE and IgA and secrete cytokines that prevent Th1 development [8]. Additional types of CD4+ cells expressing TGF-β and/or IL-10 have more recently been described. These include regulatory T cells that constitutively express the IL-2R subunit CD25, and TGF-β-producing Th3 cells located in the gut. For the purpose of this review we will refer to an immune response that is dominated by IFN-γ as a Type 1 response, and a response characterized by IL-4, IL-13 and/or IL-5 as a Type 2 immune response. An immune response characterized by abundant TGF-β will be called a ‘Type 3’ response.

The chronic persistent nature of leishmaniasis favors the development of polarized immune responses. Indeed, murine leishmaniasis caused by *L. major* provides an exquisite demonstration that Th1 and Th2 subsets can influence the course of disease toward opposite poles depending on the genetic predisposition of the murine host. Progressive *L. major* infection of susceptible BALB/c mice is promoted by expansion of Th2 cells producing IL-4, IL-10 and IL-13. In contrast, Th1 expansion in resistant mice (e.g. C3H), initiated by IL-12 and IFN-γ, causes *L. major* lesions to resolve [9–11]. Th2 cells lose their IL-12 responsiveness due to decreased expression of the β1 and the β2 subunits of the IL-12 receptor during *L. major* infection [12,13]. An early burst of IL-4 occurs in susceptible BALB/c mice and precedes disease progression [14]. This is caused by expansion of T cells expressing TCR Vα2Vβ4 specific for the *L. major* LACK antigen (homolog of mammalian RACK1) [15,16]. Indeed the response to LACK has been found to dominate the course of *L. major* disease in mice. As such, mice in which Vβ4 expressing cells are deleted with a superantigen become resistant; whereas control mice with deleted Vβ6 remain susceptible, to *L. major* infection [15]. Continuous expression of LACK in mouse tissues render them tolerant to LACK, and this inability to respond to LACK causes these mice to down-regulate their Type 2 response and heal infection [16].

3. Visceralizing *Leishmania* spp. in experimental animal models: tissue-specific immune responses and the lack of a Th1/Th2 dichotomy

Rodent models have been used extensively in the study of *L. donovani* and to a lesser extent *L. chagasi* and *L. infantum*. Mice are either genetically susceptible or resistant to infection, but even susceptible strains heal their infections [17]. Thus, they are better models of self-healing.

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**Table 1**

Major species of *Leishmania* infecting humans [156]

<table>
<thead>
<tr>
<th>Species*</th>
<th>Major disease syndrome</th>
<th>Geographic location</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. (L.) donovani</em></td>
<td>Visceral leishmaniasis, PKDLb</td>
<td>India, North and Eastern China, Pakistan, Nepal</td>
</tr>
<tr>
<td><em>L. (L.) infantum</em></td>
<td>Visceral leishmaniasis</td>
<td>Mediterranean, Middle East, Balkans, Asia, northwest China, northern and sub-Saharan Africa</td>
</tr>
<tr>
<td><em>L. (L.) chagasi</em></td>
<td>Visceral leishmaniasis, cutaneous leishmaniasis (rare)</td>
<td>Latin America</td>
</tr>
<tr>
<td><em>L. (L.) archibaldi</em></td>
<td>Visceral leishmaniasis</td>
<td>Sudan, Kenya, Ethiopia</td>
</tr>
<tr>
<td><em>L. (L.) tropica</em></td>
<td>Cutaneous leishmaniasis, visceral tropic leishmaniasis</td>
<td>Middle East, northwest China, northwest India, Pakistan, Africa</td>
</tr>
<tr>
<td><em>L. (L.) aethiopica</em></td>
<td>Cutaneous leishmaniasis, diffuse cutaneous leishmaniasis</td>
<td>Ethiopia, Kenya, Yemen</td>
</tr>
<tr>
<td><em>L. (L.) amazonensis</em></td>
<td>Cutaneous leishmaniasis, rarely mucosal leishmaniasis</td>
<td>Mexico, Central America, Texas</td>
</tr>
<tr>
<td><em>L. (L.) venezuelensis</em></td>
<td>Cutaneous leishmaniasis</td>
<td>Amazon basin, Brazil</td>
</tr>
<tr>
<td><em>L. (V.) pifanoi</em></td>
<td>Cutaneous leishmaniasis, diffuse cutaneous leishmaniasis</td>
<td>Venezuela</td>
</tr>
<tr>
<td><em>L. (V.) braziliensis</em></td>
<td>Cutaneous leishmaniasis</td>
<td>Venezuela</td>
</tr>
<tr>
<td><em>L. (V.) peruviana</em></td>
<td>Cutaneous leishmaniasis</td>
<td>Venezuela</td>
</tr>
<tr>
<td><em>L. (V.) guyanensis</em></td>
<td>Cutaneous leishmaniasis</td>
<td>Guyana, Surinam, Amazon basin</td>
</tr>
<tr>
<td><em>L. (V.) panamensis</em></td>
<td>Cutaneous leishmaniasis</td>
<td>Peru, Argentina highlands</td>
</tr>
<tr>
<td><em>L. (V.) garnhami</em></td>
<td>Cutaneous leishmaniasis</td>
<td>Panama, Costa Rica, Colombia</td>
</tr>
</tbody>
</table>

