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Immunity and immunosuppression in experimental visceral leishmaniasis

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Abstract

Leishmaniasis is a disease caused by protozoa of the genus *Leishmania*, and visceral leishmaniasis is a form in which the inner organs are affected. Since knowledge about immunity in experimental visceral leishmaniasis is poor, we present here a review on immunity and immunosuppression in experimental visceral leishmaniasis in mouse and hamster models. We show the complexity of the mechanisms involved and differences when compared with the cutaneous form of leishmaniasis. Resistance in visceral leishmaniasis involves both $CD4^+$ and $CD8^+$ T cells, and interleukin (IL)-2, interferon (IFN)-γ, and IL-12, the latter in a mechanism independent of IFN-γ and linked to transforming growth factor (TGF)-ß production. Susceptibility involves IL-10 but not IL-4, and B cells. In immune animals, upon re-infection, the elements involved in resistance are different, i.e., $CD8^+$ T cells and IL-2. Since one of the immunopathological consequences of active visceral leishmaniasis in humans is suppression of T-cell responses, many studies have been conducted using experimental models. Immunosuppression is mainly *Leishmania* antigen specific, and T cells, Th2 cells and adherent antigen-presenting cells have been shown to be involved. Interactions of the co-stimulatory molecule family B7-CTLA-4 leading to increased level of TGF-ß as well as apoptosis of $CD4^+$ T cells and inhibition of macrophage apoptosis by *Leishmania* infection are other components participating in immunosuppression. A better understanding of this complex immune response and the mechanisms of immunosuppression in experimental visceral leishmaniasis will contribute to the study of human disease and to vaccine development.

Key words
- Visceral leishmaniasis
- Immunosuppression
- T cell
- Cytokines
- Mice
- Hamster

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Introduction

Protozoa of the genus *Leishmania* cause cutaneous, mucocutaneous or visceral diseases in man depending on the species of the parasite and the host immune response. There are many different species of *Leishmania* causing different clinical manifestations, mainly in the New World. Visceral leishmaniasis is caused by *Leishmania* (*Leishmania*) *donovani* in the Old World, by *L.* (*L.*) *infantum* in the Southeast of Europe and

Mediterranean area and by *L.* (*L.*) *chagasi* in the New World, including Brazil. A spectrum of clinical manifestations occurs in visceral leishmaniasis, from asymptomatic or oligosymptomatic disease to progressive disease with severe manifestations such as hepatosplenomegaly, fever, pancytopenia, and hypergammaglobulinemia (1).

While extensive information is available about the immune response in experimental cutaneous leishmaniasis, the nature of immunity in experimental visceral leishmaniasis, which is different in many aspects, is poorly understood. In order to develop vaccines for different forms of leishmaniasis, and since there are many areas where different species and different forms of the disease overlap, a detailed knowledge of the particularity of the immune response and pathogenesis is extremely important. In the present review on immunity and immunosuppression in experimental visceral leishmaniasis we present the many important advances made in the last three decades and the complexity of the mechanisms involved. We show that there are marked differences in immunity between experimental visceral leishmaniasis and cutaneous leishmaniasis. Since updated and comprehensive reviews on immunity in cutaneous leishmaniasis are available (2), here we will mention this form of the disease only when pertinent for comparison.

Since one of the immunopathological consequences of active visceral leishmaniasis in humans is suppression of the T-cell response, many studies on experimental models are available in this field, which will be covered in the last part of this review.

Resistance and susceptibility in experimental visceral leishmaniasis

Throughout the review, when we compare data on experimental cutaneous leishmaniasis that we briefly summarize here, we do it mainly referring to the most extensively studied models of cutaneous leishmaniasis which involve certain isogenic mouse strains infected with *L.* (*L.*) *major*. In this model a paradigm was established linking a subpopulation of $CD4^+$ T cells producing mainly interferon (IFN)-γ (T helper 1 cells = Th1 cells) to resistance, and $CD4^+$ T cells producing mainly interleukin (IL)-4 and IL-10 (T helper 2 cells = Th2 cells) to susceptibility. The importance of $CD8^+$ T cells has only recently been appreciated. The importance of IFN-γ is stressed in the mechanism of resistance as both an efficient activator of macrophages and as a factor directing the differentiation of non-differentiated T helper cells to Th1 cells with participation of IL-12 and natural killer (NK) cells, and a T-box transcription factor T-bet playing a central role in this process. Conversely, IL-4, IL-13 and IL-10 are linked to susceptibility or persistence of infection, with the latter cytokine being linked to the $CD4^+CD25^+$ regulatory T-cell activation (see Ref. 2 for detailed information).

