

Protective and Pathological Functions of CD8⁺ T Cells in *Leishmania braziliensis* Infection

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Cutaneous leishmaniasis (CL) caused by *Leishmania braziliensis* is characterized by a strong Th1 response that leads to skin lesion development. In areas where *L. braziliensis* transmission is endemic, up to 15% of healthy subjects have tested positive for delayed-type hypersensitivity to soluble leishmania antigen (SLA) and are considered to have subclinical (SC) infection. SC subjects produce less gamma interferon (IFN- γ) and tumor necrosis factor alpha (TNF- α) than do CL patients, but they are able to control the infection. The aim of this study was to characterize the role of CD8⁺ T cells in SC infection and in CL. Peripheral blood mononuclear cells (PBMC) were stimulated with SLA to determine the frequencies of CD4⁺ IFN- γ ⁺ and CD8⁺ IFN- γ ⁺ T cells. Monocytes from PBMC were infected with *L. braziliensis* and cocultured with CD8⁺ T cells, and the frequencies of infected monocytes and levels of cytotoxicity markers, target cell apoptosis, and granzyme B were determined. The frequency of CD8⁺ IFN- γ ⁺ cells after SLA stimulation was higher for SC individuals than for CL patients. The frequency of infected monocytes in SC cells was lower than that in CL cells. CL CD8⁺ T cells induced more apoptosis of infected monocytes than did SC CD8⁺ T cells. Granzyme B production in CD8⁺ T cells was higher in CL than in SC cells. While the use of a granzyme B inhibitor decreased the number of apoptotic cells in the CL group, the use of z-VAD-FMK had no effect on the frequency of these cells. These results suggest that CL CD8⁺ T cells are more cytotoxic and may be involved in pathology.

Leishmaniasis is caused by infection with parasites of the genus *Leishmania*. Leishmaniasis is a neglected tropical disease; 214,000 new cases of cutaneous leishmaniasis (CL) are reported annually worldwide, and the estimated incidence of leishmaniasis is 690,000 to 1,200,000 cases. Approximately 67,000 cases are reported in South America, Central America, and the Caribbean (1). In mice, the majority of *Leishmania*-specific CD4⁺ T cells differentiate into T-helper 1 (Th1) cells that secrete gamma interferon (IFN- γ) and contribute to the elimination of the parasite through the activation of macrophages (2, 3). Although protective immunity has predominantly been related to IFN- γ -producing CD4⁺ T cells, infection with *Leishmania* also results in the activation and expansion of parasite-specific CD8⁺ T cells (4, 5).

Human CL caused by *Leishmania braziliensis* is characterized by a strong Th1 response with the production of high levels of IFN- γ and tumor necrosis factor alpha (TNF- α) (6, 7). This exaggerated Th1 response is associated with the development of lesions and the severity of the disease (6, 8–10). In patients with CL caused by *L. braziliensis*, there are more CD4⁺ than CD8⁺ T cells, but this ratio reaches an equilibrium due to the increase in CD8⁺ T cells that occurs during the healing process (11). The enrichment of *Leishmania*-reactive CD8⁺ T cells in older lesions suggests that these cells may play a role in the healing process (12). In contrast, other studies have associated CD8⁺ T cell functions with pathology. For example, the cytotoxicity mediated by CD8⁺ T cells is greater in mucosal leishmaniasis (ML), a more severe form of *L. braziliensis* infection, than in CL (13, 14). More recently, it was shown that the frequency with which CD8⁺ T cells express granzyme in the lesions of CL patients is greater than that in patients in the early phase of CL (i.e., before the ulcer has developed) and that the frequency with which CD8⁺ T cells express granzyme is directly associated with the intensity of the inflammatory reaction

observed in CL ulcers (15, 16). This controversy regarding the role of cytotoxicity in the pathogenesis of human leishmaniasis indicates that the functions of CD8⁺ T cells in different clinical forms of leishmaniasis remain to be established.

Our studies have focused on diseases caused by *L. braziliensis*, the most important causal agent of American tegumentary leishmaniasis (ATL) in South America. In areas of *L. braziliensis* transmission, CL represents more than 90% of the clinical forms of leishmaniasis (17). However, the ratio of infected individuals to those manifesting the disease is 3.7:1, indicating that the majority of subjects infected with *L. braziliensis* do not develop disease and are considered to have subclinical (SC) infections (18). Individuals with SC infection (SC individuals) are characterized by positive leishmania skin tests (LST), delayed-type hypersensitivity reactions to soluble leishmania antigen (SLA), or evidence of *in vitro* production of IFN- γ in cultures stimulated with SLA in the absence of active leishmaniasis or a history of the disease (19). These individuals produce less IFN- γ and TNF- α than do patients with

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CL (18, 20). The mechanisms by which individuals with SC *L. braziliensis* infection achieve control over the infection are not currently understood. In this study, we evaluated whether CD8⁺ T cells play a role in inducing protection or if they participate in lesion development in human *L. braziliensis* infection.

MATERIALS AND METHODS

Patients. This study was performed in the village of Corte de Pedra, an area in the state of Bahia, Brazil, where *L. braziliensis* transmission is endemic. Patients with CL ($n = 20$) had typical ulcerative skin lesions, and diagnoses were made based on parasite detection by culture aspirate histopathology or based on the presence of a typical CL lesion plus a positive LST. All patients with CL were evaluated before therapy. Household contacts of CL patients without active leishmaniasis or a history of the disease and with a positive LST and/or IFN- γ production in a lymphocyte culture stimulated with SLA were considered to have an SC infection ($n = 20$). Ten healthy controls (HC), i.e., LST-negative individuals, were also included in the study. All participants provided informed consent, and the study followed the guidelines of the Ethical Committee of the Federal University of Bahia.

Parasite culture and antigen preparation. The leishmania isolate MHOM/BR/2003/LTCP15344 was obtained from a skin lesion of a CL patient and was characterized as *L. braziliensis*. This isolate was initially cultivated in biphasic medium (NNN), and following isolation, the parasites were cryopreserved in frozen nitrogen. After selection, the parasites were expanded in complete Schneider's medium, and SLA was prepared as previously described (21).

