

# Interleukin 10–Dominant Immune Response and Increased Risk of Cutaneous Leishmaniasis After Natural Exposure to *Lutzomyia intermedia* Sand Flies

Augusto M. Carvalho,<sup>1</sup> Juqueline R. Cristal,<sup>1</sup> Aline C. Muniz,<sup>2</sup> Lucas P. Carvalho,<sup>2,3</sup> Regis Gomes,<sup>1</sup> José C. Miranda,<sup>1</sup> Aldina Barral,<sup>1,4</sup> Edgar M. Carvalho,<sup>2,3</sup> and Camila I. de Oliveira<sup>1,4</sup>

<sup>1</sup>Fundação Oswaldo Cruz, Centro de Pesquisas Gonçalo Moniz, <sup>2</sup>Serviço de Imunologia, Hospital Universitário Professor Edgard Santos, Universidade Federal da Bahia, <sup>3</sup>Instituto Nacional de Ciência e Tecnologia em Doenças Tropicais, and <sup>4</sup>Instituto de Investigação em Imunologia, Salvador, Bahia, Brazil

**Background.** Leishmaniasis is caused by parasites transmitted to the vertebrate host by infected sand flies. During transmission, the vertebrate host is also inoculated with sand fly saliva, which exerts powerful immunomodulatory effects on the host's immune response.

**Methods.** We conducted a prospective cohort analysis to characterize the human immune response to *Lutzomyia intermedia* saliva in 264 individuals, from an area for cutaneous leishmaniasis (CL) caused by *Leishmania braziliensis*.

**Results.** Antibodies were found in 150 individuals (56.8%); immunoglobulin G1 and G4 were the predominant subclasses. Recall responses to salivary gland sonicate showed elevated production of interleukin 10 (IL-10), interleukin 13, interferon  $\gamma$ , CXCL9, and CCL2 compared with controls. CD4<sup>+</sup>CD25<sup>+</sup> T cells, including Foxp3<sup>+</sup> cells, were the main source of IL-10. *L. braziliensis* replication was increased ( $P < .05$ ) in macrophages cocultured with saliva-stimulated lymphocytes from exposed individuals and addition of anti-IL-10 reverted this effect. Positive correlation between antibody response to saliva and cellular response to *Leishmania* was not found. Importantly, individuals seropositive to saliva are 2.1 times more likely to develop CL (relative risk, 2.1; 95% confidence interval, 1.07–4.2;  $P < .05$ ).

**Conclusions.** Exposure to *L. intermedia* sand flies skews the human immune response, facilitating *L. braziliensis* survival in vitro, and increases the risk of developing CL.

**Keywords.** sand fly saliva; *L. braziliensis*; cutaneous leishmaniasis; *Lutzomyia intermedia*; ELISA; cytokines; chemokines; killing assay.

Leishmaniasis are neglected diseases caused by protozoa of genus *Leishmania* that affect millions of persons worldwide. *Leishmania* parasites are inoculated into the vertebrate host by infected sand flies and take up permanent residence within macrophages, where they

replicate and cause disease. During parasite inoculation, the vertebrate host is simultaneously injected with sand fly saliva, which contains anticoagulants, vasodilators, and other molecules capable of modulating the host's immune response (reviewed in [1]).

In murine models, coinoculation of *Leishmania* plus sand fly saliva exacerbates *Leishmania* infection [2, 3]. On the other hand, immunization with sand fly saliva or with individual recombinant salivary proteins protects against subsequent challenge with inoculation of live parasites or with infected sand flies [4–6]. With regard to human leishmaniasis, an association between natural exposure to sand flies and disease outcome has been observed. For example, in an area endemic for visceral leishmaniasis (VL), individuals develop antibody responses to *Lutzomyia longipalpis* saliva and a

Received 23 September 2014; accepted 1 January 2015; electronically published 16 January 2015.

Presented in part: Fifth World Congress on Leishmaniasis, Porto de Galinhas, Pernambuco, Brazil, 13–17 May 2013.

Correspondence: Camila I. de Oliveira, PhD, Laboratório de Imunoparasitologia-Centro de Pesquisas Gonçalo Muniz-Fundação Oswaldo Cruz, Rua Waldemar Falcão 121, Candeal-Salvador, BA, Brazil 40196-710 (camila@bahia.fiocruz.br).

The Journal of Infectious Diseases® 2015;212:157–65

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DOI: 10.1093/infdis/jiv020

positive *Leishmania* skin test result against *Leishmania infantum-chagasi*-soluble antigens [7]. A follow-up study demonstrated an increased incidence of positive *Leishmania* skin test results among individuals seropositive to *L. longipalpis* saliva [8]. Experimental exposure to *L. longipalpis* sand flies also induced the development of a T-helper (Th) 1-biased immune response to saliva, promoting *L. infantum-chagasi* killing in vitro [9]. In contrast, in an area endemic for cutaneous leishmaniasis (CL), individuals naturally exposed to *Phlebotomus papatasi* sand flies developed an interleukin 10 (IL-10)-mediated response, which the authors postulated could favor *Leishmania major* infection [10].

