Published in final edited form as:

Eur J Immunol. 2010 October ; 40(10): 2816–2829. doi:10.1002/eji.201040384.

Murine model of chronic *L. (Viannia) panamensis* infection: Role of IL-13 in disease

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Abstract

Leishmania (Viannia) organisms are the most prevalent etiologic agents of human cutaneous leishmaniasis in the Americas. Nevertheless, our knowledge of the immunological mechanisms exploited by *L. (Viannia)* organisms remains limited and the mechanisms underlying disease are not well understood. Here, we report the development of a BALB/c mouse model of *L. (V.) panamensis* infection that is able to reproduce chronic disease, with persistent infection and clinically evident lesions for over 1 year. The immune response of the mouse resembles that found for *L. (V.) panamensis*-infected patients with chronic and recurrent lesions, presenting a mixed Th1/Th2 response with the presence of TNF- α , IFN- γ , IL-10 and IL-13. Using immunodeficient mice, the critical role for IL-13 and/or IL-4R α in determining susceptibility to chronic infection was evident. With the induction of healing in the immunodeficient mice, increases in IFN- γ and IL-17 were found, concomitant with parasite control and elimination. Specifically, increases in CD4⁺ (but not CD8⁺) T cells producing IFN- γ were observed. These results suggest that IL-13 represents an important target for disease control of *L. (V.) panamensis* infection. This murine model should be useful to further understand the pathology associated with chronic disease and to develop methods for the treatment and prevention of leishmaniasis caused by *L. (Viannia)* parasites.

Keywords

Chronic infection; IL-13; Leishmaniasis; *Leishmania (Viannia)*; Murine model

Introduction

The infection by organisms of the genus *Leishmania* in humans causes a broad spectrum of clinical manifestations ranging from asymptomatic and cutaneous to more serious, disfiguring and fatal conditions that can be classified as diffuse cutaneous, mucocutaneous and visceral leishmaniasis. The human disease is found throughout the world, affecting 21 nations in the Americas and 66 nations in Africa, Europe and Asia. The prevalence of the disease is approximately 12 million, with a population at risk of approximately 350 million

(http://www.who.int/leishmaniasis/burden/magnitude/burden_magnitude/en/index.html) [1, 2].

Although organisms belonging to the *Leishmania* (*Leishmania*) and *L. (Viannia)* subgenus are the etiologic agents of human leishmaniasis, the latter is the most frequent cause of human disease in the Americas [3]. Further, the characteristic human immune response across the spectrum of infection with *L. (Viannia)* organisms is marked by mixed cytokine response (Th1/Th2) with concomitant production of IFN- γ , IL-13, IL-10 and TNF- α [4–8]. Despite the public health importance and the distinct immune response to infection by organisms of the *L. (Viannia)* subgenus, we have a limited understanding of the mechanisms of disease and disease-associated pathology. Currently, our knowledge and insights regarding the immunological mechanisms that potentially drive and sustain the infection are primarily based on the human studies [4–12]. These studies are essential to our understanding but are limited in terms of the ability to explore the early phases of infection and the impact of various cell populations and mediators on disease.

The apparently poor infectivity of *L. (Viannia)* species in mice has been a major factor limiting the use of a murine model, where a self-healing transient lesion has been consistently observed [13–16]. Despite this, several authors have successfully employed the murine infection model to explore mechanisms leading to disease exacerbation; using genetic approaches, several elements/mechanisms that confer immunological resistance, including IFN- γ , IL-12, STAT4, TNF- α and iNOS have been identified.

However, a mouse model of chronic disease, as can occur in humans, has remained elusive; nevertheless, the genetic and immunological tools available for the mouse make this an advantageous model. Previous studies by Neal and Hale [17], found that BALB/c mice are susceptible to a high inoculum (10^7) of *L. (V.) panamensis* promastigotes. Therefore, we focused on the development of a chronic infection model for *L. (V.) panamensis* using numbers of parasites that would more closely approximate infection in nature. The development of infective promastigotes for infection was monitored using mAb that recognize Ag that are upregulated in the amastigote stage [18]; the inoculation of 5×10^4 organisms generates chronic lesions in BALB/c mice with persistent parasites at site of the infection and draining LN (>1 year). Throughout infection, a mixed cytokine response (IFN- γ , IL-13, IL-10 and TNF- α), consistent with cytokine profiles of infected individuals and representative of the spectrum of outcomes of infection (asymptomatic, chronic and recurrent) is observed. Our results indicate that IL-13 is critical to disease pathogenesis, as BALB/c mice genetically deficient in either IL-4R α or IL-13 were resistant to infection. Increased IFN- γ and/or IL-17 was observed in with the onset of healing in the resistant mice, suggesting a role in healing. These findings support a pivotal role for IL-13 in the regulation of *L. (V.) panamensis* infection; consequently, IL-13 signaling may represent a target for immunotherapeutic intervention.

