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Review Article

Dynamicity of Immune Regulation during Visceral Leishmaniasis

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Visceral leishmaniasis is a vector-borne tropical disease associated with a spectrum of clinical manifestations and immunosuppression leading to parasite survival. Chemokines which act as chemo-attractants for generating innate immune responses are significant in bringing innate and adaptive responses side by side for combating against infection. The independency and interdependency of regulatory T cells and Th17 cells on Th1/Th2 signalling pathways and an imbalance in immune responses could lead to disease progression. Although Interleukin- (IL-)12 is crucial in generating protective Th1 response against the disease, various members of the IL-12 family perform diverse immune functions due to their chain pairing promiscuity. This review focuses on the immunopathogenesis of VL by chronologically summarising the developments in the studies of the immune regulation as well as the mechanisms involved. A better understanding of the innate and adaptive immune functioning of the host could aid in rational control and better therapeutic intervention of the disease.

Key Words: Immunology; Leishmaniasis; Cytokine; Chemokine; Regulatory Cell; Immune Cell; Innate Immunity; Adaptive Immunity; Memory Response

Introduction

Visceral leishmaniasis (VL), also known as kala-azar, is caused by the protozoan parasites of the genus *Leishmania* that includes *Leishmania donovani*, *Leishmania chagasi* and *Leishmania infantum*. VL is prevalent in more than eighty countries (Choi and Lerner, 2001) worldwide. Of them approximately 90% of VL cases are reported in Bangladesh, India, Ethiopia, Sudan, South Sudan, and Brazil (Alvar *et al.*, 2012). The characteristic symptoms of VL include irregular bouts of fever, substantial weight loss, swelling of the spleen and liver, and anaemia. The disease is fatal if left untreated. VL is an immunosuppressive disease with most of the patients showing negative leishmanin skin test (Gidwani *et al.*, 2009). It could be an interesting topic to explore since the proliferative response of peripheral blood mononuclear cells (PBMC) from such active VL patients is poor.

In VL, the beginning of infection is marked by recruitment of innate immune cells such as, neutrophils, macrophages, natural killer (NK) cells and dendritic cells (DCs) at the site of infection due to the secretion of different chemoattractant proteins. Later these recruitments generate a mixed regulatory and inflammatory responses by secreting chemokines and cytokines. These responses are exhibited generally by low levels of IL-12 or IFN-γ and elevated production of IL-10, TGF-β, IL-4 and IL-13 in leishmanial antigens stimulated culture supernatants of VL patients. Contradictory reports are also there manifesting increased level of IFN-γ m-RNA in both liver and spleen of the infected subjects (Saha *et al.* 2007; Nylen *et al.* 2007; Sacks *et al.*, 1987). Similar observations were made in murine VL, where both IL-10 and TNF-α production were found to be elevated in the spleen. TNF-α, which is critical for the development of protective immunity, causes

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granuloma formation in the liver, but may also induce IL-10 secretion to control the tissue damage (Ato *et al.*, 2002). Enhanced IL-10, besides inhibiting macrophage activation and granuloma formation, also promotes parasite persistence through its immunosuppressive mechanisms (Ato *et al.,* 2002; Murray *et al.,* 2000; Stanley *et al.,* 2007; Buxbaum 2013; Murray *et al.,* 2002).

IL-27, a cytokine central in regulation of Th17 cells, is mainly produced by antigen presenting cells. Studies in mice have demonstrated that this cytokine can inhibit the differentiation of Th17 cells. IL-27 could also act synergistically with IL-10 and TGF-β in inducing disease pathogenicity (Chen and Liu 2009; Belkaid *et al.,* 2001). TGF-β, on the other hand, is essential for the induction of $FoxP3^+$ Regulatory T (Treg) cells. Additionally it could also inhibit the generation of Th1 cells, leading to disease progression (Batten *et al.,* 2006). Treg cells are considered as masters of immune regulation that promote bystander suppression of effector T cells and infectious tolerance (Banchereau *et al.,* 2012). Although Tregs have mostly been shown to promote Th2 response in cutaneous leishmaniasis (CL) in human and mice (Tang and Bluestone 2008; Lund *et al.,* 2008; Rodriguez-Pinto *et al.,* 2012), some reports also suggest their involvement in controlling disease manifestation by facilitating timely homing of immune effector cells to the site of infection (Rodriguez-Pinto *et al.,* 2012). In VL such reports are lacking and more studies on human and mice are required to establish the role of Tregs. Moreover, it would be interesting to investigate the possible involvement of IL-35 producing T cells in VL as it was recently reported to play a Th2 promoting response in autoimmune diseases (Collison *et al.,* 2010). In the present review the initial part deals with the current understanding of the immunology of *Leishmania* infection and the dynamicity of the host pathogen interaction. We considered different evidences available to explain the immunoregulatory role of diverse immune cells and their cytokines with the course of infection in murine VL. It is an attempt to summarise the new concepts developed so far in terms of roles played by the different subsets of T cells, Th17 cells and their cytokines during disease. These are followed by a concise description on modulation of signalling pathways by *L. donovani*. At the end, a brief description on the shortcomings in the study of immunology of VL and challenge to overcome the disease has been incorporated as future prospects.

Innate Immune Response

Infection with *Leishmania* begins when an infected female sand fly suck blood from its host. It has been found that few thousands of meta-cyclic promastigotes present in sand fly saliva are sufficient to establish disease progression causing wounding of the microvasculature that creates a hemorrhagic pool. Sand fly saliva contains well characterized molecules that have several activities, including vasodilation, inhibition of coagulation and immunomodulatory effects (Okwor *et al.,* 2009; Sacks D and Kamhawi S 2001). It also contains few uncharacterized molecules that could attract neutrophils and macrophages (Zer *et al.,* 2001) and recruit them to local inflammatory reaction (Kamhawi *et al.,* 2000).

