Regulatory T-Cell Dynamics in Cutaneous and Mucocutaneous Leishmaniasis due to *Leishmania braziliensis*

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Abstract. To evaluate the dynamics of regulatory T cells (Tregs) during tegumentary leishmaniasis, we assessed peripheral blood and biopsies from 54 patients. Patients with cutaneous leishmaniasis (CL) had a decreased proportion of Tregs in the peripheral blood, but the proportion was higher in the biopsies of lesions. During treatment of CL, circulating Tregs increased reaching normal proportions, whereas antigen-specific interferon- γ responses diminished. By contrast, circulating Tregs from mucosal leishmaniasis patients failed to normalize during treatment. C-C chemokine receptor type 5 was expressed on a large proportion of Tregs at the site of infection. These results demonstrate increased Tregs at the site of infection, possibly homing from the peripheral circulation.

INTRODUCTION

Human leishmaniasis is a neglected tropical disease caused by protozoan parasites of the genus *Leishmania*. A wide-spectrum of clinical manifestations results from complex interactions that vary with both host and parasite factors. *Leishmania braziliensis* causes lesions ranging from self-healing cutaneous ulcers to destructive mucosal disease.^{1–3}

Regulatory T cells (Tregs) are a unique population of CD4⁺ T cells that are able to modulate immune responses to self-antigens and blunt inflammatory reactions to foreign antigens. The main group of Tregs is characterized by constitutive expression of high levels of interleukin-2 receptor α (CD25) and the transcription factor Foxp3.^{4,5} Regulatory T cells exert their suppressive function through the production of anti-inflammatory cytokines, cell-to-cell contact, and consumption of proinflammatory cytokines.⁶

In murine models of *Leishmania* infection, Tregs suppress *Leishmania*-specific effector T-cell activity at the site of infection, leading to long-term persistence of the parasite. Persistence, in turn, is associated with resistance to reinfection. Transfer of Tregs is sufficient to trigger disease reactivation and prevent the expression of an effector memory response.^{7–11} In human leishmaniasis, Tregs are present in lesions and lesion-derived Tregs have been shown to be capable of suppressing proliferation and cytokine-producing effector cells.^{12–14} However, there are also other cells involved in modulating the host response in human leishmaniasis, including Foxp3-negative CD4 cells, which produce IL-10.¹⁵

Effective host defenses require appropriate T-cell migration and homing of T-cell subsets to the infection site. Several chemokines must be expressed at the infected site.^{16–19} CCL5 and its receptor CCR5 are known to mediate the recruitment and homing of Tregs during inflammation and infection.^{20–23} In a murine model of *Leishmania major* infection, C-C chemokine receptor type 5- knockout (CCR5-KO) mice lacked Tregs at the infection site and were resistant to the infection.²⁴ CCR5-KO mice showed an exaggerated antigen-specific interferon- γ (IFN) response in *Leishmania* infection that correlated with an

enhanced control of the parasite.²⁵ Few data are available on the expression of CCR5 among Tregs in human leishmaniasis.²⁶

Previous reports have not studied variations in the immune responses during treatment of *Leishmania* infection.^{27–29} There are limited data on the homing of Tregs and their effects on antigen-specific Th1 responses in human leishmaniasis. We hypothesized that Tregs home to the site of active infection, leading to a redistribution of peripheral blood Tregs, and that this redistribution reverses with antiparasitic treatment. Therefore, we studied the proportion of lymphocytes with Treg markers in peripheral blood and infected tissues from patients with tegumentary leishmaniasis before, during, and at the completion of treatment.

