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Immunology

Transforming growth factor β as a virulence mechanism for *Leishmania braziliensis*

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ABSTRACT **Transforming growth factor β (TGF-β) has potent down-regulating effects on macrophages and is thus capable of influencing the fate of intramacrophage parasites, including leishmanias. We report the development of a mouse model for the study of the human pathogen *Leishmania braziliensis* and demonstrate, both *in vitro* and *in vivo*, a key regulatory role for TGF-β in the pathogenesis of infection with this parasite. Recombinant TGF-β added to cultures of murine peritoneal macrophages led to increased intracellular *L. braziliensis* replication, whereas addition of neutralizing anti-TGF-β monoclonal antibody decreased levels of infection. Macrophages infected with *L. braziliensis* produced biologically active TGF-β, with a direct correlation between amounts of TGF-β induced by two parasite isolates and their relative virulence. *In vivo*, treatment with recombinant TGF-β rendered avirulent parasites virulent and activated latent *L. braziliensis* infection. Activation of parasite replication was observed in mice which had been infected with *L. braziliensis* 15 weeks previously but had not developed lesions or had healed lesions, depending on the parasite isolate used to infect the mice. The exacerbation of *L. braziliensis* infection *in vivo* was associated with an increase of interleukin 10 mRNA in the draining lymph node. These results demonstrate that TGF-β is able to alter the course of *in vitro* and *in vivo* infections with *L. braziliensis*, the latter being characterized by an increase in interleukin 10, an important T_{h2} helper-T-cell cytokine.**

Leishmanias are intracellular protozoan parasites transmitted to humans and other mammals by the bite of a sandfly. They cause a variety of human diseases characterized by visceral, cutaneous, or mucosal lesions. The different species and isolates of these obligate macrophage parasites vary considerably in their ability to infect and replicate in these host cells *in vitro* or *in vivo*. For example, both *Leishmania major* and *Leishmania amazonensis* multiply rapidly in human and mouse macrophages *in vitro* and readily infect mice, whereas *Leishmania braziliensis* has been reported to be relatively less infectious for cultivated macrophages or mice (1). No clear associations have been made between inter- or intraspecies properties and virulence, although the developmental stage of the parasite is important for infectivity (2).

Clinically, infections with *L. braziliensis* present as single or multiple cutaneous lesions, with a small percentage of individuals progressing to more severe mucosal disease (3, 4). While the cutaneous lesions may heal spontaneously or respond well to chemotherapy, mucosal lesions are often highly destructive and are relatively refractory to treatment. Even if the mucosal lesion heals, there is often spontaneous relapse, perhaps years later. Largely due to difficulties in establishing a mouse model, there have been relatively few studies on immunoregulatory aspects of *L. braziliensis* infections.

The present study was undertaken to determine the potential role of transforming growth factor β (TGF-β) in the control of *L. braziliensis* infection. This important cytokine has a variety of immunological effects (5–8). These include inactivation of physiological processes of macrophages (9, 10), thus influencing the ability of these cells to control intracellular parasites, including leishmanias and *Trypanosoma cruzi* (11, 12). Of particular interest is the finding that TGF-β can block the effects of interferon γ (IFN-γ), a cytokine important in mediating parasite destruction (12). In mice, a healing response to leishmanial infection has been associated with the production of IFN-γ, a T_{h1} helper-T-cell cytokine, and a non-healing response with the production of T_{h2} cytokines, including interleukin 4 (IL-4) and IL-10 (13–15).

It is apparent that the pattern of cytokine expression is closely linked to the outcome of leishmanial infection. Therefore, processes which influence this pattern play critical roles in the disease process. We demonstrate in this study that biologically active TGF-β, a cytokine capable of blocking the utilization of IFN-γ, is induced following *L. braziliensis* infection and that TGF-β plays an important role in regulating *in vitro* and *in vivo* infection with this parasite. Further, we provide evidence that TGF-β can promote the development of a non-healing T_{h2} response *in vivo*.