The data reviewed here are based on two models. The most widely studied model of visceral leishmaniasis is the BALB/c strain of mice infected with *L.* (*L.*) *donovani* or *L.* (*L.*) *chagasi*. Although this strain is considered to be susceptible and the infection progresses during the first two weeks, the infection is then controlled by the host immune response (3). As mentioned above, human visceral leishmaniasis presents a spectrum of clinical manifestations from a self-controlled infection to a progressive disease. The mouse model is comparable to self-controlled oligosymptomatic cases and therefore is useful for the study of the protective immune response. Alternatively, the better model to study the progressive disease is hamsters infected with *L.* (*L.*) *donovani* or *L.* (*L.*) *chagasi* that develop a disease similar to human progressive visceral leishmaniasis with hepatosplenomegaly, hypoalbuminemia, hypergammaglobulinemia, and pancytopenia (4). Therefore, this model is mainly used to study the mechanisms of immuno-

suppression that we will present in the final section.

Role of T lymphocyte populations

In human and experimental leishmaniasis immunity is predominantly mediated by T lymphocytes (2). Initial studies on mice using T cell-depleted mice (5) and nude BALB/c mice (3) have shown the importance of T lymphocytes for protection against *L. donovani* infection. Adoptive transfer of T cells, immune to *Leishmania* antigen, conferred resistance against *L. donovani* infection (6). Reconstitution experiments using nude BALB/c mice, and cell depletion experiments in euthymic mice using monoclonal anti-CD4 or anti-CD8 antibodies showed the necessity of both $CD4^+$ and $CD8^+$ T cells in the protection against *L. donovani* infection (7). In *L. donovani*-infected BALB/c mice, there was a time course-related participation of different cell populations: $L3T4^+$ ($CD4^+$) cells are important in the initial two weeks of infection, when the parasite replication is still occurring, mainly in the formation of hepatic granulomas, but later this cell population decreases and is replaced by Lyt 2^+ ($CD8^+$) cells when the progress of the infection is controlled (8). However, other studies point to the differences in the participation of immune elements in the protection observed in immune animals upon re-infection compared with those in naive animals with a primary infection. In immune BALB/c mice, depletion of Lyt 2^+ but not $L3T4^+$ abolishes resistance. Furthermore, granuloma formation is not affected by depletion of Lyt 2^+ or $L3T4^+$. Conversely, cyclophosphamide A treatment abolishes granuloma formation but does not interfere with parasite replication (9).

Role of cytokines

T lymphocytes participate in the immune response to *L. donovani* infection by producing different cytokines. While euthymic *L. donovani*-infected BALB/c mice are able to control infection with granuloma formation and IFN-γ and IL-2 production, nude BALB/c mice neither form granulomas nor produce IFN-γ (3). Human recombinant IFN-γ restores the ability of nude BALB/c mice to control *L. donovani* infection. Furthermore, anti-IFN-γ antibody abolishes granuloma formation (10), confirming the importance of this cytokine in protection. Moreover, depletion experiments using anti-IL-2 monoclonal antibodies and reconstitution using recombinant IL-2 showed a role for IL-2 in leishmanicidal activity apparently through the induction of IFN-γ, and in granuloma formation with involvement of $L3T4^+$ and Lyt 2^+ T cells (11). Results in immune mice differ from those described for naive animals. Neutralization of IL-2 but not IFN-γ abolishes resistance, and granuloma formation is not affected by neutralization of IL-2 or IFN-γ (9).

The role of IL-4 as a cytokine related to susceptibility has been recently questioned in experimental cutaneous leishmaniasis (2), but most studies on experimental visceral leishmaniasis had raised this question about the role of IL-4 in susceptibility from the beginning. In one study, besides predominant IFN-γ production in the initial and late phase of infection, IL-4 production was detected in the intermediary phase coinciding with peak of parasite burden in the susceptible strain, and no IL-4 production in the resistant mouse strain (12). Nevertheless other studies contradicted these findings. No IL-4 or IL-5 production was observed in three different strains of mice infected with *L. donovani* (13), and in the liver, only IFN-γ RNA was detected by Northern blot, and both Th1 and Th2 cytokine mRNAs, IL-4, IL-10, IFN-γ and IL-2 mRNA were detected by PCR (14). Furthermore, mice treated with anti-IL-4 monoclonal antibodies (14) and mice with IL-4 gene disruption (15) did not show better control of the infection. IL-10,