Infection with *Leishmania braziliensis* results in a variety of clinical phenotypes, including CL, mucosal leishmaniasis, and disseminated leishmaniasis [11, 12]. We previously found that experimental immunization with *Lutzomyia intermedia* saliva did not induce a classic delayed-type hypersensitivity (DTH) response [13], as described for the saliva of other sand fly species [5, 6]. Mice immunized with *L. intermedia* saliva displayed a mixed cytokine response and were not protected against challenge with live *L. braziliensis* [13]. In fact, immunization with *L. intermedia* saliva increased neutrophil migration and IL-10 expression on challenge with *L. braziliensis* [14]. In the endemic area, patients with CL displayed higher levels of antibodies to *L. intermedia* saliva than those with subclinical infection [13]. Collectively, these results suggest that prior exposure to *L. intermedia* saliva may negatively influence the outcome of *L. braziliensis* infection.

Based on the premise that natural exposure to *L. intermedia* sand flies modulates the immune response and impacts on the outcome of *L. braziliensis* infection, we followed a prospective cohort of individuals naturally exposed to *L. intermedia* sand flies, residing in an area endemic for CL caused by *L. braziliensis*. We evaluated their humoral immune response to *L. intermedia* saliva and characterized the cellular recall responses after peripheral blood stimulation. Coculture experiments using autologous lymphocytes and macrophages enabled us to investigate how the cytokine milieu affects the infection rate.

## METHODS

### Area of Study and Selection of Individuals

This study was conducted in Corte de Pedra, Bahia, Brazil, an area of *L. braziliensis* transmission where *L. intermedia* is present. A prospective cohort was established in January 2010 and was followed up to January 2013 [15]. Inclusion criteria for participating individuals consisted of a negative history of any type of *Leishmania* infection, established after a medical interview and examination for signs consistent with previous CL or mucosal leishmaniasis, such as scars on the skin or mucosal area. In addition, participating individuals were living in the same home as patients with CL, the latter diagnosed after parasite

isolation or a positive polymerase chain reaction finding for *L. braziliensis*. This research was conducted with the approval of the ethical committee of the Hospital Professor Universitário Edgard Santos (Salvador, Bahia, Brazil; 240/2009) and Comissão Nacional de Ética em Pesquisa (Brazilian National Ethics Committee, Brazil), and informed consent was obtained from each participant.

### Sand Flies and Preparation of Salivary Gland Homogenate

Adult *L. intermedia* sand flies were captured in Corte de Pedra, Bahia. Sand flies were morphologically identified according to the identification key proposed by Young and Duncan. Salivary glands were dissected and stored in groups of 20 pairs in 20  $\mu$ L of sodium chloride (150 mmol/L)-HEPES buffer (10 mmol/L; pH 7.4) at  $-70^{\circ}\text{C}$ . Salivary gland homogenate (SGH) was prepared and tested for the presence of lipopolysaccharide, as described elsewhere [14].

### Analysis of Anti-*L. intermedia* Saliva Antibodies by Enzyme-Linked Immunosorbent Assay

Humoral (immunoglobulin [Ig] G and IgG subclasses) response to *L. intermedia* SGH was determined as described elsewhere [9, 13]. The enzyme-linked immunosorbent assay (ELISA) cutoff value for each product was established as the mean optical density (OD) value plus 3 standard deviations, using serum samples from healthy volunteers ( $n = 80$ ) from a nonendemic area. For the detection of IgE, serum samples were preincubated with RF-Absorbent reagent (Dade Behring) to eliminate IgG antibody competition, as described elsewhere [9].

### Gel Electrophoresis and Western Blot Analysis

*L. intermedia* SGH (equivalent to 60 salivary gland pairs) was assayed on NuPAGE gel (8%–12%) (Invitrogen) for gel electrophoresis, according to manufacturer's instructions. Proteins were visualized by staining with SimplyBlue stain (Invitrogen) and transferred to nitrocellulose. Membranes were incubated with individual serum samples from exposed individuals diluted in phosphate-buffered saline/Tween/0.05% nonfat milk, followed by incubation with anti-human IgG alkaline phosphatase conjugate (Promega). Bands were visualized by adding alkaline phosphatase substrate (Promega).

### Cell Culture and Cytokine Detection

Peripheral blood mononuclear cells (PBMCs) were obtained from heparinized venous blood layered over a Ficoll-Hypaque gradient (GE Healthcare). Cells were washed and resuspended in Roswell Park Memorial Institute 1640 medium supplemented with 10% human AB serum, 100 IU/mL penicillin, and 100  $\mu$ g/mL streptomycin (all from Invitrogen). Cells ( $3 \times 10^6$ /mL) were plated in 24-well plates and stimulated with SGH (equivalent to 1.5 pair) for 72 hours at  $37^{\circ}\text{C}$  and 5% carbon dioxide ( $\text{CO}_2$ ). Cytokine levels in culture supernatants were determined by ELISA (R&D Systems).

## Flow Cytometry

For flow cytometric analysis,  $10^6$  PBMCs were stimulated with SGH (equivalent to 1.5 pair), cultured for 24 hours, and stained for CD4, CD25, and Foxp3 using the One Step Staining Human Treg Flow Kit (Biolegend). For intracellular staining, brefeldin A (Sigma) (10  $\mu\text{g}/\text{mL}$ ) was added in the last 8 hours of culture. Cells were permeabilized using 0.2% saponin and stained with anti-IL-10 antibody (Ebioscience). Alexa Fluor 488, phycoerythrin, and peridinin-chlorophyll protein isotype controls were included in all experiments. Samples were acquired in a FACS-CantoII flow cytometer (BD Pharmingen), and analysis was performed using FlowJo software (version 7.6.5; Tree Star).