Results

Amastigote Ag expression of *L. (V.) panamensis* promastigotes is associated with infectivity

Parasites of the *L. (Viannia)* subgenus are known to be poorly infectious to most laboratory strains of mice [13–16]. In initial experiments, we were able to establish infection in BALB/c mice using the $1-2 \times 10^7$ stationary promastigotes of several strains of *L. (V.) panamensis* as reported previously [17]. However, only 25–30% of the mice developed chronic lesions (data not shown), suggesting an inconsistent infection and low levels of infective organisms. A number of parameters, previously shown to lead to the development of infective promastigotes for other *Leishmania* species, including parasite resistance to complement

lysis and medium pH were evaluated. However, these parameters did not consistently correlate with persistent/chronic lesion development.

Consequently, an alternate approach was taken. Infective organisms (metacyclic) begin biochemically to resemble the amastigote stage, expressing molecules that ultimately allow for its survival within the mammalian host. Consequently, in order to assess the process of metacyclogenesis or development of infectivity of *L. (V.) panamensis*, we decided to utilize the expression of amastigote-specific Ag recognized by previously characterized mAb [18]. The mAb were used with *L. (V.) panamensis* organisms at distinct differentiation stages: (i) promastigotes from midlog phase, (ii) gradient-purified late stationary phase promastigotes and (iii) axenic amastigotes. A small fraction (1.9–4.1%) of gradient purified live promastigotes from late stationary phase and all axenic amastigotes (as expected) expressed the Ag (Supporting Information Fig. 1). Consistent with the observations of the development of *L. (V.) panamensis* promastigotes within the sand fly vector [19], the promastigotes recognized by the mAb did not appear to have a unique morphology. Optimal development of infective organisms occurred between 15 and 21 days in culture and correlated with the initiation of amastigote Ag expression.

Therefore, for experimental infections, promastigotes (5×10^4) were isolated from percoll gradients [20] after 20 days in culture and used for infection. This procedure generated reproducible/consistent infections with development of chronic lesions lasting for more than a year (Fig. 1A), with amastigotes present at the site of infection and draining LN (Fig. 1B). Appearance of average lesions in the infected BALB/c mice at 3 wk and 10 months post-infection is shown in Fig. 1C. In contrast to the unrestrained cutaneous disease of BALB/c mice by other species of *Leishmania*, lesion development appeared to plateau (50–60 days post-infection; lesion ratio, 1.7:2.1) and was maintained (1 year post-infection). Further, lesions sizes contrast to those found in the BALB/c mouse with *L. amazonensis* or *L. major* [21, 22], which at 10 wk post-infection already have ratios of 1.9:2.6 and continue to progressively develop. Consequently, although parasites persist, chronic disease caused by *L. (V.) panamensis* appears self-limited.

Although infection could be established with tenfold fewer parasites, the course of lesion development was slower (Supporting Information Fig. 2); consequently, 5×10^4 parasites were routinely used for infection. Metastases to the spleen and liver were also detected; however, this occurred in a portion of the infected mice. This is consistent with the recent observations, indicating that *L. (Viannia)* parasites can be detected at sites distal from the lesions, with little to no overt pathology [23–25].

Therefore, these results appear to differ from findings for the species *L. (V.) braziliensis* in mice, where, in general, lesions have been found to resolve spontaneously. Further study is required, however, to determine whether these apparent differences of disease development in mice reflect species variation or alternately differences in culture and isolation of infective organisms.

Human response to *L. (V.) panamensis* infection

Natural infection with *L. (V.) panamensis* produces a wide range of clinical outcomes ranging from asymptomatic infection to recurrent or chronic lesions, including mucocutaneous leishmaniasis [5, 26]. PBMC stimulated with promastigote Ag secreted a mixed Th1/Th2 profile of cytokines with significantly increased production of IL-10, IL-13, IFN- γ and TNF- α in patients with chronic or recurrent leishmaniasis (Fig. 2A) compared with asymptotically infected individuals and controls. However, IL-4 was not detected. Notably, these cytokines were also induced, though at a lower level, in PBMC from asymptomatic individuals. Hence, the mixed cytokine profile is found across the spectrum of

outcomes of human infection by *L. (V.) panamensis* and is more pronounced in individuals who have had chronic and recurrent disease.

Regression analysis showed that Th1 and Th2 cytokine production were positively correlated for infected individuals, Fig. 2B. However, the putative clinically susceptible (chronic and recurrent disease) and resistant (asymptomatically infected) individuals were distinctly distributed with susceptible individuals presenting concomitantly elevated Th1 and Th2 cytokines and a significant correlation between IFN- γ and IL-10 production. Overall, these results demonstrate concomitant increases in inflammatory–anti-inflammatory cytokines in response to *L. (Viannia)* infection.

Immune response of *L. (V.) panamensis*-infected mice parallels that found in human infection

To assess the immune response of the BALB/c mouse to infection, *in vitro* cytokine production to *L. (V.) panamensis* promastigote Ag (pLAg) stimulation was evaluated. The production of IL-10, IL-13 and IFN- γ was observed in both the acute phase and the chronic phase (Table 1) in accordance with what is observed for human infection. Notably, the IL-4 levels were low until late in infection (Fig. 3).

