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# Differential immune regulation of activated T cells between cutaneous and mucosal leishmaniasis as a model for pathogenesis

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## SUMMARY

*Cutaneous (CL) and mucosal leishmaniasis (ML) are characterized by a predominant type 1 immune response (IFN-γ and TNF-α production) and strong inflammatory response in the lesions with few parasites. This exacerbated type 1 response is more evident in ML as compared to CL. Our main hypothesis is that a differential immune regulation of T cell activation leads to over reactive T cells in ML. In the present study, we investigated immunological factors that could explain the mechanisms behind it by comparing some immune regulatory mechanisms between ML and CL patients: frequency of cells expressing co-stimulatory molecules, apoptotic markers, T cell activation markers; and ability of neutralizing antibodies to IL-2, IL-12 and IL-15 do down-regulate IFN-γ production in leishmania antigen-stimulated peripheral blood mononuclear cells (PBMC). Interestingly, in CL anti-IL-2 and anti-IL-15 significantly suppressed antigen-specific IFN-γ production, while in ML only anti-IL-2 suppressed IFN-γ production. Finally, higher frequency of CD4+ T cells expressing CD28−, CD69+ and $CD62L_{low}$ were observed in ML as compared to CL. These data indicate that an exacerbated type 1 response in ML is differentially regulated and not appropriately down modulated, with increased frequencies of activated effectors T cells, maintaining the persistent inflammatory response and tissue damage observed in ML.*

**Keywords** *activated T cells, cutaneous leishmaniasis, cytokines, human leishmaniasis, mucosal leishmaniasis*

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## INTRODUCTION

American cutaneous leishmaniasis (CL) is a disease characterized by a cutaneous ulcer predominantly on the lower limbs. In areas of *Leishmania braziliensis* transmission, ~3% of patients with active or past CL develop mucosal leishmaniasis (ML), a disease that affects predominantly the nose, leading to tissue damage and occasionally disfiguring facial lesions (1). Visceral leishmaniasis (VL) and diffuse cutaneous leishmaniasis (DCL) are associated with impaired T cell response against parasite antigens (2,3). In contrast, patients with CL and ML have a strong type 1 immune response to soluble leishmania antigen (SLA) (4). It is well known that Th1 mediated immunity is important for the control of leishmania infection and that oxidants produced by IFN-γ activated macrophages are the main final effector molecules killing leishmania (5,6). However, evidence has accumulated that an exaggerated T cell response is a cause of pathologic lesions in CL and even more in ML. This evidence includes the following: (i) lymphocytes from individuals with CL or ML produce high amounts of IFN-γ and TNF-α, two important pro-inflammatory cytokines, in peripheral blood and tissue (4,7); (ii) the lesions are characterized by a rich inflammatory infiltrate and parasites are rare or undetectable; (iii) a high frequency of cells expressing IFN-γ and low frequency of cells expressing IL-10 receptor is observed in tissue by confocal microscopy (8); (iv) during the initial stages of CL (lesions < 20 days old), granulomatus vasculitis precedes the appearance of the ulcer (9); (v) there is a correlation between the frequency of inflammatory cytokine producing T cells and lesion size (10). (vi) drugs that down-modulate the immune response associated with antimony therapy increase the cure rate and decrease the healing time of cutaneous and mucosal lesions (11,12).

The immunopathogenesis of CL and ML is dependent on a complex interplay involving parasite and host factors. Whereas in peripheral blood there is a very strong production

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of type 1 cytokines that decreases with the resolution of lesions (4), both type 1 and type 2 cytokines are observed in tissue (7,13). More recently, we demonstrated by confocal microscopy that activated CD4+ T cells producing IFN-γ is more frequently found in ML than in CL lesions. Moreover, ML lesions present lower frequency of cells expressing IL-10 receptor (13). As IL-10 is one of the cytokines that down-regulate inflammatory response, the reduced expression of its receptor would impair IL-10 ability to down-regulate immune response in ML lesions, explaining the intense inflammatory infiltrate and tissue damage observed in this disease. On the parasite side of pathogenesis, recent studies have shown that *L. braziliensis* from CL and ML are polymorphic and there is an association between genetically distinct parasite isolates and the different clinical forms of disease (7). We have previously shown that, in comparison with cells from patients with CL, SLA-stimulated peripheral blood mononuclear cells (PBMC) from individuals with ML secrete higher levels of IFN-γ and TNF-α and lower amounts of IL-10 (4). High levels of TNF-α are also detected in sera from ML patients during active disease, and these decrease after therapy (14). Moreover, the exacerbated T cell response of PBMCs from individuals with ML is not appropriately modulated by IL-10 and TGF-β *in vitro* (4). However, the mechanisms responsible for the refractory inflammatory response in ML patients are still unknown. Factors that could be involved in inducing an exaggerated T cell response in such patients include high expression of co-stimulatory molecules, decreased expression of IL-10 receptor, decreased T cell apoptosis, increased number of memory/activated T cells and/or lack of regulatory T cells. In the current study, PBMCs from CL and ML patients were compared regarding: (i) expression of co-stimulatory molecules; (ii) frequency of apoptotic cells; (iii) frequency of activated/effectors T cells and (iv) ability of anti-IL-2, anti-IL-12 and anti-IL-15 to down-modulate the antigen specific IFN-γ response.