Cell separation and frequencies of CD4⁺ and CD8⁺ T cells in CL patients and SC individuals. Heparinized blood samples from CL patients and SC individuals were separated in Ficoll-Hypaque gradients (GE Healthcare, Uppsala, Sweden), and peripheral blood mononuclear cells (PBMC) were collected. For determination of the *ex vivo* frequencies of CD4⁺ and CD8⁺ T cells, PBMC were stained with phycoerythrin (PE)-conjugated mouse anti-human CD3 and fluorescein isothiocyanate (FITC)-conjugated anti-CD8 and anti-CD4 (Sigma, St. Louis, MO) and analyzed by fluorescence-activated cell sorter (FACS) analysis.

Intracellular cytokine staining. To determine the frequencies of cells expressing cytokines, PBMC were resuspended in RPMI 1640 complete medium with 10% heat-inactivated human AB serum (Sigma, St. Louis, MO) at a concentration of 5×10^5 cells/ml in polypropylene tubes and kept unstimulated or stimulated with SLA (5 μ g/ml) for 12 h at 37°C and 5% CO₂. After this culture period, 10 ng/ml of brefeldin A (Sigma, St. Louis, MO) was added. Cells were then stained with FITC-conjugated mouse anti-human CD3 (clone SP34-2), allophycocyanin (APC)-conjugated anti-CD8 monoclonal antibody (MAb) (clone RPA-T8), PE-Cy5-conjugated anti-CD4 MAb (clone RPA-T4), and APC-conjugated anti-CD14 MAb (clone 61D3) (Sigma, St. Louis, MO). These cells were also stained with PE-conjugated mouse anti-human IFN- γ antibody (clone 4S.B3) (BD Biosciences, San Jose, CA) by use of BD Cytotfix/Cytoperm solution according to the manufacturer's instructions (BD Biosciences, San Jose, CA). A minimum of 50,000 gated events from each sample were collected in a FACSCanto II flow cytometer (Becton Dickinson and Company, Franklin Lakes, NJ) and analyzed using the FlowJo 7.6.5 program.

Cell sorting. CD8⁺ T cells were isolated from 1×10^7 PBMC/ml by use of a magnetic bead sorting system and a monoclonal anti-CD8 antibody according to the manufacturer's instructions (Dynabeads untouched human CD8 T cells; Invitrogen Dynal AS, Oslo, Norway). Monocytes (MOs) were isolated from 5×10^6 PBMC/ml by use of magnetic beads (Dynabeads untouched human monocytes; Invitrogen Dynal AS, Oslo, Norway) as previously described (22). The process was performed twice, and these cells were washed with phosphate-buffered saline (PBS) and adjusted to a concentration of 1×10^6 MOs/ml in complete RPMI medium.

The overall purity (including CL and SC subjects and healthy controls) of the resulting cells was 83% \pm 0.9% for CD8⁺ T cells and 87.3% \pm 4.5%

for MOs, as analyzed by flow cytometry using a specific FITC-anti-CD3 MAb (clone SP34-2), PE-anti-CD4 MAb (clone RPA-T4), PE-Cy5-anti-CD8 MAb (clone RPA-T8), and APC-anti-CD14 MAb (clone 61D3). Cell viability was >90% as determined by trypan blue exclusion.

Infection of monocytes. Promastigotes in the stationary phase were adjusted to 1×10^7 /ml, labeled with DDAO-SE (CellTracer FarRed, DDAO-SE; Invitrogen Molecular Probes) according to the manufacturer's instructions, and then utilized for monocyte infection. Monocytes (1×10^6) were cocultured with *L. braziliensis* promastigotes (5×10^6) in polypropylene tubes for 2 h at 37°C in 1 ml RPMI 1640. Non-ingested promastigotes were washed away with complete RPMI medium. The evaluation of infection was performed by cytometry and by microscopic evaluation of cytospin slides stained with Giemsa stain for 10 SC subjects, 10 CL patients, and 10 HC. No parasites were found outside monocytes by microscopic evaluation.

Cytotoxicity assay. The cytotoxicity assay was performed as previously described (23). Briefly, CD8⁺ T cells plus uninfected MOs or infected MOs (iMOs) were mixed in cocultures in polypropylene tubes at an effector/target ratio of 5:1 at 37°C and 5% CO₂ for 8 h. After incubation, 5 μ l PE-anti-CD14 MAb (clone 61D3; BD-Bioscience Pharmingen, San Jose, CA) was added for 15 min at 4°C. Afterwards, 5 μ l of annexin V-FITC in 1 \times annexin binding buffer (BD-Bioscience Pharmingen) was added for 20 min. Samples were analyzed on a FACSCanto II flow cytometer (BD-Bioscience Pharmingen, San Jose, CA). At least 50,000 gated events were collected and analyzed with FlowJo ThreeStar.

PE-anti-FasL MAb (clone L5178Y) and Alexa Fluor 647-anti-granzyme B MAb (clone GB11) (BD-Bioscience Pharmingen, San Jose, CA) were used to determine the levels of FasL and intracellular granzyme B expression in CD8⁺ T cells. The cytotoxicity index (CI) was calculated using the following formula: CI = number of CD14⁺ annexin V⁺ cells among CD8⁺ T cells incubated with *Leishmania*-infected monocytes minus number of CD14⁺ annexin V⁺ cells among CD8⁺ T cells incubated with uninfected monocytes (24).

Viability of *L. braziliensis* promastigotes after coculture of infected monocytes with CD8⁺ T cells. To evaluate if CD8⁺ T cells contribute to parasite killing, after coculture of infected macrophages with peripheral autologous blood CD8⁺ T cells for 8 h, the cells were washed and the medium was replaced by 0.5 ml of Schneider's medium (Sigma-Aldrich) supplemented with 10% fetal calf serum to quantify the number of viable parasites. The plates were cultured at 26°C for 5 additional days. The number of viable *L. braziliensis* parasites was estimated by the proliferation of extracellular motile promastigotes in Schneider's medium.

Granzyme B measurement. Supernatants from cocultures of cells from SC subjects, CL patients, and HC were collected after 8 h, and granzyme B levels were determined by enzyme-linked immunosorbent assay (ELISA) (BD Pharmingen and R&D Systems, Minneapolis, MN).

Functional assays. For inhibitory assays, cultures were performed in the presence of 3,4-dichloroisocoumarin (100 mmol liter⁻¹; Sigma-Aldrich, St. Louis, MO), a reactive serine esterase inhibitor (granzyme B inhibitor), and z-VAD-FMK (100 mM; Sigma), to block caspase activation (16). Phorbol myristate acetate (PMA) (10 ng/ml) plus ionomycin (500 ng/ml) was used as a positive control, and dimethyl sulfoxide (DMSO) (0.4%) was used as a negative control.

