Involvement and interactions of different immune cells and their cytokines in human visceral leishmaniasis

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ABSTRACT

Visceral leishmaniasis (VL) or kala-azar, a disseminated infection of the lymphoreticular system of the body, is marked by severe defect in immune system of the host. Successful cure of VL depends on the immune status of the host in combination with the effects of the antileishmanial drugs. The rationale approach towards eradication of this disease would be to potentiate the immune functioning of the host in addition to parasite killing. This review deals with different aspects of adaptive and innate immune responses and explores their role in protection or pathogenesis of VL. IL-10 has emerged as the principal cytokine responsible for disease pathogenesis, although evidences regarding its source during active VL remain inconclusive. On the other hand, IFNγ, under the influence of IL-12, is mostly correlated with healing of the disease. Chemokines are important in mounting cell-mediated immune response as they can prevent parasite invasion in association with cytokines. Different types of T cells like CD4, CD8 and NK T cells also contribute to the immunology of this disease. In spite of conflicting reports, the role of regulatory T cells in VL pathogenesis is important. Recently discovered Th17 subset and its different members have been reported to perform diverse functions in the course of VL and leishmaniasis as a whole. Innate immune responses, depending on the cell types, are essential in early parasite detection and subsequent development of an efficient NK cell response. Immunotherapy targeting IL-10 could be looked upon as an interesting option for the treatment of VL.

Keywords: Immunity. Human visceral leishmaniasis. Cytokines. IL-10.

INTRODUCTION

Protozoans of the genus *Leishmania* are obligate intracellular parasites causing a broad spectrum of diseases collectively known as leishmaniasis. This has emerged as the third most prevalent parasite-borne disease worldwide after malaria and filariasis[1]. Manifestations of this disorder can range from less severe cutaneous leishmaniasis (CL) characterized by self-resolving local cutaneous lesions and mucosal leishmaniasis (ML) affecting the mucus membranes of mouth, nose and throat to the more serious visceral leishmaniasis (VL), which is potentially fatal. An estimated 12 million people worldwide have some form of leishmaniasis, and another 350 million people are at risk. There are approximately 0.5 million new cases of VL each year and over 90% of these occur in Brazil, Bangladesh, India, Nepal and Sudan, although these numbers are likely to be underestimated since majority of the cases are not even reported in these and many of the other 88 endemic countries[2].

VL, caused by *Leishmania donovani* and *Leishmania infantum* (named *Leishmania chagasi* in the americas) is characterized by persistent low grade fever, hepatosplenomegaly, cachexia, pancytopenia and hypergammaglobulinemia. The parasite resides primarily within macrophages of the liver, spleen, and bone marrow, and the course of the disease indicates an underlying defect in immune defense mechanism of the host. Earlier reports on *Leishmania* infection suggested that immune response in human CL[3,4] and VL were associated with an interaction of T helper 1 (Th1)/Th2 cytokines at both cellular[5-8] as well as transcriptional[9] levels and depleted lymphoproliferative responses[4]. This suppressive nature of the immune response during active VL was mostly specific to leishmanial antigens as tests for delayed-type hypersensitivity (Leishmanin or Montenegro tests) to these antigens were negative[10]. The major players of this response were interferon gamma (IFNγ) and interleukin-4 (IL-4) in case of both VL and CL. However, unlike CL the effects of these cytokines were more of a mixed type in case of VL. As the immunological studies progressed IL-10 emerged as the most potent factor for VL pathogenesis which is evident from recent observations at cellular[11,12] and messenger RNA (mRNA) levels[13]. The scenario, however, remains the same for Th1 cytokines as current observations also report suppressed antigen specific IFNγ and IL-12 production[11,12] in active VL. The level of IFNγ in serum, however, remains high[12,14,15] suggesting that their sources could be lymphoid organs where the parasite proliferates. Indeed studies confirm high IFNγ mRNA expression in bone marrow[16], lymph node[14] and splenic[17] aspirates. Thus, these studies broadly reaffirm the previously described[17] interplay of Th1/Th2 cytokines during *Leishmania* infection. Investigation for the cellular specificity of these cytokines led to the discovery of the involvement of cluster of differentiation 4+ (CD4+), CD8+ and Natural Killer (NK)
T cells in this disease. However, as with the earlier reports\textsuperscript{5,8,18} more recent observations\textsuperscript{20-24} also failed to distinguish these T cells based on their protective or pathogenic role in VL. Another T cell essential for this disease is regulatory T cell (Treg). Although the effect of CD4\textsuperscript{+}CD25\textsuperscript{+} T cells in disease progression has already been reported\textsuperscript{9}, the role of CD4\textsuperscript{+}CD25\textsuperscript{+}FoxP3\textsuperscript{+} natural Treg cells in the disease pathology still remains a matter of debate\textsuperscript{15,25,26}. Chemokines are important members of cell-mediated immune response that exhibit antileishmanial activity through recruitment of different immune cells at infection sites (both liver and spleen). In leishmaniasis, both cytokines and chemokines interact closely to strengthen the host defense against parasite. IL-17, the signature cytokine for the recently discovered Th17 subset, was found to play a significant role in the migration, recruitment and activation of neutrophils. Since the neutrophils are known to have important regulatory and/or effector functions during \textit{Leishmania} infection, Th17 can be looked upon as an important regulator for this disease. Indeed, there have been reports of involvement of different Th17 cytokines like IL-17, IL-22\textsuperscript{27} and IL-21\textsuperscript{28} in human VL. In addition to adaptive immune response innate immune response involving cells like dendritic cells (DCs), macrophages, neutrophil/eosinophil etc. are essential in regulating the initial entry of parasites. They are involved not only in preventing parasite invasion but also in controlling their multiplication through the release of different cytokines. This review will examine the interplay of different components of adaptive and innate immune responses and their involvement in disease or elimination of human VL.