Neutrophils

Neutrophils are the first cells to arrive at the site of *Leishmania* infection (Muller *et al.,* 2001), where they release neutrophil extracellular traps (NETs). NETs are composed of filamentous DNA with antimicrobial peptides and found to be induce by *Leishmania* surface proteins like GP63 and lipophosphoglycan (LPG) (Bhowmick *et al.,* 2008; Brinkmann *et al.,* 2004). In vitro experiments using human neutrophils and *Leishmania amazonensis* promastigotes have revealed that *Leishmania* are trapped and killed by NETs. Moreover *Leishmania braziliensis* elimination is dependent on the interaction between neutrophils and macrophages and was shown to be associated with tumor necrosis factor-α (TNF-α) as well as superoxide production. Co-operation between neutrophils and macrophages towards parasite elimination was also observed in experiments performed with *L. braziliensis* infected human cells (Bhowmick *et al.,* 2008; Brinkmann *et al.,* 2004; Guimaraes-Costa *et al.,* 2009).

However, neutrophils have proved to be beneficial for survival of *Leishmania* in infected tissues. Indeed, *Leishmania* have been found to extend lifespan of these cells where they can survive for hours to days after infection. The infected neutrophils secrete chemokines like CXCL8 (also known as IL-8) (Laufs *et al.,* 2002) and macrophage inflammatory protein (MIP)-1β which play a critical role in attracting more neutrophils and macrophages, respectively, to the site of infection. These neutrophils when taken up by macrophages evade their antimicrobial responses leading to a 'silent' entry of these parasites into their host macrophages. (van Zandbergen *et al.,* 2004). Laskay and colleagues, (2003) coined a new term 'Trojan Horses' for neutrophils which help promastigotes to achieve this goal (Fig. 1). They observed that *L. major* promastigotes were readily phagocytosed by neutrophils *in vitro*, but survived within their phagosomes. The infected neutrophils were induced to undergo apoptosis and became a phagocytic meal for macrophages. As apoptotic neutrophils are phagocytosed through receptor-mediated pathways that fail to trigger macrophage defence responses, their cargoes of promastigotes are thereby efficiently and safely shuttled into the macrophage phagosomes (Laskay *et al.,* 2003; Ravichandran and Lorenz 2007). However, it has been shown in an i*n situ* imaging study that most of the infected neutrophils rapidly engulfing promastigotes, are short-lived and release the parasites before being phagocytosed by the macrophages. Moreover, the observation that the parasite burden of macrophages and DCs isolated from the mice remains unchanged even after neutrophil depletion (Peters *et al.,* 2008), suggest that the neutrophils may merely scavenge the parasites that are otherwise ignored. Although neutrophils clearly participate in the early response to infection, their role as a "Trojan Horse" has yet to be confirmed.

Macrophages

Macrophages are the second wave of cells that enter the site of *Leishmania* infection. They serve as host for parasite replication and a source of cytokines modulating the T cell-mediated immune responses. Moreover, after appropriate activation by Th1 cells, they act as effector cells for intracellular parasite killing. Promastigotes are rapidly phagocytosed by dermal macrophages (Locksley *et al.,* 1988) through a complement receptor (CR)-3 dependent mechanism (Mosser and Edelson 1985) and eventually metamorphose to amastigotes. In infected macrophages, amastigotes settle in acidic parasitophorous vacuoles that exhibit most of the features of phagolysosomes including the presence of lysosome markers such as lysosomal associated membrane protein (LAMP)-1, LAMP-2, and rab7p (small GTP-binding proteins involved in vesicular transport) (Dermine *et al.* 2005, Antoine *et al.,* 1999; Courre *et al.,* 2002). *Leishmania* parasites have evolved several mechanisms to avoid hydrolysis in the host innate environment. For example, *L. donovani* promastigotes inhibit the fusion of the phagosome with hydrolase-enriched endocytic organelles. This mechanism depends on the promastigote cell surface molecule lipophosphoglycan which prevents the formation of phagolysosome by disruption of lipid microdomains on the phagosome membrane (Dermine *et al.,* 2005).

Monocytes are attracted in the early stages of infection by products of sand fly saliva (Zer 2001) and later by chemokines such as MIP-1β (van Zandbergen *et al.* 2004). *Leishmania* also produce chemotactic factors which induces the macrophages to secrete monocyte chemoattractant protein (MCP)-1 and CXCL1 (keratinocyte derived cytokines) that act as chemo-attractants for monocytes and neutrophils (Fig. 1). CCL2 is MCP-1 homolog in human macrophages (Deshmane *et al*. 2009; Racoosin and Beverley 1997; van Zandbergen *et al.* 2002). CCL2 can also attract other cells such as NK cells and DCs that are positive for the chemokine receptor CCR2. In human leishmaniasis, CCL2 and MIP-1α appear to be responsible for macrophage activation in skin lesions (Ritter and Moll 2000). Macrophages stimulated by the synergistic action of CCL2 and IFN-γ kill parasites in localized CL (Muzio *et al.,* 2000), while the occurrence of IL-4 in diffused cutaneous leishmaniasis (DCL) lesions suppress CCL2 expression and promote progression of the disease. Therefore macrophages activated by chemokines play a pivotal role in bringing innate and

adaptive immune responses together during *Leishmania* infection.

Natural Killer (NK) Cells

NK cells come to the site of infection as early as 24 hours after parasite invasion and then migrate to the infected skin and draining LNs (Laskay *et al.* 1995). The migration of NK cells correlates with the expression of the NK cell-activating chemokine CXCL10. Moreover, treatment of susceptible BALB/c mice with recombinant CXCL10 results in significantly increased NK cell cytotoxic activity in the draining LN (Vester *et al.*, 1999). Previously NK cells were thought to be non-essential for the ultimate control of cutaneous leishmaniasis (CL) and VL as NK cell deficient mice could heal their lesions through IL-12 dependent IFN-γ production by $CD4^+$T cells (Satoskar *et al.*, 1999). But recent research emphasizes NK cells as an important source of IFN-γ that can elicit antileishmanial activity in macrophages thereby mounting a protective Th1 response against the parasite (Bogdan 2012; Martin-Fontecha *et al.*, 2004). It has been shown that antigen specific $CD4^+$ T cells are required for initiation of NK cell activation *in vivo* upon *L. major* infection (Bihl *et al.*, 2010) demonstrating a bidirectional regulation between innate and adaptive immunity.