* The subspecies *Leishmania Leishmania* or *Leishmania Viannia* are indicated on this table. The subspecies designation is not indicated throughout the text.

b PKDL—post-kala-azar dermal leishmaniasis.

c Visceral leishmaniasis, cutaneous leishmaniasis (rare) Latin America

c Evidence is accumulating that *L. infantum* and *L. chagasi* are the same species.
or subclinical infection than disseminated visceral disease. In contrast, hamsters develop progressive disease in which parasites replicate in the liver, spleen, and bone marrow eventually causing death of the host [18]. Surprisingly there are significant amounts of the Type 1 cytokines IFN-γ, IL-2, and TNF-α mRNA expressed in the spleens of hamsters infected with *L. donovani*. Although there is little or no IL-4, substantial amounts of TGF-β and IL-10 mRNAs are also present. Furthermore, there is decreased expression of NOS2 mRNA, the gene encoding iNOS, and there is little NO’ generation in infected hamsters. This may account for the defect in parasite killing [19]. Progressive hamster infection with *L. donovani* or *L. chagasi/infantum* appears to mimic human visceral leishmaniasis. However, most hamsters develop severe ascites before their surmise, and histologic studies have revealed immune complex-mediated glomerulonephritis and disseminated amyloidosis, which are thought to produce a nephrotic syndrome [20]. Whereas immune complex glomerulonephritis has been observed histologically in humans and dogs with visceral leishmaniasis, renal failure and the nephrotic syndrome are rare [21]. Thus, although visceral leishmaniasis in hamsters is more similar to progressive human visceral leishmaniasis than mouse models, the hamster is still not a perfect model of human disease.

Studies of mouse models have underscored similarities and differences between *L. major* and *L. donovani/L. chagasi/L. infantum* infections. Different strains of mice are genetically either susceptible or resistant to leishmaniasis, and there are differences between the responses of some strains to different *Leishmania* species [17,22]. Table 2 delineates strains that are susceptible or resistant to infection with *L. major* versus the visceralizing *Leishmania* sp. Strains that are susceptible to *L. donovani* correspond to those susceptible to several unrelated intracellular pathogens (*Salmonella typhimurium, Mycobacterium bovis* BCG, *M. leprae*, and *M. intracellular*). Susceptibility to these pathogens is determined by the specific allele at one locus on mouse chromosome 1 [23]. The gene at this locus was previously called *Lsh, Iry*, or *Bcg* depending on the infectious model under consideration. It has now been mapped and characterized, and renamed as *SLC11A1* (described in detail below) [24]. Curiously, susceptibility to *L. major* maps to different loci, a fact that likely determines the differences in strain susceptibility [23].

### 3.1. Contrasts with *L. major* infection

Similar to *L. major* infection of resistant mice, resistant mouse strains such as C57BL/6 and C3H develop a type 1 (Th1) response with CD4+ cells producing IFN-γ and IL-2 during *L. donovani* or *L. chagasi/infantum* infection. Also similar to *L. major* infection, susceptible mice exhibit a decrease in IFN-γ produced by liver granuloma cells [25,26].

In contrast to *L. major* infection, however, susceptible BALB/c mice resolve a high level infection with the visceralizing *Leishmania* sp. spontaneously, making them a better model of subclinical infection than of progressive disease (Fig. 1A). In these mice the effect of IL-12 is delayed for four weeks after infection, at which time lymphocytes producing antigen-specific IFN-γ develop. Liver granulomas form, and mice develop parasite-specific CD4+ and CD8+ T cell responses [27–31]. Also in contrast to *L. major* infection, susceptible BALB/c mice infected with the visceralizing species lack type 2 (Th2) antigen-specific immune responses, and as such they lack IL-4 and IL-5 producing cells despite disease progression [25,26,32]. Further illustrating the lack of a type 2 response, the parasite burden is not affected by administration of neutralizing antibody to IL-4 or IL-4 receptor, or by disruption of the IL-4 gene [33–35].

### 3.2. Organ-specific immune responses

One remarkable aspect of murine visceral leishmaniasis is the different growth rate of parasites in infected organs (Fig. 1A). *L. donovani* promastigotes inoculated into the skin of hamsters convert to amastigotes [36]. Intracellular parasites multiply during the first week after inoculation. This is followed by the formation of granulomas between 4 and 6 weeks. By the 8th week, cutaneous intracellular parasites are largely gone (Fig. 2). This histologic response is consistent with early replication of *L. donovani* in macrophages in the skin. Cutaneous infection is eventually controlled locally, coincident with granuloma formation. Despite these local responses, the organism is able to disseminate and produce symptomatic visceral leishmaniasis [36].