Statistical analysis. The Mann-Whitney U test was used to compare the frequencies of infected monocytes, results of cytotoxicity assays, granzyme B determinations, and levels of expression of apoptosis inducers after coculture. The Kruskal-Wallis test with Dunn's *post hoc* test was used to analyze the results of apoptosis of iMOs after coculture with CD8⁺ T cells, as well as the results of functional assays. The cutoff for statistical significance was set at a *P* value of <0.05. Statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA).

RESULTS

CD8⁺ T cells are the main source of IFN- γ in SC individuals. The frequencies of CD4⁺ T cells and CD8⁺ T cells were similar in

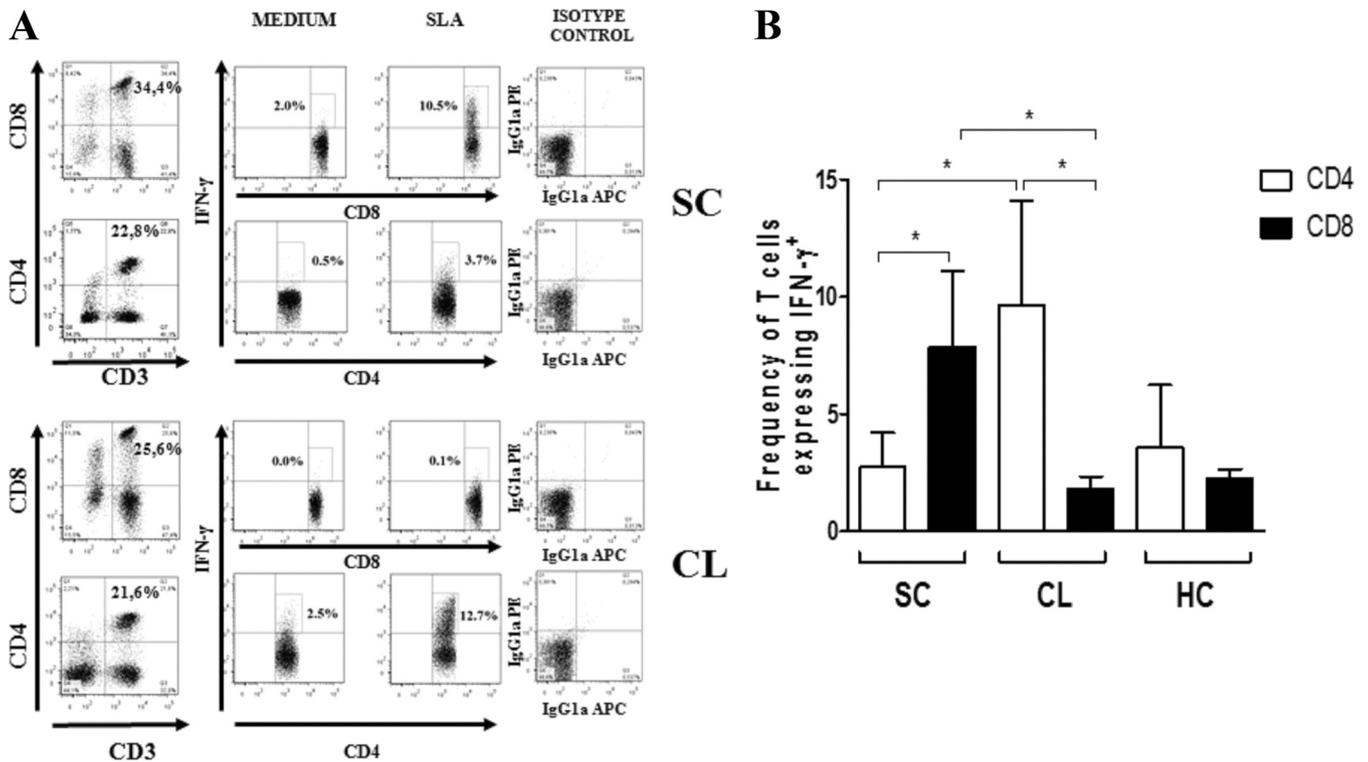


FIG 1 Frequencies of CD4⁺ IFN- γ ⁺ and CD8⁺ IFN- γ ⁺ cells after stimulation with SLA. PBMC were stimulated with SLA (5 μ g/ml) for 12 h, stained with anti-CD3, anti-CD4, anti-CD8, and anti-intracellular IFN- γ and analyzed by FACS analysis. (A) Strategies for analyses of the CD3⁺CD4⁺ and CD3⁺CD8⁺ T cell subsets from lymphocytes gated on forward scatter (FSC) and side scatter (SSC). Dot plots are representative of CL patients and SC subjects and display the frequencies of CD4⁺ and CD8⁺ T cells expressing IFN- γ in samples from SC and CL individuals. Isotype control plots for CD4⁺ T cells, CD8⁺ T cells, and IFN- γ are shown on the right. (B) Expression of IFN- γ in CD4⁺ and CD8⁺ T cells from CL patients, SC subjects, and HC. Statistical comparisons were performed with the Mann-Whitney U test. *, $P < 0.05$.

the PBMC from CL patients and SC subjects. CD4⁺ T cells represented $48\% \pm 10\%$ of lymphocytes in CL patients and $42\% \pm 14\%$ of those in SC subjects, while the frequency of CD8⁺ T cells was $20\% \pm 6\%$ in CL patients and $18\% \pm 5\%$ in SC subjects ($P > 0.05$). The flow cytometry gating strategy used to evaluate cytokine expression in CD4⁺ and CD8⁺ T cells is shown in Fig. 1A, and the frequencies of CD4⁺ and CD8⁺ T cells expressing IFN- γ after stimulation with SLA are shown in Fig. 1B. The frequency of CD4⁺ T cells expressing IFN- γ in unstimulated PBMC was $2.6\% \pm 1.3\%$ in SC samples and $2.4\% \pm 1.9\%$ in CL samples, and the frequency of CD8⁺ T cells expressing IFN- γ was $3.4\% \pm 1.1\%$ in SC samples and $1.6\% \pm 0.3\%$ in CL samples ($P < 0.05$). After stimulation with SLA, CD8⁺ T cells were the major source of IFN- γ in SC infection, whereas CD4⁺ T cells were the main cellular producers of IFN- γ in CL patients (Fig. 1B). The frequency of CD4⁺ T cells displaying IFN- γ in SC subjects ($2.7\% \pm 1.5\%$) was lower than that in CL patients ($9.7\% \pm 4.4\%$) ($P < 0.05$). In contrast, the frequency of CD8⁺ T cells producing IFN- γ in SC subjects ($7.8\% \pm 3.2\%$) was higher than that observed in CL patients ($1.8\% \pm 1.7\%$) ($P < 0.05$). These observations suggest that CD8⁺ T cells from SC individuals have a profile associated with protection.

