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Transforming Growth Factor-β in Human Cutaneous Leishmaniasis

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Transforming growth factor (TGF)-β has several downregulatory functions on the immune system: inhibition of interleukin-2 receptor induction, decrease of interferon-γ-induced class II antigen expression, inhibition of macrophage activation, as well as cytotoxic and lymphokine-activated killer cell generation. TGF-β has also been recognized as an important immunoregulator in murine leishmaniasis, for which it increases susceptibility to disease. In the present study we evaluate the involvement of TGF-β in human leishmaniasis* in vitro *and in patients with cutaneous leishmaniasis. Human macrophages produce active TGF-β after infection by* Leishmania amazonensis *(480 ± 44.7 pg/ml; mean ± SEM),* L. donovani chagasi *(295 ± 7.6 pg/ml), or* L. braziliensis *(196 ± 15.7 pg/ml). When TGF-β was added to cultures of human macrophages infected with* L. braziliensis *it led to an increase of approximately 50% in parasite numbers as compared with untreated cultures. Exogenous TGF-β added to macrophage cultures was able to reverse the effect of interferon-γ in controlling* Leishmania *growth. Even at 100 IU/ml interferon-γ the presence of TGF-β increases the number of intracellular parasites. On the other hand, TNF-α at high concentration (100 IU/ml) totally blunts the suppressive effect of TGF-β. Immunostaining for TGF-β was observed in the dermis, produced by fibroblasts and occasionally by inflammatory cells in the biopsies from human leishmaniasis lesions, being present in most of the biopsies taken from patients with early cutaneous leishmaniasis (less than 2 months of ulcer development) and in cases of active mucosal leishmaniasis. Taken together these observations suggest an important role for TGF-β in human leishmaniasis, with its production by infected macrophages being probably related to parasite establishment in the early stages of the disease. (Am J Pathol 1995, 147:947–954)

Leishmaniasis is an important public health problem in several areas of the world, including the Americas. *Leishmania* are generally divided into parasites that cause tegumentary disease (*L. braziliensis*, *L. amazonensis*, and *L. mexicana* in the New World and *L. tropica* and *L. aethiopica* in the Old World) and those agents of visceral disease (*L. donovani* and *L. donovani chagasi*). After inoculation in the skin, the flagellated promastigote penetrates into the macrophage, transforms into amastigotes, and multiplies inside the parasitophorous vacuoles. The cutaneous lesions begin at the site of parasite entrance as a small papule that develops into a nodule that ulcerates in the center. Lesions can take on aspects of papules, nodules, ulcers, tubercles, or infiltrated plaques.[1] Clinical manifestations of tegumentary leishmaniasis include the self-healing cutaneous disease (CL) and the destructive and hyper-responsive mucosal form (MCL). More rarely, diffuse cutaneous leishmaniasis (DCL) is observed. The most frequent aspect observed in CL cases is an ulcer with

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elevated borders and sharp crater. CL patients exhibit anti-leishmania cell-mediated immunity (CMI). Mucosal involvement, probably caused by hematogenous spread from the primary lesion, occurs in approximately 3% of patients infected by *L. braziliensis* and is related to multiple or extensive skin lesions above the belt and inadequate therapy of the primary lesion.[2] Initial involvement of the nasal mucosa can spread to the hard and soft palate, uvula, pharynx, gums, and upper lip. MCL is considered the hyper-responsive pole of the disease because of the potent anti-leishmania CMI responses observed in these patients. The initial lesion of DCL resembles those of CL without ulceration. The lesions are erythematous and appear as papules, nodules, tuberous lesions, infiltrated plaques, and diffuse infiltration of a part of the body. DCL, similar to lepromatous leprosy, occurs in the absence of anti-parasite CMI.[3,4]

Details of the host or parasite properties responsible for the variations in healing and severity of disease are not known. CMI responses are considered elemental for the resistance to and recovery from leishmanial infection. Leishmanicidal activity is probably a result of the increased capacity of the macrophages to produce toxic oxygen[5] and nitrogen radicals[6] in response to interferon (IFN)-γ.[7] Upon its entry, *Leishmania* induces the macrophage production of tumor necrosis factor (TNF)-α,[8] which potentiates the action of IFN-γ and promotes macrophage activation,[9,10] and transforming growth factor (TGF)-β,[11–13] linked to macrophage deactivation and inhibition of IFN-γ action.[11,14,15] Initial survival of *Leishmania* inside the macrophage may critically depend on which of these opposing cytokines predominate in the microenvironment of infection.

