Determinants for the Development of Visceral Leishmaniasis Disease

Laura-Isobel McCall, Wen-Wei Zhang, Greg Matlashewski*

Department of Microbiology and Immunology, McGill University, Montreal, Quebec, Canada

Abstract: Leishmaniasis is a vector-borne neglected tropical disease associated with a spectrum of clinical manifestations, ranging from self-healing cutaneous lesions to fatal visceral infections. Among the most important questions in Leishmania research is why some species like L. donovani infect visceral organs, whereas other species like L. major remain in the skin. The determinants of visceral leishmaniasis are still poorly understood, although genomic, immunologic, and animal models are beginning to provide important insight into this disease. In this review, we discuss the vector, host, and pathogen factors that mediate the development of visceral leishmaniasis. We examine the progression of the parasite from the initial site of sand fly bite to the visceral organs and its ability to survive there. The identification of visceral disease determinants is required to understand disease evolution, to understand visceral organ survival mechanisms, and potentially to develop better interventions for this largely neglected disease.

Introduction

Leishmaniasis is a neglected tropical disease caused by Leishmania protozoa that are transmitted to mammalian hosts by an infected sand fly. Leishmania have a dimorphic life cycle in which parasites reside as extracellular promastigotes in the vector and as intracellular amastigotes in mammalian host macrophages [1]. Leishmaniasis includes a spectrum of clinical manifestations, from cutaneous lesions at the site of sand fly bite to systemic visceral leishmaniasis (Table 1). The distinct clinical manifestations are associated with different degrees of parasite metastasis, from parasites such as L. major that remain contained at cutaneous lesions to dissemination into the visceral organs in visceral leishmaniasis caused by L. donovani. We focus here on the most severe form of the disease, visceral leishmaniasis.

Visceral leishmaniasis is ranked second in mortality and fourth in morbidity among tropical diseases, with 20,000 to 40,000 deaths per year [2] and over 2 million DALYs (disability-adjusted life years) lost [3]. Symptoms include hepatosplenomegaly, high fever, pancytopenia, and hypergammaglobulinemia, and the disease is almost always fatal if untreated [1,4]. Identification of the factors that mediate the development of visceral leishmaniasis is highly relevant to understanding disease progression and parasite survival mechanisms.

Navigating from Skin to Viscera

The sand fly vector lacerates blood vessels during feeding, so parasites are introduced intradermally into a pool of blood [5]. Free amastigotes have been detected in the bloodstream and could be directly delivered to blood-filtering organs [6]. Alternatively, the eventual spread to visceral organs could involve the movement of infected cells. Neutrophils are the earliest cells recruited to the site of the sand fly bite [7,8] and represent the first infected cell population [9]. Infected neutrophils or free parasites are then taken up by dendritic cells and macrophages, which migrate away from the site of the bite [9]. The dermal dendritic cell population infected at early time points may differ between L. major and L. donovani [7], and there is evidence that L. donovani-infected macrophages and dendritic cells leave intradermal injection sites in higher numbers than L. major-infected cells [10]. However, the route used by infected cells to eventually reach the visceral organs remains poorly understood.

Experimental subcutaneous needle infection with L. major in BALB/c mice is associated with parasite proliferation and lesion development at the site of injection, as well as parasite dissemination to visceral organs [11]. Dissemination of L. major to the visceral organs does not occur in C57BL/6 mice, indicating an important role for the host immune response in the control of visceralization [11]. In comparison, subcutaneous injection of L. donovani in BALB/c mice only causes minimal swelling at the site of injection and no dissemination to the viscera [12]. Therefore, these subcutaneous infection models do not accurately reflect the situation in humans where L. donovani, but not L. major, metastasizes to the visceral organs.

Intradermal infection models have been developed in BALB/c mice [13] and in hamsters [14] in which parasites are cleared from the skin and disseminate to the visceral organs. However, for both models, the inoculation dose is 100- to 1,000-fold higher than the natural sand fly inoculum [15]. Nevertheless, these models more closely mimic natural infection routes for visceral disease and could provide better insight into the cells and pathways involved in reaching the visceral organs.

