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Interleukin-10 and the pathogenesis of human visceral leishmaniasis

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The mechanisms underlying the failure to control the growth and systemic spread of *Leishmania* parasites in human visceral leishmaniasis (VL) are not well understood. Although the absence of antigen-specific Th1 responses in the peripheral blood mononuclear cells from VL patients is thought to be causally related to disease progression, the finding that these patients also express elevated interferon-γ mRNA in lesional tissue, as well as elevated serum levels of proinflammatory cytokines, suggests that their immunological defect cannot be explained simply by immune tolerance or Th2 polarization. As a possible homeostatic mechanism to control persistent infection-induced inflammation, elevated levels of the regulatory cytokine interleukin (IL)-10 have been reported repeatedly in clinical studies of VL. Here, we review the studies with relevance to immune responses in human VL and highlight the central role that IL-10 might have in the pathogenesis of VL and as a target for immune-based therapy.

Introduction

Leishmaniasis refers to a spectrum of vector-borne diseases caused by parasitic protozoa of the genus *Leishmania*. All *Leishmania* species are transmitted by phlebotomine sand flies as extracellular, flagellated promastigotes and replicate as intracellular, aflagellate amastigotes in mononuclear phagocytes in the mammalian host. The outcome of infection is dependent on the infecting species and the immune status of the host, ranging from subclinical infection to localized cutaneous diseases, to disseminated, visceral disease characterized by uncontrolled parasitization of the spleen, liver and bone marrow. Visceral leishmaniasis (VL) or kala-azar is caused by *Leishmania donovani* in Africa and on the Indian sub-continent and by *Leishmania infantum*/*chagasi* in the Mediterranean region, southwest and central Asia and South America.

Clinical presentation of VL typically involves long-term, low-grade fever, enlarged spleen and liver and weight loss. Laboratory findings often reveal pancytopenia and hypergammaglobulinemia. With time, untreated VL can cause severe cachexia, bleeding due to thrombocytopenia, increased susceptibility to bacterial infection and it leads ultimately to death in almost all untreated cases. The standard therapy for VL is pentavalent antimony or, in regions with a high prevalence of antimony resistance, such as India, amphotericin B. Both treatments have the disadvantages of prolonged parenteral administration (3–4 weeks) and toxicity. Liposomal amphotericin B can be effective at a lower dose administered over 1 week, but its cost is prohibitive. Newer drugs being evaluated currently include paromomycin and miltefosine, the latter being administered orally [1].

Immune responses in human VL

Mammalian host protection against leishmanial infection is dependent on the development of Th1-type immunity, which triggers enhanced leishmanicidal activity by infected macrophages. Based on extensive studies in mice, the production of interleukin (IL)-12 by antigen-presenting cells (APCs) and interferon (IFN)-γ by T cells appear to be required for control of the parasites and development of acquired resistance [2–8]. Although peripheral blood mononuclear cells (PBMCs) from humans with subclinical/asymptomatic infection (i.e. leishmanin skin-test-positive individuals, with no history of disease) respond typically to stimulation with leishmanial antigen with the production of IL-2, IFN-γ and IL-12 [9], a key immunological feature of VL is the inability of PBMCs to proliferate or to produce IFN-γ in response to leishmanial antigens [6,10]. However, there appears to be no inherent defect in antigen-induced Th1 responsiveness because cured individuals are resistant to re-infection, become leishmanin skin-test positive and mount antigen-specific IFN-γ responses *in vitro* [11,12]. Moreover, PBMCs from most patients are fully responsive to stimulation with purified protein derivative (PPD) or bacterial superantigen [10,13]. The impaired ability of PBMCs to respond to mitogenic stimulation associated with kala-azar is usually only evident in the later stages of the disease, at a point at which the frequency of T cells within the PBMC population has decreased significantly [14].

Non-curing and visceralizing forms of leishmanial disease have, based on extensive work in BALB/c mouse models of cutaneous leishmaniasis, been thought to be associated with a Th2-type immune response and, in particular, with early and sustained production of IL-4. Indeed, elevated levels of IL-4 and/or IL-13 have been associated with VL [13,15–17]. There is, however, increased production of multiple cytokines and chemokines in VL patients and much of the response appears to be proinflammatory, as indicated by the elevated plasma protein levels of IL-1, IL-6, IL-8, IL-12, IL-15, IFN-γ-inducible protein-10 (IP-10), monokine induced by IFN-γ (MIG), IFN-γ and tumor necrosis factor (TNF)-α [13,18–21].