MATERIALS AND METHODS

Study participants. We enrolled 54 patients (mean age 38.9 $\pm$ 16.4 years, 64.8% males) with newly diagnosed tegumentary leishmaniasis at the outpatient clinic of the Instituto de Medicina Tropical “Alexander von Humboldt” between June 2008 and June 2011. Forty-six patients presented with cutaneous leishmaniasis (CL) and eight with mucosal leishmaniasis (ML). *Leishmania* infection was confirmed by direct microscopic observation of the parasite and/or culture. The *Leishmania* species was assessed by polymerase chain reaction as previously described.³⁰ All isolates were confirmed to be *L. braziliensis*. Enrolled patients received standard therapy with sodium stibogluconate at 20 mg/kg/day for 20 days (CL) or 28 days (ML). Healthy normal individuals ($N = 16$) were enrolled as controls for peripheral blood samples.

Mononuclear cells isolation. Blood samples were drawn in CPT tubes (BD, Franklin Lakes, NJ) at baseline, after 10 days of treatment, and at the end of treatment. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation. Cell viability was determined by trypan blue exclusion.

At baseline, a 2- to 4-mm biopsy was obtained from the border of the lesion (skin or mucosa). Cells in the biopsy were mechanically disaggregated using a medimachine (BD Biosciences, San Jose, CA) and filtered through a 40 μ m mesh.

Flow cytometry staining. The cells were washed with RPMI-1640 (GIBCO; Invitrogen Corporation, Carlsbad, CA) and 10% fetal bovine serum and stained with antihuman CD45 peridin-chlorophyll protein complex (PERCP) monoclonal

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antibody (mAb) (BD), antihuman CD4 phycoerythrin (PE) mAb (BD Pharmingen, San Diego, CA), and antihuman CD25 allophycocyanin (APC) mAb (BD) for 30 minutes at 4°C. After permeabilization with a Perm/Fix kit (eBioscience, San Diego, CA), the cells were stained with antihuman Foxp3 fluorescein isothiocyanate (FITC) mAb (eBioscience, 30 minutes, 4°C). Peripheral blood mononuclear cells and skin mononuclear cells were resuspended in phosphate buffered saline (PBS) and 2% fetal bovine serum and acquired with a FACScalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ). Lymphocytes were acquired using CD45 and side-scattered light lymphocyte gating and analyzed with FlowJo software (Ver.8.8.4; Tree Star, Ashland, OR). Regulatory T cells were defined by the phenotype CD4⁺ CD25^{high} Foxp3⁺ (Figure 1A).

The expression of CCR5 among Foxp3-expressing CD4⁺ cells was assessed from seven CL patients. Briefly, mononuclear cells from both tissue and peripheral blood were stained using CD45 PERCP, CD4 FITC, CCR5 PE, and Foxp3 APC (Figure 1B).

Enzyme immunoassay. Peripheral blood mononuclear cells harvested at baseline, at day 10 or 14 (CL and ML respectively), and at treatment completion were stimulated with soluble-*Leishmania*-antigen (SLA-1) and incubated for 48 hours. The supernatants were collected and IFN- γ concentration measured by enzyme immunoassay (BD Biosciences).

SLA-1 was provided by Dr. Tom Rolf, Infectious Disease Research Institute, Seattle, WA.³¹ For SLA-1 production, cultures of *Leishmania (V.) amazonensis* were pooled and centrifuged at

1,200 rpm for 30 minutes. After centrifugation, the supernatants were removed and discarded. The remaining pellet was washed three times in cold PBS. The pellets were centrifuged at 3,750 rpm for 10 minutes. The supernatants were discarded and the pellet was resuspended at approximately 1×10^6 parasites per milliliter in PBS with protease inhibitor and 1 mM ethylenediaminetetraacetic. After incubation (30 minutes at 4°C), the pellet was sonicated. After centrifugation ($20,000 \times g$, 90 minutes), the supernatant was saved and the pellet discarded. The supernatant was dialyzed into PBS, filtered, and the protein concentration estimated using a Bradford assay.