Additionally, the changes in the LN cell populations in response to infection were examined, as lymphadenopathy is a common feature in the early stages of human disease caused by *L. (Viannia)* [27]. Chronic lesions were accompanied by lymphadenopathy in the draining (inguinal and popliteal) LN with an overall 3.4-fold increase in cell number by 3.5 wk post-infection. Analysis of the cell populations in the draining LN by flow cytometry indicated an expansion of CD4⁺, CD8⁺ and CD19⁺ cells in the draining LN (Table 2). However, overall the *T/B* ratio decreased from 3.3 to 1.3, indicating a preferential increase in the B-cell population. B-cell expansion continued throughout the chronic phase of the disease, with *T/B* ratio of 0.9 found at 4 months post-infection. These results are similar to what has been observed for LN cell populations in the late phase of human disease caused by *L. (Viannia)* [27] as well as in murine models of leishmaniasis [28, 29]. Interestingly, in the case of murine leishmaniasis, B cells have been found to contribute to disease exacerbation.

Consequently, the chronic infection mouse model of *L. (V.) panamensis* appears to mimic the characteristic cytokine and cellular responses observed in human disease. Given the mixed cytokine response (IFN- γ and IL-13), and B-cell expansion during chronic lesion development, the regulation of chronic infection (cytokines, B cells) was examined.

B cells in murine *L. (V.) panamensis* infection

B cells have been shown to be important in the development of the disease caused by *L. (L.) amazonensis*, *L. (L.) pifanoi*, *L. (L.) donovani* and some strains of *L. (L.) major* [22, 30, 31]. As the draining LN CD19⁺ compartment expands during the *L. (V.) panamensis* infection, we further evaluated the role of B cells in the development of chronic infection using BALB/c J_HD mice, which are deficient in B cells. Overall, in initial phase of infection (first month) the J_HD mice had similar lesion development to that observed for WT BALB/c mice. Subsequently, lesion progression is slower in the J_HD mice, with lesions being significantly smaller at 50 days post-infection, when lesion development plateaued in the WT BALB/c mice. Nevertheless, lesion development in the J_HD mice continued to increase and by 4 months post-infection no significant difference was observed (Fig. 3A). Furthermore, parasite burdens although lower in the J_HD mice at 50 days post-infection were not significantly different from the WT BALB/c mice (Fig. 3B). As expected, the parasite levels in lesions and LN at 4 months post-infection were indistinguishable between the two groups of mice (Fig. 3C). These results suggest that B cells can contribute to disease

pathology (lesion development), but do not impact significantly on parasite burden. This differs from what has been observed in the murine model with other species of *Leishmania*, where B-cell Ig and Ag presentation foster disease exacerbation [22, 30, 31].

IL-13 and/or IL-4R α play major roles in disease development and susceptibility

As IL-13 has been linked to susceptibility to *Leishmania* infection in mice [32–34] and appeared to be a major Th2-like cytokine produced in human infection (above), we investigated the role of IL-13 in the *L. (V.) panamensis* infection. BALB/c (WT or deficient in IL-13 or IL-4R α) were infected and lesion development and immune responses monitored. The lesion development in both the IL-13 $^{-/-}$ and the IL-4R α $^{-/-}$ mice was significantly reduced when compared with WT BALB/c mice (Fig. 4A). At 9 wk post-infection, the lack of lesions reflected a complete elimination of the parasite (foot and DLN) of the IL-4R α $^{-/-}$ mice. Parasites were significantly reduced in the IL-13 $^{-/-}$ mice, with a 1.5×10^4 -fold reduction in parasite numbers in comparison to WT mice (Fig. 4B and C); only 25% of the IL-13 $^{-/-}$ mice examined had detectable levels of parasites.

Given the control of parasitemia observed, we analyzed the immune response of WT BALB/c as well as the IL-13 $^{-/-}$ and IL-4R α $^{-/-}$ mice (Fig. 5) at three time points: (i) early in infection (day 10 post-infection); (ii) when the parasite burdens are relatively similar but disease is beginning to resolve in the IL-13 and IL-4R α -deficient mice (3.5 wk post-infection) and also upon (iii) resolution of disease (2 months post-infection). Lymphocytes from infected mice were stimulated *in vitro* with either concanavalin A or with pLAg. Interestingly, at 3.5 wk post-infection (beginning of disease resolution), the absence of IL-13 or IL-4R α enhances IFN- γ or IL-17 production. No differences in the levels of TNF- α were observed. Although both CD4 $^{+}$ and CD8 $^{+}$ T cells were found to produce IFN- γ (Fig. 6) in both WT and immunodeficient mice in response to infection, the increased level of IFN- γ observed was due to a preferential expansion of CD4 $^{+}$ IFN- γ $^{+}$ T cells in the immunodeficient mice ($p < 0.03$); no similar increase in the relative levels of CD8 $^{+}$ IFN- γ $^{+}$ T cells was observed.