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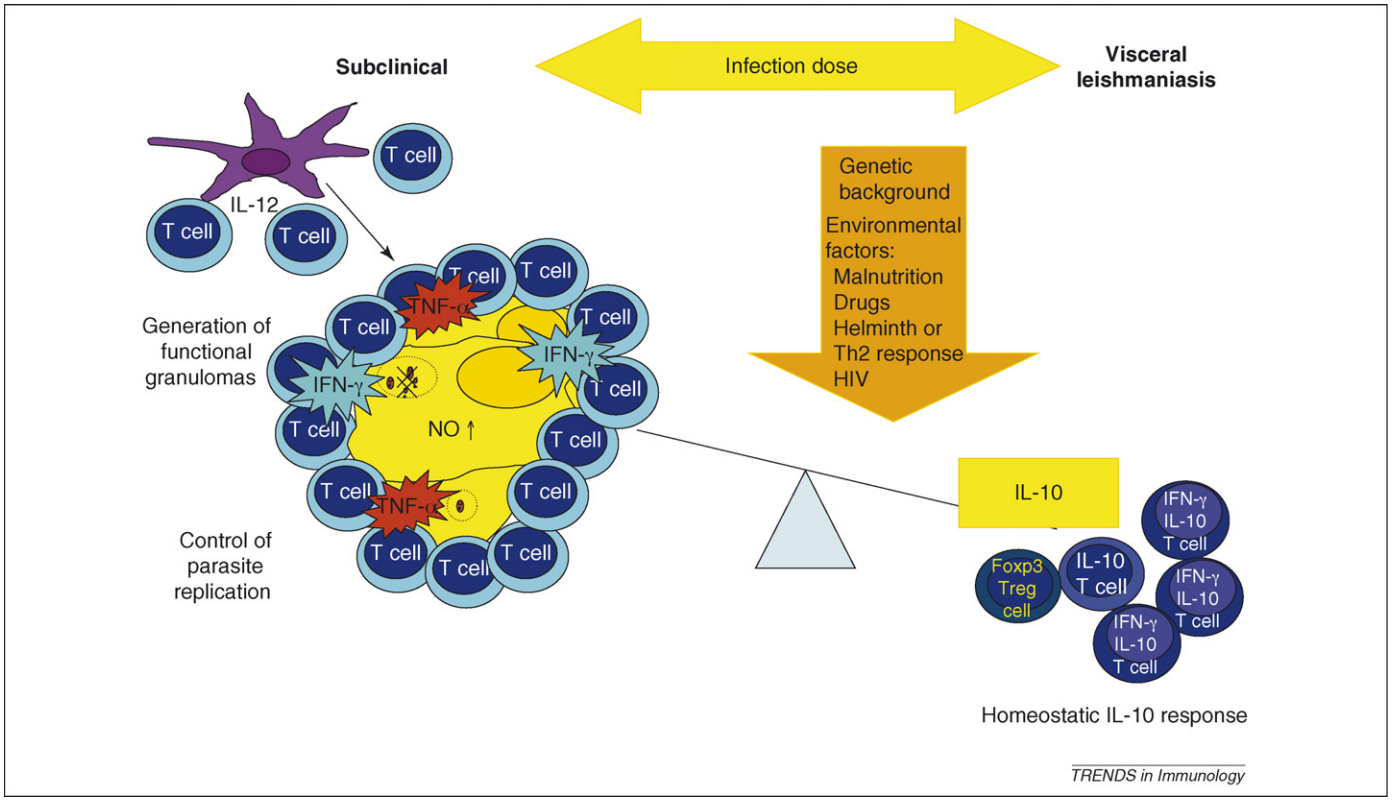

Figure 1. Induction of VL. In asymptomatic individuals (left), a Th1 type immune response is induced. Dendritic cells produce IL-12 and prime antigen-specific T cells to production of effector cytokines (IFN-γ, TNF-α). The parasite is controlled within functional granulomas (based on experimental models and the examination of asymptomatic dogs, which are natural reservoirs [25,39]). There is a balance between effector responses, which control the parasites, and regulatory cytokines and cells (e.g. IL-10, natural regulatory Foxp3 T cells), which limit collateral tissue damage. As a result of combinations of genetic and environmental factors (orange arrow), VL ensues when innate and/or acquired immune responses are inadequate to clear or control the infection (right). Persistent stimulation with parasite antigen induce high levels of pro-inflammatory cytokines, which in turn triggers anti-inflammatory control mechanisms, including differentiation and expansion of IL-10-producing T cells. The elevated levels of IL-10 promote progressive disease by the activities depicted in Figure 2.