Dendritic Cells (DCs)

Interaction of parasite reactive IgG with Fcγ receptors on DCs are essential for recognition and engulfment of *Leishmania* parasite. It appears that at later stages of infection, the balance between FcγR mediated induction of IL-12 from DCs and FcγR associated IL-10 release from infected macrophages is ultimately responsible for disease outcome (Filardy *et al.*, 2010). MHC class I- and II-restricted antigen presentation towards both $CD4^+$ and $CD8^+$ T cells, respectively, by DCs is essential for appropriate T cell priming against parasite antigens. Infected DCs are crucial for T cell priming in CL, whereas infected macrophages mainly contribute to MHC class II-restricted $CD4^+$ T cell restimulation (Kautz-Neu *et al.*, 2012). Mast cell primed DCs stimulate $CD4^+$ T cells to release IFN-γ and IL-17, demonstrating that DCs promote Th1 and Th17 responses which are protective against murine CL. Moreover, interaction of DCs with NKT cells determines Th1/Th2 differentiation (Dudeck *et al.*, 2011). DC derived IL-6 acts as a key cytokine regulating the expansion of $CD4^+CD25^-FoxP3^-IL\text{-}10^+$ T cells in vivo against *L. donovani* infection (Wiethe *et al.*, 2008; Stager *et al.*, 2006). *In vivo* studies on mice have shown that $CD11c^{hi}$ DCs, as well as a mixed $CD11c^{int/lo}$ cell population, are capable of inhibiting host resistance and promoting disease-associated pathology in murine VL (Owens *et al.*, 2012). Furthermore $IL\text{-}10^+IL\text{-}27^+$ DCs are able to promote IL-10 production by Th1 cells (Awasthi *et al.*, 2008) thereby emphasizing the importance of $CD11c^+$ cells in priming of Th1 and Th17 subsets, subsequently as a potential target for immunotherapy.

During cure in VL, IFN-γ secreting neutrophil, eosinophil and NK cells increase, accompanied by increase in IL-12 producing monocytes. There is a clear correlation between cytokine profile of the innate immune cells and VL pathology, indicating their pivotal role not only in the control of early parasite growth and systemic spreading of *Leishmania*, but also as relevant source of immunoregulatory cytokines. The co-ordination of innate immune response with adaptive immunity acts as a major relevance for both control of parasitism and morbidity (Peruhype-Magalhaes *et al.*, 2005). Thus, the innate immune cells may, in addition to other factors, contribute significantly in regulating T-cell response and cytokine microenvironment during disease progression and are critical additive to adaptive immunity for controlling the disease outburst.

Adaptive Immunity

Chemokines may have a direct role in the development of the IFN-γ mediated Th1 response. *L. donovani* infected mice lacking CCR5 or its ligand MIP-1α, demonstrate a low antigen-specific IFN-γ response during the early phases of infection. MIP-2 and CXCL1, the functional murine homologs of human IL- 8, are rapidly produced in the skin by the *L. major* infected macrophages which serve to recruit neutrophils and DC at the site of infection (Modi and

Yoshimura 1999; Racoosin and Beverley 1997). In murine VL, infected mice undergo a rapid hepatic accumulation of MIP-1α, CCL2 and CXCL10 (Cotterell *et al.,* 1999). Increased expression of CXCL10 enables accelerated liver granuloma formation and inflammatory response against the parasite. Monocytic cells attracted by MIP-1α and CCL2, following IFN-γ stimulation, could be the source of Th1-mobilizing chemokines such as CXCL10 (Farber, 1997). Unlike liver cells, spleen cells from *L. infantum* infected mice produce both Th1- and Th2-type cytokines with the Th2-type response being dominant. This is compatible with the sustained expression of CCL2 rather than CXCL10, thereby show an influx of macrophages rather than T cells in the spleen (Rousseau *et al.,* 2001). This perhaps is the reason for parasite persistence in the spleen whereas liver usually controls the infection (Kaye *et al.,* 2004). Hence, chemokines are crucial both as chemoattractant for innate immune cells, and in promoting early cell mediated immune responses during VL in both human and mice.

Th1 and Th2 Cells

Higher TNF-α, IFN-γ, IL-12, IL-4, and IL-10 levels were observed in patient serum with active lesions, whereas cured subjects produced only IFN-γ at elevated levels. These observations suggest the presence of a mixed Th1/Th2 response during active CL and a sustained Th1 response characterized by elevated IFN-γ levels, and down-regulation of IL-4 and IL-10 production while cure (Castellano *et al.,* 2009; Kedzierski *et al.,* 2008). In human, VL is associated with increased production of multiple and primarily pro-inflammatory cytokines and chemokines. Patients have been found to have elevated plasma protein levels of IL-1, IL-4, IL-10, IL-6, IL-8, IL-12, IL-15, IFN-γ inducible protein-10 (IP-10), IFN-γ, and TNF-α (Alvar *et al.,* 2012; Ansari *et al.,* 2006; Kurkjian *et al.,* 2006; Nylen and Sacks 2007) . Elevated levels of IL-10 and TGF-β have been shown from PBMC of active VL patient (Saha *et al.,* 2006; Hailu *et al.,* 2004). With treatment, IL-10 slowly decreases while IFN-γ increases in liver and spleen and attain their normal levels (Costa *et al.,* 2012). Elevated levels of IFN-γ mRNA have been found in target organs, such as the spleen and bone marrow, during the acute phase of infection, accompanied by increased levels of IL-10 (Nylen *et al.,* 2007; Nylen and Sacks 2007; Banerjee *et al.,* 2008). Some intriguing studies suggest that the pro-inflammatory cytokines are not depressed rather they are unresponsive to the stimuli of pro-inflammatory cytokines (Hailu *et al.,* 2004). The underlying causes of such immunologic unresponsiveness remain a subject of further investigation. Additionally the development of VL in human is not driven by Th2 skewing per se but some unexploited mechanisms may also contribute to the pathogenesis of the disease.

