

Review Article

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Pathophysiology of visceral leishmaniasis - some recent concepts

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Visceral leishmaniasis is characterized by diversity and complexity of clinical manifestations ranging from asymptomatic infection to life threatening illness. Experimental evidence and clinical studies indicate multifaceted role of various factors leading to parasite survival and multiplication. In early stage of infection, generation of reactive oxygen and nitrogen intermediates play significant role in curtailing the parasite multiplication while in later phase on one hand, hepatic resistance is expressed by the dominant role played by nitric oxide synthase (NOS)-2 gene regulation and on the other hand, production of inhibitors of NOS-2 gene expression, interleukin 10 (IL-10) and transforming growth factor beta (TGF β) correlate well with reduced parasite killing. The hepatic infection is usually self-limiting due to production of multiple cytokine responses including moderate level of tumour necrosis factor (TNF) while in spleen excess TNF mediates destructive pathology. CD8⁺ T cells appear to play multiple roles comprising both cytotoxic activity and secretion of cytokines and chemokines. Capacity to produce Th1 cytokines is associated with asymptomatic or subclinical self-healing infection. However, in symptomatic patients, Th I cytokine production is not depressed but there appears to be unresponsiveness to the stimuli of these cytokines. Experimental evidences indicate genetic basis for such a phenomenon.

Key words Host-parasite interaction - immunopathology - pathogenesis - promastigotes - visceral leishmaniasis

Leishmaniasis, caused by obligate intracellular protozoan parasites of genus *Leishmania* is endemic in areas of tropics, subtropics and southern Europe, in settings ranging from rain forests in America to deserts in western Asia and from rural to periurban areas¹. Visceral leishmaniasis (VL) caused by *Leishmania donovani* species complex (*i.e.*, *L. donovani* and *L. infantum* in Old World and *L. chagasi* in New World); *L. tropica* (Old World) and *L. amazonensis* (New World) is endemic in 88 countries affecting 12 million people with estimated annual number

of about 5,00,000 new cases and 350 million people at risk of infection. Over 90 per cent of worldwide cases are in Bangladesh, northeastern India Nepal and Sudan (Old World) and in northeastern Brazil (New World). In India, VL has been known to occur epidemically and endemically in well defined areas in the eastern parts of the country, mainly in Bihar, West Bengal, eastern districts of Uttar Pradesh, Assam and foothills of Sikkim². Leishmaniasis is transmitted by sandflies (*Phlebotomus* species). *Leishmania* species exist as extracellular flagellated promastigotes in the

guts of female sandflies, and transform to the amastigote form in animal and human hosts.

In visceral leishmaniasis or kala-azar the incubation period varies from 10 days to one year. The infection may be asymptomatic or may lead to fully blown kala-azar. Initially, there is low grade recurrent fever and malaise, followed by progressive wasting, anaemia and hepatosplenomegaly and if untreated, proves fatal within 2-3 yr. In some patients, the disease takes a more acute course. The cause of death is often a secondary infection. In India, 5-10 per cent of patients develop post kala-azar dermal leishmaniasis (PKDL), a syndrome resulting due to inadequate treatment. Some patients, particularly in India recover spontaneously. The confirmatory diagnosis of VL relies upon demonstration of parasite in tissue samples or tissue culture. Microscopic examinations of spleen aspirate and/or bone marrow smears offer the higher diagnostic efficacy but are associated with risk due to invasive procedure. The detection of parasite DNA in blood samples by PCR should be regarded as a promising tool, rather than the conventional invasive procedures such as splenic and bone marrow aspiration³. Immunodiagnostic methods to detect antibody or antigen are useful depending upon clinical syndrome, antigen and the assay used. The 200 kDa *L. donovani* amastigote antigenic fraction was found 96.6 per cent sensitive and 100 per cent specific. It does have prognostic significance and was found useful for differentiating active VL and PKDL⁴. Detection of antibody to rk39 antigen of *L. chagasi* is being widely accepted for the diagnosis in immunocompetent patients⁵ and may prove a better marker for detection of *Leishmania* infection in HIV infected patients⁶.

Despite the wide range of manifestations seen in this infection, all clinical and geographical varieties of the disease share a common histological feature - namely, the early accumulation of mononuclear phagocytic cells in the invaded tissues leading to hyperplasia of reticulo endothelial cell (REC) of the organs involved. The pathology of VL is dominated by the specific suppression of cell-mediated immunity; permitting the dissemination and uncontrolled multiplication of the parasite resulting in various complications, if untreated.