MATERIALS AND METHODS

Parasites. Two isolates characterized as *L. braziliensis* (MHOM/BR/88/BA-92 and MHOM/BR/90/BA-331, referred to as BA-92 and BA-331) were used in this study. Both were typed as *L. braziliensis* with monoclonal antibodies (mAbs) (courtesy of G. Grimaldi, Fundacao Oswaldo Cruz, Rio de Janeiro). The parasites were cultured as promastigotes in Schneider's medium with antibiotics and 10% fetal bovine serum.

Mouse Infection. BALB/cByJ mice (The Jackson Laboratory) were infected in the left hind footpad with 10^6 stationary-phase promastigotes in 25 μl. Parasites were suspended in saline (0.9% NaCl) containing recombinant TGF-β (rTGF-β) at 40 μg/ml (1 μg per mouse). Further doses of rTGF-β (1 μg per mouse) were administered in the infected footpad. Infected control mice were injected with the same volume of saline or with rTGF-β in the contralateral footpad. Uninfected control mice were similarly injected with saline or rTGF-β. In experiments to evaluate the reactivation of dor-

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Abbreviations: IFN, interferon; IL, interleukin; mAb, monoclonal antibody; TGF, transforming growth factor; rTGF, recombinant TGF.

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mant *L. braziliensis* infections, animals were infected in the footpad and rested without treatment for 15 weeks, then treated with rTGF-β (1 μg per mouse per dose) or saline, three times per week for 3 weeks.

Lesion progression was evaluated during the course of infection by footpad thickness measured with a dial gauge caliper (C. Starret, Athol, MA), and expressed as lesion size in millimeters (infected footpad thickness minus uninfected contralateral footpad thickness). At various times after infection, animals were killed, and infected feet were removed, fixed in buffered 10% formalin, processed, and stained with hematoxylin and eosin.

Macrophage Cultures and Infection. Thioglycolate-stimulated mouse peritoneal macrophages were plated in 24-well plates at 5×10^5 cells per well, in RPMI 1640 supplemented with antibiotics, glutamine, and 10% heat-inactivated fetal bovine serum. Macrophages were infected with stationary-phase *L. braziliensis* promastigotes (2.5×10^6 parasites per well) for 3 hr. Extracellular parasites were removed by extensive washings with warm culture medium, followed by further incubation for up to 72 hr. Cell-free supernatants were collected at 24, 48, or 72 hr after infection. Replicate monolayers were washed with phosphate-buffered saline, fixed in methanol, and stained with Giemsa reagent. The percentage of infected macrophages and the number of amastigotes per 100 macrophages were determined by microscopic examination as described (16). In some cultures rTGF-β (2 ng/ml) or 1D11.16 anti-TGF-β mAb (200 μg/ml, a gift of James Dasch, Celtrix Laboratories, Palo Alto, CA; ref. 17) was added 24 hr before infection and again after infection. The 1D11.16 mAb had <0.1 unit of endotoxin as determined by the *Limulus* amoebocyte lysate assay (Whittaker Bioproducts).

Assay for Active TGF-β. Supernatants from uninfected and leishmania-infected macrophage cultures were assayed for active TGF-β by using CCL-64 mink lung epithelial cells as described (18). Latent TGF-β will not inhibit growth of CCL-64 cells (19). The concentration of TGF-β in each sample was determined by comparison with a curve generated from TGF-β standards, computed using the Microsoft GB STAT program. The specificity of the assays was confirmed by inhibition with 1D11.16 anti-TGF-β mAb.

Cytokine PCR. RNA was isolated from mouse cells by acid guanidinium thiocyanate–phenol/chloroform extraction (20). One μg of total RNA was reverse-transcribed by the addition of 2.5 units of RNasin (Promega); 25 ng of $(dT)_{12-18}$ and 250 μM dNTPs (both from Pharmacia LKB); and 200 units of Moloney murine leukemia virus RNase H^- reverse transcriptase, 10 mM dithiothreitol, and 1× reverse transcriptase buffer (BRL/Life Technologies, Gaithersburg, MD) in 20 μl. The reaction proceeded for 1 hr at 37°C and was terminated by boiling for 10 min after the addition of 180 μl of water. Five microliters of cDNA diluted 1:10 was used for amplification in a 50-μl PCR mixture containing 200 μM dNTPs; 0.2 μM 3′ and 5′ external primers, 2.25 mM $MgCl_2$, 1× GeneAmp PCR buffer, and 1.25 units of AmpliTaq DNA polymerase (Perkins–Elmer/Cetus). PCR conditions were as follows: 96°C for 2 min, 55°C for 1 min (1 cycle); 72°C for 1 min, 96°C for 1 min, 55°C for 30 sec (25 cycles); 72°C for 2 min (1 cycle). The primers were as follows.