Monocytes from SC subjects are less susceptible to *L. braziliensis* infection. To assess the ability of CD8⁺ T cells to mediate cytotoxicity in *L. braziliensis*-infected monocytes, we performed coculture experiments. We first infected monocytes with promastigotes and established that the optimal CD8⁺ T cell/iMO ratio

was 5:1 (data not shown). The flow cytometry gating strategy for analysis of doubly positive CD14⁺ DDAO-SE⁺ events, represented in Fig. 2A, showed that a lower frequency of SC monocytes than CL monocytes were infected, despite these cells being subjected to infection with the same number of parasites as mononuclear cells isolated by microbeads. The frequency of CD14⁺ cells sorted from PBMC was $86.9\% \pm 5.1\%$ for SC samples and $89.3\% \pm 4.0\%$ for CL samples; these data are shown in a graph beside representative dot plots for separation and infection in Fig. 2A. A histogram comparing the expression of DDAO-SE on uninfected and infected monocytes from SC and CL subjects is shown at the bottom of Fig. 2A. Notably, when monocytes were analyzed after 2 h of exposure to *L. braziliensis*, we observed a lower frequency ($P < 0.05$) of infected cells in the SC group than in the CL group and the HC group. The frequency of iMOs in SC subjects ($34\% \pm 9.2\%$) was lower ($P < 0.05$) than the frequency of infected cells in CL patients ($47\% \pm 9.8\%$) and HC ($67\% \pm 18\%$) (Fig. 2B). We also determined the frequency of iMOs per 100 monocytes via counting of samples on cytopsin slides by microscopy, and the percentage of infected monocytes in SC subjects ($27\% \pm 10\%$) was lower than that in CL patients ($49\% \pm 17\%$) ($P < 0.05$). There were also smaller numbers of amastigotes per 100 monocytes from SC subjects than from CL patients (data not shown). These data indicate that monocytes from SC subjects are less permissive to leishmania infection than monocytes from CL patients.

CD8⁺ T cells induce more apoptosis in infected CL monocytes. The flow cytometry gating strategy and the frequencies of

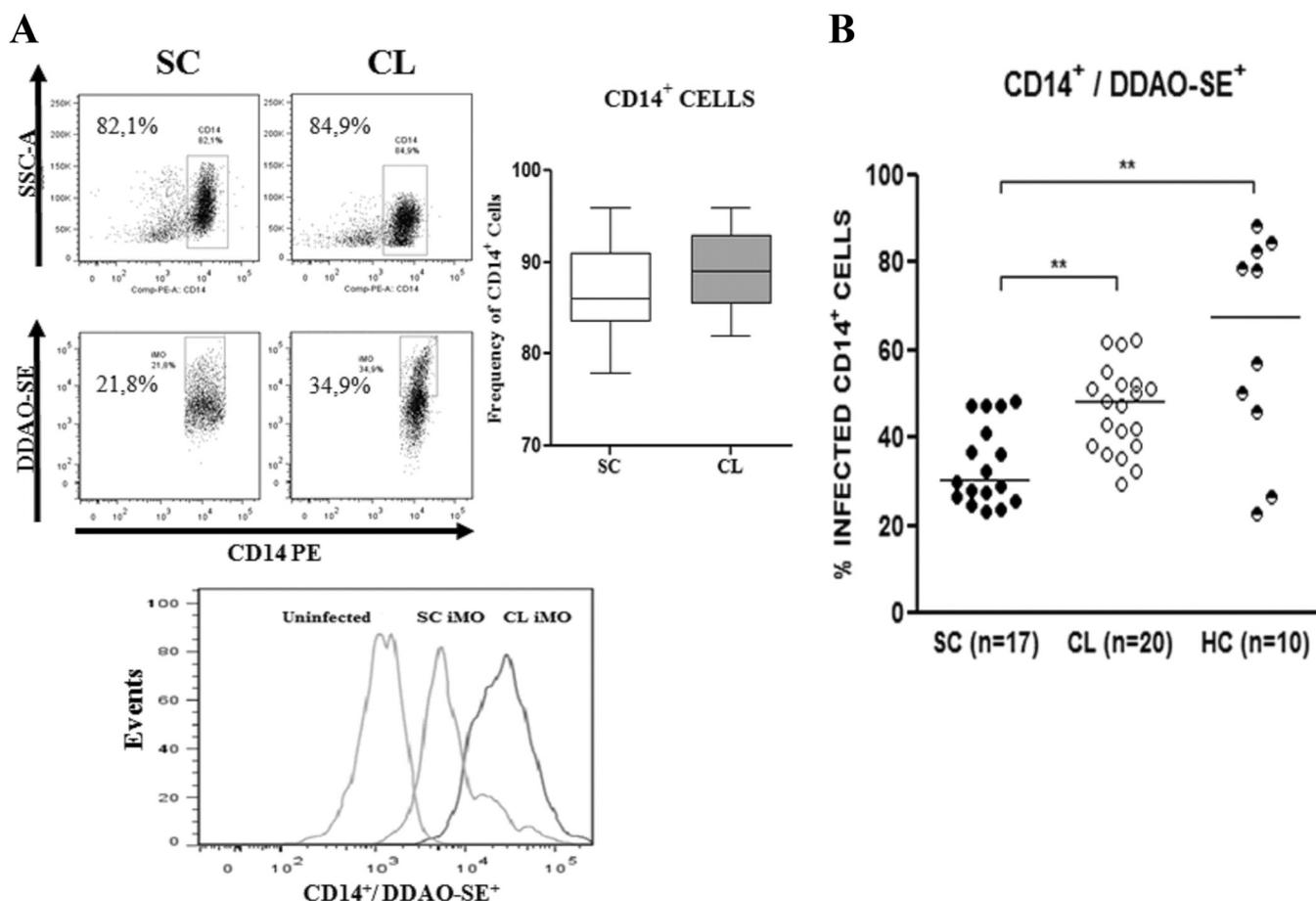


FIG 2 Frequencies of infected monocytes in samples from CL patients and SC subjects. Monocytes from SC subjects and CL patients were infected with DDAO-SE-stained *L. braziliensis* (5 promastigotes:1 monocyte) for 2 h. (A) SSC-A versus CD14⁺ plots showing monocytes sorted from PBMC from both groups (top plots) and plots showing the frequencies of infected monocytes (CD14⁺ DDAO-SE⁺ cells) representative of CL and SC subjects, selected in the iMO gate (bottom plots). The graphic on the right shows the frequencies of sorted CD14⁺ cells (purity) in SC and CL samples. The histogram at bottom shows the frequencies of infected monocytes from SC subjects and CL patients (left to right). (B) Frequencies of infected CD14⁺ cells in CL patients, SC subjects, and HC. The results are representative of those for 17 SC subjects, 20 CL patients, and 10 HC. Statistical comparisons were performed with the Mann-Whitney U test. **, $P < 0.05$.