The REC hyperplasia that follows infection with *L. donovani* affects spleen, liver, mucosa of small intestine, bone marrow and lymph nodes. The cardinal histopathological feature of hepatic resistance to visceralizing species of *Leishmania* is the development of granulomas. Diversity of granuloma structure and function in experimental VL has been extensively studied⁷, and many of the cellular and molecular components of acquired immunity necessary for the formation, maintenance, and effector function of granulomas have been characterized through the use of gene-targeted mice or *in vivo* administration of neutralizing or depleting monoclonal antibodies. Mature granulomas, showing extensive mononuclear cell cuffing, can readily be seen alongside kupffer cells that harbour intracellular amastigotes but that appear to have failed to trigger an inflammatory response. The mechanistic basis of such asynchrony is not well understood, but it may reflect intrinsic differences in kupffer cell populations with respect to chemokine production, indirect effects due to varying amastigote load or to limitation imposed by the frequency of antigen-specific T cells available for recruitment to the liver⁸. In spleen and other lymphoid organs, there may be atrophy of paracortical areas but plasma cells are numerous. The marginal zone (MZ) of the spleen is an important site for the capture of blood-borne pathogens and a gateway for lymphocytes entering the white pulp. *L. donovani* infection results in a remarkably selective loss of MZ macrophages⁹. Haematopoiesis is initially normal but later becomes depressed. The life span of leukocytes and erythrocytes is reduced, causing granulocytopenia and anaemia. The liver function is normal and few hepatocytes are invaded; and prothrombin production is decreased. Together with thrombocytopenia, the prothrombin depletion may result in severe mucosal haemorrhage. Hypoalbuminaemia is associated with oedema. Diarrhoea may be due to intestinal parasitization and ulceration or secondary enteritis. In the advanced stage, intercurrent infections are frequent, especially pneumonia, dysentery and tuberculosis and these are the common cause of death. Complement activation might contribute to development of anaemia; as there is formation of immune-complexes, though nephritis is rare. In few cases of self-healing or lymphatic VL, tuberculoid

and necrotic histological patterns have been reported¹⁰.

There is complex information concerning the events that follow the inoculation of promastigotes, which might be critical to the eventual outcome of the infection. Most of the infected individuals are asymptomatic while few develop symptomatic infection. Experimental infection in animals and *in vitro* experiments suggest the existence of several mechanisms that permit parasite survival and multiplication.

Host-parasite interaction

Although, sera from infected individuals and certain mammals contain factors capable of agglutinating or lysing promastigotes *in vitro*, yet within the host macrophages, the amastigotes are protected from antibodies and other circulating substances that might be harmful to the parasite. Parasite survival within the host must therefore depend on the capacity of the microorganism to become incorporated into these cells. However, the search for a specific organelle that might facilitate active cell invasion has so far been unsuccessful and consensus is that the amastigotes are probably taken up by simple phagocytosis and phagosomes containing parasites fuse with the lysosomes (the parasites multiply in the phagolysosomes).

Receptor-ligand interaction: The mechanism that leads to the establishment of intracellular parasitism apparently depends upon how the promastigotes enter the macrophages. The mode of entry is probably by active penetration by the flagellar end. Further, although both promastigotes and amastigotes might play an active cytochalasin inhibitable role in their initial attachment to host phagocytes, the actual ingestion occurs as per the classical "Zipper" hypothesis¹¹. One of the important events which occurs at the macrophage surface following certain receptor/ligand interactions is the triggering of the respiratory burst and production of toxic oxygen metabolites. The promastigotes and amastigotes bring about markedly different respiratory burst responses in mouse peritoneal macrophages and it can be postulated that they might be entering via

different receptors¹². Further, Remaley *et al*¹³ observed that some amastigotes caused localized inhibition of respiratory burst response and that a tartrate resistant acid phosphatase isolated from the external surface of *L. donovani* promastigotes inhibited the superoxide anion production by human neutrophils. Relative measures of reactive oxygen intermediates generated by these cells in experimental VL indicated failure of respiratory burst in resident liver macrophages and a possible role for another subpopulation of cells (immigrant macrophages) in the oxygen-dependent killing mechanisms¹⁴. Study in murine VL indicated the defective oxidative pathway of kupffer cells and the presence of morphologically and functionally different subpopulations of macrophages in liver, working through different pathways in causing tissue damage during the infection¹⁵. The differential microbicidal potentials of liver macrophages, the oxygen-dependent and oxygen independent pathways in kupffer cells and immigrant macrophages of *L. donovani* infected BALB/c mice have been investigated and it was shown that both adopt different pathways to cope with this infection¹⁶.