DIFFERENT CYTOKINES, CHEMOKINES AND CELLULAR SUBSETS: ROLE IN VL

A close look at the progressive findings in the field of immunology of human VL suggests important roles of different cytokines in disease protection and pathogenesis. Few of the very first reports on VL immunopathogenesis regarded upregulation of IL-4\textsuperscript{29} and IL-10\textsuperscript{10} and loss of function of IFN\gamma\textsuperscript{6,7} as the indication of active disease. In fact IL-4 was initially regarded as a marker for active disease\textsuperscript{8}. However, later observations exhibited its mixed response towards VL. Whereas some reports indicated lower IL-4 levels in nodal and portal areas of \textit{in situ} liver lesions as well as in serum during disease\textsuperscript{11} and its corresponding enhancement after cure\textsuperscript{12}, others suggested enhancement of IL-4\textsuperscript{-}neutrophils in active VL\textsuperscript{26}. As more investigations were carried out using different samples like tissue sections\textsuperscript{26}, plasma\textsuperscript{14,15,18,19,30-33}, whole blood\textsuperscript{14}, peripheral blood mononuclear cells (PBMC)\textsuperscript{11,12,31,35,36}, mRNA\textsuperscript{35-37} and splenic aspirate cells\textsuperscript{38} more evidences emerged suggesting IL-10 to be the principal contributor for VL pathology. Results from flow cytometric studies of cytokines also supported this finding\textsuperscript{8,29}. Post kala-azar dermal leishmaniasis (PKDL), a disease manifestation of VL, is also characterized by enhanced IL-4\textsuperscript{29} as well as IL-10 expression and perhaps the subsequent masking effect exerted on IFN\gamma by IL-10 is apparent from a generally heightened response of this cytokine\textsuperscript{6,11,14,39-41}. Like IFN\gamma, IL-12 is also responsible for a protective response. In fact it influences the production of IFN\gamma\textsuperscript{6,7,11,12,20,33} and the loss of lymphoproliferative response during active VL was formerly attributed to its lower expression\textsuperscript{47}. As already mentioned this loss of lymphoproliferation could be looked upon as a failure of response towards \textit{Leishmania} antigen and it has been proposed that PBMCs of VL patients might consist of a non-\textit{Leishmania} antigen-specific population which fails to proliferate upon \textit{Leishmania} antigen stimulation\textsuperscript{40}. Although primarily protective, IFN\gamma could sometimes be associated with disease exacerbating conditions as was evident in an alternate activation pathway of Th1 cells through Trp-Ser-X-1 (WSX-1) ligation which might also lead to activation of transcription factor for IL-10\textsuperscript{45}.

The mechanism of the suppressive activity of IL-10 in human VL was primarily attributed to reduction in migration inhibitory factor (MIF) regulated accumulation of monocyte-derived macrophages\textsuperscript{43} and inhibition of NO generation from macrophages resulting in downregulation of leishmanicidal activity\textsuperscript{44}. Recently more insights have come up regarding IL-10 expression and regulation where its production from macrophages infected with \textit{L. donovani} is reported to be negatively influenced by glycogen synthase kinase-3β (GSK-3β). GSK-3β expression, on the other hand, is inhibited by either phosphorytidinositol-3 kinase (PI3K) or activated form of serine-threonine kinase Akt\textsuperscript{46}. Another interesting observation has suggested that intracellular network of PI3K along with mammalian target of rapamycin (mTOR) signaling pathway regulate IL-10 modulation by \textit{L. donovani}\textsuperscript{45}.