apoptotic cells, measured by the expression of annexin V in uninfected MOs and iMOs cocultured with CD8⁺ T cells, are shown in a histogram for apoptotic CD14⁺ cells under the same conditions (Fig. 3A). The frequencies of apoptotic MOs and iMOs from SC subjects, CL patients, and HC in the absence and presence of CD8⁺ T cells are shown in Fig. 3B. The frequency of apoptotic iMOs in the absence of CD8⁺ T cells was $7.8\% \pm 7.2\%$ in SC samples and $18.7\% \pm 12.8\%$ in CL samples ($P > 0.05$). In the presence of CD8⁺ T cells, the percentage of apoptotic cells was $14.7\% \pm 4.9\%$ for SC samples and $29\% \pm 9.2\%$ for CL samples ($P < 0.05$), as can be seen on a representative CL dot plot (Fig. 3B, right panel). In the HC group, the frequency of apoptotic cells was very low in the absence or presence of CD8⁺ T cells.

These data indicate that CD8⁺ T cells are able to induce apoptosis in both groups but suggest that CD8⁺ T cells from SC individuals are less cytotoxic than CD8⁺ T cells from CL patients.

To determine the specific cytotoxic effects exerted by CD8⁺ T cells, the cytotoxicity index (CI) was calculated as previously described (25, 26). CL patients had a CI of 8.3 ± 5.3 , which was higher than those for SC individuals (4.8 ± 3.6) and the HC group (4.5 ± 3.6) ($P < 0.05$) (Fig. 3C).

CD8⁺ T cells from SC individuals contribute to *L. braziliensis* killing. To address whether CD8⁺ T cells are protective, we also analyzed the number of viable parasites in the coculture systems. After 8 h of coculture, the medium was replaced by Schneider's medium, and after 5 days, the numbers of motile promastigotes in supernatants were counted. The number of viable promastigotes in cultures of iMOs without CD8⁺ T cells from SC individuals (53 ± 25) was higher than that for cultures in the presence of CD8⁺ T cells (25.5 ± 20) ($P < 0.05$) (Fig. 4). However, CD8⁺ T cells from CL patients had no effect on the viability of promastigotes (89 ± 75) compared to cultures without CD8⁺ T cells (85 ± 63) ($P > 0.05$) (Fig. 4).

CD8⁺ T cells are specifically cytotoxic to infected monocytes due to the production of granzyme B. Activated CD8⁺ T cells are able to induce cytolysis of infected cells by two distinct molecular pathways: the granule exocytosis pathway and the upregulation of FasL, which can initiate programmed cell death. Efficient lysis by the granule exocytosis pathway requires delivery of perforin and granule enzymes, such as granzymes A and B (27). To determine the pathway involved in the death of infected cells, we evaluated the expression of FasL and the production of granzyme B in CD8⁺