During *L. chagasi* infection in mice, amastigotes multiply rapidly for the first four weeks in liver, but they are cleared spontaneously by week eight. Parasites grow slowly in the spleen [26]. Importantly, this ‘tissue tropism’ is similar in mice infected intradermally with *L. infantum* [37], indicating it is not merely due to parasites lodging in the first organ they encounter after intravenous inoculation. The timing of parasite growth and resolution is faster in

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Table 2

Susceptibility or resistance of different mouse strains to *Leishmania* sp. [17,22]

<table>
<thead>
<tr>
<th>Strain</th>
<th><em>L. donovani/chagasi/infantum</em></th>
<th><em>L. major</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>A/Jax</td>
<td>R*</td>
<td>R</td>
</tr>
<tr>
<td>CBA</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>C3H. HeJ</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>DBA/2</td>
<td>R</td>
<td>S</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td>C57BL/10</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td>B10.D2</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td>BALB/c</td>
<td>S</td>
<td>S</td>
</tr>
</tbody>
</table>

* R—resistant; S—susceptible.
mice infected with *L. donovani* amastigotes, but the pattern of rapid growth and spontaneous resolution in the liver is similar [38,39]. In contrast to the liver, parasites grow more slowly in the spleen and bone marrow, where they can persist for life in the animal [40]. This tissue specific growth pattern has been observed in murine disease caused by *L. donovani*, *L. chagasi*, and *L. infantum*, and when parasites are introduced to the animal either intravenously or intradermally [26,37,38] (Fig. 1). Thus the liver apparently serves as site for initial parasite expansion, and the spleen may serve as a ‘safe harbor’ for the long-term persistence of the visceralizing *Leishmania* sp.

### 3.3. Immune responses in the liver

The above observations raise the question of whether there are differences in the localized host response to *Leishmania* infection in different mammalian organs. Resolution of disease in the livers of mice infected with *L. donovani* or *L. chagasi* correlates with the local formation of granulomas (Fig. 2). Amastigotes are first observed in liver Kupffer cells, where they survive without killing [41]. Early parasite replication in the liver and spleen occurs when there is little IFN-γ and few IL-12 producing cells [25]. The kinetics of parasite growth during the first 4 weeks...
of infection is similar in wild type C57BL/6 and IL-12 p40 knockout mice, suggesting an IL-12 mediated type 1 response does not influence disease progression until late (Fig. 1B and C) [30]. This is similar to the cadence of L. major disease in C57BL/6 mice, in which there is an early phase of 'silent' parasite replication with the peak parasite load at 4–6 weeks, and the onset of immunity correlates with the emergence of cells expressing IL-12p40, IFN-γ, CD40L, and iNOS [42].

Granulomas, which normally form during disease regression in the liver, are poorly formed in immunodeficient murine and human hosts. Humans with progressive visceral leishmaniasis do not develop mature granulomas [27]. Gene knockout and antibody neutralization studies show that granuloma development in the livers of infected mice requires CD4+ and CD8+ cells, IL-12, IFN-γ, and IL-2. Parasite killing within granulomas requires infiltrating blood monocytes and TNF-α. After disease resolution, the liver is refractory to reinfection with the parasite. During rechallenge disease, granulomas form rapidly with many CD8+ cells [27,39,43,44]. This underscores the importance of CD8+ cells for long-term recall immunity to visceral leishmaniasis, a finding also observed during L. major infection [45].

Studies of immune cells in isolated liver granulomas early in L. chagasi infection have shown a local defect in IFN-γ production due to soluble factors released into these culture supernatants. In contrast, there is ample antigen-specific IFN-γ produced by splenocytes from the same animals [26]. Neutralizing antibody to TGF-β reverses the inhibition of antigen-specific IFN-γ, suggesting that TGF-β is at least in part responsible for the local inhibition [46]. Studies of IL-12 p40 knockout mice confirm the lack of an IL-4 response during progressive L. chagasi infection, and demonstrate a compensatory increase in TGF-β in the absence of IL-12. This increase is further augmented in IL-12 p40/IFN-γ double knockout mice, suggesting a reciprocal expression of IFN-γ versus TGF-β may be critical in determining the outcome of L. chagasi infection [30]. It bears mention that the compensatory response to L. major infection in IL-12KO mice differs dramatically from L. chagasi, in that IL-4 producing cells expand and dominate L. major disease, whereas IL-4 is actually suppressed during L. chagasi infection [30,47]. The biological differences between L. chagasi and L. major that lead to different host immune responses are not known.