TGF-β is a molecule with proinflammatory and immunoregulatory activities, most notably IFN-γ-induced class II antigen expression[16] and macrophage activation,[17,18] as well as cytotoxic and lymphokine-activated killer cell generation.[19,20] The important role of TGF-β in murine leishmanial infection has been recently reviewed.[21] *In vitro* infection of mouse peritoneal macrophages with *L. amazonensis*,[12] *L. braziliensis*,[11] or *L. major*[13] leads to TGF-β production. There is also an increase in local TGF-β production in mouse footpads after *Leishmania* infection, as shown by immunohistochemistry.[13] Additionally, exogenous TGF-β leads to increased parasite load both *in vitro*[11–13] and *in vivo*.[11,12]

All of these observations prompted us to investigate the role of TGF-β in human leishmaniasis, both in *in vitro* macrophage cultures and in lesions of patients with different clinical presentations (mucosal, cutaneous, or diffuse forms) of CL.

Material and Methods

Patients

We have studied lesions from 12 patients with CL for less than 2 months (early CL); 5 patients with CL with ulcerations for more than 2 months (late CL); and 8 patients with MCL, with a single ulcerated lesion in the nasal or oral mucosa. Mucosal lesions were classified as active when represented by a lesion with erythema, edema, ulceration, or hyperemic tissue exhibiting ulcerated borders. Microscopically active lesions were characterized by intense inflammatory infiltrate with abundant macrophages and lymphocytes and occasionally by the presence of the parasite. MCL without activity was characterized macroscopically by pale, shallow, non-ulcerated cicatricial lesions and histologically by a light chronic inflammatory infiltrate and fibrosis. We have also evaluated 12 biopsies (before and after treatment) of 6 patients with DCL. All patients with CL or MCL are from the endemic region of Corte de Pedra, Bahia State (Northeast Brazil). Patients with DCL are from Maranhão State (North Brazil).

A detailed evaluation including a complete physical examination was performed on each patient. The clinical diagnosis was confirmed by at least one laboratory test (serology, skin test, or parasite culture). Anti-*Leishmania* antibodies were detected by a standard alkaline-phosphatase enzyme-linked immunosorbent assay procedure as described elsewhere.[22] The reaction was considered positive when the $O.D._{402\ nm}$ was greater than 0.05 (at a 1/100 serum dilution). Montenegro's skin reaction was performed with 20 μg of protein of crude leishmanial antigen as previously described.[23] After 48 hours, the largest diameter of the indurated area was measured and considered positive if larger than 5 mm. All patients received conventional antimonial (Glucantime) treatment consisting of 20 mg/kg/day Sb^v for 20 days. Patients with MCL and DCL had received several courses of treatment at the time of study.

Reagents

TGF-β and anti-TGF-β polyclonal antibody, produced in rabbit, were gifts from Dr. D. Twardzik (Bristol-Meyers Squibb Pharmaceutical Research Institute, Seattle, WA). IFN-γ was provided by Dr. Maud Brandely (Institute Roussell Uclaf, Paris,

France) and TNF-α was provided by Dr. K. Grabstein (Immunex Corp., Seattle, WA). All cytokines were human recombinant products. Anti-TGF-β monoclonal antibody (MAb; 1D11.16) was kindly provided by Dr. L. Ellingsworth (Celtrix Corp., Palo Alto, CA).