Notably, the levels of IL-10 also increased in the IL-13 $^{-/-}$ and IL-4R α $^{-/-}$ mice at 3.5 wk post-infection as healing is developing, with higher levels occurring than for the WT BALB/c mice. However, overall the ratios of IFN- γ /IL-10 were comparable in the nonhealing BALB/c and healing (IL-4R α $^{-/-}$ and IL-13 $^{-/-}$) mice (IFN- γ ng/IL-10 pg of 0.54–0.48). At other times, post-infection, comparable levels IL-10 were observed for both the WT and the immunodeficient mice. Consequently, IL-10, in the absence of IL-13, does not appear to be sufficient for disease development and parasite persistence. Nevertheless, infection in IL-10-deficient BALB/c mice presented reduced parasite burden clearly, indicating that this cytokine also has a key role in disease outcome (Supporting Information Fig. 3). Consequently, although IL-10 alone is insufficient for disease, the absence of IL-10 can lead to healing. Interestingly, in the absence of IL-10, no changes in the IL-13 response were observed; however, increased levels of IL-17 were found. These results suggest that the lack of IL-10 or IL-13 and/or IL-4 signaling leads to increased level of the T_H1/T_H17 responses and disease resolution. Further, IL-13/IL-4R α signaling in this context appears to prevent an expansion of CD4 $^{+}$ IFN- γ $^{+}$ T cells essential to generate disease resolution.

Discussion

Clinical studies indicate that the human response to infection with *L. (Viannia)* parasites [35] differs from that which occurs upon infection with members of the *L. (Leishmania)* subgenus [36–39]. Given the public health importance of disease caused by *L. (Viannia)* parasites and the facts that asymptomatic infection as well as persistence after treatment [23, 25] and disease reactivation are known to occur [40], an understanding of the

immunological mechanisms underlying disease is essential. However, the lack of a suitable small mammalian model for the chronic form of disease caused by *L. (Viannia)* parasites has restricted experimental approaches to elucidating the mechanisms contributing to susceptibility to infection/disease. In this article, we report the development of a chronic infection model for *L. (V.) panamensis* that requires a relatively low-dose inoculum. Further, the immunological features were evaluated in the context of the main aspects of human *L. (V.) panamensis* infection to determine the general similarities and disparities between the model and the human disease and to understand mechanisms underlying susceptibility that could potentially guide improvements in treatment and/or vaccine development.

Development of chronic infection was dependent on the expression of amastigote-specific Ag [18] by stationary phase *L. (V.) panamensis* promastigotes. As metacyclic promastigotes differentiate from procyclic promastigotes and prepare to survive inside the mammalian host, the upregulation and expression of molecules that are highly selectively expressed in the amastigote stage have been observed [41, 42]. After 20 days in culture, a consistent but low percentage of the organisms ($3.2 \pm 1.1\%$ or $1.6 \pm 0.6 \times 10^3$ promastigotes/infection) expressed amastigote Ag. Consequently, it is possible that infection was established with fewer parasites. Whether these amastigote molecules are involved in the virulence of the parasite is currently not known, but will be of interest for future studies utilizing this model.

Infected mice display lymphadenopathy, with the number of amastigotes found in the draining LN proportional to the level found in the lesion. In human infection, lymphadenopathy is observed in the case of *L. (V.) panamensis* infection [43] and is one of the earliest (and sometimes the sole) clinical signs of infection caused by *L. (V.) braziliensis* [44, 45]. Accompanying the cellular expansion of the DLN upon infection, a selective expansion of B cells was observed, with an inversion of the *T/B* cell ratio. Although an expansion of the B-cell compartment upon infection is not unexpected, B-cell infiltration at the site of infection and increased Ab titers have been correlated with pathology development in the human infection with *L. (V.) panamensis* [46–49] and *L. (V.) braziliensis* [27]. Moreover, in murine models for leishmaniasis, B cells (Ab production and APC functions) have been demonstrated to be important in disease caused by infection with other *Leishmania* organisms [22, 30, 31]. Indeed, the absence of B cells resulted in delayed lesion development in the case of *L. (V.) panamensis* infection; however, the reduction in parasite numbers either at the site of infection or within the DLN was not significant. However, the plateau in lesion development observed during the chronic disease phase could mask the overall importance of the B-cell contribution to disease susceptibility (as clearly the lesion development is delayed in the B-cell-deficient mice). Overall, these results suggest that although B cells can contribute to disease pathology caused by *L. (V.) panamensis*, their role may not be as significant as found for other species of *Leishmania*.

Of major interest was the observation that the BALB/c immune response to infection with *L. (V.) panamensis* resembles the human immune response with a concomitant production of a mixed Th1 and Th2 cytokine response (primarily IL-10, IL-13, IFN- γ , TNF- α and low/absent IL-4). Our studies are consistent with the earlier studies [5, 50] of human patients, showing a mixed IL-10, IFN- γ response to *L. (V.) panamensis* infection. Interestingly, untreated active chronic patients have been found to present similar *in vitro* response (IFN- γ , IL-13, IL-10, IL-2 and TNF- α) to *Leishmania* parasites as those of treated chronic patients. On the contrary, treated historic recurrent patients produced significantly higher levels of IFN- γ and TNF- α than active recurrent patients ($p < 0.05$) [51]. Consequently, a similar cytokine profile is observed as a consequence of infection –before and after treatment. Given that drug treatment has been found not to eradicate parasites [52–54], this may reflect persistent infection.