Surviving Stress: Parasite Proliferation in the Viscera

Experimental intravenous injection of Leishmania in mice bypasses the normal requirement for the parasite to transit from...
Table 1. *Leishmania* species and disease phenotype.

| Disease Phenotype          | Common Species          | Rare Species          |
|----------------------------|-------------------------|-----------------------|
| Visceral Leishmaniasis     | *L. donovani*           | *L. tropica*          |
|                            | *L. infantum*           | *L. amazonensis*      |
|                            | *L. chagasi (=L. infantum)* |                     |
| Cutaneous Leishmaniasis    | *L. major*              | *L. mexicana*         |
|                            | *L. tropica*            | *L. braziliensis*     |
|                            | *L. amazonensis*        | *L. donovani*         |
| Mucocutaneous Leishmaniasis| *L. braziliensis*       | *L. panamensis*       |
|                            | *L. guyanensis*         | *L. amazonensis*      |

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skin to viscera and therefore focuses exclusively on parasitic survival within the liver and spleen. However, although intravenous infection models of BALB/c mice are commonly used to study visceral leishmaniasis, these do not fully reflect human visceral leishmaniasis progression. In mice, infection in the liver is self-resolving, while spleen infection is progressive, and overall infection is asymptomatic [16]. In contrast, infection of hamsters is associated with symptomatic disease and can be fatal [17].

Nevertheless, intravenous infection with cutaneous species such as *L. major* is associated with limited liver and spleen parasite burden, while intravenous infection with visceralotropic *L. donovani* and *L. infantum* results in high levels of visceral infection [18]. Therefore, regardless of the mechanism to exit the skin, cutaneous and visceral species differ dramatically: visceral species are much better adapted to survive and proliferate in visceral organs than cutaneous species.

Temperatures within infected mouse footpad dermis vary between 28°C and 32°C [19], whereas fever in visceral leishmaniasis exceeds 40°C [20]. Viscerotropic parasites must therefore withstand higher temperatures than cutaneous ones, and indeed, promastigotes from cutaneous species are considerably more sensitive to heat shock than promastigotes of visceral species [21,22]. In addition, transfection of some *L. donovani* genes into *L. major* enhanced *L. major* survival at higher temperatures and visceralization [18,21,23].

Fever itself can also augment the immune response by increasing dendritic cell and neutrophil migration, pro-inflammatory cytokine production, and Th1 cell activity [24]. Given that fever increases oxidant production by phagocytes [24], visceralotropic species are expected to be more resistant to oxidants than cutaneous species: *L. donovani* is more resistant to nitric oxide (NO) and hydrogen peroxide than *L. major* [25].

The host macrophage population targeted by *Leishmania* also differs between cutaneous and visceral species: cutaneous species infect inflammatory monocyte-derived macrophages and dendritic cells [9,26], while visceral species infect Kupffer cells, spleen macrophages, and bone marrow macrophages [27]. These different macrophage populations express different levels of cell surface molecules [28] and of NRAMP1, a cation transporter associated with resistance to *Leishmania* [29]. They also differ in their response to IFNγ stimulation, and in their capacity to produce cytokines, activate T lymphocytes, and kill pathogens [30–32]. Therefore, cutaneous and visceral species have adapted to replicate in distinct host macrophage environments. However, no direct comparisons of the susceptibility and killing potential of these different macrophage populations have been performed during *Leishmania* infection.

**Host Determinants of Visceral Leishmaniasis**

The ratio of subclinical to symptomatic visceral leishmaniasis is estimated at up to 18 to 1 [33], demonstrating that many people infected with visceral *Leishmania* species develop an effective immune response and do not manifest clinical disease. The host genetic background influences the development of disease [34,35] (reviewed in [36–38]). In particular, NRAMP1 plays a key role in susceptibility to visceral disease [39]. A number of cytokines, chemokines, and their receptors (TNFα [40]; IL4 [41]; TGFβ [42]; IL2 receptor [43]; CXCR2 [44]), as well as mannose-binding lectin [45] and the Delta-like 1 ligand for Notch 3 (DLL1) [46], have also been associated with symptomatic versus asymptomatic disease. However, it is largely unknown how deeply polymorphisms in these host immune response genes penetrate to cause visceral leishmaniasis in the human population of endemic regions.