IL-10	5′, CTC-TTA-CTG-ACT-GGC-ATG-AGG-ATC
	3′, CTA-TGC-AGT-TGA-TGA-AGA-TGT-CAA-ATT
IFN-γ	5′, CTG-GCT-GTT-ACT-GCC-ACG-GCA-CAG-TC
	3′, TCG-GAT-GAG-CTC-ATT-GAA-TGC-TTG-GCG-CT
IL-2	5′, CTT-CAA-GCT-CCA-CTT-CAA-GCT
	3′, CCA-TCT-CCT-CAG-AAA-GTC-CAC-C
IL-4	5′, TCT-CTC-GTC-ACT-GAC-GGC-ACA-GAG-CT
	3′, TCC-ATT-TGC-ATG-ATG-CTC-TTT-AGG-CT
β-actin	5′, GAC-TTC-GAG-CAG-GAG-ATG-GCC-AC
	3′, CTC-CTG-CTT-GCT-GAT-CCA-CAT-C

PCR products were run in 1% agarose gels and transferred to Nytran nylon membranes (Schleicher & Schuell) for probing. A DNA probe for IL-10 was made with 50 ng of denatured IL-10 insert DNA and a random-primer DNA labeling kit (Boehringer Mannheim). Probes for IFN-γ, IL-2, and IL-4 were made by ^{32}P-labeled PCR with the following internal primers.

IFN-γ	5′, TAG-ATG-TGG-AAG-AAA-AGA-GTC-TCT-TCT
	3′, TCT-GAG-GTA-GAA-AGA-GAT-AAT-CTG-GCT
IL-2	5′, CTG-AAA-CTC-CCC-AGG-ATG-CTC
	3′, TCC-AAG-TTC-ATC-TTC-TAG-GCA-CT
IL-4	5′, TCA-TGG-AGC-TGC-AGA-GAC-TCT-TTC-GGG-CT
	3′, TCC-AGG-AAG-TCT-TTC-AGT-GAT-GTG-GAC-T
β-actin	5′, GAG-CTG-CCT-GAC-GGC-CAA-GTC-ATC-A
	3′, GTC-AAC-GTC-ACA-CTT-CAT-GAT-GG

One microliter of each template, from 3′ and 5′ external-primer PCRs, was used for amplification in a 20-μl PCR mixture containing 200 μM dNTPs, 1 μM 3′ and 5′ internal primers, 2.25 mM $MgCl_2$, 1× GeneAmp PCR buffer, and 2.5 units of AmpliTaq DNA polymerase. PCR was performed as above, for 35 cycles. Unincorporated label was removed by Sephadex G-50 spun column chromatography. Labeled probes were boiled for 10 min, placed on ice for 3 min, and then added directly to the prehybridization mixture at 2×10^6 cpm/ml and incubated at 50°C for 16 hr. Post-hybridization washes (3) were at 55°C for 20 min with 0.3 M NaCl/0.03 M sodium citrate, pH 7/0.1% SDS and were followed by 5 min of rinsing in water at room temperature.

RESULTS

TGF-β Regulates *in Vitro* Infection by *L. braziliensis*. *In vitro* infection of mouse macrophages by *L. braziliensis* is characterized by persistent but static numbers of intracellular parasites. The effects of rTGF-β or neutralizing anti-TGF-β mAb on the course of *in vitro* infection were examined. When macrophages were treated with rTGF-β prior to infection, a progressive increase in numbers of intracellular amastigotes occurred (Fig. 1). This suggested that the macrophages were normally capable of controlling the intracellular replication of *Leishmania*, and that the mechanism(s) responsible for this control was inhibited by added TGF-β. The regulation of