another Th2 cytokine, however, was related to progressive disease in human visceral leishmaniasis (16) and was shown to have a role in susceptibility in experimental visceral leishmaniasis. A progressive increase in IL-10 mRNA level in tissues during infection suggested a role in susceptibility (17). In addition, the control of parasite growth in inner organs in BALB/c IL-10$^{-/-}$ mice and in normal mice with IL-10 receptor blockade by antibodies confirmed the role of IL-10 in susceptibility (18,19). Since IL-10 receptor blockade increased serum IFN-γ levels, a protective effect was initially attributed to the non-suppressed leishmanicidal effect of IFN-γ. However, suppression of parasite growth with IL-10 receptor blockade even in IFN-γ gene-disrupted mice suggested a broader effect of IL-10 on the suppression of multiple leishmanicidal mechanisms (20). We should emphasize, however, that visceral leishmaniasis in mice is a self-controlled infection; therefore IL-10 is probably not responsible for uncontrolled progressive increase in the parasite burden, which does not occur in this model. Its role should rather be considered similar to that in cutaneous leishmaniasis in resistant strain of mice, in which important findings have been recently obtained. In *L. major*-infected resistant C57BL/6 mice, IL-10 was shown to be important for the persistence of the parasite in the lesion, preventing its complete clearance from the lesion despite the presence of a protective immune response (21). Furthermore, this apparently undesirable persistence of the parasite was shown to be of the utmost importance for the maintenance of protective immunity against re-infection, with CD4$^+$CD25$^+$ regulatory T cells with IL-10-dependent and IL-10-independent mechanisms probably involving transforming growth factor (TGF)-ß being involved in the suppression of IFN-γ production (22,23).

In contrast, IL-12 was shown to be linked to protection against the infection. IL-12 treatment of *L. donovani*-infected BALB/c mice significantly reduced the parasite burden with the participation of CD4$^+$ and CD8$^+$ T cells, NK cells and IFN-γ, IL-2 and tumor necrosis factor (TNF)-α (24). But a distinct antimicrobial effect of IL-12, independent of IFN-γ, was also demonstrated in experiments using IFN-γ gene-disrupted mice. These mice, as expected, show a progressive infection for the first eight weeks but in the late phase develop a capacity to reduce the parasite burden with the participation of TNF-α induced by IL-12 (25). Neutralization of IL-12 with anti-IL-12 monoclonal antibody (26) or IL-12 deficiency (IL-12$^{-/-}$) (27) shows the fundamental role of this cytokine in the control of infection in susceptible mice. Furthermore, increased levels of TGF-ß were observed in IL-12$^{-/-}$ mice and were further increased in IL-12/IFN-γdouble knock-out mice, without expansion of the Th2 response (28).

Role of B cells and immunoglobulins

Polyclonal B cell activation is present both in human and experimental visceral leishmaniasis (29,30), but the actual role of B cells or immunoglobulins in the immunity in visceral leishmaniasis has been poorly evaluated. Most of the data in this area have been obtained with cutaneous leishmaniasis models, where resistance was observed with depletion of B cells using anti-IgM antibody (31) or in BALB xid mice, lacking B-1 B cells (32), and susceptibility was increased by transfer of B cells (33) or administration of B-cell hematopoietic factor, IL-7 (34). In visceral leishmaniasis, enhanced resistance was recently shown in mutant mice that lack mature B cells (35).

To distinguish the effect of B cells from that of immunoglobulins on susceptibility, experiments were performed using mice genetically altered to contain no circulating antibody, with or without functional B cells, and mice defective in Fc receptor. These studies showed that the circulating antibody is crucial for susceptibility to the develop-

ment of cutaneous leishmaniasis (36). Furthermore, amastigotes from the lesion of cutaneous leishmaniasis were shown to be coated by IgG, and internalization of immunoglobulin-coated amastigotes by macrophages was shown to lead to IL-10 production and consequent enhancement of intracellular parasite growth *in vitro* (37). Similar mechanisms might be acting in visceral leishmaniasis.