IL-10, which has been suggested to be induced by high levels of TNF-α (Ato *et al.,* 2002) may serve to control the tissue damage caused by TNF-α. But at the same time IL-10, secreted from macrophages, promotes parasite persistence by inhibiting macrophage activation (Murray 2002). IL-10 is a regulatory cytokine, presumed to be induced as a part of homeostatic network to protect tissue from collateral damage caused by excessive inflammation. Primarily it has suppressive effects on immune function, targeting multiple activation and antigen presentation pathways of macrophage and DC. It renders macrophage unresponsive to activation signals and acts on DCs causing them to down regulated CCR7 and thereby loosing migratory capacity preventing them from proper access of T cell areas and effective priming of T cell responses (Ato *et al.,* 2002). Moreover, treatment with pentavalent antimonials could inhibit IL-10 production leading to rapid granuloma formation and parasite killing with subsequent increase in IFN-γ production from Th1 cells. This is followed by enhanced expression of IL-12, NO and inducible nitric oxide synthase (iNOS) in infected tissue further enhancing effectiveness of the drugs (Buxbaum *et al.,* 2013; Murray *et al.,* 2003; Hailu *et al.,* 2004; Banerjee *et al.,* 2008).

IFN-γ, the most important cytokine for Th1 response, is likely to be produced in lymphoid organs where *Leishmania* proliferates. IFN-γ along with IL-12 has potential therapeutic efficacy and may enhance vaccine efficiency. Immunotherapy with IFN-γ/IL-12

prior to the infection restricts the early phase of the infection in CL (Ota *et al.* 2008). Vaccination against a progressive *L. donovani* infection could increase the production of IFN-γ, IL-12 and NO and downregulate IL-4 secretion thereby resulting in decrease of parasite burden in liver and spleen (Bhowmick and Ali 2009; Mazumdar *et al.,* 2004). Similar results were also obtained by IFN-γ immunotherapy manifesting an enhanced Th1 response accompanied with down regulation of disease promoting IL-4 and IL-10 (Bhowmick *et al.,* 2007).

IFN-γ may act as a double edged sword while it can stimulate macrophages and limit amastigote replication when coupled with lipopolysaccharides, it also promote amastigote survival and growth when coupled with TNF-α (Colmenares *et al.,* 2002; Qi *et al.,* 2004). Thus, IFN-γ may play a bidirectional role at the level of parasite-macrophage interactions. It has a protective effect against infection when optimally associated with other factors such as IL-12 and NO and in the absence of such synergy it promotes amastigote growth.

Accumulating reports from mouse model of nonhealing or disseminating forms of leishmaniasis has reinforced pathogenic mechanisms that take into account the presence of parasite-driven Th1 responses suppressed either in magnitude or function by IL-10 (Anderson *et al.,* 2009). In mice, activated *Leishmania* specific $CD4^+$ T cells are detected in draining LN as early as 16 h after infection. These cells rapidly expand and differentiate into cytokine secreting cells (Malherbe *et al.,* 2000). Majority of *Leishmania* specific $CD4^+$ T cells differentiate into Th1 cells that secrete IFN-γ and/or TNF-α which efficiently contribute to the parasite elimination through different mechanisms including the activation of macrophage. In contrast, genetically susceptible BALB/c mice show T cell response dominated by Th2 cells that secrete IL-4, IL-10, IL-5, and / or IL-13. IL-4 initiates induction of Th2 differentiation during progressive leishmaniasis in resistant mice but may display beneficial responses in *L. major* susceptible BALB/c mice. IL-4 is crucial in priming CD8+ T cells and protection against VL during vaccination. IL-4 has major role in differentiation and activation of CD4+ T cells, B cells and macrophage during infection (Biedermann *et al.,* 2001; Ghalib *et al.,* 1993; Stager *et al.,* 2003). Infection with *Leishmania* also results in the activation and expansion of parasite specific $CD8^+$ T cells. Upon infection expansion of T cells and their rapid recruitment to lymph nodes as well as the restriction of IL-13 and IL-10 production leading to higher IFN-γ/IL-10 ratio play an important role in protection against *Leishmania*.

Central *Versus* Effector Memory Cells

Memory cells have been broadly classified into two types-central and effector. Central memory cells home to primary lymphoid organs and proliferate to perform effector function only after secondary stimulation. In contrast, effector memory cells do not proliferate but secrete effector cytokines due to antigenic stimulation (Lanzavecchia and Sallusto 2005). Central memory $CD4^+$ T cell population generated during *L. major* infection is capable of developing into either Th1 or Th2 effectors. Under the influence of IL-12, *Leishmania* specific central memory $CD4^+$ T cells get differentiated into IFN-γ producing Th1 cells (Pakpour *et al.,* 2008). Low dose *L. major* promotes a transient Th2 response that is downregulated by IFN-γ producing $CD8^+$ T cells which may contribute to rapid resolution of secondary lesions (Uzonna *et al.,* 2004; Wang *et al.,* 1993). It has been reported that *L. donovani* infection augments vaccine induced immunity to *Listeria*, enhancing the host protective antigen specific memory $CD8^+$ T cell responses (Polley *et al.,* 2005). In VL, generation of antigen specific effector memory responses help in protection against the disease by proliferation and augmentation of $CD62L^-CD127^+$ $CD4^+/CD8^+$ T cells (Mazumder *et al.,* 2011a; Ravindran *et al.,* 2012; Roychoudhury *et al.,* 2011; Mazumder *et al.,* 2011b; Mazumder *et al.,* 2010; Bhowmick *et al.,* 2008; Mazumdar *et al.,* 2004). It appears that effector memory responses are more crucial than central memory response for protection against VL but further studies are required to clarify their roles.