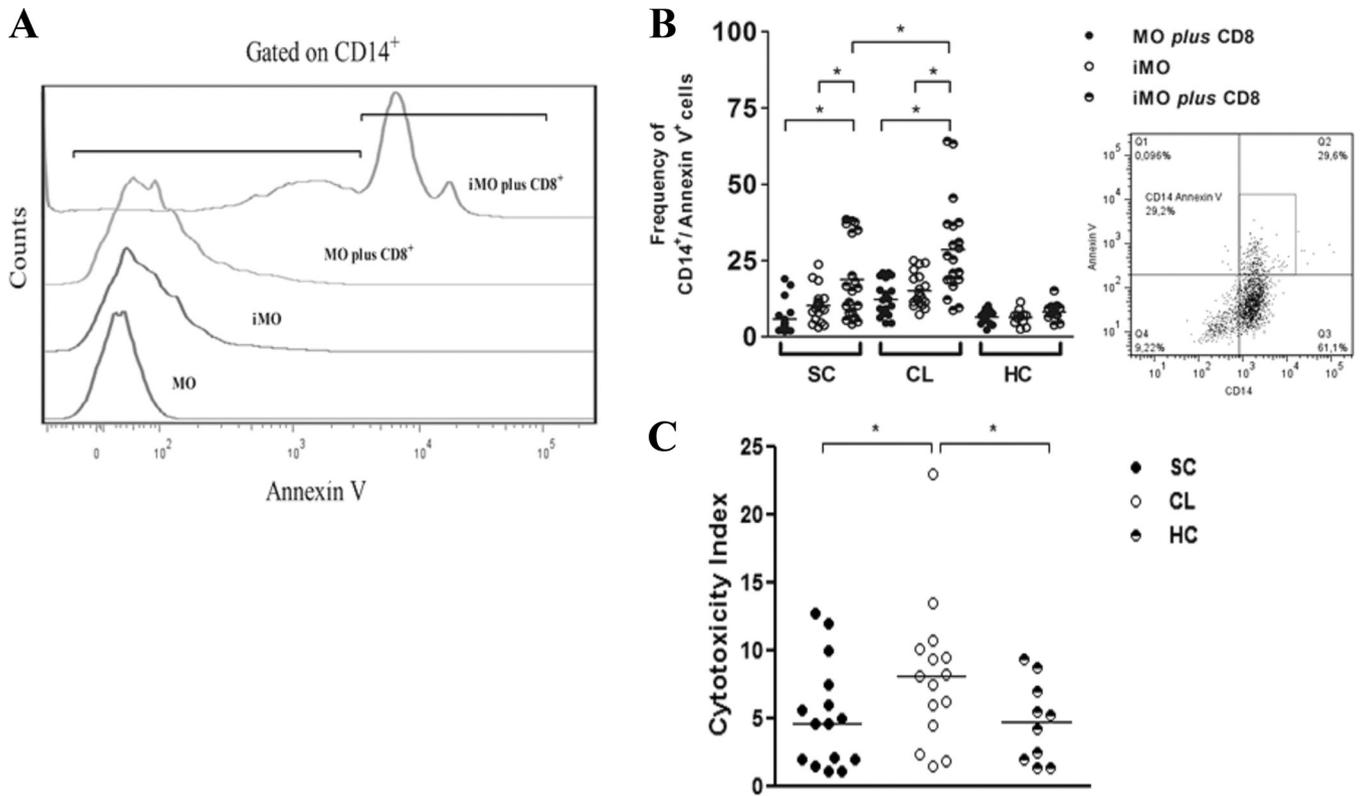


FIG 3 CD8⁺ T cells from CL patients induce more apoptosis of infected monocytes than do CD8⁺ T cells from SC subjects. Monocytes were infected with DDAO-SE-stained *L. braziliensis* promastigotes for 2 h and cocultured with CD8⁺ T cells for 8 h. Cocultured cells were stained with anti-CD14 and anti-intracellular annexin V antibodies and analyzed by FACS analysis. The strategy used for the evaluation of annexin V expression in CD14⁺ cells was the same as that shown in Fig. 2A. (A) Plots gated on SSC-A versus CD14⁺ show the frequencies of apoptotic cells in uninfected cultures (MO), infected cultures (iMO), uninfected cultures plus CD8⁺ cells, and infected cultures plus CD8⁺ cells. (B) Frequencies of CD14⁺ cells expressing annexin V on uninfected monocytes plus CD8⁺ T cells, infected monocytes, and infected monocytes plus CD8⁺ T cells. A representative histogram gated on CD14⁺ cells shows the frequencies of apoptotic monocytes cocultured with CD8⁺ T cells (from a CL patient sample). (C) Cytotoxicity indexes (CI), calculated as described in Materials and Methods, for 17 SC subjects, 20 CL patients, and 10 HC. Statistical analyses were performed with the Kruskal-Wallis test with Dunn's *post hoc* test. *, *P* < 0.05.

T cells. The frequencies of CD8⁺ T cells expressing FasL were similar in cocultures with MOs and iMOs across both groups. When MOs were cocultured with CD8⁺ T cells, the frequency of expression of FasL was 31% ± 23% in SC samples and 24% ±

25.7% in CL samples (*P* > 0.05). For iMOs cocultured with CD8⁺ T cells, the frequency of CD8⁺ T cells expressing FasL was 31% ± 23.7% in SC samples and 26% ± 19.5% in CL samples (*P* > 0.05). These results suggest that the Fas-FasL pathway is not involved in the cytolysis of *L. braziliensis*-infected monocytes. To evaluate whether the cytotoxicity was mediated by the release of granule enzymes, we evaluated the intracellular expression of granzyme B in CD8⁺ T cells and the production of this molecule in the supernatants of the cocultures. The frequencies with which CD8⁺ T lymphocytes expressed granzyme B when cocultured with MOs are shown in Fig. 5A. The frequency of cells expressing granzyme B in coculture with MOs plus CD8⁺ T cells was higher in CL samples (25% ± 5.7%) than in SC samples (11% ± 6.3%) (*P* < 0.05), suggesting that this granule is stored in CL cells. However, in cocultures of iMOs and CD8⁺ T cells, the frequencies of CD8⁺ T cells expressing granzyme B were similar for the two groups (20% ± 10% in SC samples and 20% ± 17% in CL samples; *P* > 0.05). To evaluate whether the decrease in the frequency of CD8⁺ T cells expressing granzyme B after coculture was due to the granzyme B production of CD8⁺ T cells, we measured this molecule in the supernatants of cocultures (Fig. 5B). The presence of iMOs in the cultures of CD8⁺ T cells increased the production of granzyme B in the SC group (14 ± 6 pg/ml versus 25 ± 6 pg/ml; *P* < 0.05) and the CL group (26 ± 3 pg/ml versus 51 ± 5 pg/ml; *P* < 0.05).

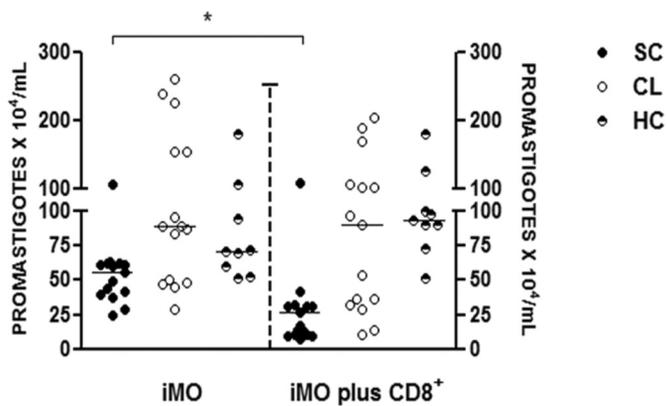


FIG 4 CD8⁺ T cells from SC subjects contribute to parasite killing. After coculture of infected macrophages with peripheral autologous blood CD8⁺ T cells for 8 h, the cells were washed, and the medium was replaced by 0.5 ml of Schneider's medium. After 5 additional days, the number of viable promastigotes was evaluated. Statistical comparisons were done using the Mann-Whitney U test. *, *P* < 0.05.