Cytokine responses: Pathogenesis appears to be related to T-cell cytotoxicity and control of VL depends on the magnitude of T helper 1 and multicytokine responses early in the course of infection. During progressive infection in mice, Th2-type CD4+ T cells expand and secrete interleukin-4 (IL-4), resulting in polyclonal B-cell activation. Later, fully established VL is associated with cellular anergy. Inappropriate antigen presentation and communication between the antigen-presenting cells and T cells, as well as the induction of IL-10 and IL-4 might explain this anergy.

In endemic areas, infected subjects may or may not develop classic signs and symptoms. Capacity to produce IL-2 and interferon-gamma (IFN- γ) (Th1 response) is associated with asymptomatic or subclinical self-healing infection. In contrast, individuals whose lymphocytes do not proliferate and, thus, do not produce IFN- γ when stimulated by *Leishmania* antigen, will develop acute VL or a subclinical infection that progresses to classical disease. Immunological abnormalities could be found

in monocyte and T-cell function, such as diminished production of tumour necrotic factor-alpha (TNF- α) and IL-1 after lipopolysaccharide (LPS) or *Listeria* stimulation, decreased production of IL-2 and IFN- γ by lymphocytes with *Leishmania* antigen. There is absence of delayed-type hypersensitivity to *Leishmania* antigen-stimulation and a decreased capability of T-cells to activate macrophages and kill *Leishmania*. All these abnormalities may account for parasite multiplication and progression of the disease¹⁷. By the antigen specific lymphocyte population, plasma levels of IFN- γ , IL-12 p40, IL-18, IL-15, interferon-gamma inducible protein (IP-10) and monokine induced by IFN- γ are markedly elevated in symptomatic VL patients as compared to individuals with asymptomatic infection, and significant decrease of plasma levels of IFN- γ and all mediators has been observed after treatment of such patients. In these patients, production of type I cytokines is not depressed, but there appears to be an unresponsiveness to the stimuli of type 1 cytokines¹⁸. *L. donovani* amastigote components have been shown to induce the production of colony-stimulating factors in experimental infection and thus it may play an important role in the pathogenesis of VL¹⁹.

Tumor necrosis factor (TNF): TNF has variety of roles in experimental infection. Some benefit host resistance while others mediate host pathology. It is critical for the control of VL. CST BL/6 mice deficient in TNF or lymphotoxin (LT) alpha have increased susceptibility to hepatic *L. donovani* infection. Production of inducible nitric oxide synthase has been found to be deficient in TNF- and LT alpha-deficient infected mice. The results demonstrated that both LT alpha and TNF are required for control of infection in noncompensatory ways²⁰.

A subset of liver natural killer (NK) T cells is activated during *L. donovani* infection by CD1d-bound lipophosphoglycan and reports suggest an important role for the CD1d-NK T cell immune axis in the early response to VL infection²¹.

Genetic basis: The mouse model has been particularly helpful in mapping the genes, which have

major influence on the course of the disease. This gene designed as "*Lsh*" is expressed at the macrophage level and is identified for its role in controlling early growth of *L. donovani* in inbred mice²². This gene is also expressed in resident liver macrophage population for *L. donovani* and thus the interplay of drug and organ dependent immunity is of paramount importance. Carter *et al*²³ have demonstrated a drop in parasite number in liver macrophages with little or no effect in spleen and bone marrow macrophages. Experimental infection is initiated by intravenous administration of amastigotes of either *L. donovani* or *L. infantum*. The outcome of infection in mice has a clear genetic basis. Early amastigote replication in tissue macrophages is regulated by the phagosomal proton-cation antiporter encoded by the *Slc1 Ia1* gene (formerly *Nramp 1* or *Lsh/Bcg/Ily*)²⁴. Early parasite growth could be controlled in Slc1 Ia1 wild type mice (*e.g.*, CBA) while it is unrestrained in Slc1 Ia1 mutant mice (BALB/c and CST BL/6 strains). Expression of this natural resistance is independent of T cells. In a recent study, where several chromosomal regions containing disease resistance genes were typed, linkage was established between VL and the 5' (CA) repeat polymorphism of the SLCA1A1 promoter²⁵.