## MATERIALS AND METHODS

### Patients

Twenty-two ML patients were selected and matched by age (± 5 if < 20 years old and ± 10 if > 20 years old), gender and disease duration with 22 CL patients. Patients were recruited from the health post of Corte de Pedra, a region with high transmission of *L. braziliensis* in southeast Bahia, Brazil. This health post is the reference centre for cases of leishmaniasis in 22 municipalities. Every year, approximately 20 ML cases and 800 CL cases are diagnosed in the health post. Inclusion criteria included the diagnosis of CL and ML, based on the presence of characteristics cutaneous skin ulcer or mucosal lesion, respectively, a positive skin test reaction (> 5 mm) to *Leishmania* antigen, and either isolation of the parasite or histopathological findings characteristic of these diseases. All patients had between 30 and 90 days of disease duration, making the two groups more homogeneous. All patients were evaluated prior to therapy. Exclusion criteria included age < 5 and > 60 years, malnutrition, HIV infection, diabetes mellitus and pregnancy. Informed consent was obtained from all patients and controls or guardians of minors. The Ethics Committee of Hospital Professor Edgard Santos, the national committee for ethics in research (CONEP) and NIH-registered IRB committee approved this study. After collecting blood for the immunological evaluation the patients were treated with pentavalent antimony (Glucantime) and all had a good response to treatment. Healthy subjects ($n = 6$) with positive delayed-type hypersensitivity test for purified protein derivated from Mycobacteria (PPD positive) were used as controls in some experiments.

### Antigen

*Leishmania braziliensis* SLA was obtained from an isolate of a ML patient prepared as previously described (15). Briefly, parasites were harvested in stationary phase of growth, freeze and thawed five times, sonicated, and centrifuged to remove insoluble materials. The protein concentration was measured in the supernatant of this SLA. In some cultures a tuberculin purified protein derivative (PPD-CT68; Connaught Laboratories, Ontario, Canada) was used.

### Cell culture and cytokine assays

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood by Ficoll-Hypaque gradient centrifugation. After washing three times in 0·9% NaCl, the PBMC were resuspended in RPMI 1640 culture medium (GIBCO BRL, Grand Island, NY) supplemented with 10% human AB serum, 100 IU/mL of penicillin and 100 mg/mL of streptomycin. Because enough cells were not obtained to perform all type of experiments in all patients, the experiments with samples from matched CL and ML patients were performed in the same day. For cytokines assays, PBMCs from ML and CL patients were adjusted to $3 \times 10^6$ cells/mL, placed in 24-well plates, and stimulated with SLA (10 μg/mL). Monoclonal antibodies anti-IL-2 (clone 533421), anti-IL-12 p70 or anti-IL-15 (clone 34505·11), or recombinant CTLA-4/FC Chimera (R&D Systems, Minneapolis, MN) were added to some SLA-stimulated, PPD-stimulated or unstimulated cultures. Because the cell numbers from the patients were insufficient to perform all these experiments, unequal numbers of patients were analyzed for each set of experiment.


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The number of patients is specified in each of the specific sections. Dose-response curves were performed, and showed that optimal concentrations for monoclonal antibodies was 20 µg/mL and the optimal concentration for recombinant CTLA-4 was 5 µg/mL. Cultures were incubated for 72 h at 37°C with 5% $CO_2$. Supernatants were collected and stored at −70°C. The levels of IFN-γ were measured by the ELISA sandwich method using commercial reagents (Duoset, R&D Systems, Minneapolis, MN). The results are expressed in pg/mL or in mean of the percentage of IFN-γ suppression in the case of the experiments with neutralizing antibodies (anti-IL-2, IL-12, IL-15) and CTLA-4.