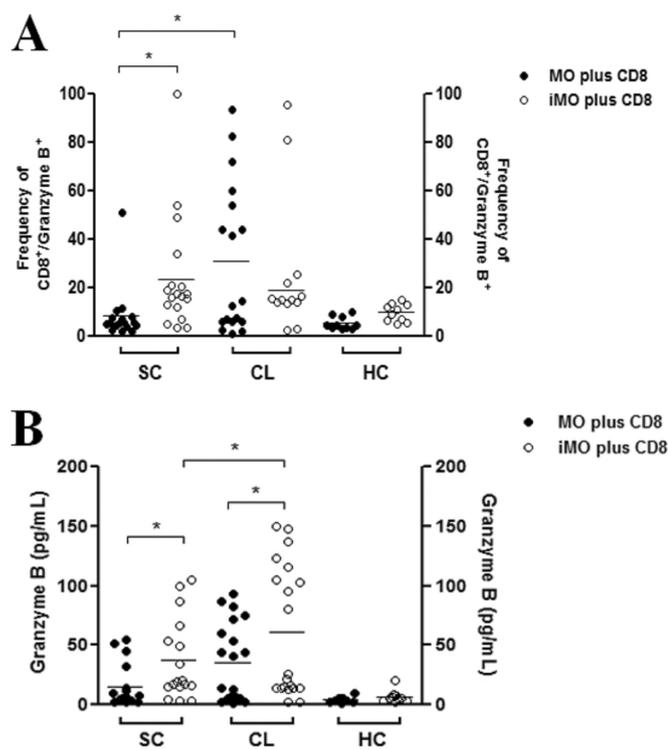


FIG 5 CD8⁺ T cells from CL patients display increases in granzyme B production after coculture with iMOs compared to CD8⁺ T cells from SC subjects. CD8⁺ T cells were cocultured with MOs and iMOs, supernatants were collected, and levels of granzyme B were measured by ELISA. Additionally, CD8⁺ T cells were collected and stained with anti-CD3, anti-CD8, and anti-intracellular granzyme B, and the frequencies of CD8⁺ granzyme B⁺ T cells were determined by FACS analysis. (A) Production of granzyme B by CD8⁺ T cells after coculture with MOs and iMOs from CL patients, SC subjects, and HC. (B) Frequencies of CD8⁺ granzyme B⁺ T cells from CL patients, SC subjects, and HC cocultured with uninfected and infected monocytes. Statistical analyses were performed with the Mann-Whitney U test in both cases. *, $P < 0.05$.

Additionally, there was less production of granzyme B in samples from SC individuals (25.7 ± 2.7 pg/ml) than in samples from CL patients (51.0 ± 4.9 pg/ml) ($P < 0.05$). HC samples showed a lower frequency of CD8⁺ granzyme B⁺ T cells and less granzyme B production than those of SC and CL samples.

To confirm these results, we performed experiments using a granzyme B inhibitor and a pan-caspase inhibitor to inhibit the interaction between Fas and FasL (z-VAD-FMK) in the coculture assays. In the presence of the granzyme B inhibitor, the number of apoptotic cells decreased in the CL group (from $24.6\% \pm 5.3\%$ to $5.3\% \pm 1.0\%$; $P < 0.05$), while this inhibitor had no effect on the frequency of apoptotic cells in the SC group (changing only from $12.7\% \pm 1.3\%$ to $7.6\% \pm 1.8\%$) ($P > 0.05$) (Fig. 6A). The negative control (0.4% DMSO) showed very similar results to those for the SC, CL, and HC groups ($2.0\% \pm 1.0\%$, $10.0\% \pm 0.5\%$, and $4.0\% \pm 2.0\%$, respectively), and the positive control (PMA and ionomycin) induced more apoptosis in the CL group, with a significant apoptosis reduction after the granzyme B block. In the presence of z-VAD-FMK, there were no differences in the frequencies of apoptotic cells in both groups or in the negative and positive controls between groups, without a significant apoptosis reduction after use of the caspase inhibitor (Fig. 6B). These

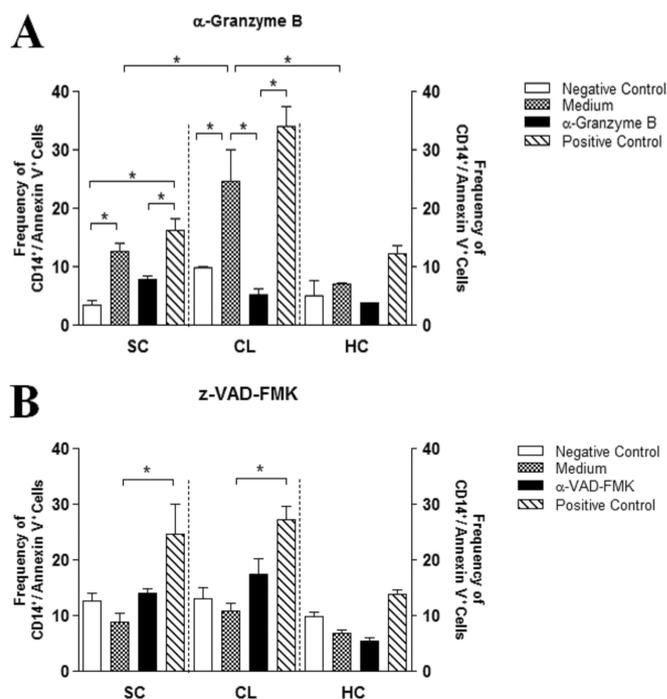


FIG 6 Granzyme B mediates CD8⁺ T cell cytotoxicity against *L. braziliensis*-infected monocytes. CD8⁺ T cells from CL patients, SC subjects, and HC were cocultured with iMOs in the absence or presence of a granzyme B inhibitor or z-VAD-FMK. PMA (10 ng/ml) plus ionomycin (500 ng/ml) was used as a positive control, and 0.4% DMSO was used as a negative control. (A) Frequencies of CD14⁺ annexin V⁺ cells in cocultures with the granzyme B inhibitor. (B) Frequencies of CD14⁺ annexin V⁺ cells in cocultures with z-VAD-FMK. Statistical comparisons were done using the Kruskal-Wallis test with Dunn's posttest. *, $P < 0.05$.

observations suggest that granzyme B production is an important pathway involved in the cytolysis of *L. braziliensis*-infected monocytes.

DISCUSSION

The pathogenesis of human CL is not well understood. In *L. braziliensis* infection, patients develop a strong Th1 immune response, and this exaggerated inflammatory response results in tissue damage (28). Both IFN- γ and TNF- α are highly produced in CL, but while IFN- γ may have a protective function (16), there are many lines of evidence that support a role for TNF- α in the pathology of cutaneous and mucosal lesions (9, 10, 29–32). Little attention has been given to the role of CD8⁺ T cells in leishmaniasis. A few studies have evaluated the role of CD8⁺ T cells in the pathogenesis of CL (16), but CD8⁺ T cell functions have not been determined for subjects with SC *L. braziliensis* infection. These individuals, who are able to control leishmania parasites without developing disease, produce less IFN- γ and TNF- α than do CL patients (18, 20). Here we showed that while CD8⁺ T cells in SC infection were the major source of IFN- γ , the CD8⁺ T cells from CL patients produced more granzyme B and had more cytotoxic activity than the cells from SC samples. Moreover, we observed that monocytes from subjects with SC infection were less permissive to leishmania penetration than those from subjects with CL. These data argue for a key role of these cells in the control of leishmania infection in SC individuals and provide additional ev-

idence supporting the role of CD8⁺ T cells in the pathogenesis of CL ulcers.