3.4. Immune responses in the spleen

Parasite replication begins late and remains at a low level in the spleen, where amastigotes can persist throughout the life of the animal [41]. Paradoxically, type 1 cytokines and inhibitory cytokines are co-expressed in splenic cultures [26]. Thus, IL-10, IL-12, IFN-γ, and TGF-β are detected by immunohistochemistry in the spleens of L. donovani infected BALB/c mice, although IL-4 remains absent [25].

Culture of total spleen cells during L. chagasi infection show there are antigen-responsive lymphocytes producing IFN-γ even at the peak of liver infection when comparable cells are absent from the liver [26,48]. IFN-γ stimulates macrophages to produce iNOS catalyzing formation of NO+, a final effector molecule necessary for intracellular parasite killing. It was therefore not immediately apparent why parasites survive in this organ [49–51]. The answer may come in studies of the trafficking of amastigotes and dendritic cells within the spleen.

During a normal antigen-specific response in normal spleens, dendritic cells migrate from the marginal zone to the periarteriolar lymphoid sheath (PALS), a zone containing mostly T cells. In the PALS the dendritic cells interact with T cells yielding an antigen-specific response. The chemokines CCL21 and CCL19 attract mature dendritic cells expressing surface CCR7 [41,52].

During the first few hours of L. donovani infection, parasites localize in marginal zone macrophages (MZXs) of the splenic white pulp, which is devoid of mature dendritic cells and T cells [48]. After a week, parasites are found in the red pulp which is macrophage-rich. There is a redistribution of dendritic cells, with failure to move from the marginal zone to the PALS where the antigen-specific response occurs. Causes of this mis-localization include: [1] DCs fail to express surface CCR7 due to increased levels of IL-10, and [2] TNF-α mediates a decrease in CCL21 and CCL19 release from PALS cells. The consequence of mis-localization of parasite antigen-expressing DCs is likely a failure of dendritic cells to interact normally with T cells and elicit an antigen-specific response. Late in L. donovani infection, the splenic architecture is disrupted with loss of MZMs in wild type mice expressing normal amounts of TNF-α. There is progressive destruction of follicular DCs, and eventual loss of germinal centers [41,48]. This pattern differs from chronic L. major infection in which dendritic cells localize normally [53]. MZMs specialize in polysaccharide uptake, and resistance to encapsulated bacteria. Thus, these changes could affect B cell reactivity and antibody production, which are known to be dysregulated during visceral leishmaniasis [41].

3.5. Inhibitory factors in visceral leishmaniasis

Although they exhibit similar organ-specific immune responses, according to murine studies there might be different inhibitory factors favoring the progression of L. donovani versus L. chagasi infantum infection. During resolution of L. donovani infection, cells secreting the type 1 cytokines IL-12 and IFN-γ increase, and cells expressing IL-10 reciprocally decrease. Instead of decreasing, cells producing TGF-β paradoxically increase as disease resolves [25]. This contrasts with the reciprocal expression of IFN-γ (induced by IL-12) and TGF-β during L. chagasi infection [30]. It is unknown what the identity of the TGF-β producing cells is, and whether they might be CD4+.
regulatory T cells. Whether the different immune responses are due to biological differences between L. donovani and L. chagasi/infantum, or due to the fact that responses were studied in the spleen versus the liver, respectively, has not been examined.

The effect of CTLA-4 blockade is similar for L. chagasi and L. donovani, but quite different from L. major infection. T cell activation requires two signals: ligation of the TCR and ligation of a co-stimulatory molecule (CTLA-4 or CD28) on T lymphocytes. L. major infection is exacerbated and there is a Th2 bias after CTLA-4 blockade [54,55]. In contrast, L. donovani infection is reduced and the block in IFN-γ production by splenocytes is reversed by CTLA-4 blockade [54,56].

CTLA-4 ligation delivers a negative signal to T cells resulting in inhibition of both type 1 and type 2 cytokines. CTLA-4 engagement also stimulates T cell TGF-β production. TGF-β blocks the initial development of both Th2 and Th1 cells, by inhibiting both GATA-3 and T-bet transcription factors, respectively. TGF-β also inhibits cytokine release from mature Th1 cells, but not from Th2 cells [57–59]. The different effects of CTLA-4 blockade in L. major versus L. chagasi/infantum infection could be due to the prominence of Th2 cells in the former infection, which could expand and augment disease in the absence of inhibitory CTLA-4 engagement. During L. chagasi/infantum infection one would expect only an expansion of Th1 cells after inhibition of TGF-β production by CTLA-4 blockade.