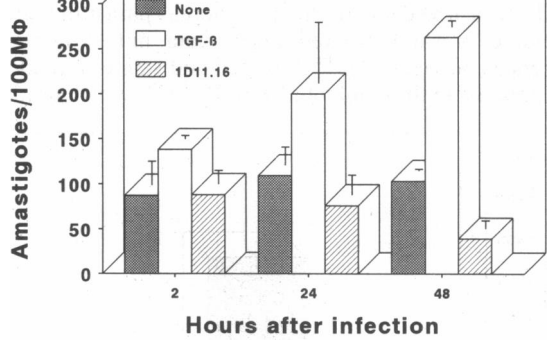

FIG. 1. Effects of TGF-β or anti-TGF-β on the *in vitro* replication of *L. braziliensis*. Mouse peritoneal macrophages (Mφ) were infected with BA-92 and cultured with TGF-β (2 ng/ml; open bars) or 1D11.16 anti-TGF-β mAb (200 μg/ml; hatched bars) 24 hr before and continuously after infection (but not during infection) or in medium alone (cross-hatched bars). Numbers of intracellular parasites were determined 2, 24, and 48 hr after infection. Significant increases in parasite numbers were seen at 48 hr after infection in the TGF-β-treated cultures ($P < 0.05$), whereas significant decreases in parasite numbers were seen in the anti-TGF-β-treated cultures ($P < 0.05$). Comparisons were by Student's *t* test. Representative results from two separate experiments are shown.

intracellular parasite replication by TGF-β was further demonstrated by the progressive reduction of amastigotes in macrophages treated with anti-TGF-β mAb. This observation suggested a role for leishmania-induced TGF-β in the persistence of this parasite and that amounts of TGF-β produced following infection may be important.

Active TGF-β Is Produced *in Vitro* by Macrophages Infected with *L. braziliensis*. Macrophages are both host cells for leishmanias and important producers of TGF-β. We analyzed the production of TGF-β by peritoneal macrophages from normal mice infected *in vitro* with the BA-92 and BA-331 isolates of *L. braziliensis*. Not only was biologically active TGF-β produced by mouse peritoneal macrophages following *in vitro* infection (Fig. 2), but the amounts of active TGF-β produced differed significantly between the two parasite isolates, even though *in vitro* infection levels did not differ significantly (data not shown). TGF-β was not observed in replicate cultures of parasites alone or in macrophages incubated with comparable numbers of heat-killed parasites. This finding confirmed our earlier observation on the production of active TGF-β by leishmania-infected macrophages (21) and suggested a potentially important and quantifiable difference related to infection with different isolates of a given species.

Exacerbation of *in Vivo* Leishmanial Infection by TGF-β. The preceding *in vitro* observations suggested a differential ability of leishmania isolates to induce active TGF-β production, as well as a role for TGF-β in the control of *L. braziliensis* infection. To determine whether the ability to induce TGF-β correlated with parasite virulence, the isolates were used to infect mice, with and without the administration of rTGF-β. Each of these isolates produced characteristic self-limiting infections in saline-treated mice (Fig. 3). However, there was a direct correlation between lesion size and TGF-β levels induced by the two parasite isolates. BA-331 was a relatively weak inducer of TGF-β *in vitro* and produced no measurable lesion, whereas BA-92 induced measurable but self-limiting lesions as well as significantly higher *in vitro* levels of TGF-β. This observation suggested a role for TGF-β production in leishmanial infectivity and/or intracellular replication *in vivo*. To test this, exogenous TGF-β was administered to groups of mice infected with either of these two *L. braziliensis* isolates. rTGF-β treatment led to a significant increase in mean lesion size in all mice (Fig. 3). Histologically, the lesions were characterized by large numbers of heavily parasitized macrophages, whereas parasites were not detectable in saline-treated mice (data not shown). The differences between rTGF-β-treated and saline-treated mice were greatest in those infected with BA-331, which produced