Immunosuppression in experimental visceral leishmaniasis

One of the immunopathological consequences of active visceral leishmaniasis in humans is suppression of the T-cell responses mainly to *Leishmania* antigen (38). Although the *L. donovani*-infected mouse is not a good model for the study of immune suppression, negative *Leishmania* antigen-induced delayed-type hypersensitivity can be observed coinciding with the peak of parasite burden in the susceptible mouse strain (39) and therefore some studies have been conducted using this model. However, the better model to study this aspect is hamsters infected with *L.* (*L.*) *donovani* or *L.* (*L.*) *chagasi* that develop progressive visceral leishmaniasis. We have studied immunosuppression in *L.* (*L.*) *chagasi*-infected hamsters and have observed a concanavalin A-induced lymphoproliferative response in all experimental periods but the total absence of a *Leishmania* antigen-induced response (40). In the literature, the *Leishmania* antigen-induced response was found to be suppressed in all studies (4,41), but there was disagreement about the concanavalin A-induced response. Some studies showed that the response was preserved during the experiment (4,29) while others did not observe a response after 42 days of infection (41). Antigen-specific T-cell anergy present during active disease recovers after treatment and cure (42).

Various factors have been reported to cause immunosuppression in studies using either mouse or hamster models. Studies on mice have indicated T cells (43) and others Th2 cells and adherent cells (39) as being responsible for suppression. Macrophage-mediated suppression is reported to lead to increased parasite growth and to be linked to either defective antigen presentation, suppression of class I and class II major histocompatibility complex molecule expression or mediation by prostaglandin-like substances (44-46). In *L.* (*L.*) *donovani*-infected hamsters, adherent splenic cells have been shown to be important in the suppression of lymphoproliferation and in defective antigen presentation (4). TGF-ß produced by adherent antigen-presenting cells from infected hamsters was implicated in immunosuppression since a high level of TGF-ß was observed in the cell culture supernatant when the *Leishmania* antigen-induced lymphoproliferative response was inhibited (47). We have studied the effect of another growth factor, insulin-like growth factor-I, and we have shown its effect on *in vitro Leishmania* growth but also on enhancement of the lesion in cutaneous leishmaniasis (48). More recent data suggest that it is a suppressor factor of macrophages leading to the decreased production of nitric oxide in *Leishmania*-infected macrophages *in vitro* (49). As mentioned in a previous section, cytokine IL-10 has been studied as a susceptibility factor in cutaneous leishmaniasis, but in our opinion it should also be considered within the context of immunosuppression. The absence of data in this field may be due to the difficulty to study hamsters, which is the most appropriate model, for which no reagents for cytokines are available. Only recently RT-PCR primers for some cytokines have been developed (50), and using these primers, no qualitative change in the expression of different cytokine RNA was observed during visceral leishmaniasis in hamsters (50), suggesting the necessity to develop a more sensitive method for evaluation such as quantitative PCR.

On the basis of elements shown to participate in suppression, such as T cells and TGF-ß and possibly IL-10, it becomes attractive to speculate on the possible participation of $CD4^+CD25^+$ regulatory cells in immunosuppression during visceral leishmaniasis.

Another aspect addressed in a number of studies of immunosuppression is the initial interaction between antigen-presenting cells and T cells. Decreased expression of costimulatory molecules B7-1 (46) and Th1-specific M150 protein (51) in antigen-presenting cells has been associated with immunosuppression. However, apparently paradoxical were the data observed with the blockade of B7-1 or B7-2 molecules that led to restoration of T-cell response and to increased IFN-γ and IL-4 production and parasite clearance, respectively, in *L. chagasi*- and *L. donovani*-infected mice (52,53). The use of different ligands for B7 molecules, searched in sequence, explained this contradiction since there are two receptors for the B7 molecules, CD28 for T-cell activation and CTLA-4 for termination of T-cell activation. Indeed, blockade of CTLA-4 has led to the recovery of resistance against infection, suggesting expression of the CTLA-4 molecule during visceral leishmaniasis (53,54). Furthermore, it has been shown that the effect of CTLA-4 linkage resulted in the production of TGF-ß, a factor that favors parasite growth within macrophages (55). All of these data demonstrate a role of CTLA-4 in immunosuppression, favoring parasite growth, but there are other reports showing its role in the development of a Th1 response in *Leishmania major* infection in mice transfected with the CTLA-4 gene (56). These paradoxical findings were elucidated in a review showing a dual role of the CTLA-4 molecule with activation of Th1 cells when T cells involved were naive, but with activation of Th2 cells when memory cells were involved (57). This dual role of the CTLA-4 molecule is a crucial point to be further analyzed in an eventual study aiming at vaccine development. We should also emphasize the importance of TGF-ß in susceptibility and immunosuppression since recent data have indicated it as one of the most important factors, maybe a determinant factor, leading to Th2 development through inhibition of T-bet in leishmaniasis (23).