## In Vitro Killing Assay

PBMCs ( $3 \times 10^6$ ) were plated onto 4 well Lab-Tek chamber slides (Thermo Scientific) and incubated for 30 minutes at  $37^\circ\text{C}$  and 5%  $\text{CO}_2$ . Nonadherent cells (autologous T cells) were removed and frozen; adherent cells were cultured for another 6 days. Macrophages were infected with stationary phase *L. braziliensis* (MHOM/BR/00/2000) [16] (5 parasites to 1 macrophage) for 2 hours at  $37^\circ\text{C}$  and 5%  $\text{CO}_2$ . Noninternalized parasites were removed, and infected macrophages were further cultured alone, with autologous lymphocytes (2 lymphocytes to 1 macrophage), in the presence of autologous lymphocytes plus *L. intermedia* SGH (equivalent to 1.5 pair) or the autologous lymphocytes plus *L. intermedia* SGH (equivalent to 1.5 pair) plus anti-IL-10 (1  $\text{ng}/\text{mL}$ ) (Ebioscience). After 72 hours, slides were washed, stained with hematoxylin-eosin, and analyzed with light microscopy for determination of the percentage of infected cells and the number of amastigotes per 100 cells.

## Statistical Analysis

Categorical data were compared using the Fisher exact test. Comparisons between 2 groups were performed by Mann-Whitney test and those among 3 or more groups by Kruskal-Wallis test followed by Dunn multiple comparison tests. The

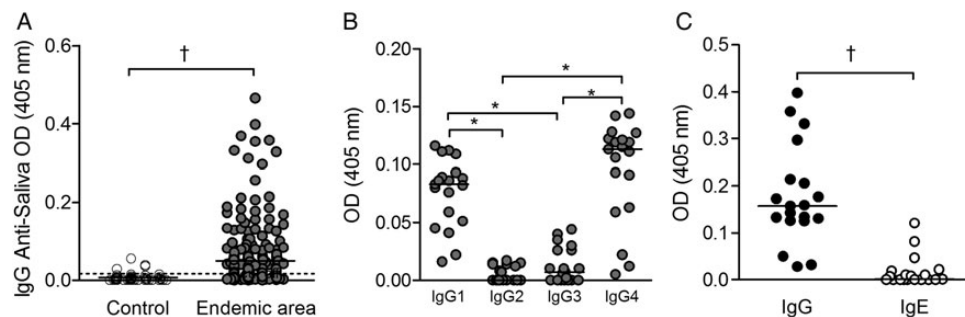
Wilcoxon paired test was used to assess differences between variables in the same subjects. The relative risk (RR) was calculated using the following formula:  $\text{RR} = I_1/I_0$ , where  $I_1$  is the incidence of CL in exposed individuals (seropositive to *L. intermedia* SGH), and  $I_0$  the incidence of CL in nonexposed individuals (seronegative to *L. intermedia* SGH). Analyses were conducted using GraphPad Prism version 5.00 for Windows (GraphPad Software), and differences were considered significant at  $P < .05$ .

## RESULTS

### Humoral Immune Response in Individuals Exposed to *L. Intermedia* Sand Flies

The IgG response against *L. intermedia* saliva was evaluated by ELISA in 264 household contacts of patients with CL with a negative history of *Leishmania* infection. Negative serum samples were obtained from residents ( $n = 46$ ) of a nonendemic area. Individuals exposed to *L. intermedia* displayed a higher ( $P < .001$ ) anti-saliva IgG response than controls: 150/264 individuals (56.8%) tested positive for anti-saliva IgG (Figure 1A). The demographic and epidemiologic features of the population studied, according to the presence of anti-saliva IgG, are shown in Table 1. There was no significant difference between individuals who were seropositive and those who were seronegative to *L. intermedia* saliva regarding age, sex, and most epidemiologic features (Table 1). One exception was the documentation that seropositive individuals more frequently arrived home after 4 PM ( $P = .01$ ; Fisher exact test).

Next, we selected the 19 individuals with the highest IgG values against *L. intermedia* (group median OD, 0.1570; Figure 1A) saliva and evaluated the IgG isotypes within this group. IgG1 and IgG4 were the principal isotypes found (Figure 1B): IgG1 and IgG4 levels were higher ( $P < .05$ ) than IgG2 and IgG3 levels, which were also detected in seropositive individuals (Figure 1B). The relative OD of anti-saliva IgG4 was higher than for IgG1, IgG2, and IgG3. We found pairwise correlations between IgG



**Figure 1.** Humoral immune response to *Lutzomyia intermedia* saliva in a cutaneous leishmaniasis (CL)-endemic area. A, Total immunoglobulin (Ig) G response in residents of a nonendemic area ( $n = 46$ ) (white circles) and a CL-endemic area ( $n = 264$ ) (gray circles). B, IgG subclasses in a subset of residents ( $n = 19$ ) from the endemic area represented in A who were seropositive to *L. intermedia* saliva. C, Comparison of IgG and IgE levels in the same subset. Circles represent individual values; horizontal lines, median optical density (OD) values; dotted line in A, cutoff level. \* $P < .05$ ; † $P < .001$ .