Th17 Cells

It was Harrington *et al*., (2005) who first reported the presence of IL-17 (Harrington *et al.,* 2005) and classified Th17 subset as another member of CD4+T cell lineage. The development of Th17 cells as a distinct population from CD4+T cells is controlled by a combination of cytokines like IL-6, IL-21, IL-23, IL-1β and TGF-β (Acosta-Rodriguez *et al.,* 2007; Chen *et al.,* 2007; Liu and Rohowsky-Kochan 2008). IL-23 is essential for generation and establishment of IL-17 producing CD4+ T whereas TGF-β and IL-6 are required for their priming (Cruz *et al.* 2006, Bettelli *et al.,* 2006; Mangan *et al.,* 2006). Cytokine-driven activation of the signal transducer and activator of transcription (STAT) 3 pathway is an essential step in Th17 cell differentiation (Holland *et al.,* 2007). This leads to the activation of a cascade of transcription factors such as retinoid-acid receptor-related orphan receptor-γt (ROR-γt), chemokine (C-C motif) receptor 6 (CCR6), Interferon regulatory factor (IRF) 4, basic leucine zipper transcription factor (BATF), FOXP3, T-bet, peroxisome proliferator activated receptor (PPAR) γ, Fatty acid binding protein and suppressor of cytokine signalling (SOCS) protein (Hwang *et al.,* 2010). Among them CCR6 and ROR-γt are well studied markers for the identification of Th17 cells (Hirota *et al.,* 2007).

Initially reports from murine CL suggested the role of Th17 similar to Th2 responses as IL-17 was observed to synergise with TGF-β in increasing parasite load and disease pathology (Santarlasci 2009; Bacellar 2009; Cezario 2011; Manel 2008). But as studies progressed, Th17 response has emerged as an additive to Th1 response which promotes initiation of inflammatory response through neutrophil chemotaxis and NO production (Reinhard *et al.,* 2011; Dudeck *et al.,* 2011). This ultimately helps in reducing parasite burden in CL, PKDL, VL of mice and human. Other members of Th17 subset like IL-22 and IL-23, along with IL-17, also play a significant role in protection against different forms of leishmaniasis like VL, CL, MCL, American tegumentary leishmaniasis, PKDL and various other diseases like tuberculosis, lung infection and rheumatoid arthritis (Pitta *et al.,* 2009; Khader *et al.,* 2007; Katara *et al.,* 2012; de Assis *et al.,* 2013; Ghosh *et al.,* 2013). Suppression of IL-17 by IL-10 and IL-27 during *Leishmania* infection (Anderson *et al.* 2009; Gonzalez-Lombana *et al.,* 2013; Owens *et al.,* 2012) furthermore supports the association of IL-17 with disease alleviation. Additionally, exacerbation of *Leishmania* suppresses LPS induced IL-17 production due to rise in Th2 responses (Lapara NJ 3rd and Kelly 2010). IL-17 being a promoter of Th1 response has been used in a combination with vaccine and drugs for improving their effectiveness. Injection of recombinant IL-17 with curdlan enhances its therapeutic effect, thereby causing a marked increase in generation of IFN-γ along with NO and a significant suppression of the organ parasite burden during murine VL (Ghosh *et al.,* 2013). CpG DNA vaccine in the presence of live *L. major* causes activation of neutrophil which leads to specific induction of Th17 cells for IL-17 secretion leading to enhancement in the development of a protective cellular immunity against the parasite (Wu *et al.,* 2010). Even in the absence of Th1 response, Th17 shows some protection against leishmaniasis through an unconventional pathway for activating effector T cell responses (Pitta *et al.,* 2009; Soong *et al.,* 2012). In addition to Th17 cells, IL-17 is shown to be secreted from other cells like B cells, $CD8^+$ T cells, γδ T cells and neutrophils as well (Bermejo *et al.,* 2013; Li *et al.,* 2010). IL-17, secreted from $CD8^+$ cells is demonstrated to participate in the inflammatory response to mucosal leishmaniasis (Boaventura *et al.,* 2010).

It is intriguing to note that Th17 cells have close developmental link with $CD4^+$ $FOXP3^+$ regulatory T cells (Tregs). FOXP3 and ROR-γt can directly interact via a DNA-independent mechanism, and during Th17 cell development FOXP3 is transiently expressed (Zhou *et al.,* 2008). There are increasing evidences for the existence of $FOXP3^+$ cells that could secrete IL-17 (Ayyoub *et al.,* 2009; Kryczek *et al.,* 2011). Recent studies in murine CL advocate a role of CCR6 in Treg cell rather than Th17 cell recruitment during parasitic infections which depends on cell-mediated immune response as the predominant protective immune mechanism (Barth *et al.,* 2012). Further, it is shown that a balance

between Treg and IL-17 control hepatic immune responses. Increase in either of these two will lead to parasite susceptibility (Zhao *et al.,* 2010). But how these two populations are interacting with each other and whether their collective effect or individual contribution is crucial for disease regression could be a topic for future investigation.

Regulatory T Cells

Regulatory T cells are divided into "natural (or constitutive)" and "induced (or adaptive)" Treg cells, which may have complementary and overlapping functions in immune regulation. Natural Treg (nTreg) cells are developed in the thymus whereas inducible Tregs (iTregs) are derived from the periphery under the influence of DCs (Sakaguchi *et al.,* 2010) and can be either $CD4^{+}CD25^{+/-}$ or $CD8^{+}CD25^{+/-}$. IL-10 secreted from APCs such as DCs or macrophages in the periphery induce naïve T cells to become $FOXP3^{-}$ Treg cells. This subset of regulatory cells secretes IL-10 for active participation in immuno-modulation and termed as Tr1 cell (Groux *et al.,* 1997).

Additionally, TGF-β secreted from APCs can induce the transformation of naïve T cells to a TGF-β secreting Treg cells known as Th3 cells (Bilate and Lafaille 2012). These two sub sets (*viz*. Tr1 and Th3) of T cells are indispensable for maintaining immune homeostasis. Studies on the mechanism of action and phenomenology of different types of Tregs (Roncarolo *et al.,* 2006; Workman *et al.,* 2009) as well as immune regulation by other T cell populations like $CD8^{+}$ T cells (Chang *et al.* 2002), $CD4^{-}CD8^{-}$T cells (Zhang *et al.* 2000), γδ T cells (Hayday and Tigelaar 2003) could help us in understanding the mechanism involved in immunosuppression.