In addition to genetic factors, acquired characteristics, such as AIDS (reviewed in [47]), preexposure to the parasite in utero [48], malnutrition [49,50], and youth [51,52], can also increase the risk of developing symptomatic leishmaniasis. All of these are associated with impaired immune responses against the parasite. Visceral leishmaniasis in HIV-co-infected individuals can be caused by strains and species that normally cause cutaneous disease [53] and is associated with atypical disease manifestations, such as the presence of cutaneous lesions as well as visceral parasitemia [54].

Overall, these observations indicate that an effective Th1 cellular immune response is required to control infection. This response involves the release of IL12 by antigen-presenting cells, leading to the differentiation of Th1 cells. These release IFNγ, resulting in macrophage activation and the production of leishmanicidal NO [55]. Decreased NO production promotes visceral dissemination following cutaneous infection with *L. major* in mice [56]. Conversely, subcutaneous self-limiting infection by *L. donovani* has been associated with strong NO production [12]. However, the Th1/IFNγ-mediated response alone is not sufficient to protect against disease, since visceral leishmaniasis patients are able to produce IFNγ in response to *Leishmania* antigen [57]. Disease is strongly correlated with the production of high amounts of IL10 [57,58], an immunosuppressive cytokine that inhibits leishmanicidal immune functions (reviewed in [59]).

**Vector Determinants of Visceral Leishmaniasis**

*L. chagasi* transmitted by *Lutzomyia longipalpis* sand flies can cause cutaneous or visceral leishmaniasis in parts of South America. Saliva of sand flies from a cutaneous region caused low levels of vasodilatation and promoted footpad swelling following subcutaneous infection in mice, whereas saliva of sand flies collected in a visceral region caused higher vasodilatation but did not enhance cutaneous lesion formation [60]. This suggested that higher vasodilatation promoted better parasite access to the visceral organs and that vector species may influence disease development. However, this may not be a general difference between vectors of cutaneous and visceral *Leishmania* species: sequence analysis of salivary gland proteins from a *Phlebotomus* species associated with cutaneous *L. infantum* cases in the Old World showed that its...
The role of the sand fly in disease pathology is an area that has been associated with protection against visceral leishmaniasis in hamsters [14] and may also be protective in humans [62–64]. The number of parasites transmitted by sand flies could also influence disease outcome. A higher infective dose may promote a stronger local immune response that limits the parasite spread, thereby preventing dissemination to the visceral organs [13].

Finally, immunity to sand fly salivary proteins has been associated with protection against visceral leishmaniasis in hamsters [14] and may also be protective in humans [62–64]. The role of the sand fly in disease pathology is an area that requires more attention.

**Parasite Determinants of Visceral Leishmaniasis**

Although vector and host characteristics influence the progression to symptomatic disease, parasite characteristics are the most important determinant that distinguishes cutaneous from visceral disease. For example, although *L. infantum* has been associated predominately with visceral leishmaniasis, some subspecies can also cause cutaneous leishmaniasis [65]. Subspecies-specific *L. infantum* differences were maintained following intravenous infection of inbred mice: *L. infantum* strains from cutaneous patients were unable to establish visceral infection, unlike *L. infantum* strains from visceral patients [66]. This demonstrated that genetic differences between *L. infantum* subspecies determine disease pathology. Differences in species-specific genes, gene polymorphisms, pseudogenes, and expression levels for virulence and stress response genes can all contribute to differences in disease pathology.