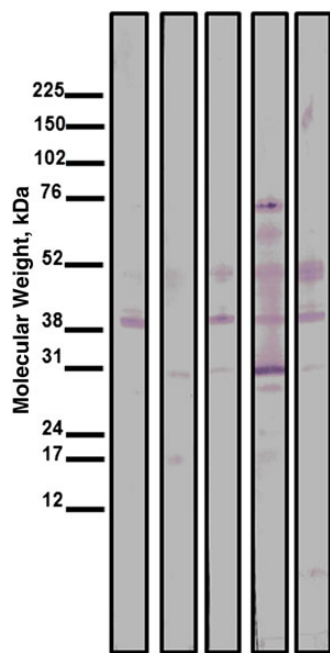
**Table 1. Demographic and Epidemiologic Features of Individuals From a CL-Endemic Area, by Seropositivity to *Lutzomyia intermedia* Saliva**

| Characteristic                                      | Seropositive<br>(n = 150) | Seronegative<br>(n = 114) | P<br>Value <sup>a</sup> |
|---|---------------------------|---------------------------|-------------------------|
| Median age, y (range)                               | 14 (2–53)                 | 15 (2–63)                 | .15                     |
| Male sex, No. (%)                                   | 74 (49.3)                 | 48 (42.1)                 | .20                     |
| DTH to <i>Leishmania</i> , No. (%)                  | 15 (10)                   | 18 (15.7)                 | .17                     |
| Occupation, No. (%)                                 |                           |                           |                         |
| Agriculture   | 21 (14)                   | 23 (20.1)                 | .21                     |
| Domestic  | 35 (23.3)                 | 30 (26.3)                 |                         |
| Student/other                                       | 94 (62.6)                 | 61 (53.5)                 |                         |
| Time living in endemic area,<br>median (range), y   | 14 (2–53)                 | 14.5 (2–63)               | .18                     |
| Time living in the same house,<br>median (range), y | 9 (0–53)                  | 9 (0–56)                  | .63                     |
| Home arrival after 4 PM, No. (%)                    | 38 (25.3)                 | 15 (13.1)                 | .01                     |

Abbreviations: CL, cutaneous leishmaniasis; DTH, delayed-type hypersensitivity.

<sup>a</sup> Continuous variables were compared using the Mann–Whitney test, categorical variables using the Fisher exact test.

and IgG1 ( $r = 0.5$ ;  $P = .009$ ) and between IgG and IgG4 ( $r = 0.6$ ;  $P = .004$ ) (Supplementary Figure 1A and 1B, respectively). In the subset of 19 seropositive individuals, anti-*L. intermedia* saliva total IgG levels were higher ( $P < .05$ ) compared with IgE (Figure 1C). Western blot analysis revealed that seropositive



**Figure 2.** Detection of *Lutzomyia intermedia* salivary proteins by Western blot analysis. *L. intermedia* saliva was submitted to sodium dodecyl sulfate polyacrylamide gel electrophoresis and tested against serum samples from residents ( $n = 5$ ) of a cutaneous leishmaniasis endemic area.

individuals preferentially recognized *L. intermedia* proteins of approximately 52, approximately 38, and approximately 30 kDa (Figure 2).

### Recall Response in Individuals Exposed to *L. intermedia* Sand Flies

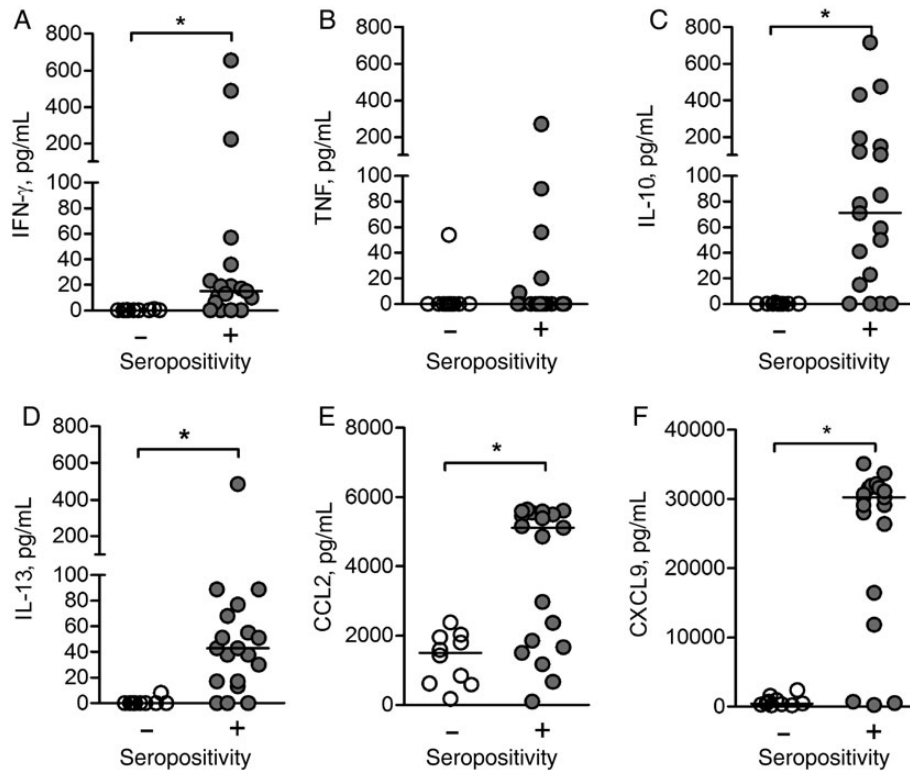
PBMCs from the same subset of 19 seropositive individuals were stimulated in vitro with *L. intermedia* saliva, and the presence of cytokines and chemokines was evaluated by ELISA. Controls consisted of 10 residents of the endemic area seronegative to *L. intermedia* saliva (Figure 1A). The levels of interferon (IFN)  $\gamma$  were higher ( $P < .05$ ) in individuals who were seropositive to *L. intermedia* saliva than in those who were seronegative (Figure 3A). Tumor necrosis factor (TNF) levels were similar in both groups (Figure 3B); TNF was detectable in 5 of 19 tested culture supernatants (Figure 3B). On the other hand, IL-10 (Figure 3C) and interleukin 13 (IL-13) (Figure 3D) levels were higher ( $P < .05$ ) in seropositive than in seronegative individuals. With regard to chemokines, CCL2 (Figure 3E) and CXCL9 (Figure 3F) levels were also higher in those who were seropositive. Therefore, individuals exposed to *L. intermedia* displayed a mixed response (Supplementary Figure 2A) with a predominance of IL-10, evidenced by elevated IL-10/IFN- $\gamma$  and IL-10/IL-13 ratios (Supplementary Figure 2B).