Treg cells are professed to use many cellular processes to control immunosuppression. Critical of them are two core phenomena known as bystander suppression and infectious tolerance. Suppressive activity of Treg cells requires their prior activation through their T cell receptors. Once potentiated, Treg cells can induce immuno-suppression in an antigen-nonspecific way called 'bystander suppression' (Qin *et al.,* 1993). Thus, Treg cells with one antigen specificity can suppress T effector cells with many other distinct antigen specificities (Wing and Sakaguchi 2010). By infectious tolerance, one population of suppressor T cells create a regulatory milieu that promotes the outgrowth of a new population of Treg cells with antigen specificities distinct from those of the original Treg population. This tolerant state induced by Treg cells is maintained even after loss or removal of the original Treg cell population (Qin *et al.,* 1993; Sakaguchi *et al.,* 2010; Tarbell *et al.,* 2007). Thus, through the processes of bystander suppression and infectious tolerance, Treg cells effectively maintain a state of stable tolerance and immune regulation.

Several examples are there demonstrating the immunosuppressive role of Treg cells in autoimmune and infectious diseases, such as arthritis (Chen *et al.,* 2013), type-1 diabetes (Ryba-Stanislawowska *et al.,* 2013), asthma (Nawijn *et al.,* 2013), HIV (Li *et al.,* 2013) and tuberculosis (Sharma *et al.* 2009; Ebinuma *et al.,* 2008). Studies of suppressive cells in leishmaniasis go back to early 1970s when Bryceson reported the emergence of antigen-specific immune suppression during *Leishmania* infection (Bryceson 1970). It was further extended by Arredondo *et al.* that the spleen cells from infected animal suppressed normal lymphocytes (Arredondo and Perez, 1979) and Howard *et al.* who tried to characterize suppressive T cell in murine models of *L. tropica* infection (Howard *et al.,* 1980; Howard *et al.,* 1981). Association of B cells with the development of suppressive T cell (Sacks *et al.,* 1987) and their active involvement against both the inductive and expressive phases of DTH response in CL were reported (Dhaliwal *et al.,* 1985). A potentially novel immunoregulatory role for $CD8^{+}$ T cells following DNA vaccination has been reported in the last decade (Gurunathan *et al.,* 2000). Our unpublished work on murine VL also indicates the existence of $CD8^{+}CD25^{+}FOXP3$ cells as potential regulatory cells through secretion of IL-10 during the development of the disease. $CD8^{+}$ cells eliciting IL-10 have been found to possess remarkable immunosuppressive activity in autoimmune and infectious diseases (Noble *et al.,* 2006). Since 1995 when Sakaguchi and colleagues defined $CD4^{+}$ $CD25^{+}$ cells as the most important population of regulatory cells (Sakaguchi

et al., 1995), the attention of studying Treg cells has been restricted mainly to this population. $CD4^+CD25^+$ regulatory T cells suppress both Th1 and Th2 cells and these regulatory T cells have a profound therapeutic potential against CL (Rodrigues *et al.,* 2013). Reports have recognized the involvement of $CD4^+CD25^+$ Tregs in progression of CL caused by both *L. major* (Belkaid *et al.,* 2002) and *L. amazonensis* in mice (Ji *et al.,* 2005) and *L. braziliensis* in humans (Campanelli *et al.,* 2006). Treg cells show suppressive role against human acute and chronic CL by way of secreting IL-10 and suppressing IFN-γ (Bourreau *et al.,* 2009). With healing of CL, the frequencies of $CD4^+CD25^{hi}CD127$ Treg cells increased, showing their contribution in the resolution of chronic dermal lesions (Tang *et al.,* 2008). During *L. infantum* infection in BALB/c mice, enrichment of $CD4^+CD25^+FOXP3^+$ Treg cells have been shown in the draining lymph nodes and spleen cells (Rodrigues *et al.,* 2009) suggesting their association with the disease. Involvement of $CD4^+CD25^+$ Treg cells in progression of human VL and PKDL has also been reported (Saha *et al.,* 2007; Katara *et al.,* 2011). In PKDL a correlation of nTreg cells both with IL-10 levels and parasite burden emphasize their role in disease severity (Katara *et al.,* 2011). Interestingly, reports from both human and murine CL suggest an association between increased frequencies of $CD4^+CD25^{hi}CD127$ Treg cells and resolution of dermal lesions (Saha *et al.,* 2007; Ji *et al.,* 2005) thereby indicating the contribution of Treg cells towards cure of CL.

Till date the role of Tregs as the primary source of IL-10 is controversial. It has been shown in human VL that rather than $FOXP3^+$ T cells, Tr1 cells are the major IL-10 producing cells and they are important in suppressing antileishmanial immunity and conditioning host macrophage for enhanced parasite survival and growth (Nylen *et al.,* 2007; Stager *et al.,* 2006; Anderson *et al.,* 2009; Maurya *et al.,* 2010). But recent reports advocate $CD4^+CD25^+FOXP3^+$ cells as the major source of IL-10 in human VL having high potential to suppress antileishmanial immune responses (Rai *et al.,* 2012). Similar observations have also been made recently by Tiwanathagorn *et al*., 2012 that $CD4^+FOXP3^+$ Treg cells induce IL-10 mediated *L. donovani* persistence in murine VL (Tiwananthagorn *et al.,* 2012).

Thus detailed investigations are required regarding the immunosuppressive role played by different regulatory T cell populations (viz., Tr1, Th3, nTregs, iTregs and other cells) and the source(s) of IL-10 for disease pathogenesis. Understanding the nature of the major immunoregulatory cells and their mechanism during leishmaniasis may not only unlock new avenues towards novel target but also lead to better intervention for disease cure.

Friends and Foes Among the Members of IL-12 Family

IL-12 family consists of heterodimeric cytokines, with several distinctive features. It has many molecular and functional characteristics that provide unique opportunities for positive and negative feedback control. Chain pairing promiscuity is a common feature of this group's member which includes IL-12, IL-23, IL-27 and IL-35 (Collison and Vignali 2008; Jones and Vignali 2011). Despite many structural similarities of these cytokines, their receptors and downstream signalling components, they have very different biological activities that challenge their symphony. IL-12 and IL-23 are mainly proinflammatory cytokines with key roles in the development of the Th1 and Th17 subsets of T cells, respectively (Kastelein *et al.,* 2007; Pflanz *et al.,* 2002, Langrish *et al.,* 2004). On the other hand IL-27 and recently identified IL-35 are known to play regulatory roles both in human and mice (Collision *et al.,* 2010; Wojno and Hunter, 2012; Murugaiyan *et al.,* 2009; Perona-Wright *et al.,* 2012; Collison, 2007; Vignali *et al.,* 2008). These observations establish a functionally balanced dichotomy in this family, with IL-12 and IL-23 being positive and IL-27 and IL-35 as negative regulators.