Weight loss, which is a characteristic sequelae of kala-azar, is associated with the overproduction of the potentially catabolic cytokines IL-1 and TNF-α/cachectin. Furthermore, even during the acute phase of disease, elevated levels of IFN-γ mRNA have been found in lesional tissue, such as the spleen and bone marrow [13,22–24].

These findings suggest that unfavorable clinical outcomes are not related to Th2 dominance or a Th1-response defect *per se* but that other immunosuppressive or immune-evasion mechanisms contribute to the pathogenesis of VL (Figure 1).

Susceptibility to VL

The majority of people who become infected with visceralizing *Leishmania* spp. never develop disease. The factors that influence susceptibility to VL remain an area of intense interest but are still largely a matter of speculation. As mentioned, many of the cytokines measured at elevated levels in VL patients indicate that the immune system responds appropriately but that other factors render these responses inadequate to contain the infection effectively. There is some evidence to suggest that genetic and/or extrinsic influences might compromise the ability of macrophages to kill the parasites or impair the formation or maintenance of functional granulomas [25,26]. Studies have linked VL to a polymorphism in the *NRAMP1/SLC11IA* gene, which is involved in the ability of macrophages to limit the replication of intracellular pathogens, including *Leishmania* spp. [27], and also to polymorphisms in the *IL-2Rβ* gene, which regulates T-cell activation [28]. VL also tends to affect poor populations in whom nutritional status is often low and in whom helminth infections might be common, especially in rural settings, where VL is in fact prevalent. Malnutrition can impair both innate immunity and T-cell functions [29,30], and helminth exposures can alter the Th1/Th2 balance in favor of the *Leishmania* parasite [31,32]. In addition, after puberty, young and adult men are over-represented among VL patients [13,33,34], suggesting that hormonal changes could influence susceptibility. Experimentally, testosterone enhances parasite replication [35].

The regulation of TNF-α production appears to be important because it has a role in granuloma formation and maintenance [36], and anti-TNF-α treatment has resulted in the development of VL as a reactivation process in patients being treated for arthritis [37–39]. However, when produced at very high levels, TNF-α might have a disease-enhancing effect; one genetic study has identified a linkage between VL and a polymorphism in an allele associated with elevated serum TNF-α [40]. High levels of TNF-α might promote the generation of IL-10-producing T cells as a homeostatic response to excessive inflammation [41,42]. The role of IL-10 in VL pathogenesis will be discussed in depth below.

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IL-10 and immunosuppression in VL

The association of IL-10 and VL is now firmly established. Patients with active disease have elevated levels of IL-10 in serum as well as enhanced IL-10 mRNA expression in lesional tissue (Table 1). IL-10 is a regulatory cytokine that can be produced by T cells, B cells, macrophages, dendritic cells (DCs) and epithelial cells. It has pleiotropic, primarily down-modulating, effects on innate as well as acquired immune responses. IL-10 is thought to be induced as part of a homeostatic network that protects tissues from collateral damage caused by excessive inflammation [43,44]. This can be illustrated in critically ill patients with polymorphisms in the IL-10 promoter, resulting in low IL-10 production; these patients are more likely to die from sepsis compared with patients who produce high levels of IL-10 [45]. However, polymorphisms resulting in elevated levels of IL-10 have been associated with greater susceptibility to HIV and faster progression to AIDS [46]. Moreover, systemic treatment with IL-10 in patients with chronic hepatitis C virus (HCV), although shown to reduce liver fibrosis, also promoted viral replication and decreased the number of HCV-specific T cells [47]. Thus, although the high levels of IL-10 observed in VL patients might help to limit immune-mediated pathologies, especially in the liver, the immunosuppressive activities of the cytokine might promote parasite replication and disease progression (Figure 2).

Direct evidence for the role of IL-10 as a key immunosuppressive factor in VL is thus far based primarily on mouse models of VL, for which IL-10-deficient BALB/c and C57BL6 mice are highly resistant to *L. donovani* infection [48,49]. The main disease-promoting activity of IL-10 in VL might be to condition host macrophages for enhanced survival and growth of the parasite. IL-10 can render macrophages unresponsive to activation signals and inhibit killing of amastigotes by down-regulating the production of TNF-α and nitric oxide [50,51]. Amastigote replication is promoted in IL-10-treated human macrophages [52] and we reported recently that the IL-10 in serum from VL patients enhances parasite replication in human macrophages and that blocking IL-10 reduced parasite growth [13].