Analysis. The proportion of Tregs and CCR5 and the concentration of IFN- γ were not normally distributed and are, therefore, presented as medians with interquartile ranges. We performed a Wilcoxon signed rank test to assess the differences between the proportions of Tregs in PBMCs compared with skin biopsies. We used the Mann-Whitney *U* test to detect the differences in peripheral blood Treg proportions between healthy controls and tegumentary leishmaniasis patients. In addition, a Friedman test was used to compare peripheral blood Tregs at baseline, during treatment, and upon treatment completion. Using a Wilcoxon signed rank test, we assessed the differences in IFN- γ concentration between baseline and at the end of treatment. *P* values < 0.05 were considered statistically significant. Analyses were performed using Prism 7 (Graph Pad Software, San Diego, CA).

Ethical consideration and human subject protection. The Institutional Review Board of Universidad Peruana

FIGURE 1. Flow cytometry gating and analysis. All lymphocytes were identified using a CD45 peridin-chlorophyll protein complex (PERCP) and side scatter. (A) Foxp3 and CD25 expression was evaluated from CD4⁺-expressing lymphocytes. (B) The expression of CCR5 and Foxp3 was assessed from CD4⁺-expressing lymphocytes. This figure appears in color at www.ajtmh.org.

TABLE 1
Clinical and demographic characteristics

	Cutaneous leishmaniasis (N = 46)	Mucosal leishmaniasis (N = 8)	P value
Male (n, %)	29 (63%)	6 (75%)	0.51
Age (years)	36 (23–480)	62.5 (32.5–71.5)	0.02
Disease duration (months)	2.5 (2–3)	24 (12–36)	0.03
Hemoglobin (mg/dL)	14.7 ± 0.2	13.9 ± 0.8	0.2
White blood cells	6.2 (5.2–6.8)	5.85 (4.75–7.55)	0.75
Neutrophils (%)	59.1 ± 10.7	57.4 ± 6.3	0.67
Neutrophils (n)	3.7 ± 1.2	3.4 ± 0.6	0.64
Eosinophils (%)	4 (2–6)	5.5 (2–11)	0.39
Eosinophils (n)	0.2 (0.1–0.3)	0.3 (0.1–0.7)	0.5
Lymphocytes (%)	29.6 ± 9.3	30.5 ± 6	0.8
Lymphocytes (n)	1.8 ± 0.6	1.9 ± 0.6	0.7

Cayetano Heredia reviewed and approved the study. All patients signed a consent form before enrollment.

RESULTS

Clinical features and demographics. Important clinical characteristics are presented in Table 1. Mucosal leishmaniasis

patients were older ($P = 0.02$) and had a longer duration of disease compared with CL patients ($P = 0.03$). However, no statistically significant differences were noted for hemoglobin, white blood cell count, or white blood cell count differential between CL and ML groups.

Regulatory T cells are increased in lesions of patients with tegumentary leishmaniasis. The proportion of Tregs

FIGURE 2. Proportion of regulatory T cells from peripheral blood (A) and blood compared with infection site (B). Regulatory T cells were characterized by their constitutive expression of CD25 and Foxp3 ($CD4^+ CD25^{high} Foxp3^+$). The proportion of regulatory T cells in blood was significantly lower for both cutaneous and mucocutaneous cases compared than that in controls (*mucosal leishmaniasis [ML] vs. controls $P = 0.02$, **cutaneous leishmaniasis [CL] vs. controls $P = 0.04$, Mann-Whitney). Regulatory T cells are increased at the infection site when compared with peripheral blood in CL and ML, respectively (*CL $P < 0.0001$ **ML $P = 0.0003$). This figure appears in color at www.ajtmh.org.