Parasites

L. braziliensis (MHOM/BR/88/Ba92), *L. chagasi* (MHOM/BR/90/Ba307), and *L. amazonensis* (MHOM/BR/89/Ba276) were used in this study. All of them have been typed by serodeme with a panel of MAbs and by isoenzymes (courtesy of Drs. Gabriel Grimaldi and H. Momem, Fundação Oswaldo Cruz, Rio de Janeiro, Brazil). Serodeme analysis was performed by using panels of anti-*Leishmania* MAbs specific for members of the *L. braziliensis*, *L. amazonensis*, or *L. donovani chagasi* complexes already described[24–26] in an indirect radioimmune binding assay.[24]

Macrophage Cultures

Macrophage cultures were performed with peripheral blood mononuclear cells from normal volunteers. Cultures were performed in round coverslips placed inside 24-well plates. Briefly, 1×10^6 peripheral blood mononuclear cells in RPMI 1640 without serum were plated overnight in a volume of 0.5 ml/well and cultured at 37°C in a humid atmosphere with 5% CO_2. Nonadherent cells were removed by washing with warm phosphate-buffered saline (PBS) and medium replaced for RPMI 1640 with 10% normal human AB serum (RPMI10H). Culture was maintained for 5 to 7 days and then infected with stationary-phase promastigotes at 10^6 parasites/well, during 3 hours at 35°C. Extracellular parasites were removed by extensive washings with warm medium, followed by additional incubation for up to 72 hours in RPMI10H, at 37°C in a humid atmosphere with 5% CO_2. Monolayers (in duplicate) were washed with PBS, fixed in acetone-methanol, and stained with Giemsa. The percentage of infected macrophages and the number of amastigotes per 100 macrophages were determined by microscopic examination.

TGF-β Production and Assay

Cell-free supernatants from macrophage cultures were obtained 72 hours after infection and from uninfected cultures kept for a similar duration under the same conditions. Supernatants (with and without previous acid treatment) were assayed for TGF-β by a CCL-64 mink lung epithelial cell bioassay as described.[27] Cells were plated at 10^5 cells/well into 96-well microtiter plates for 1 hour at 37°C in a volume of 100 μl of Dulbecco's minimal essential medium containing 1% heat-inactivated fetal calf serum. After 1 hour, TGF-β standards ranging from 2 ng to 0.49 pg or test supernatants were added at appropriate dilutions to a final volume of 200 μl/well. The concentration of TGF-β in each sample was determined by comparison with a curve generated from TGF-β standards. The specificity of the assays was confirmed by inhibition with 1D11.16 anti-TGF-β MAb.

Immunohistochemistry for TGF-β

We used the technique as previously described, with slight modifications.[12] Tissues obtained from biopsies from lesions or normal skin were used after fixation in buffered formaldehyde and embedded in paraffin. Sections were treated with hyalunoridase (1 mg/ml; Sigma Chemical Co., St. Louis, MO) for 10 minutes at room temperature, blocked with 1.5% normal goat serum in PBS containing 5% bovine serum albumin for 1 hour, and incubated with anti-TGF-β rabbit MAb (15 μg/ml) overnight at 4°C. An incubation with biotinylated goat anti-rabbit IgG (Sigma Chemical Co.) at room temperature for 30 minutes was followed by avidin-alkaline phosphatase (Biogenex, San Ramon, CA; 1:10 for 20 minutes). The substrate Fast Red (1 mg/ml; Sigma Chemical Co.) in Tris-HCl buffer (pH 8.2) with 0.02% naphthol AS-MX phosphate (Sigma Chemical Co.) was applied for 20 minutes, and the slides were counterstained with Mayer's hematoxylin. Controls for the immunohistochemistry included the use of no primary antisera or the use of normal rabbit IgG in place of the primary antibody and the use of normal human skin. All three controls always gave negative results.

Statistical Analysis

Comparison of the effect of different cytokines on leishmanial macrophage infection was done by one-way analysis of variance followed by Scheffé's test.