IL-12, the most extensively studied member of this family is a key pro-inflammatory cytokine and effective stimulant for the induction of an IFN-γ mediated Th1 response during VL. Phagocytic cells like monocytes, macrophages, neutrophils and DCs are the major source of IL-12 production (Scott P

2003; Chan *et al.,* 2006; dos Santos *et al.,* 2008; Zaph and Scott P 2003). IL-12 has a central role in generating strong Th1 response with elevation in IFN-γ level in both murine and human VL (Saha *et al.* 2007; Banerjee *et al.* 2008; Zhang *et al.,* 2013). Susceptibility to experimental *L. mexicana* infection was also attributed to IL-12 insensitiveness (Rodriguez-Sosa *et al.,* 2001). Similar observations were made in human CL as well where IL-12 unresponsiveness was reported to be responsible for the persistence of infection (Bourreau *et al.* 2001). With the progression of disease, Th1 response decreases and suppressive cytokines like IL-4, IL-10 and TGF-β increase promoting Th2 response. In the absence of IL-12, increase in parasite load is accompanied by decrease in IFN-γ, TNF-α and iNOS production (Adhikari *et al.,* 2012). During successful therapy and vaccination, IL-12 promoting Th1 response corresponds with increase in IFN-γ and NO production leading to downregulation of Th2 responses accompanied by reduction in parasite burden (Bhowmick *et al.,* 2009; Mazumdar *et al.,* 2004). It has also been reported that even in the absence of IFN-γ, IL-12 strongly promotes healing (Murray *et al.,* 2000; Adhikari *et al.,* 2012; Satoskar *et al.,* 2000) emphasising indispensability of IL-12 in the induction of protective immunity against VL.

Another important and widely studied member of this family is IL-27. It is an ongoing debate that whether IL-27 drives a Th1 response or has a pleiotropic effect on Th1, Th2 and Th17 as a key regulatory cytokine that controls the balance between immunity and pathology and their regulation (Anderson *et al.,* 2009; Hunter *et al.,* 2004). Both the hypotheses are supported by adequate evidences. IL-27 has been shown to be critical for differentiation and timely initiation of efficient anti-parasite Th1 immunity with enhanced IFN-γ during infection (Kamiya *et al.,* 2004; Takeda *et al.,* 2003; Zahn *et al.,* 2005). Potential contribution of IL-27 along with IL-17 in the control of *L. braziliensis* infection (Novoa *et al.,* 2011) by suppressing Th2 response (Yoshimoto *et al.,* 2007) has been shown. But recent studies suggest IL-27 as a protective cytokine which promotes antitumor immunity (Natividad *et al.,* 2013) as well as acts as a potent Th2 cytokine which promotes tumor growth by inducing Treg cell differentiation (Hunter and Kastelein 2012; Hall *et al.,* 2012; Murugaiyan and Saha 2013). Enhanced plasma level of IL-27 both in human and mice during healing and non-healing CL further support this hypothesis (Tolouei *et al.,* 2012).

In VL, significantly elevated levels of IL-27 at both mRNA and plasma of VL patients suggested it as a strong Th2 cytokine (Ansari *et al.,* 2011). Augmentation of IL-27 along with IL-21 leads to expansion of IL-10 thereby causing disease progression and parasite persistence. IL-27 has also been reported to be involved in mediating susceptibility to *L. donovani*, through interaction with its T cell cytokine receptor (Rosas *et al.,* 2006). Several recent studies have shown IL-27 to mediate anti-inflammatory activity through its ability to suppress Th17 cells (Batten *et al.,* 2006; Stumhofer *et al.,* 2006) and induction of IL-10 from activated $CD4^+$ T cells. It is now being accepted that IL-27 is critical for the generation of IL-10 producing $CD4^+$T cells *in vitro* (Awasthi *et al.,* 2007; Fitzgerald *et al.,* 2007; Stumhofer *et al.,* 2007), via signalling pathways dependent on STAT3. Optimal production of IL-27 requires co-ordination of c-Maf (Musculoaponeurotic Fibrosarcoma a proto-oncogene), inducible co-stimulator (ICOS) and IL-21 expression (Apetoh *et al.,* 2010). Emerging evidences also suggest that IL-27 may directly alter methylation patterns around the *il10* promoter in $CD4^+$T cells, thus allowing greater IL-10 expression (Hedrich *et al.,* 2010). IL-27 also favours the production of IL-10 by IFN-γ producing Th1 cells through an alternate signalling pathway that involves STAT1, STAT4 and Notch (Batten *et al.,* 2008; Rutz *et al.,* 2008).

IL-35, a recently identified member, produced by thymus-derived Treg cells has been reported to be one of the potent regulatory cytokine in autoimmune disease both in human and mice (Collison *et al.,* 2010; Collison *et al.,* 2007; Vignali Collison *et al.,* 2008). IL-35 association has been shown with atherosclerosis (Lin *et al.,* 2012), hepatitis B (Liu *et al.,* 2011); leukemia (Wu *et al.,* 2012), and tumor (Olson *et al.,* 2012; Wang *et al.,* 2013), but its

regulatory role in infectious diseases is yet to be established. Association of IL-35 with the progression of VL may lead to new targets for better intervention and control of the disease.