### Evaluation of surface cell markers and apoptosis

Analysis for surface markers was performed using fluorescence-activated cell sorter (FACS), according to a previous publication (16). Peripheral blood mononuclear cells (PBMC) from eight LC and eight ML patients obtained by separating whole blood over Ficoll and washing three times with medium RPMI 1640 (GIBCO BRL, Grand Island, NY) supplemented with 10% human AB Rh+ serum (SIGMA Chemical Co. St. Louis, MO) 100 IU/mL penicillin and 100 µg/L streptomycin. $2 \times 10^5$ PBMC were incubated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE)-labelled antibody solutions for 20 min at 4°C. After staining, preparations were washed with 0·1% sodium azide PBS fixed with 200 µL of 2% formaldehyde in PBS and kept at 4°C until data acquired using a FACS (Becton Dickinson, Palo Alto, CA). The antibodies used for the staining were anti-CD80, anti-CD86, anti-CD40, anti-CD40L, anti-CTLA-4, anti-CD69, anti-CD62L, anti-CD28 or anti-HLA-DR FITC-labelled and CD4, anti-CD8 and anti-CD14 PE-labelled (PharMingen, San Diego, CA). Isotype control antibodies were IgG1 and IgG2a. Fluorescence-activated cell sorter (FACS) data were based upon two gated regions based on cell size and granularity: R1, which contained only lymphocytes and blasts; and R2, a monocyte region. A total of 20 000 events were acquired for each sample. Apoptosis was detected by binding of Annexin V to CD4+ and CD8+ cells by flow cytometry using a commercial kit (Becton Dickinson, Palo Alto, CA).

### Statistical analysis

Considering that the values did not have a Gaussian distribution, the Mann–Whitney test was used to compare all data between CL and ML patients. To compare IFN-γ production in PBMC cultures from the same patients incubated with media alone or media plus CTLA4/FC or with antibodies anti-cytokines, paired analyses were performed by Wilcoxon matched-pairs test.

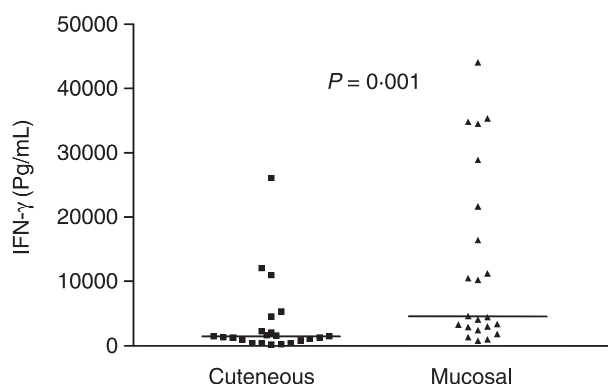


**Figure 1** IFN-γ production on SLA-stimulated PBMC supernatants from CL and ML patients. PBMC cultures from 22 CL and 22 ML patients were incubated for 72 h in the absence or in the presence of SLA (10 µg/mL). IFN-γ levels were measured in PBMC culture supernatants by ELISA sandwich technique and results expressed in pg/mL based on a standard curve with recombinant IFN-γ. The results represent the levels of IFN-γ from SLA-stimulated cultures subtracted by levels on unstimulated cultures. Statistical comparisons between CL and ML groups were done by Mann–Whitney U test.

## RESULTS

### Inflammatory profile of ML and CL patients

In order to confirm our previous findings concerning the exacerbated inflammatory nature of the cellular immune response in ML as compared to CL patients (4), we determined the levels of IFN-γ in supernatants of mononuclear cells from cultures of 22 CL and 22 ML patients. IFN-γ levels in SLA-stimulated PBMC supernatants, subtracted by the background (PBMC cultured in media) are presented in Figure 1. IFN-γ production by PBMCs from ML patients was significantly higher than from CL patients (12 751 ± 13 871 pg/mL vs. 3567 ± 5973 pg/mL; $P = 0{\cdot}001$). Next, using flow cytometry, we also confirmed our previous observation (4,17) that CD4 T cells were the major source of this IFN-γ from both CL and ML patients (data not shown).