4. Immune responses in human visceral leishmaniasis

Manifestations of human visceral leishmaniasis can range from asymptomatic infection, documented with a positive skin test to Leishmania antigen (Montenegro test), to progressive and potentially fatal visceral disease. Active visceral leishmaniasis is characterized by fever, weight loss, enlargement of the liver and spleen, and compromised immunity with increased susceptibility to bacterial super-infections [60]. Laboratory studies often reveal pancytopenia, elevated gamma globulins, and low eosinophil counts. Reminiscent of murine models of disease, the Montenegro skin test is negative during active disease, whereas antibodies to Leishmania antigens are high [21]. Positive Leishmania serology, and in particular positive antibodies to the Leishmania k39 antigen, are characteristic and help in the diagnosis of active disease [61,62]. Other manifestations of visceral leishmaniasis vary with geographic region, and include lymphadenopathy in the Sudan and Mediterranean region, and post-kala-azar dermal leishmaniasis (PKDL) in the Sudan and India [21]. There are reports of subclinical disease with a mild self-resolving syndrome that never progresses to severe visceral leishmaniasis [63]. Asymptomatic infection is commonly recognized by a positive Montenegro in individuals with no history of disease. In endemic regions the ratio of asymptomatic to active infection ranges from 5:1 in Kenya to 6.5:1 in children in Brazil [63–65].

Human immune responses to the visceralizing Leishmania spp. vary depending on the form of disease. Peripheral blood mononuclear cells (PBMCs) from individuals with asymptomatic or subclinical infection respond to Leishmania antigen with proliferation and production of IL-2, IFN-γ, and IL-12. Neutralizing antibody to IL-12 abrogates both proliferation and IFN-γ responses. Those individuals who will progress to active visceral leishmaniasis can be predicted by a failure of their PBMCs to proliferate and produce IFN-γ [66].

In contrast to the responses of cured or asymptomatic exposed persons, PBMCs from visceral leishmaniasis patients exhibit defects in antigen-specific proliferation and production of IFN-γ [67,68]. The ability of PBMCs from cured visceral leishmaniasis patients to proliferate to Leishmania antigen is suppressed by co-culture with PBMCs from the same patient prior to cure, suggesting there are immunosuppressive factors produced by these PBMCs [69]. IL-10 has been strongly implicated as an immunosuppressive factor. Thus, bone marrow and lymph node cells from Sudanese individuals with acute visceral leishmaniasis simultaneous express IL-10 and IFN-γ transcripts, and IL-10 decreases after resolution of disease [70,71]. Other immunosuppressive factors are implicated in reports of elevated IL-4 and increased IgE [72], and reports of elevated IL-13. Defects in proliferation and IFN-γ production by PBMCs from patients are corrected by neutralizing antibody to IL-10; anti-IL-4 synergizes with this effect [67,68,73]. Nonetheless, Leishmania-specific T cells recovered from cured visceral leishmaniasis patients in the Sudan have been found to express IFN-γ, IL-4, or both IFN-γ and IL-10 [74]. Defective immune responses can also be corrected by the addition of IL-12 [68], suggesting that IL-12 receptors are expressed during progressive human infection, in contrast to murine hosts. Thus, the type 1-type 2 paradigm does not universally apply in human leishmaniasis.

Other potentially immunosuppressive factors reported during human visceral leishmaniasis include soluble IL-4 receptor in the sera of infected Kenyans [75], high serum IL-6 during VL in Sudan [76], and soluble IL-2 receptor [77]. Although the role of TGF-β has not been well established, elevated levels are found in bone marrow aspirates [51] and high levels of active TGF-β are found in the serum during symptomatic disease [78] (Fig. 3).

For unclear reasons visceral leishmaniasis due to L. chagasi is more prevalent among children than in adults. Visceral leishmaniasis also affects more males than females, although the gender skew appears only during and after the teenage years [79,80]. It is of interest to speculate whether the gender bias is due to differences in exposure, or to biologic differences between males and females. Rodent models support the latter hypothesis. Male mice of three different strains are more susceptible to L. major infection...
than females. Males become more resistant after orchectomy, and testosterone treatment renders females more susceptible to infection [81]. Male DBA/2 mice are more susceptible to subcutaneous \textit{L. mexicana} infection than females, possibly due to differences in IFN-\(\gamma\) expression. [82]. Male hamsters develop larger lesions and more metastases due to \textit{L. (Viannia) panamensis} and \textit{L. V. guyanensis} infection than females, and the female resistance can be reversed with androgens [83,84]. Whether these male–female differences will also apply to human disease bears verification.

5. Effects of the parasite on immune factors

\textit{Leishmania} do not behave as inert particles in their hosts. Rather, they actively secrete proteases and other factors that affect immune cells and cytokines of the host. Examples include \textit{Leishmania} GP63, which has been shown to cleave host complement [85], to cleave CD4 molecules [86], and to digest intracellular MARCKS related protein [87]. Parasite-derived cysteine proteases have also been associated with virulence [88]. Studies of null mutants lacking cathepsins L and B document a role for each cathepsin in \textit{L. mexicana} virulence [89–91]. Treatment of infected mice with specific protease inhibitors suggests that cathepsin B (of either the parasite or the host) promotes disease progression [92,93]. The mechanism through which the cathepsins promote parasite virulence was previously unknown.