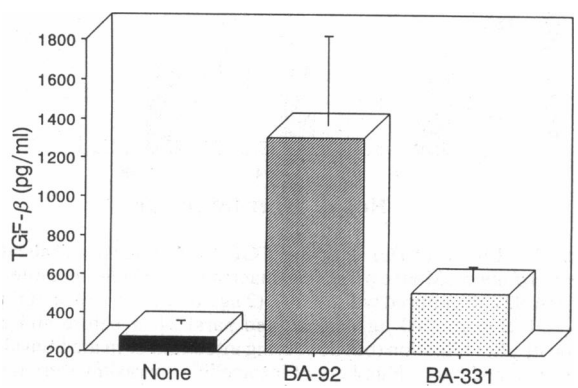

FIG. 2. Production of active TGF-β by leishmania-infected macrophages. Cultures of mouse peritoneal macrophages were infected with *L. braziliensis* (BA-92 or BA-331) and supernatants were collected 72 hr later. Data (mean ± SEM) are shown from triplicate cultures.

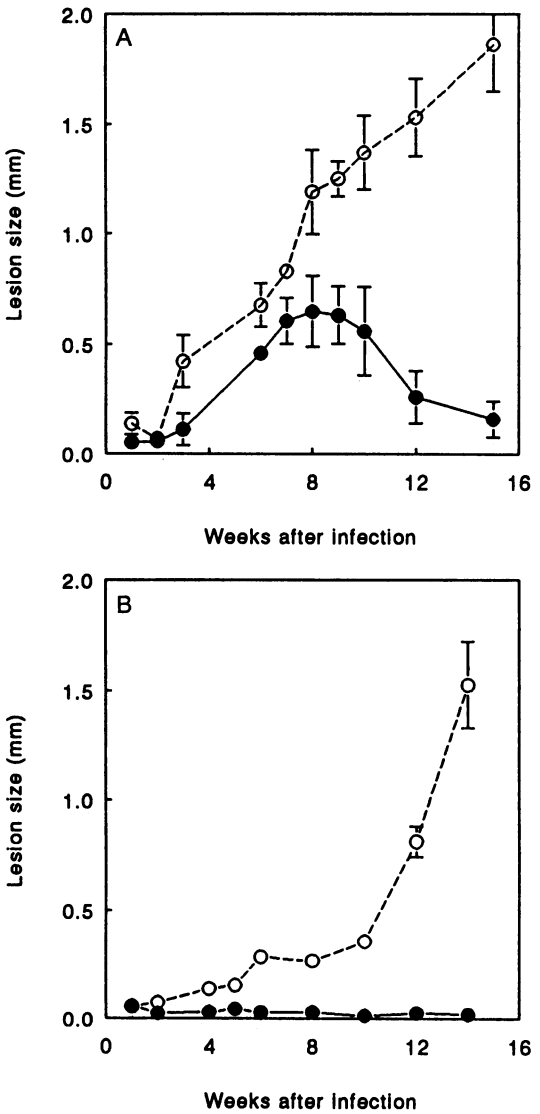

FIG. 3. (*A*) Effects of exogenous TGF-β on the course of *L. braziliensis* infections in BALB/c mice. Infections were with BA-92 (5×10^6 stationary-phase promastigotes) diluted in saline or saline containing TGF-β at 40 μg/ml for a dose of 1 μg per mouse. After infection, mice were treated with TGF-β (1 μg per dose; ○) or saline (●), injected into the infected footpad, once per week for 10 weeks. (*B*) BALB/c mice were infected with BA-331 and treated with TGF-β or saline (infection and treatment as in *A*).

measurable lesions only in the presence of TGF-β (Fig. 3*A*). By inducing or exacerbating lesions in *L. braziliensis*-infected mice, a role for TGF-β in the pathogenesis of leishmaniasis was established.

TGF-β Administration *in Vivo* Leads to Exacerbated Infection Accompanied by Increased IL-10 and Decreased IFN-γ mRNA Production in the Draining Lymph Node. RNA of unstimulated cells from the draining popliteal lymph nodes from mice infected with *L. braziliensis* BA-331 and treated with either rTGF-β or saline as in Fig. 3*B* was examined by PCR for the production of cytokines at 10 weeks after infection (Fig. 4). Serial dilutions of cDNA were used for the PCR to establish a semiquantitative relationship between the groups. Mice infected with *L. braziliensis* and treated with rTGF-β appeared to have lower levels of IFN-γ and IL-2 mRNA than did similarly infected, saline-treated mice. The most noticeable difference between the two groups was in IL-10 mRNA, which was not detected in uninfected or infected, saline-treated mice with self-healing lesions but was