Results

TGF-β Production in Vitro

Active TGF-β was measured in 72-hour supernatants of human monocyte-derived macrophages, infected with different *Leishmania* strains. Figure 1 shows that *L. amazonensis* induced the highest production (480 ± 44.7 pg/ml, mean ± SEM), *L. donovani chagasi*

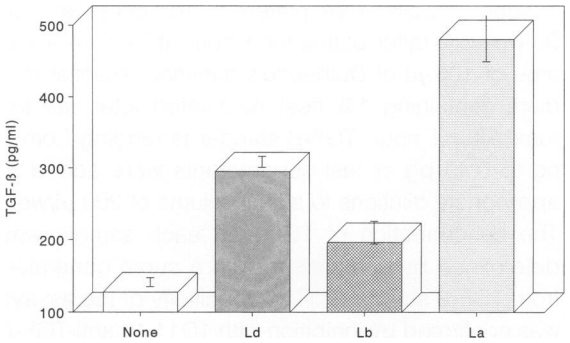

Figure 1. *TGF-β* in vitro *production by human monocyte-derived macrophages upon infection with different leishmania strains. Human macrophages were cultured at a concentration of 1 × 10⁶ peripheral blood mononuclear cells/well on 24-well plates for 7 days before infection. Infection was performed with 1 × 10⁶ stationary-phase promastigotes per well for 3 hours at 35°C. Supernatants (in triplicate) were harvested 72 hours after infection and TGF-β measured in a bioassay with CCL-64 mink lung epithelial cells. Ld,* L. donovani; *Lb,* L. braziliensis; *La,* L. amazonensis.

induced 295 ± 7.6 pg/ml, and the lowest production was induced by *L. braziliensis* (196 ± 15.7 pg/ml). The experiment was repeated twice with similar results.

Effect of TGF-β on Parasite Growth inside Macrophages in Vitro

TGF-β added to cultures of human macrophages infected with *L. braziliensis* led to an increase of approximately 50% in parasite numbers as compared with untreated cultures (Figure 2). By contrast, the addition of either TNF-α or IFN-γ resulted in a reduction in parasitism (Figure 2). Differences among the three treatments were statistically significant when tested by analysis of variance ($F = 22.26$; $P < 0.0001$). Differences between TGF-β and IFN-γ (Scheffé, 18.89; $P < 0.01$) and TGF-β and TNF-α (Scheffé, 15.20; $P < 0.01$) were statistically significant, whereas the difference between IFN-γ and TNF-α was not. The experiment was performed on different occasions with cells from 10 different normal volunteers as the source of macrophages.

TGF-β Blocks the Effects of IFN-γ or TNF-α on Leishmania *Killing*

To evaluate the capacity of TGF-β in blocking the effects of IFN-γ or TNF-α macrophages, parallel cultures were set up with or without TGF-β at a concentration of 10 ng/ml. Both sets of cultures were also exposed to different doses of IFN-γ (6 doses in the range of 0 to 100 IU/ml) or TNF-α (4 doses ranging from 0 to 100 IU/ml). The suppressive effect of TGF-β is observed across the whole dose-response curve