TGF-\(\beta\) has potent immunosuppressive properties that can affect the outcome of infectious and autoimmune diseases [94]. TGF-\(\beta\) enhances the progression, or prevents the cure of leishmaniasis in murine models [46,56,95–97]. High levels of total TGF-\(\beta\) were previously reported in tissues and cultured immune cells from mice and hamsters infected with \textit{L. donovani} or \textit{L. chagasi} [46,56,95–98]. A direct effect of \textit{Leishmania} on the abundance of biologically active TGF-\(\beta\) is supported by two recent publications. Somanna et al. cloned the cathepsin B homologue from \textit{L. chagasi} and showed this is expressed in both the amastigote and promastigote forms of the parasite. They found that both parasite lysates and recombinant \textit{L. chagasi} cathepsin B were able to release activated TGF-\(\beta\) from the latent precursor [99]. Using sensitive bioassays, Gantt et al. showed that live promastigotes and promastigote culture supernatants were able to directly activate latent TGF-\(\beta\), and that activated TGF-\(\beta\) can prolong parasite survival in cultured primary macrophages capable of parasite killing. Activation occurred at least in part through the cysteine family protease, cathepsin B [100]. Together these reports suggest that the role of cysteine proteases in disease pathogenesis may be, in part, due to local activation of the immunosuppressive cytokine TGF-\(\beta\).

The role of \textit{Leishmania} lipophosphoglycan (LPG) and proteophosphoglycans (PPGs) in leishmaniasis has been reviewed elsewhere and will not be a focus of this publication [101,102]. Suffice it to say that LPG is a multifunctional molecule expressed only in the promastigote stage, which has been suggested to promote parasite survival through several mechanisms including retrograde progression through the gut of the sand fly vector, resistance to complement mediated lysis, inhibition of oxidative responses, evasion of phagosome-lysosome fusion, and inhibition of protein kinase C [102–104]. Secreted PPGs may affect the efficiency of sand fly transmission, the responses of macrophages to activating stimuli, and the local availability of complement component C3 [101]. These functions are not unique to the visceralizing \textit{Leishmania} species, and the effect of different LPG and PPG structures from different species on immune cell responses has not been delineated.

6. Microbicidal responses

Ultimate cure of all forms of murine and human leishmaniasis requires activation of parasite-laden macrophages to kill intracellular parasites. Activation requires priming with the type 1 cytokine interferon-\(\gamma\) (IFN-\(\gamma\)) plus a second signal that can be TNF-\(\alpha\). Parasite killing proceeds through the IFN-\(\gamma\)-inducible effector molecules nitric oxide (NO) generated from arginine by the inducible nitric oxide synthase (iNOS), and superoxide (\(\cdot O_2^-\)) generated by the NADPH oxidase [50,105–108]. However, there are differences in the relative contributions of these effectors to the outcomes of human versus murine disease. Nitric oxide produced by iNOS is believed to be of prime importance in
cure of murine leishmaniasis, and in activating murine macrophages to kill intracellular parasites [109]. This is perhaps best illustrated by studies of iNOS gene knockout mice, which do not self-resolve disease [108]. Nonetheless, mice lacking the gene for the gp91phox subunit of the NADPH oxidase also developed exacerbated infections albeit there was eventual self-cure. This shows that 'O_2-' and H_2O_2 also contribute to cure of murine infection [107,108]. 'O_2-' has been long recognized as essential to Leishmania killing by human macrophages, but it was argued that nitric oxide is not detectable and may not play a significant leishmanicidal role [110]. A few publications have recently made it evident that NO' contributes in some fashion to parasite killing by human macrophages. First human peripheral blood monocytes incubated for 24–48 h in vitro and stimulated with IgE or specific antibody to ligate the low affinity IgE receptor CD23 produce detectable levels of NO_2- derived from NO', according to the Griess reaction [111]. Inhibition of NO' production with L-NMMA in either anti-CD23-treated [111] or IFN-γ-treated 48-hour human monocytes enhanced the intracellular growth of L. major or L. chagasi, respectively [51]. Nonetheless the effects of the superoxide scavenger tempol was greater in human than in murine, and the effect of iNOS inhibition with L-NMMA was greater in murine than in human cells [51]. Thus, both oxidants are important but to differing extents in the two hosts. It must be stated that both reactive nitrogen and reactive oxygen species can also act as intracellular signaling molecules. NO' is essential for IL-12-induced signal transduction through Stat4 [112]. 'O_2-' and H_2O_2 play distinct roles in NFκB activation and TCR induced Fas ligand activation, and ERK1/2 phosphorylation [113,114]. Thus the above inhibitor and gene knockout data do not prove that the roles of these oxidants are limited to their direct microbicidal functions.