The mixed Th1/Th2 cytokine profile is also common to human infection with other *L. (Viannia)* species [4–8]. Notably, both IL-10 and IL-13 have been implicated in the negative regulation of *L. (Viannia)* infection in human patients [8, 55]. The consistent (human and mouse) mixed cytokine response suggests common mechanisms to sustain infection for this subgenus are present in this murine model of chronic *L. (V.) panamensis* infection.

Although IL-4 production in response to leishmanial Ag is observed in some cases of human *L. (V.) braziliensis* infection [4, 27], IL-13 and IL-10 appear to be the predominant “Th2” cytokines [6, 8, 11] found. Notably, an IL-10 response occurred throughout infection in both the WT and the IL-13- and IL-4R α -deficient mice, suggesting that IL-10 alone is insufficient to maintain disease and indicating a critical role for IL-13. IL-13 has been shown in murine models of cutaneous leishmaniasis to be important in the susceptibility and maintenance of nonhealing response to *L. (L.) major* and *L. (L.) mexicana* infection [32, 33, 56]. IL-4 and IL-13 signal through an overlapping network of receptors that shares a functional signaling receptor chain, namely IL-4R α [57]. As IL-4R α -deficient mice controlled infection more rapidly than IL-13-deficient mice, it is possible that both IL-4 and IL-13 may contribute to disease. However, deficiency in IL-13 alone closely approximates the effect seen in IL-4R α -deficient mice, suggesting that IL-4 may play a relatively minor role in host susceptibility to *L. (V.) panamensis* infection. However, further experiments are required to verify this point.

The increased susceptibility to infection in BALB/c mice that is dependent on IL-13 and IL-4R α correlates with an increase of the IFN- γ and IL-17 responses observed with the initiation of disease resolution in the absence of IL-13 or IL-4R α . The importance of IL-13 in reducing the IFN- γ response has been previously observed in murine *L. (L.) major* and *L. (L.) mexicana* infection [32, 33]. The targeted cell populations and mechanisms by which IL-13 and IL-4R α ensure host permissiveness are not explored in this study. However, in studies of murine *L. (L.) major* infection, IL-4R α has been shown to be important for alternative activation of macrophages [58] and also to act through IL-4 on the development of CD4⁺ T cells [34]. Considering that IL-4 appears to have a minimal role in the regulation of *L. (V.) panamensis* infection and that IL-13 deficiency alone is sufficient to abrogate susceptibility, it is possible that IL-13 effects on macrophage activation are playing a role in determining infection outcome. In the case of *L. (L.) mexicana* infection, although infection in the absence of IL-13 led to partial disease amelioration, this was accompanied by an increase in IL-12R β 2 expression and IFN- γ production. These results are consistent with the studies of *L. (V.) braziliensis* in mice [15, 59], where IL-12 deficiency leads to overt disease; notably, deficiency in IFN- γ led to a more severe disease [59], suggesting that the multiple IFN- γ -dependent processes might be critical to parasite containment. Interestingly, in the case of human leishmaniasis caused by *L. (V.) guyanensis*, IL-13 has been found to regulate the expression of IL-12R β 2, although changes in the IFN- γ response were not noted [11]. Whether similar changes in IL-12 responsiveness occur in the case of *L. (V.) panamensis* remain to be determined and are of interest. However, the downregulation of IFN- γ production as a consequence of IL-13 (whether through changes in IL-12 signaling and/or macrophage function) is likely critical for disease containment.

Although an IL-17 response appears to be upregulated in the case of mucosal disease caused by *L. (V.) braziliensis* [10], IL-17 is also found in response to limited cutaneous disease. Hence, the role of IL-17 in human cutaneous leishmaniasis caused by *L. (V.) braziliensis* remains unclear. In the case of murine cutaneous leishmaniasis, IL-17 has been found to exacerbate murine leishmaniasis caused by *L. (L.) major* [60]. On the contrary, IL-17 has been detected in response to *L. (V.) braziliensis* infection of murine dendritic cells [61] and has been (together with IFN- γ) observed in mice infected with *L. (V.) braziliensis* [61] to correlate with healing in response. These results are consistent to those found here in IL-13- and IL-10-deficient mice and suggest that IL-17 together with IFN- γ may be important for

disease resolution for disease caused by infection with *L. (Viannia)* parasites. Interestingly, Th17 cells have been shown to express IL-13 receptor and to be downregulated by IL-13 [62]. Further study is required to dissect the role of IL-17 in New World leishmaniasis.

In summary, we have demonstrated a key role for IL-13 in host permissiveness to *L. (V.) panamensis* infection. Our results suggest that IL-13 acts in part through downregulation of IFN- γ and/or IL-17, which are important for infection control and parasite elimination. Hence, the IL-13 signaling pathway appears, as for asthma, to represent a promising target for immunotherapy [63]. We believe that the *L. (V.) panamensis* murine model will be useful for further studies of the host–parasite interaction and will aid in understanding the immunological mechanisms underlying disease and healing.