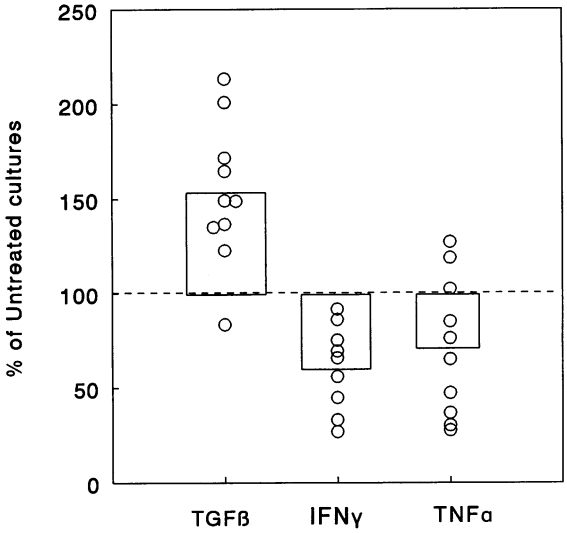

Figure 2. *Effect of TGF-β, IFN-γ, or TNF-α on* in vitro *parasite multiplication inside human macrophages. Human monocyte-derived macrophages were cultured and infected with* L. braziliensis *stationary-phase promastigotes as indicated in Figure 1. Immediately after infection cultures were treated with TGF-β (10 ng/ml), IFN-γ (50 IU/ml), or TNF-α (100 IU/ml). Number of amastigotes per 100 macrophages were determined 48 hours after infection. Results are shown as percentage of the number of parasites counted in cultures left without treatment. Each point represent a different volunteer used for obtaining macrophages. In a typical experiment the number of amastigotes per 100 macrophages was as follows: untreated cultures, 296 ± 54, and treatments with TGF-β, 426 ± 161; TNF-α, 197 ± 46; and IFN-γ, 143 ± 70.*

with IFN-γ. Even at 100 IU/ml of IFN-γ, the presence of TGF-β increases the number of intracellular parasites, although at lower levels than in cultures without or with small amounts of IFN-γ. TNF-α at high concentration (100 IU/ml) totally blunts the suppressive effect of TGF-β.

Immunohistochemistry for TGF-β in Lesions

The presence of TGF-β was observed diffusely in the dermis and occasionally in epidermis appendages. We focused our attention, however, on TGF-β observed in association with the inflammatory infiltrate. Immunostaining for TGF-β was observed in the dermis, produced by fibroblasts and occasionally by inflammatory cells in the biopsies from human leishmaniasis lesions (Figure 4A). The presence of TGF-β was observed in the majority (approximately two-thirds) of lesions obtained from patients with disease for less than 2 months (Table 1). The rate of positivity dropped in the lesions from cutaneous patients with longer disease periods and was present in approximately one-half of mucocutaneous lesions (Table 1). The mucocutaneous lesions with detectable TGF-β were obtained from cases with active disease. TGF-β was observed in three cases of DCL even after treatment (Table 2). Although DCL is character-

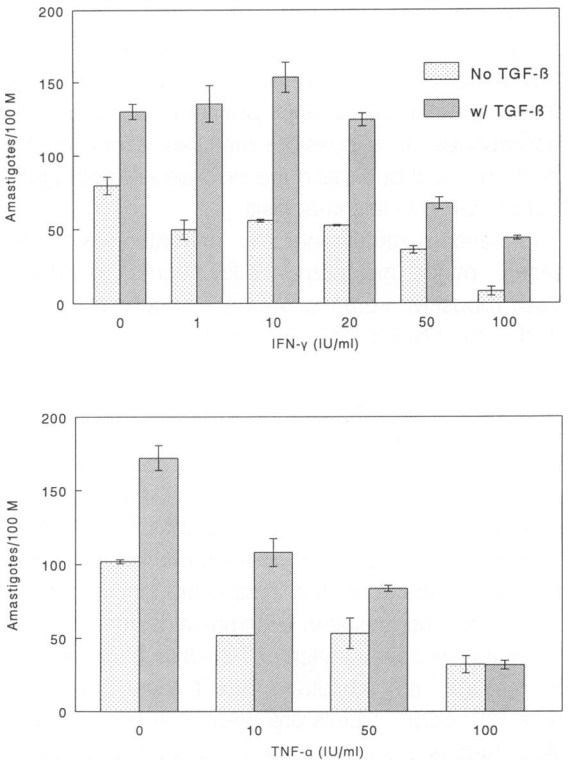

Figure 3. *Effect of TGF-β on* in vitro *intra-macrophage* Leishmania *killing induced by different doses of IFN-γ* (upper panel) *or TNF-α* (lower panel). *Human monocyte-derived macrophages were cultured and infected with* L. braziliensis *stationary-phase promastigotes as indicated in Figure 1. Immediately after infection cultures were treated with TGF-β (10 ng/ml), IFN-γ (50 IU/ml), TNF-α (100 IU/ml), or a mixture of TGF-β (10 ng/ml) plus the indicated amounts of either IFN-γ or TNF-α. Number of amastigotes per 100 macrophages were determined 48 hours after infection.*

ized by intense parasite growth, TGF-β was detected with high intensity in only one of six cases examined (Table 2). Immunohistochemistry for TGF-β in normal skin was negative.