The macrophage phenotype also plays a role in activation and intracellular Leishmania killing. Macrophage activation through classical signals such as LPS, IFN-γ, or TNF results in enhanced production of microbicidal effector molecules. However, activation by IL-4 instead induces 'alternative' activation and drives the pathway of arginine metabolism toward arginase with production of polyamines that enhance parasite growth [115,116]. Furthermore, ligation of Fc receptors stimulates a 'type II' activation pattern causing macrophages to produce IL-10, which in turn down-modulates the IFN-γ response [117]. Finally, there is evidence that intracellular L. donovani directly inhibits signaling through the IFN-γ receptor pathway, due to up-regulation of the phosphatase SHP-1 [118–120].

7. Genetic susceptibility to leishmaniasis

Human infection with L. chagasi or L. donovani results most often in asymptomatic infection, whereas a minority of infected individuals go on to develop progressive, symptomatic visceral leishmaniasis [63–65]. Risk factors associated with the development of visceral leishmaniasis include nutritional status, which increases the risk of L. chagasi infection in humans and mice [65,121,122]. Immunosuppression caused by HIV-1 infection or malignancy can predispose to visceral leishmaniasis, sometimes with unusual manifestations or caused by non-pathogenic L. infantum strains [123]. Children are at increased risk for visceral leishmaniasis if they have a sibling with visceral leishmaniasis [79,124], a fact that could be due to shared environment, shared genetic background or both. Indeed, in addition to the multiple environmental risks there is increasing evidence that genetic factors contribute to the outcome of human Leishmania infection.

Mouse models lend credence to the hypothesis that there may be a genetic component to susceptibility to visceral leishmaniasis. For more than two decades it has been known that different strains of mice are susceptible or resistant to initial infection with L. donovani, Salmonella typhimurium, Mycobacterium bovis, and M. lepraeurum. Susceptibility or resistance manifests during the early stages of infection. Using backcrosses in inbred strains of mice, susceptibility was mapped to a single locus on chromosome 1 called LSH, BCG, or ITY depending on the disease under discussion [125]. The susceptibility gene was later positionally cloned and identified as NRAMP1, now called SLC11A1 [24]. Late stage cure of infection is not determined by the SLC11A1 genotype, but is instead linked to H-2 genes [126,127].

As would be surmised by differences in the susceptibility of mouse strains (Table 2), genetic determinants of murine cutaneous leishmaniasis map to different regions in the mouse genome. In BALB/c-B10.D2 back-crosses L. major lesion development and visceralization has been mapped to a 2 cM region on mouse chromosome 11 (syntenic with human 5q23.3-q33). This locus encodes a number of Th2 cytokines [128,129]. However, congenic mice containing this region from BALB/c mice remain resistant to L. major infection [130] suggesting the locus contributes to, but is not the only determinant of, disease susceptibility. Back cross studies between B10.D2 and BALB/c mice have also indicated resistance/susceptibility loci on mouse chromosomes 5, 6, 7, 10, 11, 15, 16 [131]. Additionally, there are suggestions of interacting loci on chromosomes X, 9, 17 and at the H2 locus [132–134].

Evidence is emerging that genetic factors also influence the outcome of human infection with L. donovani or L. chagasi. There are profound racial differences in the ratio of asymptomatic (detected by a positive Leishmania skin test) to symptomatic infection [135,136]. Familial aggregation of asymptomatic L. chagasi infection [80] or of active and asymptomatic L. chagasi infection [124] has been documented several times in Brazilian populations. In favor of a genetic contribution to familial aggregation pattern, close relatives are more likely to share phenotypes than more distant or unrelated individuals living in the same local environment [80].
Segregation analysis is a complex statistical means of examining how a trait is transmitted over generations, and whether there is evidence for a major gene effect, polygenic effects or shared environmental effects on the variability of the disease or trait. Segregation analyses have found evidence for a major gene controlling susceptibility of cutaneous leishmaniasis caused by *L. peruviana* in migrant populations in Bolivia, modified significantly by environment [137,138]. Another segregation analysis of asymptomatic *L. chagasi* infection led to similar conclusions [139]. Finally, segregation analysis of symptomatic visceral leishmaniasis in a Brazilian population revealed evidence that a major gene or polygenic effect may control susceptibility to visceral leishmaniasis [140,141].

Based on these data, a number of groups have hypothesized that functional polymorphisms in genes encoding immune function proteins are associated with, and influence the different outcomes of, infection with several *Leishmania* sp. Approaches to this hypothesis have included examination of candidate genes that might be associated with disease phenotypes [142–147], as well as genome-wide scans of populations exposed to the agents of visceral leishmaniasis [144,145]. Genes or loci suggested by these groups to be associated with infection with leishmaniasis, both visceral and other forms, are summarized in Table 3.

Polymorphisms in the TNFa and TNFb genes in the MHC class III region, which encode TNF-α and lymphotoxin-α, respectively, have been associated with increased risk for mucosal or cutaneous leishmaniasis due to *L. braziliensis* [142]. Polymorphisms in the HLA locus are associated with different forms of cutaneous leishmaniasis [147–149], but not with visceral leishmaniasis in Brazilians [150].