### Production of IL-10 by CD4<sup>+</sup> T Cells From Individuals Exposed to *L. intermedia* Sand Flies

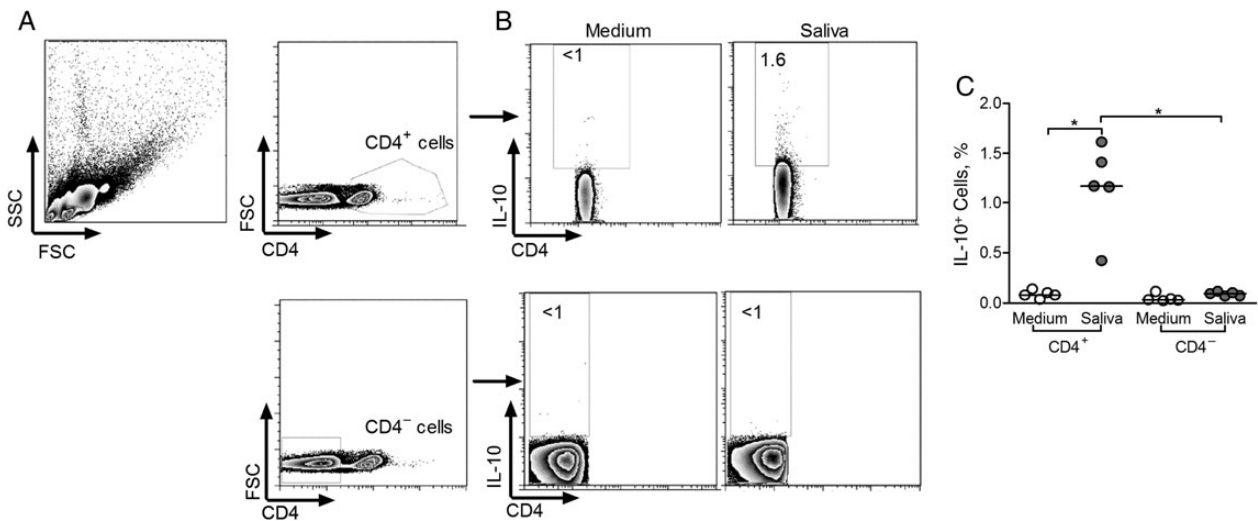
Next, PBMCs from 5 seropositive individuals were restimulated with *L. intermedia* saliva and the CD4<sup>+</sup> and CD4<sup>-</sup> T-cell subsets were analyzed by flow cytometry for the production of IL-10 (Figure 4A and 4B). In the presence of *L. intermedia* saliva, the frequency of CD4<sup>+</sup>IL-10<sup>+</sup> cells increased ( $P < .05$ ) compared with control (unstimulated) cultures (Figure 4C). Within CD4<sup>-</sup> cells, the frequency of IL-10-secreting cells did not change despite stimulation with saliva (Figure 4C). Similar experiments were performed with cells obtained from control individuals residing in nonendemic areas, and the frequency of IL-10-secreting cells did not change after stimulation with *L. intermedia* saliva (Supplementary Figure 3). Moreover, within the CD4<sup>+</sup>CD25<sup>+</sup> compartment, we detected the presence of both Foxp3<sup>+</sup> and Foxp3<sup>-</sup> cells secreting IL-10 (Figure 5A). After stimulation with *L. intermedia* saliva, the frequency of both CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>-</sup> (Figure 5B) and CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> subpopulations (Figure 5C) increased significantly, as did the frequency of IL-10-secreting cells within these 2 subpopulations (Figure 5D). Therefore, both Foxp3<sup>-</sup> and Foxp3<sup>+</sup> cells contribute to IL-10 production after stimulation with *L. intermedia* saliva.