IL-10 also suppresses multiple antigen-presentation functions of DCs and macrophages. It inhibits the maturation of DCs from monocyte precursors, downregulates MHC II and co-stimulatory molecules and, perhaps most importantly, inhibits IL-12 production [44]. The antigen-specific unresponsiveness in PBMCs from VL patients with respect to T-cell proliferation and IFN-γ production has, in South American and Sudanese studies, been observed to be reversed by treatment with anti-IL-10 antibodies [6,22,53]. It is not clear from these studies, however, which cells were proliferating or the source of IFN-γ. Because the numbers of patients evaluated were few, the concentration of antibody used was extremely high or the study lacked adequate controls, the effect on recovery of antigen-specific T-cell responses is not so clear. In our own studies of a large series of VL patients from India, we were unable to detect antigen-driven IL-10 production by PBMCs or to recover an antigen-specific response with anti-IL-10 treatment [13]. Further studies are clearly needed to elucidate whether antigen-specific T cells are present but suppressed in active VL, if they are lost and/or never generated appropriately or if they are recruited to the sites of infection and are therefore not detectable in the peripheral blood.

Based on analyses of splenic aspirates, T cells are the main leukocyte subsets in the spleen of VL patients during the early stages of active disease [13,54], in contrast to healthy donors in whom B cells dominate [13,55]. This might be a consequence of the selective recruitment and/or expansion of T cells in the spleen. Clinical studies indicate that there is a selective loss of memory T cells from the peripheral compartment because alterations in the CD45RA:RO ratio of $CD4^+$ T cells has been observed

Table 1. Clinical studies associating visceral leishmaniasis with IL-10

Tissue examined	Findings	Origin of patient	Refs
Spleen	Elevated mRNA pre-treatment compared with post-treatment and healthy spleen cells	India	[13]
Plasma	Elevated compared with endemic controls. VL plasma stimulated amastigote replication, which was reversed by anti-IL-10 treatment	India	[13]
PBMCs	No effect of anti-IL-10 treatment	India	[13]
Blood cells	Higher number of IL-10^+ $CD8^+$ and IL-10^+ $CD19^+$ cells	Brazil	[67]
Serum	Elevated levels compared with asymptomatic and 'non-infected'		
Serum	Elevated compared with subclinical, preclinical (developed VL within 1 year) and patients treated recently	Bangladesh	[20]
Serum	Elevated pre-treatment compared with post-treatment and controls	India	[18]
Plasma	Elevated pre-treatment compared with post-treatment	Brazil	[4]
Plasma	Elevated pre-treatment compared with post-treatment, asymptomatic, malaria patients and endemic controls	Ethiopia	[56]
PBMCs	Detected in γδ T cells	Italy	[93]
Serum	Elevated pre-treatment compared with post-treatment	Brazil	[19]
Spleen	Elevated mRNA pre-treatment compared with post-treatment	India	[24]
Serum	Elevated pre-treatment compared with post-treatment	Italy	[94]
PBMCs	Neutralization of IL-10 enhanced IFN-γ production by acute VL PBMCs Recombinant human (rh)IL-10 blocked IL-12p40 by convalescent VL PBMCs	Sudan	[6]
PBMCs	Elevated mRNA expression pre-treatment compared with post-treatment Anti-IL-10 treatment enhanced IFN-γ production and proliferative response in acute VL PBMCs	Brazil	[53]
Lymph node	Elevated mRNA pre-treatment compared with post-treatment		
PBMCs	Anti-IL-10 treatment enhanced proliferative response in acute VL PBMCs rhIL-10 blocked proliferation of convalescent VL PBMCs	Sudan	[22]
Bone marrow	Elevated mRNA pre-treatment compared with post-treatment and healthy donors	Sudan	[23]