Modulation of Signalling Pathways by *L. donovani*

Leishmania dampens host cell immune response by modulating several key signalling pathways such as nuclear factor-κB (NF-κB) and janus kinase (JAK)-signal transducer and activator of transcription (STAT) dependent pathways. NF-κB has a critical role in initiating innate immune response and in protective Th1 mediated immunity. The *Leishmania* parasite has been observed to evade NF-Kb activation in infected macrophage and/or DC by promoting cleavage of its RelA sub unit. As a result production of pro-inflammatory cytokines and antimicobial mediators such as iNOS is inhibited that allows parasite survival and persistence within the host cell (Reinhard *et al.* 2012). *L. donovani* infection modifies NF-kB signalling by causing a specific cleavage of p^{65}RelA subunit. p^{35}RelA, a cleavage fragment of p^{65}RelA then migrates to the nucleus and binds to DNA as a heterodimer with NF-kB p^{50}. p^{35}RelA/p^{50} dimer alters the transcription of several pro-inflammatory cytokine and chemokine genes, thereby subverting macrophage's effector functions (Gregory *et al.* 2008). Modulation of JAK/STAT pathway by *L. donovani* attenuates IFN-γ dependent macrophage response. In addition, it downregulates IFN-γ protein receptor expression and upregulate a transient expression of SOCS3 protein, a negative regulator of IFN-γ signaling. Amastigote form of the parasite has been reported to hinder nulear translocation of STAT1α in response to IFN-γ and compromises STAT1α-nuclear transport adaptor importin-α5 interaction (Matte and Descoteaux 2010). Together, modulation of these two pathways results in downregulation of Th1 response with enhanced survivability of the parasite.

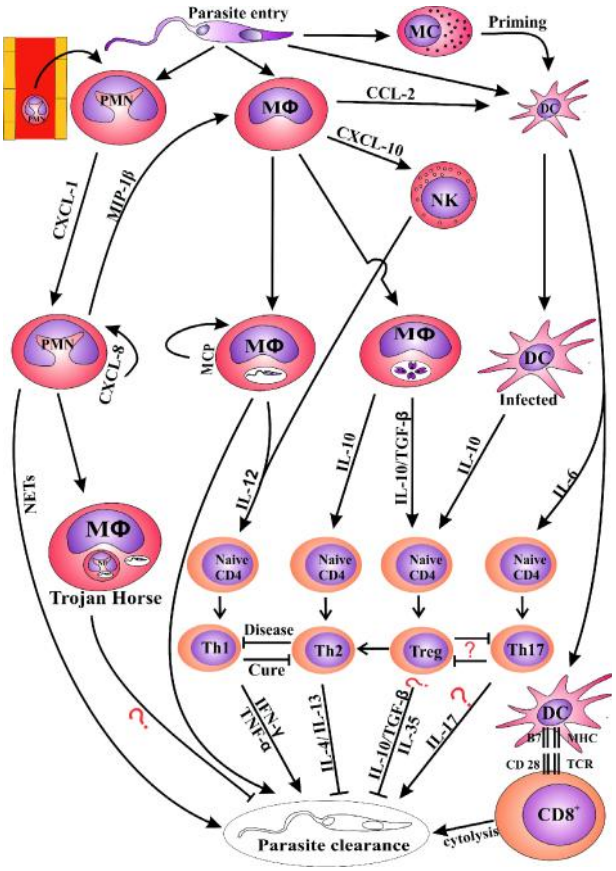

Fig. 1: Host immune response against *L. donovani*. With the parasite entry polymorphonuclear neutrophils (PMNs) and macrophages (MΦs) are recruited at the site of infection and promastigotes are engulfed into phagosomes. Neutrophils, secrete chemoattractants, CXCL-1, MIP-1β and CXCL-8 to engage more PMNs, MΦ and other immune cells. MΦ actively take up parasites either from fresh inoculum or released from PMN. They may also take up PMNs containing phagosytosed promastigotes surving as Trogan horses. Chemoattractants, CCL-2 and CXCL-10 secreted by MΦ activate DCs and NK cells respectively, for generation of various anti-leishmanial responses, such as NK cell-mediated IL-12 secretion, and mast cell (MC) primed DC-induction of $CD8^+$ cells for cytolysis. $CD4^+$ cells under the influence of IL-12, IFN-γ, IL-10 and TGF-β cytokines from MΦ and DCs differentiate to give rise to (i) IFN-γ and TNF-α secreting Th1 cells (ii) IL-17 secreting Th17 cells (iii) IL-4 and IL-13 producing Th2 cell and (iv) IL-10 and TGF-β producing Treg cells for parasite clearance/survival

Future Prospects

VL is a neglected tropical disease with a complex immune mechanism. It remains a challenge to understand its biology. Host immune defence mechanism, triggered by the bite of sand fly, recruits neutrophils and macrophages followed by NK cells and DCs for the phagocytosis of the parasite. But the mechanism involved in the establishment of *L. donovani* infection and how these responses fail to control the parasite propagation is not well

understood. Currently, the Trojan horse concept explaining parasite survival within the neutrophils during *L. major* infection could provide an answer, but contradictory reports exist regarding its role in protective response against infection. Moreover, there is no such report available for its involvement in VL. The understanding of the involvement of chemokines as chemoattractants and cytokines to abridge innate and adaptive immunity is important in *Leishmania* infection. Studying the role of these chemokines in clearing *L. donovani* parasites and modulating the immune response against the parasite may unravel future targets for immunotherapy. Among the cytokines, IFN-γ is crucial for priming protective Th1 responses, and along with IL-12, suppresses Th2 cytokines like IL-10 and IL-4. Nevertheless, there is a lacuna in the use of such knowledge for the development of contemporary approaches towards combating the disease. Although IL-10 is proven to be the principal cytokine involved in VL pathology, and the use of anti IL-10 antibodies remains one of the potent forms of immunotherapy against this disease, contradiction still exists regarding its source(s). Moreover, our understanding of the role of different immune cells and their mechanism of action against VL is still unclear. Although Tregs have emerged as the most important cellular subset responsible for disease pathogenesis, their nature and mode of action for regulating the immune response remains elusive. Recent research suggests Th17 as an additive to Th1 response, but to what extent this is true in controlling VL is unanswered. Involvement of TGF-β in both Th17 cell priming and development of Treg could also be looked upon as an important issue. Members of IL-12 family are exceptionally versatile, consisting of three α and two β chains, of which one α and one β chain combine to give highly diverse cytokines which could act in antagonism or synergism in the generation of Th1 response. Studying their unexploited combinations could lead to the discovery of new cytokine networks and prospective immunotherapeutic targets to combat the disease.

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