### Lack of Parasite Killing Upon Coculture of Autologous Lymphocytes and *L. braziliensis*-Infected Macrophages

We next asked whether saliva-induced IL-10 secretion modulated *L. braziliensis* infection in vitro. Monocyte-derived macrophages obtained from exposed individuals were infected with *L.*

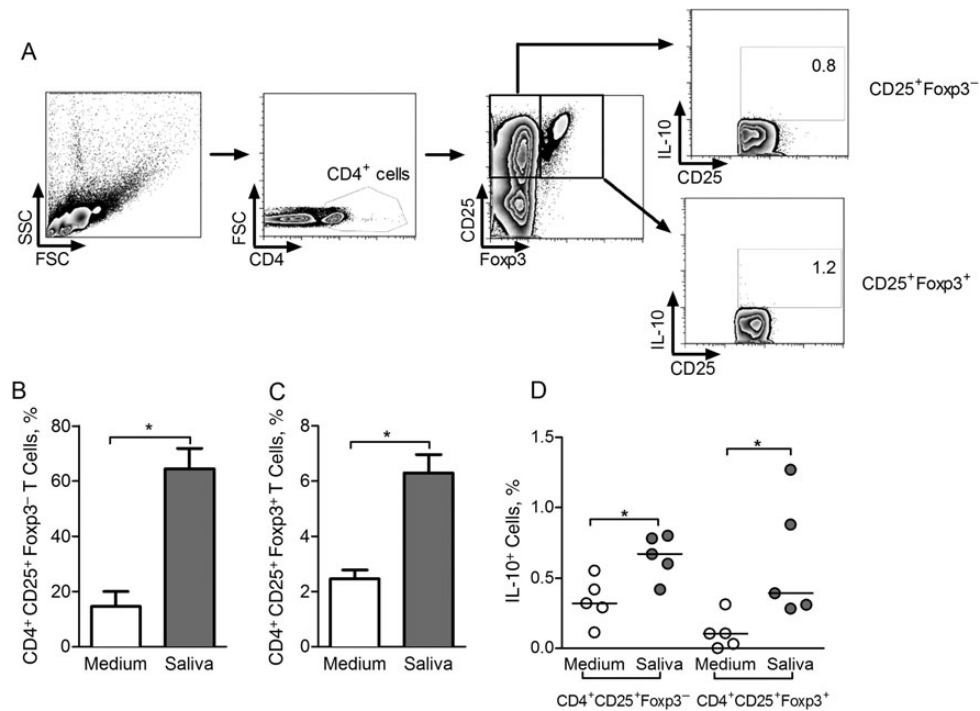


**Figure 3.** Cellular immune response to *Lutzomyia intermedia* saliva in a cutaneous leishmaniasis-endemic area. Peripheral blood mononuclear cells from a subset of residents who were seronegative ( $n = 10$ ) (white circles) or seropositive ( $n = 19$ ) (gray circles) to sand fly saliva were restimulated with *L. intermedia* saliva (equivalent to 1.5 pair) for 72 hours. Cytokine levels of interferon (IFN)  $\gamma$  (A), tumor necrosis factor (TNF) (B), interleukin 10 (IL-10) (C), interleukin 13 (IL-13) (D), CCL2 (E), and CXCL9 (F) were determined by enzyme-linked immunosorbent assay after 72 hours. Horizontal lines indicate median levels.  $*P < .05$ .



**Figure 4.** Frequency of interleukin 10 (IL-10)-producing cells in individuals exposed to *Lutzomyia intermedia* sand flies in a cutaneous leishmaniasis-endemic area. A, Gating strategy for flow cytometry analysis of CD4<sup>+</sup> and CD4<sup>-</sup> cells after restimulation of peripheral blood mononuclear cells with *L. intermedia* saliva. In this sample gating, cells were first gated for size and granularity (FSC  $\times$  SSC). The gated cells were further analyzed for expression of CD4. B, IL-10 expression was determined in both CD4<sup>+</sup> and CD4<sup>-</sup> gated populations after culture in medium alone (control) or in the presence of *L. intermedia* saliva. C, Frequency of IL-10<sup>+</sup> cells in CD4<sup>-</sup> and in CD4<sup>+</sup> populations, in control (white circles) and saliva-stimulated (gray circles) cultures from 5 seropositive individuals. Circles represent individual values; horizontal lines, indicate median levels.  $*P < .05$ . Abbreviations: FSC, forward scattered light; SSC, side scattered light.





**Figure 5.** Frequency of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>-</sup> and CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T cells in individuals exposed to *Lutzomyia intermedia* sand flies in a cutaneous leishmaniasis-endemic area. **A**, Gating strategy for flow cytometry analysis of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> cells after restimulation with *L. intermedia* saliva. In this sample gating, cells were first gated for size and granularity (FSC × SSC). CD25 and Foxp3 expression were determined in CD4<sup>+</sup> gated cells and, within these, interleukin 10 (IL-10) expression was determined. **B**, Frequency of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>-</sup> cells after culture in medium alone (white bar) or in the presence of *L. intermedia* saliva (gray bar). **C**, Frequency of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> cells after culture in medium alone (white bar) or in the presence of *L. intermedia* saliva (gray bar). Data are shown as median with interquartile range. **D**, Frequency of IL-10<sup>+</sup> cells in Foxp3<sup>-</sup> and in Foxp3<sup>+</sup> populations, in control (white circles) and saliva-stimulated (gray circles) cultures from 5 seropositive individuals. Circles represent individual values; horizontal lines, median levels. \**P* < .05. Abbreviations: FSC, forward scattered light; SSC, side scattered light.

*braziliensis* and cocultured with autologous lymphocytes, in the presence or absence of *L. intermedia* saliva. Addition of saliva to cocultures increased the percentage of infected cells (Figure 6A), although not significantly. However, the number of intracellular amastigotes was significantly increased in comparison with macrophages cultured alone or with autologous lymphocytes (Figure 6B). This effect was reversed in the presence of a neutralizing IL-10 antibody (Figure 6A and 6B). Representative photographs confirm the elevated number of intracellular amastigotes in the presence of saliva and the opposite effect in the presence of anti-IL-10 (Figure 6C). ELISA confirmed increased levels (*P* < .05) of IL-10 in cocultures stimulated with saliva (Supplementary Figure 4A), corroborating the higher parasite load (Figure 6B), whereas IFN- $\gamma$  levels were not altered (Supplementary Figure 4B). Importantly, *L. intermedia* saliva per se does not alter *L. braziliensis* infection in human macrophages (Supplementary Figure 5).