Although most Slc1 Ia1 mutant mice eventually control their hepatic infection, the rate and effectiveness of this control are determined largely by major histocompatibility complex (MHC) haplotype; indicating an important role for acquired immune responses. The mechanisms underlying this host resistance to both *L. donovani* and *L. infantum* infection have been studied most extensively in the liver. Because CD4+ and CD8+ T cells are both required for optimal resistance in normal mice, resistance as expected is absent in nude, severe combined immunodeficiency disease (SCID), or recombination activating gene (*Rag*)^{-/-} mice, and reconstitution experiments suggest that both populations of T cells can effectively transfer resistance with varying degrees of efficacy⁸. Association between rate of cure and relative T-helper 1 (Th1): Th2 bias has been difficult to detect. Most Slc1 Ia1 mutant mouse strains have a mixed T-cell cytokine profile, apparent throughout the infection. By enzyme linked immunospot (ELISPOT)

assay analysis, IFN- γ and IL-4- producing cells are found at a ratio of approximately 2-3:1, with only minor variations over time and in different organs⁸.

Organ specific pathogenic mechanisms

Studies on experimental infection have clearly indicated contrasting outcomes in liver and spleen depending upon the compartmentalized immune response with tissue specific regulation of the quality and quantity of response. The hepatic infection is usually self-limiting and hepatic immune response is a good example of a mononuclear cell-dominated granulomatous inflammatory response involving resident kupffer cells, monocytes, CD₄⁺ and CD₈⁺ T cells. Multiple cytokine responses including IFN- γ , IL-12, IL-4 and moderate levels of TNF contribute to host protection in liver with focal TNF production readily observed in hepatic granulomas. By contrast, simultaneously with the resolution of hepatic infection, control of amastigote growth in the spleen is lost and destructive pathology is the norm. Strikingly, excess TNF in this lymphoid environment mediates much of the architectural damage and immunological dysfunction associated with this chronic inflammatory state. Although, the issue of mononuclear phagocyte heterogeneity appears to be of paramount importance, yet many questions *e.g.*, receptors governing amastigote internalization by the heterogenous subpopulations of mononuclear phagocytes found in spleen, liver and other target tissues, key immunological events that switch the spleen into a state of chronic inflammation, local regulation of TNF in liver and spleen, *etc.*, remain unresolved⁹.

Hepatic resistance correlates well with the generation of reactive oxygen and reactive nitrogen intermediates. In early stages of infection (up to 12 day post infection), both play significant roles in curtailing the parasite growth, as determined by studies in *phox*^{-/-} and nitric oxide synthase (NOS)-2^{-/-} mice, and this significance might relate to the T cell dependent recruitment of both neutrophils and monocytes. During later phase of infection, when hepatic resistance is expressed by declining amastigote number, *NOS-2* gene regulation appears to play the more dominant role, and the generation

of NO mainly reflects T cell dependent macrophage activation. Although efficiency of macrophage activation may reflect cytokine-mediated cross-regulation at the level of Th-cell differentiation, cross regulation at the level of *NOS2* gene expression in macrophages themselves appears to play a dominant role, with production of the inhibitors of *NOS-2* gene expression, IL-10 and transforming growth factor β (TGF β) correlating well with reduced parasite killing⁸. These two counterprotective cytokines might be temporarily regulated, with TGF β appearing rather late in infection compared to IL-10. To date, the range of cellular sources of these cytokines in VL has not been fully characterized, though it is clear that T cells contribute both to TGF β production and to IL-10 production⁸.

Further, it is suggested that spleen is the source of *Leishmania*-specific T lymphocytes that migrate to the liver, where parasite replication is highly active. In liver, these pre-activated cells become effector T lymphocytes. Strong regulation of CD8⁺ T cells effector function has been observed, probably preventing hepatic tissue damage. Comparing mice strains with ‘cure’ and ‘non-cure’ phenotype, an imbalance between protective and pathogenic CD4⁺ subsets in animals might be involved in the evolution of non-healing infection²⁶. Recently, a prominent role for CD8⁺ T cells in immunity against pathogens has emerged. The mode of action of CD8⁺ T cells in murine VL and their contribution to the clearance of the parasite has been addressed. CD8⁺ T cells appear to play multiple roles comprising both cytotoxic activity and secretion of cytokines and chemokines during the course of the experimental infection. Cytotoxic clones specific for *L. infantum* antigens developed in the spleen of susceptible BALB/c mice, showed an activated phenotype and became susceptible to apoptotic cell death late in the course of the disease²⁷. CD8⁺ T cells exhibited considerable cytotoxic activity against cells expressing *Leishmania* antigens. This activity was mediated by both the perforin and the Fas/FasL pathway, as judged from *in vitro* and *in vivo* assays. The CD8⁺ T cells also up-regulated mRNAs for cytokine (IFN- γ and TNF- α) and C-C chemokines (RANTES and MIP-L alpha), which have a major role in