FIGURE 3. CCR5 expression among tissue-derived Foxp3⁺ cells. Biopsies from seven cutaneous leishmaniasis patients were evaluated for Foxp3 and CCR5 expression. The proportion of regulatory T-cells expressing CCR5 was higher among tissue-derived lymphocytes than in peripheral blood. * $P = 0.015$. This figure appears in color at www.ajtmh.org.

was significantly higher in biopsies than in peripheral blood (for CL tissue: 7.5% [5.3–10.8]; blood: 3.6% [2.4–4.8] $P < 0.0001$; for ML tissue: 8.6% [4.5–16.6]; blood: 2.5% [2.0–4.3] $P = 0.004$) (Figure 2). Furthermore, the total number of lymphocytes was increased in diseased skin compared with limited numbers in normal skin, suggesting increased Tregs at the infection site. In addition, at baseline the Treg proportion in blood for both CL and ML was lower than that in healthy

controls (4.7% [3.4–5.7]) ($P = 0.03$ and $P = 0.01$, respectively) (Figure 2).

CCR5 was expressed by a higher proportion of Tregs from the infection site compared with Tregs from peripheral blood (CL: tissue: 71.3% [65.1–80.9] versus blood: 43.5% [41.30–50.9] $P = 0.015$) (Figure 3). We were unable to isolate sufficient numbers of lymphocytes from normal skin to determine whether the proportion is increased compared with that in normal skin. However, CCR5 expression is a characteristic of homing cells, and the increased proportion at the site could be because of Treg recruitment and homing. This could also be due to local proliferation of Tregs.

Interestingly, no statistically significant differences were noted between CL and ML patients in Treg proportion either in the tissues or the peripheral blood ($P = 0.70$ and $P = 0.20$, respectively) (Figure 2).

Anti-Leishmanial treatment affects Treg proportions and IFN- γ production. Peripheral blood Treg proportion progressively increased with treatment in the CL group (baseline: 3.8% [2.2–5.1]; during treatment: 4.0% [2.8–5.6]; at completion: 4.5% [3.8–5.9], Friedman test $P = 0.0003$). Furthermore, at completion the proportion was not different from levels in healthy controls (CL: 4.5% [3.9–5.9]; healthy controls: 4.7% [3.4–5.7], $P = 0.88$), consistent with a normalization of Treg distribution with treatment.

Interestingly, this was not the case for ML. For ML patients, peripheral blood Tregs proportions were consistently lower than that in healthy controls (ML: baseline: 2.2% [2.0–3.7]; during treatment: 3.1% [2.4–3.3]; at completion: 1.8% [1.3–4.3]; healthy controls: 4.7% [3.4–5.7]; treatment completion versus healthy controls: $P = 0.035$). Also, peripheral

FIGURE 4. Normalization of peripheral blood Tregs during treatment of cutaneous leishmaniasis (CL). The solid line represents proportions of peripheral blood regulatory T cells from CL patients; the dotted line represents the same proportion from mucosal leishmaniasis (ML) patients and the dashed line represents the same proportion from healthy controls. At baseline, a lower proportion of peripheral blood mononuclear cells from both CL and ML were CD4⁺ CD25^{hi} Foxp3⁺ compared with healthy controls (baseline: CL vs. healthy controls $P = 0.03$; ML vs. healthy controls $P = 0.01$). During treatment, the proportion of CD4⁺ CD25^{hi} Foxp3⁺ cells in peripheral blood increased and normalized at treatment completion in CL patients. The proportion of CD4⁺ CD25^{hi} Foxp3⁺ cells from peripheral blood of ML patients failed to normalize and remained at lower proportions at treatment completion (treatment completion: CL vs. controls $P = 0.88$ ML vs. controls $P = 0.04$). Error bars represent the standard error of the mean.

blood Treg proportions in ML did not vary when comparing baseline versus treatment completion ($P = 0.63$) (Figure 4).

Although peripheral blood Treg proportions increased with treatment in CL patients, the antigen-specific IFN- γ response tended to decrease (baseline versus treatment completion, Wilcoxon test $P = 0.095$).