Apoptosis of T cells has been reported in experimental visceral leishmaniasis. More than 40% of $CD4^+$ T cells from susceptible but not from resistant mice undergo apoptosis, accompanied by a significant decrease in IL-2 and IFN-γ secretion, and unaltered IL-4 secretion during *L. donovani* infection, findings that were also related to immunosuppression (58). In addition, apoptosis was detected in inflammatory cells in the liver and spleen during *L. donovani* infection, but when the role of CD95-CD95 L was assessed using CD95 ligand-deficient mice, increased parasite growth, but no effect on apoptosis, was observed in the CD95 ligand-deficient mice, suggesting a role of CD95 L in the control of parasite growth that is independent of host cell apoptosis (59). Since apoptosis of host lymphocytes may have a role in immunosuppression leading to parasite growth, we addressed this question in the hamster model. We observed apoptosis of inflammatory cells in the liver and spleen of *L. chagasi*-infected hamsters that was induced by *Leishmania* antigen stimulation in the early period of infection. We have not seen so far a direct time-related correlation with the *Leishmania* antigen-induced suppression of the lymphoproliferative response since apoptosis was present in the initial phase of the infection and the suppression of the lymphoproliferative response throughout the experimental period (40). Based on these observations, we can speculate on the occurrence of selection of the lymphocyte populations, with apoptosis of *Leishmania* antigen-specific lymphocytes in the initial phase and survival of nonspecific ones in the later phase. This may explain the absence of a *Leishmania* antigen-specific lymphopro-

liferative response throughout the study period but the presence of apoptosis only in the initial phase. These are some of the scattered lines of evidence about the role of apoptosis in immunosuppression that are still indirect and incomplete and that should be explored in the future.

Macrophages are not only the habitat of *Leishmania* but also the main cells involved in the leishmanicidal process. Survival of *Leishmania* depends on the integrity and supply or proliferation of macrophages in the lesion, besides a suppression of the leishmanicidal machinery. It was shown that *in vitro* infection of macrophages by *Leishmania* renders them resistant to apoptosis (60). We studied this phenomenon *in vivo* in hamsters with visceral leishmaniasis and observed that apoptosis is induced in macrophages by *L.* (*L.*) *chagasi* infection in the initial phase. However, as the infection progresses, apoptosis of macrophages disappears from both the liver and spleen, suggesting protection of macrophages by *Leishmania* infection (40).

Soluble factors other than immunoglobulin have been involved in immunosuppression. Inhibition of concanavalin A-induced lymphoproliferative responses of splenic cells was seen in the presence of serum obtained during the acute phase of visceral leishmaniasis infection. Increased serum triglyceride levels were detected 60 days after infection and the suppression was abolished when serum was delipidated (41).

Concluding comments

The immunological responses induced during experimental visceral leishmaniasis are markedly different from those induced in cutaneous leishmaniasis. Although the amount of data is still modest when compared with that for cutaneous leishmaniasis, the studies have disclosed interesting facets of the immune response, even contradicting some dogmas such as the role of IFN-γ in protection. These particularities should be studied in order to understand how different species of *Leishmania* by determining different forms of disease can generate such different immunological responses. Furthermore, detailed analysis of the differences is very important in view of the fact that the ultimate goal in the fight against any endemic parasitic disease including leishmaniasis, is the development of an efficient vaccine that is effective in leishmaniasis caused by different species of *Leishmania*.

Identification of the differences is the initial step directed at the development of an efficient vaccine for leishmaniasis, and further extensive studies are needed to identify parasite- or host-related factors leading to these differences. Parallel studies on human visceral leishmaniasis become imperative also to compare and identify differences related to the host. In this context, studies of the mechanisms of immunosuppression can also contribute to a better understanding of this undesirable consequence of *Leishmania* infection, and indicate a type of immune response that should be avoided when induced by a candidate vaccine, for example directing the enrollment of CTLA-4 molecule in the activation of Th1 cells, and avoiding responses leading to IL-10 and TGF-ß production. Furthermore, the understanding of the mechanisms of immunosuppression, if proven similar in patients, can also contribute to the planning of their treatment. In patients resistant to conventional treatment or in those who present recurrences, like HIV/*Leishmania* co-infected subjects, eventual interference blocking the effects of IL-10 and TGF-ß may be considered in the future.

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