### Expression of co-stimulatory molecules

To evaluate whether the higher IFN-γ production observed in cultures from ML patients was due to an increase in co-stimulatory molecules, the frequency of cells expressing HLA-DR, CD80, CD86, CD40, CD40L and CTLA-4 was determined in PBMCs of eight CL and eight ML patients by FACS analysis. The frequency of cells expressing HLA-DR was 100% in both groups of patients. The frequency of CD14+ antigen presenting cells (APCs) expressing CD80, CD86, CD40 and of CD4+ T cells expressing CD40L was higher in ML (4·6 ± 5·94, 60·6 ± 35·89, 82·4 ± 10·67, 63·43

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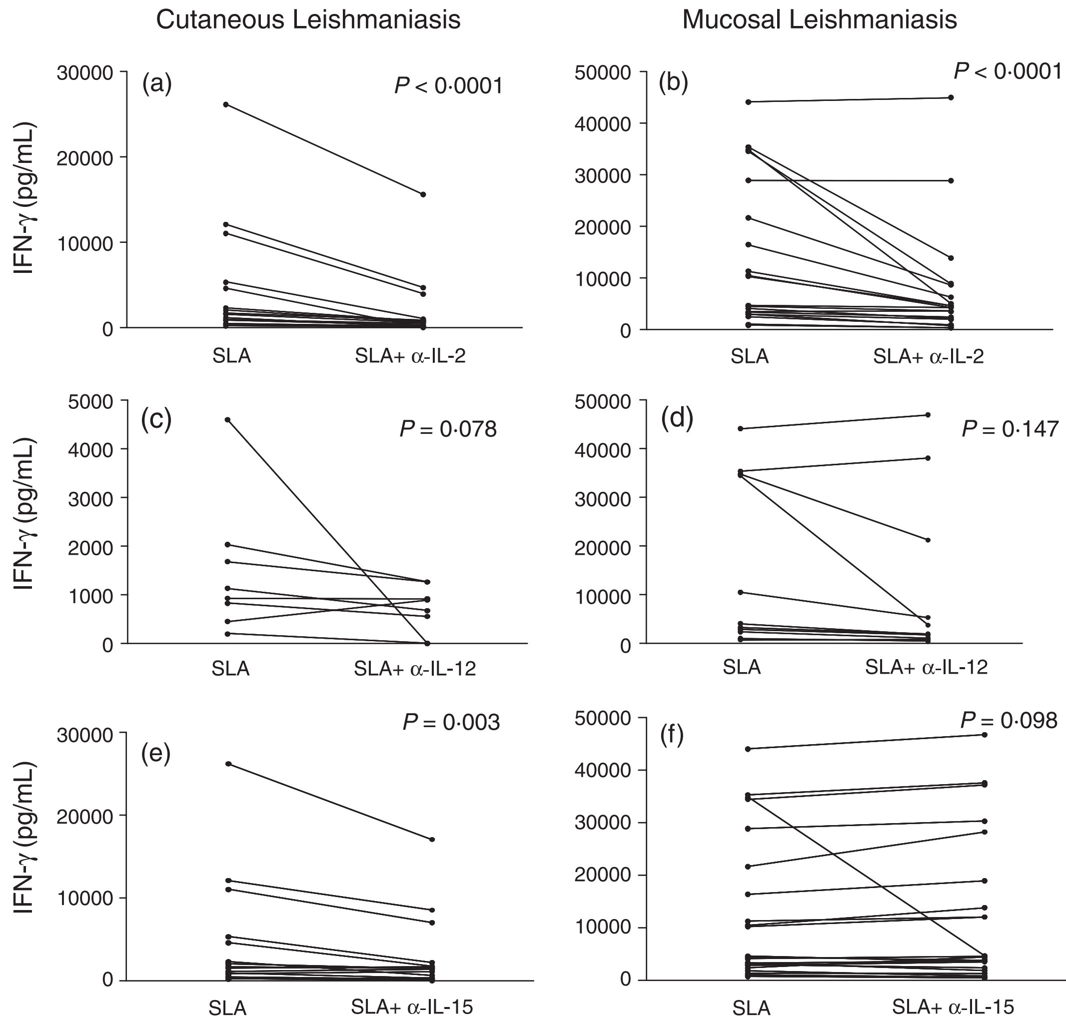


**Figure 2** Ability of anti-IL-2 (20 μg/mL), anti-IL-12 (20 μg/mL) or anti-IL-15 (20 μg/mL) in suppress SLA (10 μg/mL)-induced IFN-γ production in CL and ML patients. Anti-IL-2 and anti-IL-15 were added to SLA-stimulated PBMC cultures from 16 each CL and ML patients and anti-IL-12 was added to SLA-stimulated PBMC of eight CL and nine ML patients. IFN-γ levels were determined by ELISA on PBMC supernatants. a, c and e show mean ± SD of results using cultured PBMCs from CL patients; b, d and f show results using PBMCs from ML patients. All conditions were tested in triplicate in each experiment. In all these experiments, a control with antibody of the same isotype was done, and they do not inhibit IFN-γ production. Statistical comparisons were done using the Wilcoxon matched-pairs test.