Table 1. *Immunohistochemistry to TGF-β in Lesions from Human Cutaneous and Mucosal Leishmaniasis*

Disease		Anti-TGF-β reaction	
Form	Duration	Intensity	Positivity
Early cutaneous	15 d	++++	
	15 d	+	
	15 d	++	
	15 d	+++	
	1 m	+	
	1 m	++	
	1 m	++	8/12
	1 m	Neg	
	2 m	+	
	2 m	++	
	2 m	Neg	
	2 m	Neg	
Late cutaneous	4 m	+	
	5 m	+	
	5 m	Neg	2 /5
	8 m	Neg	
	3 y	Neg	
Mucosal	6 m	Neg	
	8 m	Neg	
	1 y	++	
	2 y	Neg	
	3 y	+	4 /8
	12 y	Neg	
	15 y	+++	
	18 y	++++	

Intensity of staining was graded on a scale of 0 to ++++. d, days; m, months; y, years; Neg, negative.

Discussion

The production of active TGF-β by monocyte-derived human macrophages after infection by *Leishmania* extends to the human system previous observations made with mice.[11–13] Latent TGF-β may be produced by uninfected macrophages, but the presence of the active molecule may bear importance for parasite survival and infectivity. *Leishma-*

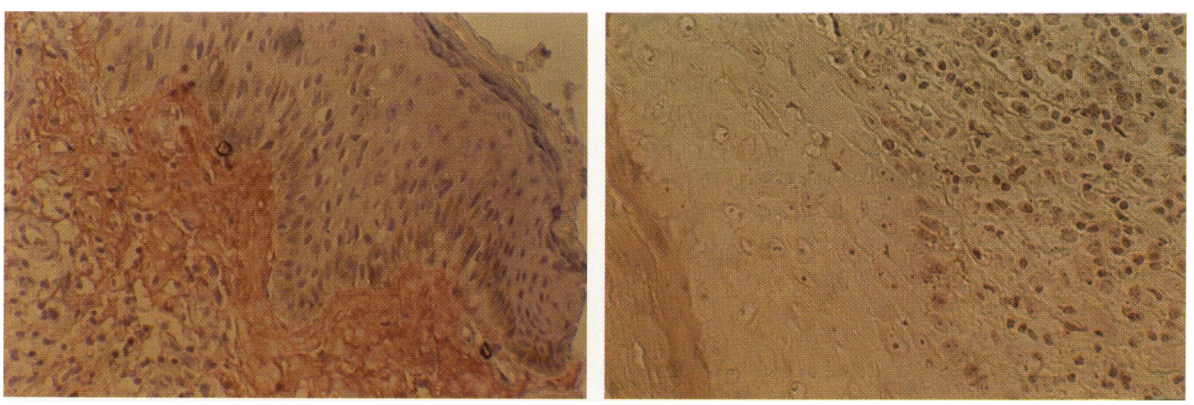

Figure 4. *Immunohistochemical demonstration of TGF-β in human cutaneous leishmaniasis lesion, obtained from a CL case with 15 days of duration and considered* +++. Left panel: *Positive stain (red) for TGF-β in the dermis of a tissue section from a leishmanial skin lesion treated with anti-TGF-β polyclonal antibody.* Right panel: *Negative control, showing absence of stain in a section treated with normal rabbit IgG.*

Table 2. *Immunohistochemistry to TGF-β in Pre- and Post-Treatment Lesions from Human Diffuse Cutaneous Leishmaniasis*