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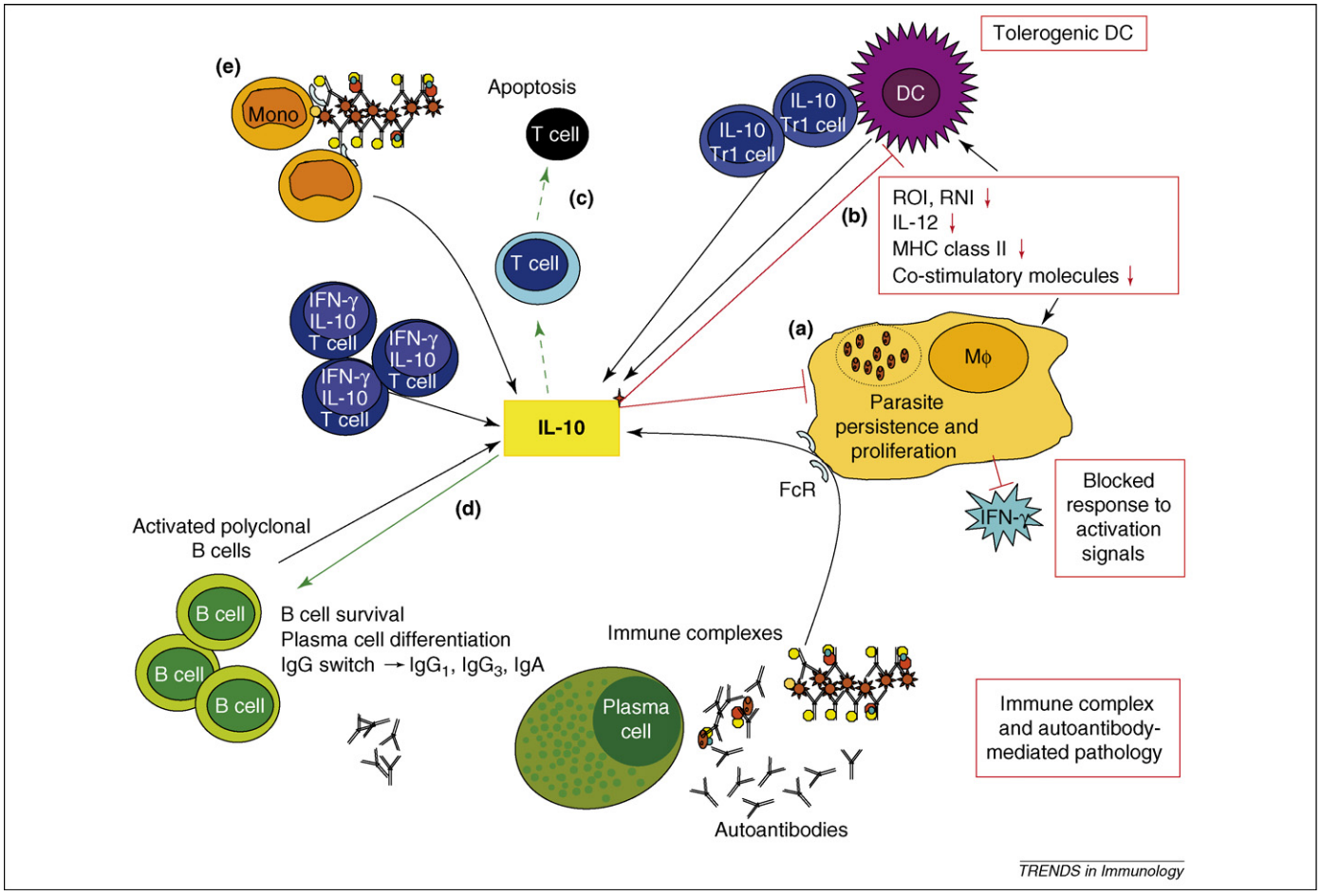

Figure 2. IL-10 and VL pathology The actions of IL-10 might contribute to VL pathology in multiple ways. **(a)** IL-10 renders macrophages unresponsive to activation signals (i.e. IFN-γ) and inhibits the induction of reactive nitrogen and oxygen metabolites (RNI and ROI). **(b)** In antigen-presenting cells (DCs and macrophages), IL-10 down-regulates the expression of MHC class II, co-stimulatory molecules and IL-12, which will inhibit the effective generation and/or maintenance of antigen-specific Th1 cells. Moreover, IL-10 inhibits DC maturation and migration [41] and can induce tolerogenic DCs, which produce IL-10 and promote the generation of IL-10-producing Tr1 cells. **(c)** IL-10 might also contribute to enhanced activation-induced T-cell death and **(d)** promote B-cell survival and plasma-cell differentiation. As disease progresses, B cells, which might also be a source of IL-10, and antibodies could be important contributors to VL pathology because self-reactive antibodies and immune-complex deposition might cause tissue damage. **(e)** Moreover, immune complexes can stimulate the macrophages and monocytes to produce IL-10 as well as proinflammatory cytokines (e.g. IL-6, TNF-α), a loop that will promote the generation of more immune complexes and more IL-10. Black arrows indicate sources of IL-10; the red lines indicate blocking/down-modulating activities of IL-10; the green arrows indicate differentiation/apoptosis promoted by IL-10. Abbreviations: ROI, reactive oxygen intermediates; RNI, reactive nitrogen intermediates.