± 48·88) than in CL (3·4 ± 2·96, 47·6 ± 30·12, 50·96 ± 40·05, 25·9 ± 32·86), but these differences did not reach statistical significance. There was also a tendency towards decreased frequency of CD4+ T cells expressing CTLA-4 in ML (1·2 ± 0·59) compared to CL patients' PBMCs (5·6 ± 5·63).

### Ability of anti-IL-2, anti-IL-12, anti-IL-15 or CTLA-4 to suppress IFN-γ production

IL-12 is a cytokine produced by APCs and is involved in Th1 cell differentiation. IL-2 is the main T cell growth factor and IL-15, in addition to inducing lymphocyte proliferation, protects cells from apoptosis. To determine the role of IL-2, IL-12 and IL-15 in SLA specific IFN-γ production, monoclonal antibodies were used to neutralize these cytokines in SLA-stimulated cultures (Figure 2). Addition of anti-IL-2 to SLA-stimulated PBMC from 16 CL patients (Figure 2a), decreased IFN-γ production by 70 ± 17% (range 41–100%; $P < 0·0001$), while in SLA-stimulated PBMC from 16 ML patients, anti-IL-2 decreased IFN-γ production by 47 ± 28% (Figure 2b, range 0–79%; $P < 0·0001$). Neutralization of IL-12 had a weak effect, decreasing SLA-induced IFN-γ production from PBMC of eight CL patients by 42 ± 38% (Figure 2c, range 0–100%; $P = 0·078$) and from PBMC of nine ML patients by 30 ± 24% (Figure 2d, range 0–57%; $P = 0·147$). A differential immune regulation between CL


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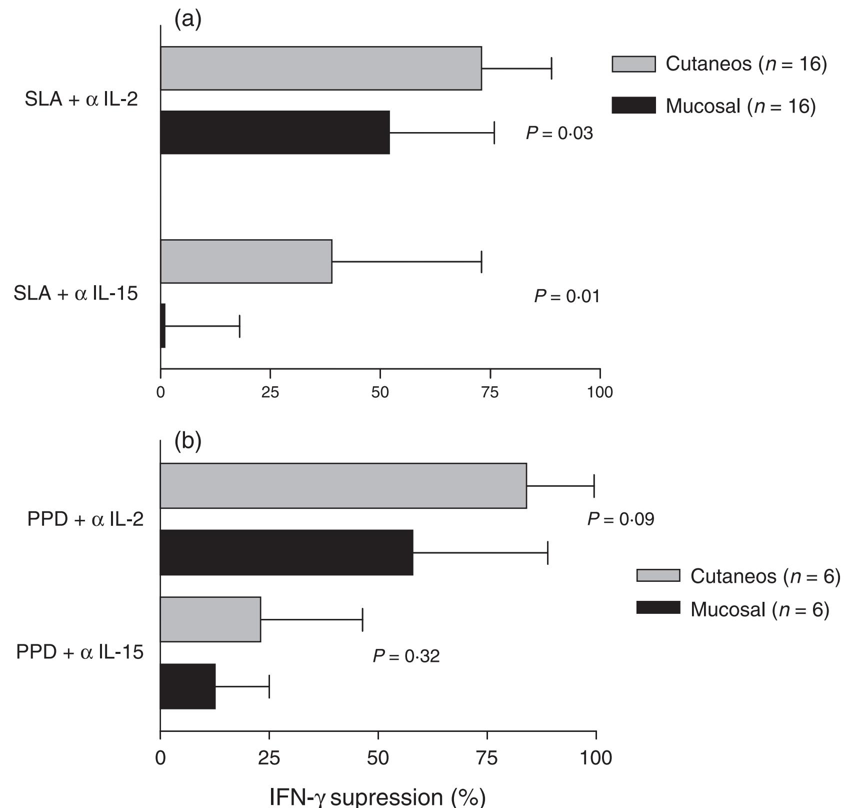