Some genetic determinants implicated in human visceral leishmaniasis localize to loci that are syntenic with those associated with murine leishmaniasis or mycobacterial infections [141]. Using a Brazilian population, markers in the TNF locus were found to associate with the development of overt visceral leishmaniasis as opposed to asymptomatic *L. chagasi* infection, detected by a positive skin test [146]. The TNF locus has also been associated with development of cerebral malaria and with several autoimmune diseases [151,152]. There are conflicting reports of the functional consequence of TNF polymorphisms, particularly a −308 promoter polymorphism (actually located at −307 with respect to the transcription start site) [153,154]. The allele associated with both visceral and mucosal leishmaniasis, the TNF2 allele, has been associated with higher levels of TNF-α transcription in some but not all studies [155].

*SCL11A1 (NRAMP1)*, the mouse susceptibility gene for *L. donovani* infection, has been associated with both visceral leishmaniasis and post kala azar dermal leishmaniasis (PKDL) in the Sudan [144,145]. In addition the abovementioned the human chromosome 5q23-31 ‘Th2 cytokine locus’, comes up repeatedly as containing candidate susceptibility genes for infectious and autoimmune diseases. This region contains a number of genes whose products are augmented during a Type 2 immune response, including IL-4, IL-5, IL-13 and IL-9. The syntenic region on murine chromosome 11 includes the genes for GM-CSF, IL-3, IL-4, IL-5, IL-13, IRF1, ITK (IL-2 inducible T-cell kinase), and Tcf1 (T cell factor 1)(http://www.ensembl.org/). The Th2 locus was identified as potentially associated with visceral leishmaniasis in Sudan [144].

### Table 3

Proposed loci associated with susceptibility to infection with the *Leishmania* sp

<table>
<thead>
<tr>
<th>Country</th>
<th>Phenotype</th>
<th>Locus</th>
<th>Nearby Genes</th>
<th>Significance</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brazil</td>
<td>CL, MCL</td>
<td>6p21.3</td>
<td>TNFa, TNFb</td>
<td>Case control</td>
<td>[142]</td>
</tr>
<tr>
<td>Sudan</td>
<td>VL</td>
<td>2p35</td>
<td>(SCL11A1) NRAMP1</td>
<td>lod 1.32, <em>p</em> = 0.007</td>
<td>[143]</td>
</tr>
<tr>
<td>Sudan</td>
<td>VL, PKDL</td>
<td>5q31-q33 2q35</td>
<td>IL4 etc. NRAMP1</td>
<td>lod1.84, <em>p</em> = 0.0008</td>
<td>[144]</td>
</tr>
<tr>
<td>Sudan</td>
<td>VL</td>
<td>2q12 2q23-q24</td>
<td>IL2RαNRAMP1</td>
<td>lod3.5, <em>p</em> = 0.00003 lod1.0, <em>p</em> = 0.015</td>
<td>[145]</td>
</tr>
<tr>
<td>Brazil</td>
<td>Asymptomatic</td>
<td>TNFA-308</td>
<td>TNFa, TNFb</td>
<td>TDT <em>p</em> = 0.0006</td>
<td>[146]</td>
</tr>
<tr>
<td>Brazil</td>
<td>CL</td>
<td>6p</td>
<td>HLA</td>
<td></td>
<td>[147]</td>
</tr>
</tbody>
</table>

8. Summary

Studies of infections with the visceralizing *Leishmania* spp., *L. donovani* and *L. infantum/chagasi*, have underscored the fact that host responses to these parasites differ significantly from *L. major* infection. In rodent models the Th1/Th2 paradigm is important in determining the outcome of murine *L. major* infection. This dichotomy is not as influential during murine *L. donovani* and *L. chagasi* disease, in which curative Type 1 responses are instead suppressed by IL-10 and TGF-β. *L. chagasi* directly affects its local environment by activating latent TGF-β, and both *L. donovani* and *L. chagasi* suppress host macrophage responses to IFN-γ. There are localized immune responses in the liver and spleens of infected animals, which lead to apparent tissue ‘tropism’ and unique patterns of localized growth or cure of parasite infection. Studies of *L. donovani* infection in inbred strains of mice have elucidated a major susceptibility gene, and studies in humans are beginning to show a contribution of genetics to the outcome of *L. donovani* and *L. chagasi* infection as well.
The application of human and parasite genome projects to studies of visceral leishmaniasis will likely continue to help us understand why the outcome of human infection with the different *Leishmania* sp. are so dramatically different. The authors would like to acknowledge support from the National Institutes of Health (R01 AI45540, R01-AI48822, R01 AI32135, P50 AI-30639), the VA Medical Center (MEW Merit Review grant), and a grant from the Brazilian Conselho Nacional de Desenvolvimento Científico e Tecnológico (SMBJ).

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