#### Increased Risk of CL in Individuals Exposed to *L. intermedia* Sand Flies

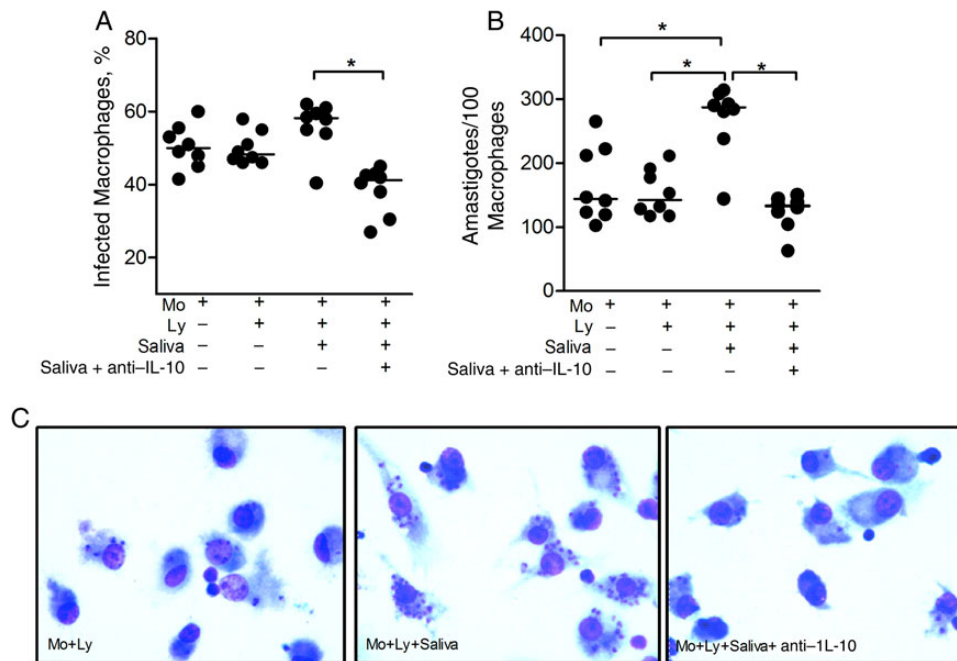
During the 3-year follow-up, 38 of 264 residents of the endemic area (14.3%) developed CL. Importantly, 28 of 150 (18.6%) who were seropositive to saliva developed CL, compared with only

10 of 114 (8.7%) who were seronegative. Based on these results, individuals naturally exposed to *L. intermedia* saliva are 2.1 times more likely to develop CL than nonexposed individuals (RR, 2.1; 95% confidence interval, 1.07–4.2; *P* < .05).

## DISCUSSION

In the present work, we investigated how natural exposure to *L. intermedia* sand flies shapes the human immune response and whether such exposure affects the development of human CL. We conducted our study in a large cohort of 264 individuals residing in an area endemic for CL caused by *L. braziliensis*, transmitted by *L. intermedia* sand flies. A seropositive response to saliva was detected in 56.8% of individuals, confirming our earlier findings regarding the immunogenicity of *L. intermedia* saliva [13] and also results obtained in areas of *P. papatasi* [17] and *L. longipalpis* [7]. We also found an association between seropositivity to *L. intermedia* saliva and home arrival after 4 PM, indicating that the antibody response correlates with the increased presence of sand flies at dusk [18].

We found that IgG1 and IgG4 subclasses predominated, similar to results reported after experimental exposure to *L.*



**Figure 6.** Infection rate of *Leishmania braziliensis*-infected macrophages after coculture with autologous lymphocytes and *Lutzomyia intermedia* saliva. Macrophages (Mo) from 8 individuals seropositive to *L. intermedia* saliva were infected with *L. braziliensis* promastigotes. Cells were cocultured with autologous lymphocytes alone (Ly), with Ly plus *L. intermedia* saliva, or with Ly plus saliva plus anti-interleukin 10 (IL-10). After 72 hours, glass coverslips were stained with hematoxylin-eosin and assessed with light microscopy for the percentage of infected macrophages (A) and for the number of amastigotes per 100 macrophages (B). Circles represent individual values; horizontal lines, median levels. \* $P < .05$ . C, Representative photomicrographs of cultures shown in A and B. Magnification  $\times 40$ . Data are shown individually, horizontal lines indicate median levels.

*longipalpis* [9] and, in the case of IgG4, similar to results after *P. papatasi* exposure [19]. We found positive correlations between total IgG/IgG1 and total IgG/IgG4, suggesting that both IgG1 and IgG4 are markers of exposure in serologic assays. In contrast to mosquito saliva [20], exposure to *L. intermedia* did not induce a strong IgE response, indicating lack of allergic reaction, nor was there a correlation between IgE and IgG4 levels. Antibodies to *L. intermedia* were directed mainly against approximately 52, approximately 38 and approximately 30 kDa proteins, suggesting that yellow-related protein, apyrase (putative), and proteins from the 33-kDa Phlebotomine family are the immunodominant antigens in *L. intermedia* saliva. Indeed yellow proteins and apyrase were also immunodominant in VL-endemic areas [21, 22].