The A2 gene family represents the prototype example of a gene required for visceralization. It is expressed in *L. donovani* and *L. infantum*, whereas it is a pseudogene in some cutaneous species such as *L. major* and *L. tropica* [67,68]. A2 genes are arranged in tandem arrays on chromosome 22 [69] and encode a family of proteins from 42 to 100 kDa that are made up almost entirely of 40 to 90 copies of the same repetitive ten amino acid sequence [70]. Downregulation of A2 by antisense RNA [71] or partial knockout of A2 genes [18] resulted in decreased liver parasite burden in BALB/c mice. Conversely, introducing A2 genes into *L. major* enhanced the ability of *L. major*-infected cells to migrate out of the dermis and increased parasite survival in visceral organs [10]. Likewise, expression of *L. donovani* A2 in *L. tarentolae* (a lizard *Leishmania* species) enhanced *L. tarentolae* survival in mouse visceral organs [72]. Finally, A2 expression is downregulated in human post kala-azar dermal leishmaniasis (PKDL) where the *L. donovani* parasite relocates to the skin following successful treatment [73]. Overall, these results indicate a key role for A2 in parasite survival in the visceral organs. A2 gene expression is induced by promastigote to amastigote differentiation [74] and by a variety of stresses, including heat shock [21], unfolded protein stress (UPR) [75], and misfolded protein stress [76]. A2 protects from heat shock [21] and oxidative stress [77], and this may allow the parasite to survive in the warmer visceral organs as well as withstand host defences. It is noteworthy that A2 is now a leading candidate for a visceral leishmaniasis vaccine in dogs and potentially humans [78].

Complementation of the *L. major*, *L. donovani*, *L. braziliensis*, and *L. mexicana* genomes [79] has highlighted only 19 *L. donovani*-specific genes (out of over 8,000 genes) that are absent or found as pseudogenes in cutaneous species [80]. The ability of some of these genes to promote parasite survival in visceral organs has been investigated by ectopically expressing them in *L. major* and monitoring spleen and liver parasite burden in BALB/c mice [81–83]. Interestingly, three *L. donovani*-specific genes could promote *L. major* survival in the viscera, including the orthologues of LinJ28.0340, LinJ15.0900, and LinJ36.2480 [82]. A list of genes identified in this way as potentially involved in visceral disease is presented in Table 2. Knocking out LinJ28.0340 in *L. donovani* also decreased parasite survival in the visceral organs [82]. LinJ28.0340 is a cytosolic protein of unknown function [82], LinJ15.0900 is a nucleotide sugar transporter localized in the Golgi apparatus [83], and LinJ36.2480 is a cytotoxic glyceraldehyde-3-phosphate dehydrogenase, a rate limiting enzyme involved in glucose metabolism and ATP production [82]. The impact of LinJ36.2480 on parasite survival in the visceral organs suggests that energy production may be an important difference between cutaneous and visceral species [82]. However, apart from LinJ28.0340, these genes are present in *L. mexicana* [82,83], and several of them also promote increased footpad swelling, making them general virulence factors, rather than visceralization-promoting factors.

Although transfection of these *L. donovani*-specific genes into *L. major* increased parasite survival in the visceral organs, none of them were able to fully restore *L. major* virulence to the same level as *L. donovani*. This argues that combinations of *L. donovani*-specific genes and other factors such as gene amplifications, polymorphisms, and differences in posttranscriptional regulation could all play important roles in visceral infection [82].

**Additional Genetic Determinants of Visceral Disease**

There are a large amount of chromosome copy number variations between *L. donovani* strains and between *Leishmania* species [79,84]. Gene dosage effects may alter protein expression levels between cutaneous and visceral species, some of which could influence parasite tropism and pathology. Microarray studies also showed significant differences in mRNA profile between *L. major* and *L. infantum* for proteases, kinases, antioxidants, enzymes involved in carbohydrate and lipid metabolism [85], and surface proteins such as gp46 and proteophosphoglycan family members [86]. However, protein expression in *Leishmania* is largely regulated posttranscriptionally, and only a weak correlation exists between mRNA and protein levels [87].