**Figure 3** Down-modulation of SLA-induced IFN-γ production by anti-IL-2 (20 μg/mL) and anti-IL-15 (20 μg/mL) is more evident in CL (*n* = 16) than in ML (*n* = 16) patients. A comparative analysis of the percentage of suppression modulated by anti-IL-2 or anti-IL-15 is shown as the mean ± SD suppression in SLA-stimulated PBMC from CL vs. ML patients. Data are compiled from the raw ELISA data shown in Figure 2(a,b,e,f). Ability of anti-IL-2 (20 μg/mL) or anti-IL-15 (20 μg/mL) to suppress PPD (10 μg/mL)-induced IFN-γ production from PBMC cultures from six CL and six ML patients. Shown here is the mean ± SD suppression. IFN-γ levels were measured by ELISA on supernatants of PPD-stimulated PBMC cultures with or without addition of anti-IL-2 or anti-IL-15 (4b). All conditions were tested in triplicate in each experiment. In all these experiments, a control with antibody of the same isotype was done, and they do not inhibit IFN-γ production. Statistical comparisons between the degrees of suppression in CL vs. ML groups were done by Mann–Whitney U test.

and ML was observed when IL-15 was neutralized by specific antibody. While addition of the monoclonal antibody anti-IL-15 to SLA-stimulated PBMC decreased the IFN-γ production from 16 CL patients by 39 ± 29% (Figure 2e, range 0–87%; $P = 0{\cdot}003$) neutralization of IL-15 in 16 ML patients only decreased by 11 ± 18% the IFN-γ production (Figure 2f, range 0–53%; $P = 0{\cdot}098$). Moreover, while 59% CL patients presented an inhibition of IFN-γ production over 30%, only 18% of ML patients presented an inhibition of IFN-γ production over 30%.

In spite of the ability of anti-IL-2 to modulate SLA-induced IFN-γ production in both CL and ML patients, IFN-γ suppression was more pronounced in cultured PBMCs from CL than from ML patients after neutralization of IL-2 (Figure 3a, $P = 0{\cdot}03$). Moreover, whereas anti-IL-15 down-modulated IFN-γ production in PBMC cultures from individuals with CL, there was no significant decrease of IFN-γ levels in PBMCs from ML patients after neutralization of IL-15 (Figure 3a). To determine whether the diminished ability of monoclonal antibodies anti-IL-2 or anti-IL-15 to down-regulate IFN-γ production in PBMCs from ML patients was antigen-specific, we neutralized IL-2 or IL-15 in PPD-stimulated PBMC cultures from PPD skin test positive CL (*n* = 6) and ML patients (*n* = 6). As shown in Figure 3(b), neutralization of IL-2 suppressed PPD-induced IFN-γ production in PBMC culture supernatants from CL patients 84 ± 15% (range 62–100%) and in cultured PBMCs from ML patients by 57 ± 31% (range 4–91%). Anti IL-15 had lower suppressive effect in PPD-induced IFN-γ productions in both CL (23 ± 23·4) and ML (12·6 ± 12·43; Figure 3b). There were no differences in the suppression mediated by anti-IL-2 and anti-IL-15 in PPD-stimulated PBMC, between ML and CL patients ($P = 0{\cdot}09$ and 0·32, respectively). However, similar to the observation in SLA-stimulated PBMC, the suppression of IFN-γ production was more pronounced in PPD-stimulated PBMC from CL than ML patients.

CTLA-4 is a down regulatory molecule, which competes with CD28 binding to the co-stimulatory molecules CD80 and CD86. Addition of CTLA-4 poorly down-modulate IFN-γ production in SLA-stimulated PBMC of nine CL and six ML patients (Mean ± SD = 18 ± 19·7% and 9 ± 15·8%, respectively), while it suppressed IFN-γ production from PPD-stimulated PBMC of six health controls 56 ± 43·3% (Figure 4).

## Apoptosis

The frequency of apoptotic cells was evaluated in PBMC preparations from five CL and five ML patients. Annexin V

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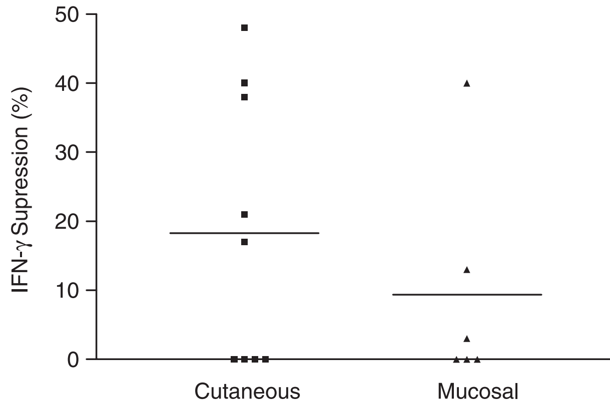


**Figure 4** Suppression of SLA-induced IFN-γ in PBMC from CL ($n$ = 9) and ML ($n$ = 6) patients by addition of CTLA-4 (5 µg/mL). The bars represents mean ± SD of the suppression levels of the groups. Differences between CL and ML are not statistically significant. Control experiments were performed with PPD-stimulated PBMC of six PPD positive healthy subjects, and show a suppression of 56 ± 43·3%.