Materials and methods

Mice

Female BALB/c mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA) or National Cancer Institute (Frederick, MD, USA). Mice were housed in the Yale University School of Medicine facilities, which are approved by the American Association for Accreditation of Laboratory Animal Care-facilities. The experiments were approved by Yale University Committee on Use and Care of Animals (Assurance number A3230-01). Breeding pairs of IL-4R $\alpha^{-/-}$ (BALB/c IL4r $\alpha^{\text{tm1Sz/J}}$) and IL-10 $^{-/-}$ (C.129P2-IL-10 $^{\text{tm1Cgn/J}}$) mice were purchased from Jackson Laboratories. Breeding pairs of IL-13 $^{-/-}$ and B-cell-deficient J μ D mice BALB/c mice were the kind gifts from Dr. Marsha Wills-Karp (University of Cincinnati College of Medicine) and Dr. Mark Shlomchik (Yale University), respectively.

Indirect immunofluorescence assay

Indirect immunofluorescence of *L. (V.) panamensis* organisms was performed using standard methodology [18]. Digital images were taken in a Leitz Orthoplan 2 fluorescence microscope immunofluorescence microscope.

Leishmania culture, mouse infection and parasite burden analyses

L. (V.) panamensis (strain MHOM/CO/1995/1989), isolated from a human case of cutaneous leishmaniasis in Colombia, was used for the studies in mice. The parasites were routinely checked either by PCR or by mAb to verify that these were *L. (V.) panamensis* [64, 65].

L. (V.) panamensis was grown in Schneider's Medium supplemented with 10 or 20% heat-inactivated FBS and 17.5 $\mu\text{g/mL}$ gentamycin (GIBCO BRL®). Axenic amastigotes were maintained in the same medium at 33°C, and were obtained from promastigotes, as described previously [18].

Promastigotes were grown at 22°C. Promastigotes, recently transformed from amastigotes isolated from infected tissue, were used for infection. Late stationary phase (15–21 days in culture) promastigotes provided for optimal infectivity and consistent chronic lesion development; organisms harvested earlier (7–10 days), whereas infective, did not generally result in chronic disease. Infective promastigotes were purified using a step percoll gradient (Sigma Chemical, St. Louis, MO, USA; PBS containing 20 mM EDTA) that was a modification of the previously described method [20]. Briefly, infective promastigotes were purified from a 45/60% percoll interface; washed promastigotes were then employed for intradermal infection (10 μL , 5×10^4). At different time points after infection, four to five mice were sacrificed and the number of parasites in various tissue sites was determined by limiting dilution analysis, as described previously [20].

Immune response analyses – BALB/c mice

Single-cell suspensions were obtained from either spleen or LN tissues. Cells (5×10^6 cells/mL) were then incubated at 37°C/5% CO₂ for 72 h alone or stimulated with either concanavalin A (5 µg/mL), or *L. (V.) panamensis* promastigote freeze-thaw lysates (Lag – equivalent to 1 or 5×10^6 organisms/mL). Supernatants were collected and stored at –80°C until used. Cytokine levels (TNF-α, IL-10, IL-4 and IFN-γ) were determined by sandwich ELISA, according to the manufacturer's specifications, as described previously [20]. IL-17 was determined using a kit from eBiosciences (San Diego, CA, USA). For IL-13 measurements, paired Ab (MAB413 and BAF413 from R&D Systems, Minneapolis, MN, USA) and rmIL-13 (eBiosciences) were used.

For flow cytometric analyses, LN cells were directly employed for *ex vivo* analysis or, alternately, cells stimulated/or not with pLAg for 72 h (as indicated above) were cultured for 4 h in the presence of Brefeldin-A (BD Biosciences Pharmingen) according to manufacture's instructions. The cells were then stained for surface markers (CD3ε (145-2C11), CD4 (L3T4), CD8α (Ly-2), CD19 (1D3) and CD11b (M1/70)), washed and fixed. Subsequently, cells were permeabilized and stained for FOXP3 (FJK-16S, eBiosciences), or IFN-γ (XMG1.2). Controls were performed with the respective isotype controls: Hamster-IgG, Rat-IgG2a and Rat-IgG1 from eBiosciences; and Rat-IgG2a (R35-95), Rat-IgG2b (A95-1) from BD Biosciences Pharmingen. Data were acquired on a BD FACSCalibur (BD Biosciences), using software CellQuest™ Pro, and collected data were analyzed on FlowJo Software (Tree Star).

Human study population

The study population consisted of 57 individuals between 15 and 58 years of age, of either gender. They were classified into four groups: (i) Historical Chronic Disease, individuals who had previously presented parasitologically confirmed lesions of >6 months of evolution that had cured after treatment with Glucantime® (Specia Rhone-Poulenc Rorer, Paris, France); (ii) Historical Recurrent Disease, individuals who had presented a new leishmanial lesion after healing of a documented, parasitologically confirmed leishmaniasis. Neither historical groups presented active lesions at the time of the study; (iii) Asymptomatic Infection, subjects living in an endemic area of transmission of dermal leishmaniasis (Tumaco, Nariño, Colombia), without active or healed lesions or clinical history of leishmaniasis, having a positive Montenegro skin test [66] or *in vitro* proliferative response to *Leishmania* Ag. These participants were identified from the previous studies of incidence and prevalence of leishmaniasis [67] and (iv) Healthy Controls, laboratory personnel without history of exposure to areas of transmission of leishmaniasis and negative lymphoproliferation response to *Leishmania* Ag. All participants of the study were seronegative for HIV-1/HIV-2 and HTLV-1 virus determined by ELISA (Abbott Laboratories, Abbott Park, IL, USA).