Our results show that stimulation of PBMCs from seropositive individuals induced IFN- $\gamma$ , IL-10, and IL-13 production. This mixed response recapitulates our previous results after immunization of BALB/c mice with *L. intermedia* saliva [13] and, importantly, shows a predominance of IL-10 over IFN- $\gamma$  (Supplementary Figure 2). Individuals exposed to *P. papatasi* also developed an IL-10-dominant response [10], possibly resulting from the presence of adenosine and adenosine monophosphate in sand fly saliva [23, 24]. Adenosine increases IL-10 production [25] and decreases interleukin 12 [26]. However, individuals

exposed to *Phlebotomus duboscqi* were categorized as Th1, Th2, or mixed responders [27] and those exposed to *L. longipalpis* produced IFN- $\gamma$ , IL-10, and TNF- $\alpha$  [9]. In a study of *P. papatasi*-exposed individuals [10], the authors also found that saliva-stimulated cells did not produce IFN- $\gamma$  but, rather, induced IL-10 secretion, primarily from CD8<sup>+</sup> T cells. In contrast, we found that the source of IL-10 was both CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> and Foxp3<sup>-</sup> cells. Foxp3 is a transcription factor expressed in naturally occurring regulatory T cells (Tregs), which suppress effector T cells and are important for maintenance of peripheral tolerance [28]. Tregs mediate suppression by distinct mechanisms, including IL-10 production [29]. In CL caused by *L. braziliensis*, the presence of Tregs (CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup>) suggested suppression of the effector response, facilitating parasite survival [30, 31].

In the current study, coculture of lymphocytes from *L. intermedia*-exposed individuals with autologous *L. braziliensis*-infected macrophages significantly increased infection rates and amastigote numbers, findings that were associated with elevated IL-10 and, importantly, reversed on neutralization of IL-10. Therefore, we suggest that exposure to *L. intermedia* sand flies stimulates a regulatory or suppressive response, favoring *L. braziliensis* replication. These results differ from those reported for *L. longipalpis* [9] and *P. papatasi* sand flies [4, 5]. In fact, they argue against the hypothesis that the initial response to whole

*L. intermedia* saliva can alter the immune response to *Leishmania*, in the context of protection against CL caused by *L. braziliensis* (reviewed in [1]).

In addition to natural (CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup>) Tregs, it is also possible that activated CD4<sup>+</sup> T cells or even Treg type 1 (Tr1) cells (CD4<sup>+</sup>CD25<sup>-</sup>Foxp3<sup>-</sup>) [32] cells contribute to IL-10 production. Likewise, Tr1 cells suppress effector T cells through IL-10 production [33] or through contact-dependent mechanisms. Interestingly, IL-10 secreted by Tr1 cells regulates IgG4 secretion [34], and we found that IgG4 levels were increased in individuals exposed to *L. intermedia*. Because IgG4 cannot properly bind complement [35], preventing antibody-mediated complement activation and pathogen uptake and destruction, this could be another mechanism by which *L. intermedia* salivary molecules modulate the host's immune response.

Individuals who were seropositive to *L. intermedia* saliva displayed increased CCL2 and CXCL9 production. These data corroborate our recent findings regarding elevated CXCL9 messenger RNA in response to exposure to *L. intermedia* saliva [36]. Lesions of patients with CL also display high expression of CXCL9 [37]. Finally, both *L. intermedia* [14] and *L. longipalpis* [38] saliva induce CCL2 production in murine cells. Given that CCL2 induces macrophage recruitment, the definitive host cells for *Leishmania* parasites, this could also affect *Leishmania* infection.

In studies conducted in VL-endemic areas, a significant correlation was found between presence of antibodies to *L. longipalpis* saliva and a positive cellular response (DTH) to *Leishmania* [39]. Later, a prospective cohort study showed that the incidence of anti-*Leishmania* DTH was higher in subjects who also presented antibodies to *L. longipalpis* saliva [8]. These findings indicated that mounting an immune response to *L. longipalpis* saliva paralleled the development of a cell-mediated immune response to *Leishmania*. Moreover, immunization with *L. longipalpis* salivary proteins confers protection against experimental VL [6, 40]. On the contrary, in the current study we showed that exposure to *L. intermedia* saliva increases the risk of developing CL in an endemic area for *L. braziliensis*.

This finding recapitulates our previous results in the experimental model of infection [13] but, more importantly, points to a yet unappreciated role regarding natural exposure to *L. intermedia* sand flies. The presence of antibodies to *P. papatasi* saliva was also associated with increased risk of CL in a Tunisian endemic area [19]. Given that a minority of sand flies are infected with parasites, it is likely that residents will be constantly exposed to non-infected bites. In this scenario, the IL-10-biased immune response developed by individuals exposed to *L. intermedia* sand flies adds yet another layer of complexity to CL caused by *L. braziliensis*.

## Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (<http://jid.oxfordjournals.org>). Supplementary materials consist of

data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

## Notes

**Acknowledgments.** We thank Phillip Scott for critical review.

**Financial support.** This work was supported by the National Institutes of Health (grant AI 30639 to E. M. C.), the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq; grant 564689/2008-4 to C. I. d. O.), and Fundação de Amparo à Pesquisa da Bahia (fellowships to A. M. C. and J. R. C.). A. B., E. M. C., and C. I. d. O. are senior researchers at CNPq.

**Potential conflicts of interest.** All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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