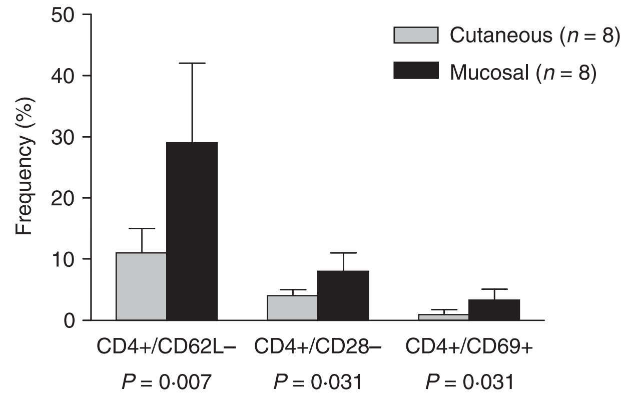


**Figure 5** Frequency of CD4+ T cells expressing cell activation markers *ex vivo*. The mean ± SD of the frequency of CD4+ cells in PBMC from eight CL vs. eight ML patients expressing activation markers ($CD62_{low}$, CD28–, CD69) were compared. Conditions were tested in triplicate. Statistical comparisons were done using the Mann–Whitney test.

staining was assessed using flow cytometric analysis of gated cells in the blast and lymphocyte regions to analyze PBMCs cultured in presence or absence of SLA. There were no statistically significant differences between the frequencies of total apoptotic cells in CD4+ or CD8+ T cells population from CL or ML patients (Data not shown).

### Activation of lymphocyte populations from CL and ML patients

The frequency of CD28, CD69 and CD62L expressing T cells was evaluated *ex vivo* using PBMC from eight CL and eight ML patients by flow cytometry (Figure 5). As a measure of direct recent T cell activation, the marker CD69 was used which demonstrated a higher frequency of CD4+ CD69+ cells in cultured PBMCs from ML (3·3 ± 1·8%) than from CL patients (0·93 ± 0·85%; $P$ = 0·031). Moreover, a higher frequency of CD4+CD28– T cells was also seen in PBMC from ML (8 ± 3%) as compared to CL patients (4 ± 1%; $P$ = 0·031). Finally, a higher frequency of CD4 + $CD62L_{low}$ cells was observed in PMBCs from ML (29 ± 13%) patients when compared to CL patients (11 ± 4%; $P$ = 0·007).

## DISCUSSION

Cell-mediated immune response is known to be important to control *Leishmania* sp. protozoa infection. An impairment in type 1 immune responses can lead to parasite multiplication and dissemination (2,7,18). However, in CL and even more so in ML patients, lymphocytes produce large amounts of IFN-γ and TNF-α, although few parasites can be found at the lesion site (8). The concepts that T cells may participate in leishmaniasis pathology in humans have been suggested from previous studies by our group and others, comparing the immune responses of ML and CL patients (4,17). More recently, these concepts have been supported also by animal studies (19). The current study extends these previous observations from our group showing that type 1 immune responses are elevated in both CL and ML. During both diseases, CD4+ T cells are the main source of IFN-γ, although also CD8+ and double negative cells also produce this cytokine (4,17). Despite the evidences that an exacerbated type 1 T cell response participates in the pathogenesis of tegumentary leishmaniasis the mechanisms involved in the exacerbated type 1 immune response have not been determined.

In the classical model of experimental CL, susceptible BALB/c mice infected with *L. major* develop a type 2 response that suppresses the curative type 1 immune response. In contrast resistant C57BL/6 mice develop a type 1 immune response and control infection (5,6). In CL and ML there is a predominant type 1 immune response but IL-4 and IL-5 are produced by PBMC and are also expressed at the lesion site (19–21). Therefore, Th1 polarization is not observed. In fact we have shown that during the initial phase of *L. braziliensis* infection there is a decrease in the type 1 immune response (18). Very quickly, however, the response changes and a strong antigen-specific production of IFN-γ and TNF-α is documented (18). We hypothesize that a persistent and non modulated inflammatory response might be the major factor responsible for the extensive tissue damage observed in ML.