immunity against the pathogen. CD8⁺ T cells thus displayed a Tc1 pattern of differentiation in visceral leishmaniasis model comprising both cytotoxic activity and secretion of cytokines and chemokines²⁷. Chemokines are a group of chemoattractant cytokines that play important roles in physiological as well as pathological processes and it is becoming increasingly clear that chemokines play a major, perhaps decisive role in *Leishmania* infections²⁸.

Ca²⁺ signaling can be an appropriate candidate for a second messenger during the transformation of promastigotes to amastigotes²⁹. It has recently been recognized that sandfly components might enhance the ability of promastigotes to enter macrophages and thus increase the virulence^{1,30}. Sandfly saliva was demonstrated to induce the expression of IL-6 and TNF- α in murine macrophages and was chemoattractive for murine macrophages and enhanced *L. amazonensis* infection by modulating IL-10 production³¹.

If spontaneous recovery occurs, the patient's cell-mediated immunity improves. Such patients develop a delayed hypersensitivity response as indicated by positive Leishmanin or Montenegro test. If the individual is unable to mount an appropriate immune response, the parasite disseminates in the reticuloendothelial cells of the host. Alternatively, the parasite may remain dormant and not present itself until one's immune system is compromised³².

Sodium stibogluconate does not seem to alter the immune status of the host³³. The macrophages from drug treated animals tackled the parasites themselves by their microbicidal mechanisms and the *in vitro* infection was tackled by the drug *in vitro*. This indicated that a well developed specific immunity in leishmaniasis helps in the antileishmanial activity of these drugs³⁴.

Leishmania-HIV interaction

Both *Leishmania* and HIV are able to infect and multiply in monocyte/macrophage cells. Both pathogens can establish latent infection following primary infection. The immunopathogenic

mechanisms by which *Leishmania* and HIV may interact and affect the immune system to influence the expression of one another have been emphasized. Possible effects of *Leishmania* that favour HIV replication appear to be transactivation of HIV in latently infected monocytes and in both antigen-specific manner and antigen non-specific manners in latently infected CD₄⁺ T cells. The other evidences indicate polyclonal B-cell activation, enhancement of activity of Th2 cells, decreased HIV-specific CD₈⁺ cytotoxic T cell response and depression of activity of Th1 cells. Possible effects of HIV that favour amastigotes multiplication are deactivation of macrophage function, depression of activity of TH I cells, inhibition of phagosome-lysosome fusion and enhancement of activity of Th2 cells^{35,36}.

In conclusion, the major insights into the different spectrum of leishmaniasis have derived from animal studies, primarily using in bred mouse strains. The parasite, which replicates in quiescent macrophages, is killed by activated macrophages. The outcome of infection is determined by the nature and magnitude of the T-cell and cytokine responses in infection. Organ specific pathogenic mechanisms appear to be due to heterogenous subpopulations of mononuclear phagocytes.

In susceptible BALB/c mice, infection results in the preferential activation and/or maturation of CD4⁺ T cells into effector CD4⁺ T lymphocytes expressing the Th-2 phenotype. Maturation to the Th-1 or Th-2 phenotype may be function of how antigen is presented by the antigen presenting cell (APC). Once Th-2 cells have expanded, soluble factors are released that interfere with the expansion of antigen-specific Th-1 cells and disease progress. In resistance mice, APC's present antigen in such a manner that CD4⁺ precursors are activated to mature to effector Th-1 cells, the soluble products of Th-1 cells interfere with Th-2 cell expansion and successful activation of macrophages and resolution of infection occur. The reports suggest genetic basis for such Th-1/Th-2 paradigm. However, the T cells and cytokine responses in infected human beings are more 11 complex and less polarized than in mice and the immune responses differ among the leishmanial syndromes and species. The complexities of clinical manifestations require further studies.

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