DISCUSSION

Here we demonstrate an increase in the proportion of Tregs at the site of tegumentary *Leishmania* infection compared with the peripheral blood along with increased numbers of lymphocytes at the site of infection. Previous reports have suggested that ML is associated with lower Treg activity at the infection site. However, we have found no differences in the proportions of intralesional Tregs between ML and CL.^{32,33} Also, there are other cells involved in immunomodulation, such as Foxp3-negative IL-10-producing cells.¹⁵ The high proportion of Tregs at the infection site and the decreased proportion in the circulation could be due to the active migration of Tregs to the site of infection. However, earlier human leishmaniasis studies noted that Treg antigen specific anti-inflammatory cytokines were strongly associated with active lesions, and high intralesional Foxp3 expression was associated with unresponsiveness to treatment.^{14,34–37}

The homing of Tregs is further supported by CCR5 expression. The migration and homing of Tregs to the infection site are thought to be the result of the expression of several chemokines. In a murine model of *Leishmania* infection, CCR5 was required for Treg chemoattraction. Mice lacking this receptor presented inefficient Treg migration.²⁴ We have extended that observation to human leishmaniasis.²⁶ Regulatory T cells from the infection site had an increased proportion of CCR5⁺ cells compared with peripheral blood Tregs. In that sense, selective targeting of CCR5 may pose as a novel immunomodulatory strategy for *Leishmania* treatment.^{18,26} Although we were unable to isolate sufficient lymphocytes from normal skin to determine whether this is an increased proportion in diseased skin compared with normal skin, clearly there are increased numbers of CCR5⁺ Foxp3⁺ cells at the site of infection.

The subsequent normalization of Tregs in peripheral blood with successful treatment is also consistent with the hypothesis of Treg homing. Thus, as the parasite load at the infection site diminishes, the Treg “homing” stimulus could wane. This could lead to the redistribution of the Treg pool and normalization of peripheral blood Tregs during CL. Thus, we were unable to determine if the proportion was increased compared with that in normal skin, there is clearly a marked increase in total number of lymphocytes at the site of infection, and thus the total number of Tregs per area of skin.

Surprisingly, peripheral blood Tregs from ML individuals remained lower than that from controls even with treatment completion. This may reflect a host defect that predisposes to ML or may reflect an incomplete treatment response. Alternatively, the patients with mucosal lesions were older, which might have modified their ability to increase Treg proportions.

The immunological responses in human leishmaniasis have been addressed in several reports.^{38–40} Although previous studies discuss the immunological profiles during leishmaniasis treatment, these studies examined CD4⁺ T cells and CD8⁺ T cells proportions.^{27,28,41} Here we expanded these

results by examining the proportions of CD4⁺ Foxp3⁺ T cells. Others have noted that there are other immunomodulatory cells besides CD4⁺ Foxp3⁺ T cells. For example, Nylen and colleagues identified CD4⁺ Foxp3[−] cells producing IL-10 in human visceral leishmaniasis.¹⁵ Similar cells have been identified in murine models of CL.^{42,43}

Our findings are consistent with a pattern of recruitment, or homing, of Treg from the circulation to the site of infection, and a role in modulating the immune response during tegumentary leishmaniasis. Further studies are needed to clarify the mechanisms directing Tregs homing and the functional role of the different CD4⁺ T-cell subsets in human tegumentary leishmaniasis.

Received August 4, 2017. Accepted for publication November 13, 2017.

Published online February 5, 2018.

Acknowledgments: We thank the IMT Leishmania and Malaria Study Group for coordinating clinical procedures and providing blood and biopsy samples and Jaime Zaldivar for sample processing.

Financial support: The experiments reported in this article have been supported by funds from the National Institute of Health (NIH) grant (1R01 A1075575-01A1), and a Fogarty Training grant (D43TW006569).

Disclaimer: Abstract poster presentation at the IDSA 44th annual meeting, October 12–15, 2006, in Toronto, Canada, the IDSA 48th annual meeting, October 21–24, 2010, in Vancouver, British Columbia, Canada, and the IDSA 49th annual meeting, October 20–23, 2011, in Boston, Massachusetts.

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