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In the current study, we showed that the enhanced IFN-γ production observed in PBMC cultures from ML patients is not appropriately down-modulated by neutralization of cytokines involved in T cell activation and proliferation. Additionally, ML patients presented a higher frequency of activated effectors CD4+ T cells than CL patients. These data suggest that peripheral blood CD4+ T cells are more activated in ML than in CL patients *ex vivo,* possibly explaining why they are not easily modulated *in vitro.*

Several immunological parameters are consistent with the more exacerbated CD4+ T cell response in ML than in CL. Expression of co-stimulatory molecules is important for T cell activation. It is known that blocking CTLA-4 in experimental leishmaniasis enhances IL-12 and IFN-γ secretion (22) as well as antimony-associated leishmania killing (23). So, we would expect that rCTLA 4 would block the binding of B7·1/B7·2 with CD28 and reduce the IFN-γ production. CTLA-4 decreased IFN-γ production in SLA-stimulated PBMC by only 20% in CL and 10% in ML, while it decreased IFN-γ production induced by PPD in PBMC from health subjects by 56%. Since the co-stimulation between B7·7/B7·2 and CD28 is necessary for the early events of T cell activation, this data suggest that the majority of T cells from both CL and ML patients are already pre-activated in peripheral blood. The reduced capability of antibodies anti-IL-12 and anti-IL-15 to down-modulate IFN-γ production also support the presence of pre-activated CD4+ T cells in peripheral blood of these patients.

A decrease in the rate of apoptosis can also explain the exacerbated immune response by maintaining activated cells after antigen stimulation. Although we did not detect a difference in the frequency of apoptotic CD4+ T cells between CL and ML patient, ML patients did express enhanced numbers of activated/memory T cells.

Cytokines involved in T cell differentiation and proliferation (IL-2, IL-12 and IL-15) are important in the maintenance of T cell activation. IL-12 is important for T cell differentiation and proliferation. Neutralization of IL-12 suppressed *Staphylococcus epidermidis*-induced IFN-γ release (24) and exogenous addition of IL-12 restored *in vitro* T cells responses in VL (25). However, besides IL-12, other cytokines produced by APCs such as IL-18, IL-23 and IL-27 also induces T cell differentiation to Th1 (12,26,27). During the current study the addition of anti-IL-12 did not modulate SLA-induced IFN-γ production in both ML and CL patients, likely reflecting the fact that these cells are already differentiated toward a type 1 phenotype and no longer depend on IL-12. However, this data might also suggest that other cytokine from the IL-12 family (IL-23, IL-27, IL-18) is more importantly produced during T cell activation by APCs in CL and ML. In animal models, there are evidences of a differential production of these cytokines in response to distinct intracellular pathogens and each of these cytokines leads to a peculiar T cell response. For instance, IL-23 induces activation of a T cell that produces IL-17, which induces a very high inflammatory response in the tissue. Future studies should be performed to evaluate the role of these cytokines in human CL and ML.

IL-15 induces lymphocyte proliferation and protects cells from apoptosis, thereby generating memory T cells (28) and anti-IL-15 can down-modulate lymphocyte proliferation (29). In our study, neutralization of IL-15 down-modulated SLA-induced IFN-γ secretion by PBMC from CL patients but had a weaker effect on PBMC from ML patients. The difference in SLA response between CL and ML patients could indicate that many of these cells are recently activated, or that, similar to the PPD-responsive memory cells, these have become IL-15 independent. (26,30) Our data concerning the higher expression of CD69+ *ex vivo* in these patients PBMC supports this hypothesis.

As a consequence of antigen stimulation, lymphocytes become activated and later differentiate to effectors memory T cell. Increasing number of cells expressing activation markers have been documented in other chronic inflammatory diseases (31,32). We found the frequency of CD4+ T cells expressing activation markers *ex vivo* was significantly higher in ML than CL patients. It is likely that these are effectors CD4+ T cells, which could explain not only the increased IFN-γ production in ML, but also the differences in the ability of neutralizing antibodies against IL-15 between ML and CL, as well as the inability of IL-10 and TGF-β in down-modulate IFN-γ production in ML patients previously shown by our group (4).

The data from this study document the extent of the exaggerated type 1 immune response in PBMC from ML patients in comparison to CL patients.

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