This study was conducted in accordance with national (Resolution No. 008430, 1993, República de Colombia, Ministry of Health) and international (Declaration of Helsinki and amendments, World Medical Association, Edinburgh, Scotland, October 2000) guidelines for protection of human subjects involved in research. The study was approved and monitored by the CIDEIM Institutional Review Board. Informed, signed consent was provided by each participant.

Isolation of human blood mononuclear cells and lymphoproliferation assays

PBMC were isolated from heparinized blood by centrifugation over Ficoll-Hypaque (Sigma Chemical). After washing, PBMC were cultured (1×10^6 /mL) in RPMI 1640 (Nunc, St. Louis, MO, USA) containing 10% pooled human serum free of IL-10, in medium alone or in

the presence of Ag from 2.5×10^4 /mL lysed promastigotes of *L. (V.) panamensis* (MHOM/COL/1982/LS94), as described previously [46].

Human cytokine quantification

Cytokine levels were determined using culture supernatants of 2.0×10^6 PBMC/mL of human patients cultured in the absence and presence of 2.5×10^4 /mL *L. (V.) panamensis* promastigotes. Based on the kinetic assays to determine the optimal time to evaluate each cytokine, IFN- γ , IL-10 and IL-13 were measured at 72 h and TNF- α at 18 h. ELISA immunoassays of IFN- γ , IL-10 and TNF- α were performed as described previously [5]. IL-13 (PharMingen, San Diego, CA, USA) was determined in accordance with manufacturer's instructions.

Statistical analysis

Statistical analyses of human PBMC's cytokine production were carried out using SPSS software. Cytokine concentration was normalized by conversion to a base 10 logarithm. One-way ANOVA was performed to establish statistical differences among the clinical groups and Duncan procedure was used to determine multiple comparisons. Statistical analysis of the BALB/c data was performed in Kaleidagraph using Student's *T* and/or unpaired Wilcoxon tests. Values of *p* less than 0.05 ($p < 0.05$) were considered as significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank Rafael Tovar for performing the statistical analyses of the human PBMC's cytokine production data and patient data management, and Harold Golston for technical support. This work was supported through a grant from NIAID, U19 AI65866 (D. Mc. -P.).

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Figure 1. Stationary phase *L. (V.) panamensis* promastigotes generate chronic lesions in BALB/c mice. Mice were intradermally infected (5×10^4 late log phase percoll-purified organisms) on the top of the right rear hind foot. Course of infection was monitored by measuring (A) ratio of lesion development in BALB/c footpad compared with the contralateral uninfected foot ($n = 10$), and by (B) parasite burden analyses of feet (diagonal-stripped bars) or pooled draining popliteal and inguinal LN (open bars, $n = 4-5$). (C) Representative median lesions of infection at 3 wk or 10 months after infection. Data show mean $\pm$ SEM and are representative of at least two independent experiments.

Figure 2.

Human natural infection presents a clinical spectrum (from chronic and recurrent lesions to asymptomatic infection) characterized by an immune response constituted by a mixed pro- and anti-inflammatory cytokine profile. (A) Cytokine production by PBMC from patients with active chronic (CH) and recurrent disease (RE), asymptomatically infected individuals (AS) and healthy controls (HC). ANOVA ($p < 0.001$ for all cytokines) and Duncan's test for multiple comparison, * $p < 0.01$. (B) Regression analyses of cytokine responses across the spectrum of disease caused by *L. (V.) panamensis*. Regression analyses of cytokine production were conducted only in infected individuals (chronic, recurrent and asymptomatic) using Pearson correlation analysis. p -Values of less than 0.05 are considered significant.

Figure 3.

L. (V.) panamensis infection in BALB/c does not require B cells. (A) Lesion development was monitored by lesion development of infected footpad in BALB/c WT (squares) or B-cell-deficient, JhD (circles) mice, infected with 5×10^4 stationary phase purified promastigotes, a minimum of ten mice of each group was analyzed for lesion development. Results are representative of three independent experiments. Parasite burden was determined at (B) 2 or (C) 4 months post-infection ($n = 4/\text{group/determination}$). Data show mean $\pm$ SEM, * $p < 0.05$ by unpaired Wilcoxon test.

Figure 4.

L. (V.) panamensis infection in BALB/c requires IL-13 and IL-4R α . Course of infection was monitored by (A) footpad lesion development in WT (squares), and in IL-4R α (triangles) or IL-13- (open circles) deficient BALB/c mice (data show at least four mice *per* group and are representative of three independent experiments). * (IL-13 KO) and + (IL-4R α KO) indicate $p < 0.001$ by unpaired Wilcoxon test compared with WT controls. Parasite burden was determined at (B) 25 days and (C) 60 days post-infection in BALB/c WT (filled bars), IL-13 KO (diagonally striped bars) and IL-4R α KO (open bars). The numbers of mice with positive parasite burden in each group are indicated beneath the bars. * $p < 0.03$ by unpaired Wilcoxon test and error bars represent standard error of the mean ($n = 4$). Similar results were observed at 180 and 360 days post-infection.