IFN\gamma and IL-10 might be the major players of VL pathology, but various other cytokines also have significant role in disease persistence. Some of them are traditionally known whereas others have emerged through progressive research. One such member, IL-6, was known to be associated with both active VL\textsuperscript{14,15,46} and PKDL\textsuperscript{14} conditions. It is an important pro-inflammatory cytokine related to Th2 differentiation\textsuperscript{47}, inflammation and Th1 response\textsuperscript{48} as well as development of Th17 cells\textsuperscript{49}. Thus IL-6 maintains a balance between T cell responses during active disease. Involvement of transforming growth factor beta (TGF\beta) in VL pathology was earlier reported by Saha et al.\textsuperscript{11}, which later on got confirmed by immunohistochemical observation of Kupffer cells\textsuperscript{50} and polymorphism study of a particular locus of TGB1 gene\textsuperscript{51}. Mechanism of action of TGF\beta, in active \textit{L. donovani} infection, was thought to be associated with overexpression of ubiquitin-editing enzyme A20 through tyrosine phosphatase response ensuring transient activation of inflammatory signaling pathways in macrophages\textsuperscript{52}. It was also known to contribute towards PKDL manifestation\textsuperscript{11,14}.

Another important cytokine tumor necrosis factor alpha (TNF\alpha), known for exerting cytotoxic effects on invading pathogens, along with its receptor TNFR is also closely associated with VL pathogenesis and PKDL manifestation. This was previously reported by Medeiros et al.\textsuperscript{53} and further supported by more recent observations\textsuperscript{18,32,38,41,46,54-57} in both VL and PKDL patients. Unique cytokines have also emerged in implicating disease progression as recent observations indicate IL-27 to be the regulator of IL-10 producing T cells through induction of IL-21\textsuperscript{35}. 
Chemokines, which are chemotactic cytokines, also play crucial roles during *Leishmania* infection. They help in eliciting antileishmanial activity by promoting cell-mediated immune response through recruitment of different subsets of leukocytes at infection site and subsequently stimulating them. Members of the chemokine family involved in this process depend on the stage of infection and pathogens involved; the cell populations recruited at the early stage are essential in defining the outcome of the disease. In one study, elevated levels of chemokines, chemokine (C-X-C motif) ligand 9 (CXCL9) and CXCL10, were reported in serum during active infection and it was suggested that these chemokines along with IFNγ play an important immunopathogenic role during disease. Since not many reports are available regarding the role of chemokines in human VL, we will mainly discuss the importance of different chemokines in experimental VL models where they are known to mount a Th1 response through their influence on IFNγ production. Indeed *L. donovani* infected mice lacking chemokine receptor chemokine (C-C motif) receptor 5 (CCR5) or its ligand macrophage inflammatory protein (MIP) 1α exhibits a low antigen-specific IFNγ production during the initial phases of infection. The higher expression of MIP-1α along with chemokine (C-C motif) ligand 2 (CCL2) in liver cells of *L. donovani* infected mice during early stages of infection is responsible for the recruitment of monocytes. Monocytes thus recruited could produce Th1 mobilizing chemokine CXCL10 under the influence of IFNγ at the late phase of infection enabling hepatic granuloma formation and lymphocyte recruitment. *L. donovani* infection could also downregulate CCR7 expression, thereby impairing DC migration to draining lymph nodes and facilitating disease progression. The scenario is somewhat different in spleen cells of *L. infantum* infected mice where Th2 type response predominates over Th1. This response is regulated by persistent CCL2 expression suggesting that there is an invasion of macrophages rather than T cells in the spleen.

Investigations are also being carried out to determine the source of these various cytokines so that particular cell types could be attributed to VL infection. Previously CD4⁺CD8⁻ and CD8⁺CD4⁻ T cell populations were reported to be major contributors of disease manifestation. They still remain so, however, further observations identify different CD4 sub-populations like chemokine receptor-5 (CCR5) CD4⁺CD4⁺ and CD2⁺CD4⁺ to be the markers of active VL. Human immunodeficiency virus (HIV) co-infection has emerged as a potent threat in VL patients and their involvement with different T cells is established with reports of higher CXCR4, a co-receptor of HIV, expression on CD4⁺T cells and lower CD8⁺ T cell count in patients with HIV-VL. Subpopulations of CD8⁺ T cells also become important as lower expression of bone-marrow CD8⁺CD18⁺CD45RO⁺ lymphocytes can be looked upon as the biomarker of acute VL. NK T cells (CD3⁺CD161⁺Vα24), formerly known for clearance of parasite from liver by direct and indirect lysis and playing a regulatory role in VL, are also reported to produce IFNγ in response to *L. donovani* antigens at the disease site.