[13,54,56]. This might in part be owing to sequestration of $CD45RO^+$ CD4 T cells in lesional tissue. In mice, both heterologous T cells and those with specificity for the parasite accumulate in the VL spleen [57]. The specificity of splenic T cells that accumulate in the human VL spleen has not been addressed to date. By contrast, the cytokine responses of these cells have been profiled recently and will be discussed below.

Regulatory T cells in VL

The expansion or accumulation of IL-10-producing regulatory T cells (Tregs) is associated with a number of chronic infections, including HCV, HIV and *Leishmania* [43]. Several IL-10-producing T-cell subsets have been described, including the $CD4^+CD25^+Foxp3^+$ T cells and $CD4^+CD25^-Foxp3^-$ T cells, variably referred to as natural or endogenous Tregs, and adaptive or Tr1 cells, respectively [58]. Natural Tregs are generated during their thymic development, whereas adaptive Tregs emerge following an encounter with antigen in the periphery. In murine cutaneous leishmaniasis, natural Tregs modulate the development of effector responses and prevent complete elimination of *L. major* parasites from the skin after clinical cure [59]. Natural Tregs, capable of producing large amounts of IL-10, accumulate in the skin of humans infected with *L. braziliensis* [60]. It cannot be out ruled that $CD4^+CD25^+Foxp3^+$ Tregs contribute to immune suppression during the earliest stages of infection, however, extensive analyses of blood and splenic aspirates from Indian VL patients do not support a major role for natural Tregs during overt disease. Natural Tregs were not elevated in the blood and did not accumulate at the site of infection (spleen) in active VL cases; no changes in frequencies were observed with treatment. Moreover, antigen-specific IFN-γ responses were not rescued by depleting the PBMCs of $CD25^+$ cells [13]. Importantly, the high levels of splenic IL-10 mRNA was found primarily in the $CD25^-$FoxP3 $mRNA^{low}$ T cells, which accumulate in the spleen of VL patients. Thus, parasite-driven adaptive regulatory/Tr1 cells [61] appear to be more important than natural Tregs in the suppression of anti-*Leishmania* immunity in human VL. In line with these clinical findings,

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in *L. donovani*-infected mice, IL-10 production by splenic $CD4^+CD25^-$ T cells correlates strongly with disease progression [62].

In two recent articles [63,64], it was demonstrated that infection-driven IFN-γ-producing Th1 cells are a crucial source of IL-10. IL-10 production by antigen-specific Th1 cells was necessary and sufficient to suppress immunity to a non-healing form of *L. major* in conventionally resistant mice, and, in *Toxoplasma gondii*-infected mice, the cells were required as a protective feedback mechanism to prevent lethal inflammation. Because splenic $CD25^-$ T cells from VL patients have elevated expression of both IFN-γ and IL-10, it is possible that at least some are IL-10–IFN-γ double producers, activated in a strong inflammatory setting as a mechanism of feedback control. Simultaneous production of IFN-γ and IL-10 by human T cells clones (both CD4 and CD8) can be induced by IL-12 [65], which is elevated at systemic levels in VL patients [13].

A role for $CD8^+$ T cells as an important source of IL-10 is suggested in patients with HCV, in whom less fibrosis was detected in those areas of the liver where virus-specific IL-10-producing CD8 T cells had accumulated [66]. Limitations in the amount of splenic material that can be obtained from VL patients have so far precluded the separation of CD4 and CD8 T cells for analysis. Nonetheless, a recent study found that the number of $IL\text{-}10^+$ CD8 T cells in the peripheral blood was higher in VL patients compared with asymptomatic or non-infected donors [67]. Moreover, CD8 clones generated from patients with active VL produced IL-10 and suppressed effector T cells isolated from the same patient after cure [68].