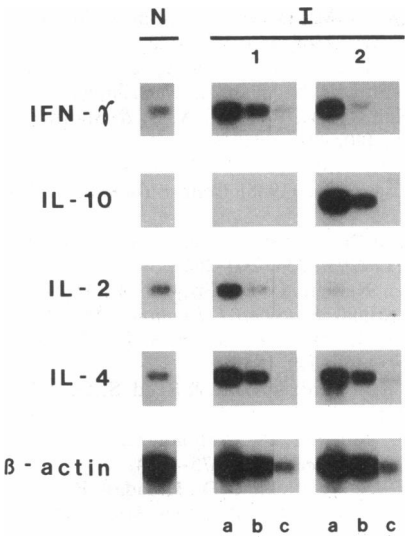

FIG. 4. Cytokine mRNA production in the draining lymph nodes of mice infected with *L. braziliensis* and treated with TGF-β. Popliteal nodes from mice treated as in Fig. 3*A* were removed 10 weeks after infection, and RNA was prepared for cytokine PCR analysis as described in *Materials and Methods*. N, uninfected mice; I, infected mice treated with saline (panel 1) or TGF-β (panel 2). cDNA dilutions of 1:10 (a), 1:50 (b), and 1:500 (c) were used for PCR.

relatively abundant in rTGF-β-treated mice with exacerbated lesion growth. No difference in IL-4 mRNA levels was detected. None of these cytokine mRNA levels were elevated in the contralateral lymph nodes from infected mice or in the popliteal nodes of uninfected mice treated with rTGF-β (data not shown). Thus, *in vivo* administration of rTGF-β led to the development of a lesion from *L. braziliensis* infection, as well as to an increase in IL-10 mRNA in the lymph node draining the infected feet in which lesion development occurred.

Activation of Latent *L. braziliensis* Infections by Administration of Exogenous TGF-β. Clinically, *L. braziliensis* infection is often characterized by prolonged intervals between relapsing disease. In mice, *L. braziliensis* infection leads to transient or no lesion development, as illustrated in Fig. 3. To determine whether parasites persisted following infection, and whether rTGF-β could influence prior infections, mice were treated with rTGF-β for a 3-week period beginning 15 weeks after infection, a time at which no demonstrable lesion was present. In both groups injection of rTGF-β, but not saline, led to lesion development, detected by 2–3 weeks after the initiation of treatment (Fig. 5). Even though rTGF-β treatment was continued for only 3 weeks, progressive lesion growth continued throughout the 15-week observation period. Saline-treated mice exhibited no change in lesion size. These experiments illustrated the persistent latency of *L. braziliensis* infection in mice and further demonstrated the ability of TGF-β to mediate disease resistance and susceptibility.

DISCUSSION

The potential importance of TGF-β in infections with macrophage parasites was suggested by studies which documented the down-regulating effects of this cytokine on class II major histocompatibility complex expression (22) and oxidative metabolism (9). Silva *et al.* (12) subsequently reported that TGF-β could block macrophage activation in *T. cruzi* infections and was capable of altering resistance and susceptibility to this organism *in vivo*. Cytokines which can inactivate macrophage functions, particularly TGF-β and IL-10, are proving to play key roles in the regulation of

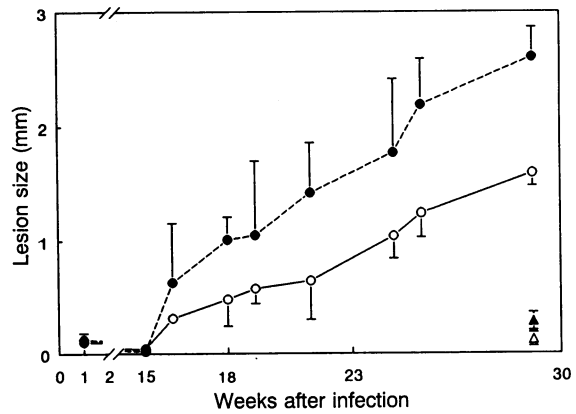

FIG. 5. Effects of TGF-β on previous infection with *L. braziliensis*. BALB/c mice were infected with either BA-92 (●) or BA-331 (○) as in Fig. 3*A* and maintained without treatment for 15 weeks. Mice were then treated with TGF-β (1 μg per injection) or saline for a 3-week period, with three injections per week. Saline-injected control mice (▲, △) did not develop lesions.

infections with certain intracellular parasites, including *Leishmania* (11) and *T. cruzi* (12, 23).