Further exploration of the cellular origin of cytokines led to the discovery of a distinct T cell subset known as regulatory T cells (Treg). Elemental role of Treg cells include homeostasis and prevention of excessive inflammation mostly due to IL-10 production. This can be broadly divided into two categories: thymus derived CD4⁺CD25⁺FoxP3⁺ natural Treg (nTreg) cells, and inducible Tregs that are derived from periphery and are either FoxP3⁺ iTreg or adaptive FoxP3⁺ type-1 Treg cells (Tr1). There are contradictory reports regarding the role of nTregs in VL progression. Whereas some observations downplayed the importance of nTregs as the population responsible for IL-10 secretion and disease pathogenesis, others suggested it to be the source of IL-10. Role of CD4⁺CD25⁺ T cells in PKDL pathogenesis was reported by Saha et al. This observation was further extended in reports of enhanced lesional CD4⁺CD25⁺FoxP3⁺ expression in PKDL patients. More conclusive evidence came from the demonstration of accumulated nTreg cells at infection site with a correlation of both IL-10 and nTreg levels with parasite burden, thus implying their role in disease severity in PKDL.

### TH17 CELLS: SIGNIFICANCE IN PROTECTION AND PROGRESSION OF LEISHMANIASIS

Th17 subset of T cells was only identified in 2005 and mainly associated with different autoimmune diseases like psoriasis, multiple sclerosis (MS), rheumatoid arthritis (RA) and inflammatory bowel disease (IBD) as well as allergic reactions including asthma. Th17 cell lineage development is induced by cytokines such as IL-6, IL-23 and TGFβ which in turn activate the signal transducer and activator of transcription (STAT)3 pathway leading to the expression of the transcription factor retinoic-acid orphan related receptors (ROR-α and ROR-γt). Th17 cells mainly produce IL-17A and IL-17F cytokines, in addition to other cytokines such as IL-21 and IL-22. IL-17 performs an important function in neutrophil development and since neutrophils are regarded as an important effector role in disease severity in *Leishmania* infection, it would be possible that Th17 cells might have a significant role in this disease. However, the exact function they perform in this disease depends largely on the parasite species and the genetic background of the host.

First report of the involvement of Th17 subset in human VL came from studies of Pitta et al. when they suggested that IL-17 and IL-22, along with Th1 cytokines, play complementary roles in protective response against VL and any defect in this mechanism could increase the risk of this disease. More recent observations, however, indicate IL-21 to be the part of a feedback control mechanism that could lead to the expansion of IL-10-producing T cells thereby representing its role as a disease-promoting cytokine. Increased lesional expression of IL-17 and IL-23 in PKDL patients and their subsequent downregulation following cure establish the importance of Th17 cells in this disease.

Since not many reports are available regarding the involvement of Th17 cells in human VL, we will include experimental VL as well as other forms of leishmaniasis in our discussion. Disease promoting role of IL-17 has been recognized in IL-17 deficient mice model of CL which show lesser infection severity than IL-17 positive mice. This effect was attributed mainly to IL-17 because comparable levels of other cytokines...
like IL-10, IFNγ etc. was observed in both IL-17 positive and depleted mice whereas reduced neutrophil recruitment was reported only in IL-17 deficient mice77, thereby establishing the association between increased neutrophil recruitment and severity of disease.

Conflicting roles of IL-17 in disease pathology was also evident in other Leishmania species. Whereas studies with experimental models of L. braziliensis78 and L. panamensis79 infections exhibited induction of healing in association with elevated IL-17 as well as IFNγ levels, reports from human ML suggested that IL-17 was involved in ML pathogenesis80.

Vaccine studies provide evidence for the protective role of Th17 cells as live L. major plus CpG DNA vaccine led to enhanced proliferation of CD4+ Th17 cells thereby preventing lesion development and providing long-term immunity85. Similar observations were made in experimental VL where protection induced by CpG stimulated bone marrow-derived DCs pulsed with L. infantum kinetoplastid membrane protein 11 (KMP-11) peptide was correlated with secretion of IL-1781.