It is difficult to draw conclusions from proteomic comparison of *L. major* and *L. donovani* due to high variability between species [88]. However, proteomic analysis may be more informative if these techniques are applied to strains of the same species that cause different disease manifestations, such as for example distinct *L. infantum* isolates causing cutaneous and visceral disease [65]. Finally, changes in posttranslational modifications may also be important. Indeed, a different phosphorylation profile was observed for virulent and avirulent *L. donovani* strains during heat shock [89]. Application of these and other proteomic techniques to carefully chosen *Leishmania* isolates may therefore be more effective in identifying relevant determinants of visceral disease.

It is also of interest to consider how genetically distinct *Leishmania* species alter host macrophage gene expression. *L. donovani* or *L. major* induced remarkably similar macrophage gene expression profiles, although *L. donovani* induced higher levels of Cox2 and prostaglandin E synthase than *L. major* [90]. Both of these enzymes are part of the PGE2 biosynthetic pathway, which has been associated with increased visceral organ infection levels [91]. Spermine/spermidine N1-acyl transferase 1, a rate-limiting enzyme of polyamine metabolism, was also higher in *L. donovani*-infected cells [90], and this may increase polyamine production to promote higher levels of parasite growth [92].
**Table 2.** *Leishmania* genes implicated in the development of visceral disease.

| *L. infantum* | *L. major* | *L. mexicana* | *L. braziliensis* | Name and function | Localization | Effect of introduction into *L. major* | Effect of knockout in *L. donovani* | Ref. |
|--------------|-----------|--------------|------------------|------------------|-------------|--------------------------------------|----------------------------------|-----|
| Uni.15.0900  | pseudogene | LmxM.15.0840 | absent            | nucleotide sugar transporter | Golgi | Liver: 18 × increase | increased footpad swelling | No change [83] |
|              | LmjF.15.0840 |              |                  |                  |             | Spleen: 20 × increase |                                    |                  |
| Uni.22.0670  | pseudogene | LmxM.22.0691 | absent            | A2               | Endoplasmic reticulum | Spleen: 3 × increase | 6–8 × decrease | Partial knockout: 2–3 × decrease in LDU [10] |
|              | LmxM.22.0692 |              |                  |                  |             |                                     | Antisense: 10–25 × decrease in LDU [18] |
|              | LmjF.28.0420 | LmxM.28.0420 | LbrM.28.0430     | Hypothetical protein | Cytosol | Liver: 14 × increase | 2 × increase in footpad swelling | Liver and spleen: 200 × decrease [82] |

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Perspectives for Future Work

Our understanding of the determinants of visceral disease and the interplay of host, vector, and parasite factors has progressed significantly, and this has led to the conclusion that the genetic differences between species are the main determinant for cutaneous or visceral disease. There are however a number of questions to answer to clarify the evolution of visceral disease and the key parasite genetic differences, and these are outlined in Table 3.

One of the most promising new approaches to identify new determinants of visceral disease involves crossing of different Leishmania species in infected sand flies [96,97]. This approach could identify key regions of the L. donovani genome required for visceral disease. Another promising approach is to closely examine the genome of rare L. donovani isolates that cause cutaneous disease, such as for example in Sri Lanka, where L. donovani is responsible for an epidemic of cutaneous leishmaniasis [98]. It will be important to determine how these L. donovani strains behave in animal models and, through genome sequencing, to identify potential deletions, pseudogenes, and polymorphisms. It may be possible to experimentally repair genetic defects in these attenuated L. donovani isolates to identify key visceral disease–associated genetic determinants.

One of the challenges of studying the genome of Leishmania is that the majority of the genes do not have homology with genes of known function from higher eukaryotes. Indeed, although several species-specific genes are associated with visceral disease in animal models [10,81,82], the role of the majority of them remains to be determined. Gene function determination is required, potentially aided by the identification of relevant protein-protein interactions.

Visceral leishmaniasis is one of the most lethal neglected tropical diseases [3] and is closely associated with poverty [99]. A better understanding of the factors that mediate visceral disease will help guide the identification of better drug targets, immunomodulators, and epidemiologic markers for virulence and potential vaccines, which will support disease elimination efforts that would have a significant impact in the poorest regions of endemic countries.

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