In a previous study, we demonstrated that TGF-β was produced both *in vitro* and *in vivo* following infection of mouse cells with *L. amazonensis* and that TGF-β administered *in vivo* could exacerbate infection with either *L. amazonensis* or *L. braziliensis* (21). The present study significantly expands these earlier findings by establishing a direct correlation between the amount of TGF-β produced following *in vitro* infection and parasite virulence, by demonstrating that exogenous TGF-β could activate abeyant *L. braziliensis* infections, and by establishing a correlation between TGF-β-induced lesion development and increased IL-10 mRNA production. These results link TGF-β-mediated leishmania-induced pathogenesis with the production of a T_{h2} helper-T-cell response (24). Increased IL-10 mRNA has also been associated with pathology due to experimental *L. major* infection (15). However, there have been no previous reports on the pattern of cytokine production in *L. braziliensis* infections, due primarily to the lack of a mouse model to study this parasite. The present study provides evidence for a link between two key cytokines, IL-10 and TGF-β, which are associated with down-regulating immune responses. TGF-β treatment of uninfected footpads did not increase IL-10 mRNA in the draining lymph nodes. This suggests that the infections exacerbated by TGF-β treatment led to increased IL-10 production. Whether increased IL-10 may itself mediate increased leishmania replication remains to be determined. One possibility is that TGF-β is essential for regulating early events in determining intramacrophage parasite growth and that uncontrolled growth promotes the development of a T_{h2} response.

Leishmanias are obligate macrophage parasites. A variety of species infect humans, and most or all of these can cause experimental infection, but not always disease, in mice. An example of the latter is *L. braziliensis*, for which there has been no good model of the chronic and often recurring disease seen in humans. Our demonstrations that TGF-β can exacerbate leishmanial infection and lead to lesion development following injection of normally avirulent (for mice) parasites implicate TGF-β as a key factor in the establishment and control of macrophage infection. Further, the finding that production of active TGF-β follows leishmanial infection of macrophages demonstrates that this may be an important mechanism by which intracellular parasites up-regulate molecules which favor their survival in host cells. In a related study, it was shown that an uncharacterized com-

ponent(s) of the saliva from the insect vector exacerbated *L. braziliensis* infection in mice (25). The relationship between sandfly material and TGF-β, including the possibility that vector-associated components can induce macrophage TGF-β production, could be an interesting area of study. Previous work has associated macrophage activation with increased TGF-β production (26). The mechanisms by which induction and activation of TGF-β occur during leishmanial infection are not understood. Of particular interest was the observation that local injection of rTGF-β could activate previous infection in which no lesion had developed or in which a healing response had already occurred. This suggests that TGF-β and/or other cytokines with similar or related activities may be responsible for the recurrence of quiescent *L. braziliensis* infections in humans. The concept that TGF-β may play a key role in the latency of *L. braziliensis* infections is further supported by results of *in vitro* studies, in which neutralizing anti-TGF-β mAb was found to lead to a decrease in otherwise static parasite levels (Fig. 2). Taken together, the experiments suggest a role for parasite-induced TGF-β in establishing and maintaining macrophage infection, as well as the importance of TGF-β levels in determining the degree of pathology.

Macrophage-activating cytokines, principally IFN-γ and granulocyte/macrophage-colony-stimulating factor (16, 27–29), as well as inactivating cytokines, including IL-10 and TGF-β, can tightly control the intracellular replication of both leishmanias and *T. cruzi in vitro* and *in vivo*. The clinical implications of these findings are exciting and are especially important in parasitic diseases, for which the development of safe and effective therapeutics has been neglected.

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