CD4⁺ T cells are the main source of IFN- γ in the initial phase of the disease caused by *L. braziliensis* (33). Here we showed that rather than CD4⁺ T cells, CD8⁺ T cells are the main cellular producer of IFN- γ in subjects with SC infection. Since IFN- γ is the major macrophage-activating cytokine, these data suggest that CD8⁺ T cells may participate in the control of *L. braziliensis* in subjects with SC infection by producing IFN- γ . In an area of *L. major* transmission, the immune response of subjects with self-healing CL who are protected against further *Leishmania* infection is characterized not only by increases in antigen-specific IFN- γ -producing CD4⁺ Th1 cells but also by increases in IFN- γ -producing CD8⁺ T cells (34). Recently, in BALB/c mice vaccinated with Toll-like receptor (TLR) ligands (DNA priming) plus TLR1 and TLR2 agonists as an adjuvant, protection against *Leishmania (Viannia) panamensis* was found to be dependent on the memory of CD8⁺ T cells and on IFN- γ production (35). Indeed, in subjects immunized with a killed *L. amazonensis* vaccine, CD8⁺ T cells comprised the majority of responder cells after *in vitro* stimulation of PBMC with *L. braziliensis* antigen. These data provide support for our finding of the possible participation of CD8⁺ T cells in the prevention of progression from infection to disease in SC subjects.

Macrophages play a central role in the pathogenesis of *Leishmania* infection (36). We previously showed that while macrophages from both CL and ML patients exhibit predominantly pro-inflammatory profiles and secrete significantly more CXCL9, CXCL10, and TNF- α than do macrophages from SC subjects, macrophages from SC subjects have a greater ability to kill *L. braziliensis* than macrophages from CL or ML patients (37). For *L. panamensis* infection, others have shown that monocytes from SC individuals are less permissive to leishmania invasion or phagocytosis than monocytes from patients with relapsed episodes of CL (38). In this study, by evaluating the penetration/phagocytosis of *L. braziliensis* by monocytes, we used flow cytometry and microscopy to show that the number of amastigotes was decreased in samples from SC subjects compared to samples from CL patients. These data provide further support for the role of monocytes/macrophages in the control of *L. braziliensis* infection. Studies aimed to evaluate the mechanisms that allow the monocytes of SC subjects to be less permissive to *L. braziliensis* infection are ongoing.

While our data suggest that CD8⁺ T cells may have a protective role in SC infection, evidence has been accumulating for a role of CD8⁺ T cells in the pathology of CL. Previously, by comparing the tissue infiltration in patients with early cutaneous leishmaniasis (E-CL) with that of CL patients who had classical ulcers, we observed a 5-fold increase in CD8⁺ T cells in the CL ulcers compared to the E-CL lesions (15). Moreover, the CD8⁺ T cells from patients with classical ulcers expressed significantly higher levels of granzyme A than did the cells from the papular lesions of E-CL patients (15). Regarding cytotoxicity, Brodskyn et al. showed that the cytotoxicity mediated by CD8⁺ T cells, as measured by the lysing of infected macrophages, was greater in ML than in CL, suggesting that cytotoxicity may be involved in the pathology of tegumentary leishmaniasis (14). Herein we showed that after exposure of iMOs to CD8⁺ T cells, the frequency of apoptotic cells in CL patients was higher than that in SC individuals. It has also been reported that CD8⁺ T cells do not contribute to the killing of

parasites in cocultures with *L. braziliensis*-infected macrophages from CL patients (16). In this study, we demonstrated that while in coculture assays the use of CD8⁺ T cells from SC individuals decreased the number of viable promastigotes, CD8⁺ T cells from CL patients had no effect on the viability of the parasites, suggesting that CL CD8⁺ T cells do not contribute to the killing of parasites.

Moreover, we also determined the mechanism of the cytotoxicity mediated by CD8⁺ T cells. While there was no evidence that the Fas-FasL pathway participated in this process, we showed that the frequency of CD8⁺ T cells expressing granzyme B and the levels of granzyme B in the supernatants of MOs cultured with CD8⁺ T cells were elevated in samples from CL patients compared to samples from SC individuals. We also demonstrated that in the presence of a granzyme B inhibitor, the number of apoptotic cells decreased in the CL group, while no effect on the frequency of apoptotic cells in the SC group was observed. Also, in the presence of an inhibitor of the interaction between Fas and FasL, there were no differences in the frequency of apoptotic cells in both groups. These data and the observations that granzyme B levels increased in the supernatants of MOs cocultured with CD8⁺ T cells and that the frequencies of CD8⁺ T cells expressing granzyme B decreased after exposure to iMOs are further indications of the participation of this enzyme in the cytotoxicity response mediated by CD8⁺ T cells.

In this study, we showed that while CD8⁺ T cells in subjects with SC are the major source of IFN- γ , these cells have less cytotoxicity than CD8⁺ T cells from CL patients. Therefore, it is likely that although CD8⁺ T cells exhibit decreased cytotoxicity in *L. braziliensis*-infected cells in SC infection, these cells help to control the infection by activating macrophages to kill leishmania parasites. Moreover, since the cytotoxicity in SC infection is mild, tissue damage does not occur, and ulcers do not develop. Alternatively, the greater cytotoxicity observed in CL patients may participate in pathology through the killing of cells infected with leishmania parasites or the expression of *L. braziliensis* antigen. A study of the participation of IFN- γ and perforin in the pathogenesis of heart disease during experimental *Trypanosoma cruzi* infection provides support for this dual role of CD8⁺ T cells in the pathogenesis of intracellular infection. In that study, CD8⁺ IFN- γ ⁺ cells were shown to exert a protective function, whereas CD8⁺ perforin⁺ cells were shown to have a possible detrimental role in *T. cruzi*-elicited heart injury (39). In conclusion, our data reveal the roles of monocytes and CD8⁺ T cells in the control of leishmania parasites in subjects with SC *L. braziliensis* infection and the role of CD8⁺ T cells in the pathogenesis of CL. The monocytes of subjects with SC infection were less permissive to leishmania penetration because the production of IFN- γ by CD8⁺ T cells from these individuals increased the abilities of monocytes and macrophages to kill leishmania parasites. In contrast, the enhanced cytotoxicity response observed in samples from CL patients did not contribute to the killing of the parasite and may have induced tissue damage and facilitated the development of cutaneous ulcers.

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