IL-10 and polyclonal B-cell activation in VL

IL-10 also promotes B-cell survival and plasma-cell differentiation. Thus, not surprisingly, overproduction of IL-10, polyclonal B-cell activation and hypergammaglobulinemia tend to go hand in hand in many systemic human diseases, including VL. IL-10 also promotes IgG isotype switch towards IgG_1 and IgG_3, a feature that has been observed in human VL patients [4,69]. Apart from being useful in diagnosis of disease, the antibody response in VL patients does not appear to be of benefit to the patient. There are several clinical case reports of autoantibody production and immune complex formation in VL patients [70–73]. Signs of autoreactivity, such as vasculitis [74,75] and decreased renal function [76,77], have been observed. Furthermore, there is a negative correlation between anti-leishmanial IgG and delayed-type hypersensitivity (DTH) responses [78]. Experimental models of VL indicate that B cells and antibodies contribute to parasite persistence because B-cell-deficient mice have enhanced resistance to *L. donovani* infection [79]. Circulating antibodies and Fc receptors have a crucial role in the pathogenesis of non-healing lesions due to *L. mexicana* complex parasites in mice [80,81] and immune complexes precipitated from VL serum induce higher levels of IL-10 and other proinflammatory cytokines in PBMCs compared with control sera [70]. Moreover, immune complex formation contributes to high level IL-10 production by both human and mouse macrophages [78,82].

Thus, although T cells appear to be the main source of IL-10 during the early stages of disease, the evolution of high antibody titers and immune complex formation in VL might drive higher IL-10 production by macrophages and other cells, contributing to the progressive decline in the immune status of VL patients and to the fatal outcome in untreated cases. Because IL-10 promotes activation-induced death mediated by Fas-ligand in the PBMCs from systemic lupus erythematosus (SLE) and HIV patients [83,84], it is interesting to speculate that IL-10 might have a role in promoting T-cell death in VL. In fact, accelerated rates of apoptosis have been observed for peripheral blood T cells in patients with VL [85], as well as in experimental models of VL [86]. Spleen cells from VL patients express elevated levels of Fas and FasL [87]. This could enhance the subsequent systemic loss of T cells and contribute to immune suppression in VL. As the disease progresses, plasma cells and macrophages become the more predominant cell subsets found in the affected organs. Bone marrow aspirates from VL patients showed an increased frequency in phagocytic cells and plasma cells [88] and parasite loads correlated with the increase in the plasma cells [89]. Postmortem examination of spleen and lymph nodes from VL patients revealed a reduction of the white pulp owing to loss of small lymphocytes. This was accompanied by an accumulation of infected histiocytes and plasma cells both in red and white pulp and paracortical areas of the lymph node [90].

IL-10: a target in future VL therapy

Treatment of VL remains unsatisfactory and there is an urgent need to develop new therapies to reduce drug toxicity and long-term hospitalization and also to prevent drug resistance. Successful treatment of kala-azar is thought to depend, at least in part, on alterations in the host immune response to the parasite, therefore, direct manipulation of the immune response, either alone or in combination with drugs, might be a useful strategy for improving treatment regimens for VL. The accumulated clinical and experimental data discussed here support IL-10 as a target for VL therapy. By inhibiting IL-10, the balance between effector and regulatory cytokines can be shifted and thereby the length of treatment and/or the dose patients receive can be reduced. Neutralizing IL-10 would enable infected macrophages to respond appropriately to IFN-γ and thereby promote the killing of parasites and it would also enhance APC function. In addition, IL-10 blockade might promote the generation and/or survival of effector T cells and prevent the pathological consequences of B-cell responses. Experimental models of VL have demonstrated the potential benefits of IL-10 inhibition clearly. Treatment of mice with an anti-IL-10 receptor antibody promotes the rapid control of *L. donovani* infection and enhances the leishmanicidal activity of pentavalent antimony dramatically [91]. The benefit of IL-10 neutralization in a clinical setting has been shown in SLE, an immune complex disorder, in which anti-IL-10 treatment decreased disease activity and restored impaired T-lymphocyte function [92].

Although the pathogenesis of visceral leishmaniasis appears to be a result of, at least in part, a shift in the

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balance of effector and regulatory factors in favor of the latter, efforts to control infection by immunological manipulations have focused on the former (e.g. therapeutic vaccination with antigen or treatment with IFN-γ). By targeting IL-10 or its cellular sources transiently, the aim is to unleash the host's own immune response, an approach that might find application in other chronic infections.

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