Figure 5.

Response to infection with *L. (V.) panamensis* of WT and IL-13 or IL-4 α -deficient mice. Cytokine responses of WT, IL-13- or IL-4 α -deficient BALB/c mice at different times post-infection, as indicated. ELISA analyses for cytokines were performed on supernatants of spleen-derived lymphocytic cells stimulated with Con A or pLAG (as indicated in the *Materials and methods* section). Data show mean $\pm$ SEM. * (IL-13 KO) and + (IL-4 α KO) indicate $p < 0.05$ by Student's *T*-test. Similar results were obtained in another independent experiment at 3.5 wk. The IL-4 production by lymphocytes of IL-4 α -deficient mice is not shown. However, consistent with the previous observations [68], IL-4 levels were found to be >300 pg/mL at all time points.

Figure 6.

Lack of IL-13 signaling leads to increases in CD4⁺IFN-γ⁺ T cells. IFN-γ responses of WT or IL-13-deficient BALB/c mice at 3.5 wk post-infection. FACS analyses for IFN-γ were performed as indicated in the *Materials and methods* section (gating strategy is shown in Supporting Information Fig. 4) using spleen lymphocytes stimulated with pLAg. Lymphocyte populations (based on the side and forward scatter) were sorted for either surface (A) CD4 or (B) CD8 expression (as indicated) and examined for IFN-γ synthesis. Results are representative of two independent experiments ($n = 4$ mice/group). (C) Ratio of % IFN-γ⁺ cells divided by % IFN-γ⁺ cells in the WT group is shown (average of points from multiple experiments), error bars indicate SD, and * $p < 0.03$ by Wilcoxon test.

Table 1Cytokine responses of *L. (V.) panamensis* infected BALB/c Mice^{a)}

Cytokine	3 Wk post-infection	4 Months post-infection	8 Months post-infection
IFN- γ	9.6 $\pm$ 0.3 ng/mL	43.6 $\pm$ 3.1 ng/mL	36.5 $\pm$ 0.02 ng/mL
IL-10	0.27 $\pm$ 0.04 ng/mL	1.5 $\pm$ 0.4 ng/mL	0.48 $\pm$ 0.02 ng/mL
IL-13	0.11 $\pm$ 0.03 ng/mL	0.31 $\pm$ 0.06 ng/mL	0.16 $\pm$ 0.01 ng/mL
TNF- α	Not determined	0.24 $\pm$ 0.03 ng/mL	Not determined

^{a)}Lymphocytes (DLN) from *L. (V.) panamensis*-infected BALB/c mice (three to five *per* group) were stimulated for 72 h with soluble leishmanial Ag and cytokine production was assessed by ELISA.

Table 2

Draining LN cell populations in BALB/c WT or IL-13-deficient mice^{a)}

Cell Population	WT BALB/c		IL-13-deficient BALB/c		Ratio: Infected/naïve
	Naïve	Infected	Naïve	Infected	
CD3e ⁺	1.45 ± 0.01 × 10 ⁶	3.69 ± 0.01 × 10 ⁶	1.36 ± 0.01 × 10 ⁶	2.13 ± 0.01 × 10 ⁶	1.57
CD4 ⁺	9.70 ± 0.03 × 10 ⁵	2.66 ± 0.03 × 10 ⁶	9.93 ± 0.06 × 10 ⁵	1.52 ± 0.05 × 10 ⁶	1.53
CD8 ⁺	4.19 ± 0.05 × 10 ⁵	1.05 ± 0.01 × 10 ⁶	3.52 ± 0.01 × 10 ⁵	5.97 ± 0.02 × 10 ⁵	1.70
CD19 ⁺	4.37 ± 0.05 × 10 ⁵	2.77 ± 0.03 × 10 ⁶	2.68 ± 0.01 × 10 ⁵	1.00 ± 0.01 × 10 ⁶	3.73
CD11b ⁺	2.04 ± 0.01 × 10 ⁴	5.00 ± 0.0 × 10 ⁴	1.68 ± 0.04 × 10 ⁴	4.00 ± 0.22 × 10 ⁴	2.38
CD4 ⁺ FOXP3 ⁺	1.03 ± 0.02 × 10 ⁵	2.68 ± 0.03 × 10 ⁵	7.26 ± 0.29 × 10 ⁴	1.72 ± 0.03 × 10 ⁵	2.37

^{a)} Values represent the absolute number of LN cells (average of six to ten pooled sets of popliteal and inguinal LN) at 3.5 wk post-infection that are positive for each cell marker (CD4, CD8, CD19, CD3e and CD11b) and FOXP3. Staining was performed in duplicate and the standard deviation for each measurement is presented. The experimental data are representative of two independent experiments.