IL-27 is known to be an important regulator of the expression of Th17 cell lineage. It belongs to the IL-6/IL-12 cytokine family and its suppressive effect on Th17 subset was earlier reported in experimental autoimmune encephalomyelitis82 and murine influenza infection83. This observation was further extended in murine CL model where IL-27 was found to maintain a critical balance between disease protection and pathogenesis by inducing IFNγ and IL-10 from CD4+ T cells and suppressing inappropriate Th17 development84. Interesting observations regarding IL-27/T-cell cytokine receptor (TCCR) pathway has been made for experimental VL where, unlike CL, disease resistance was linked to IL-27/TCCR deletion87 rather than their wild-type counterparts85. However, these results were attributed to liver immunopathology and no Th17 component was examined.

INNATE IMMUNE RESPONSE

Innate immune response against Leishmania infection plays a pivotal role in predicting the outcome of the disease. It not only helps in preventing the early parasite growth and spreading of infection but also acts as a source of different immunoregulatory cytokines. Leishmania being an opportunistic parasite can employ several strategies for its survival inside the macrophage phagolysosomes. Macrophage, one of the major players of innate immunity, was primarily known to exert its effects against L. donovani infection through the production of inducible nitric oxide synthase (iNOS) in liver86. Although one report suggested the anti-parasitic effect of macrophages in liver to be iNOS-independent87, the mechanism by which it exerts its antileishmanial activity remained mostly iNOS-dependent in both liver and spleen88,89. Studies are, in fact, being carried out to discover the iNOS-independent parasiticidal mechanism of macrophages. Leishmania donovani, on the other hand, is known to evade the iNOS-dependent killing in macrophages through the induction of arginase 1 in an IL-10 mediated fashion80.

DCs, another important contributor of the innate response, can be of two types- myeloid (mDC) and plasmacytoid (pDC). Of these mDCs are known to exert cytotoxic effects on L. infantum parasites by producing IL-12 under the influence of Toll-like receptor (TLR) 9 and inducing NK cells to release IFNγ90. pDCs, however, are unable to phagocytose parasites, but they can produce IL-12 in a TLR-9 dependent manner suggesting their relevance during later stages of infection91. Studies in VL patients suggested a reduced secretory activity of neutrophils and eosinophils attributable to the deficiencies in the production of their potent activators IL-8 and eotaxin, respectively89. Cytokines secreted by these cells during infection show a predominant Th2 response with an enhanced IL-4+ neutrophil and IL-10+ eosinophil and reduced IFNγ+ and IL-12+ eosinophil frequencies29 (Figure 1).

![Figure 1 - Interaction of different components of innate and adaptive immune systems and their possible role in parasite persistence/clearance.](www.scielo.br/rsbmt)
CONCLUSIONS

The immunological studies that have been carried out so far on human VL clearly identify IL-10 to be the most important factor for disease pathogenesis. The immunosuppressive effect exerted by this cytokine is mainly due to the deactivation of monocyte-derived macrophages promoting enhanced survival and growth of the parasite. However, the cellular sources responsible for its induction and the exact mechanism behind the establishment of persistent infection still remain unclear. Present chemotherapeutic approaches against VL are unsatisfactory due to the problems of resistance, toxicity and long-term hospitalization. Since successful treatment is thought to depend, in part, on potential immune functioning of the host, direct manipulation of the immune response, either alone or in combination with drugs, through neutralization/blockade of IL-10 and targeting of its cellular source(s) might be considered as a novel therapeutic intervention. Chemokines released after parasite invasion could attract different subsets of leukocytes. Whether these cells act as reservoir of the parasites or contribute to their virulence depends on the nature of chemokine involved. Thus developing inhibitors of the chemokine system could help us to explore the mechanism of immunopathogenesis.

Experimental observations regarding the involvement of Th17 subset with leishmaniasis clearly signify the importance of IL-22 and IL-23 along with IL-17. These studies also provide evidences against the so-far known purely pro-inflammatory role of Th17 cells. IL-27, known for its general suppressive effects on Th1, Th2 and Th17 subsets, has also emerged as an important cytokine as it could induce IL-10 production from Th1 cells and inhibit effector T cells. Thus it would be interesting to further investigate the role of IL-27 and its downstream cytokine IL-21 in pathogenesis of VL. The control of L. donovani multiplication in the macrophages could be achieved by both iNOS-dependent and independent pathways depending on the stage of infection. The activation of NK cells in VL is the outcome of its close interaction with mDCs, which are triggered by the parasites for the production of IL-12 in a TLR9-dependent fashion. The exact role of pDCs and their TLR9-dependent release of IL-12 require further investigation.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

FINANCIAL SUPPORT

Indian Council of Medical Research (ICMR), India and Council of Scientific and Industrial Research (CSIR), India.

REFERENCES


immune response in human visceral leishmaniasis