Patient	Clinical status	Intensity
1	Pretreatment	+
	Post-treatment	+
2	Pretreatment	Neg
	Post-treatment	Neg
3	Pretreatment	++++
	Post-treatment	+
4	Pretreatment	Neg
	Post-treatment	Neg
5	Pretreatment	Neg
	Post-treatment	Neg
6	Pretreatment	+
	Post-treatment	+

Intensity of staining was graded on a scale of 0 to ++++. Neg, negative.

nia-induced production of TGF-β by murine macrophages has been observed with *L. amazonensis*[12] or *L. braziliensis*.[11] In the case of *L. braziliensis* a higher production of active TGF-β was obtained with a more virulent isolate as compared with a less virulent one.[11] It is premature to draw firm conclusions relating quantifiable differences in TGF-β production and *Leishmania* virulence, as it is very difficult to compare the severity of disease caused by different isolates in man.

When evaluated by immunohistochemistry in human tissues from leishmanial lesions, the presence of TGF-β was observed in 8 of 12 early (2 months or less) human cutaneous lesions and in active mucosal leishmaniasis. These data are consistent with a role for TGF-β in the initiation of *Leishmania* infection.

It is of importance that TGF-β was still detectable in several cases of DCL even after therapy. Shortly after treatment, DCL patients exhibit transient periods of marked clinical improvement, followed by relapses; post-treatment biopsies were taken during this period. TGF-β has been shown in the epidermis of human viral infection[28] and may indicate that local depression of immune mechanisms are important in allowing the pathogen to grow, as observed for *Leishmania* in the present study. In human neoplastic diseases, the presence of TGF-β in lesions has been correlated with the advancing edge of primary tumors[29] or depth of tumor invasion,[30] also indicating the role of TGF-β in lesion progression. A recent study demonstrated a higher expression of mRNA for TGF-β in human CL lesions with more than 4 months of disease than in earlier lesions.[31] It is possible that the continued production of TGF-β in some cases is implicated in the persistence of the *Leishmania* and in disease progression.

The presence of *Leishmania* inside the macrophage interferes with antigen presentation[32] and may influence the course of the disease. As TGF-β allows greater *Leishmania* proliferation inside the macrophage, it is possible that this is one of the mechanisms that explain the disease-enhancing effect of TGF-β in leishmaniasis.

In several immunologically mediated skin diseases, major histocompatibility complex (MHC) class II-positive keratinocytes are observed associated with IFN-γ production by the surrounding T cells. Besides antigen presentation, those cells seem to be able to produce cytokines and can maintain clonal expansion of activated lymphocytes. MHC class II-positive keratinocytes have been observed in CL but not in DCL lesions, suggesting a lower IFN-γ production in DCL lesions.[33] TGF-β inhibits IFN-γ-induced MHC class II expression[16] and may be implicated in decreased keratinocyte induction of CMI responses in leishmanial lesions.

Another possible action of TGF-β in leishmaniasis is through its inhibitory effect over cytotoxic cells.[19,20] $CD8^+$ T cells are present in both CL and MCL human lesions but not in delayed-type hypersensitivity reactions elicited by *Leishmania* antigen, suggesting a role for these cells in the destruction of infected cells.[34] We have shown the presence of cytotoxic cells in the peripheral blood of CL and MCL patients and its down-modulation by TGF-β or interleukin-10.[35] We recently developed an assay with autologous infected macrophages both as antigen-presenting and target cells. In this system, MCL patients exhibited cytotoxic activity mediated by $CD8^+$ and/or NK cells, and this response is down-modulated by TGF-β (manuscript in preparation).

After *Leishmania* penetration, the human macrophage produces not only TGF-β, as shown in this study, but also other cytokines such as TNF-α,[8] which promotes macrophage activation for *Leishmania* killing.[9,10] Other products, such as interleukin-12, that are produced early after *Leishmania* infection probably also participate in this balance.[36] The outcome of the initial infection will likely depend on the predominance of host-protective or deleterious cytokines; however, what determines the predominant production on any opposing group remains to be investigated.

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