TH1/TH2 CYTOKINE PROFILE IN HIV AND LEISHMANIA CO-INFECTED PATIENTS
IN BRAZIL

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Abstract

To evaluate the effects of HIV on immune responses in cutaneous leishmaniasis (CL), we quantified cytokine levels from plasma and stimulated PBMCs from individuals infected with HIV and/or CL. IFN-\(\gamma\), IL-13, and the ratio of IFN-\(\gamma\)/IL-10 produced in response to stimulation with soluble Leishmania antigens were significantly lower in HIV/Leishmania co-infected patients.

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The clinical outcome of *Leishmania* infection depends on the balance between Th1 and Th2 cytokines (4, 9, 31). HIV infection alters the Th1/Th2 balance in patients with visceral leishmaniasis, resulting in increased Th2 cytokine responses and decreased levels of IL-12 and IL-18 (31). Here we evaluate the effects of HIV on cytokine responses in cutaneous leishmaniasis (CL).

Eight HIV-1 infected patients with CL, 9 HIV-1 seronegative patients with CL, and 21 HIV-infected patients were studied. Institutional review board approval was obtained from Comissão Nacional de Ética em Pesquisa (CONEP), FIOCRUZ and UCSD’s Human Research Protection Program.

Peripheral blood mononuclear cells (PBMC) were separated by Ficoll-Hypaque centrifugation (Amersham Biosciences) and resuspended in RPMI-1640 (Sigma-Aldrich) supplemented with 2 mM L-glutamine, penicillin (100 U/mL), streptomycin (100 µg/mL) (GIBCO) and 5% heat-inactivated human AB serum. Cells were stimulated with soluble *Leishmania* antigen (SLA) at 10µg/ml (11), HIV p24 antigen at 2µg/ml (Protein Sciences, Meriden, CT), or medium alone and incubated for 48 hours at 37°C with 5% CO₂. Supernatants were collected, stored at -20°C and analyzed for IL-2, IL-4, IL-10, IL-13, TNF-α, and IFN-γ using the Luminex® 200 system (Austin, TX, USA) (8). The dynamic range of this multiplex assay is from 0.2 pg/ml to 32,000 pg/ml. Only 6 HIV-1 infected subjects with CL, 9 HIV-1 subjects and 5 subjects with CL had supernatant analyzed for IL-13. Plasma samples were analyzed using the human Th1/Th2 cytokine kit II, BD® cytometric bead array (San Jose, CA, USA) (6). The limit of detection for this assay ranged from 2.6 pg/ml for IL-4, 2.8 pg/ml for IL-10 and TNF-α, and 7.1 pg/ml for IFN-γ. IFN-γ/IL-10, IFN-γ/IL-13 and TNF-α/IL-10 ratios were calculated using measured values. Patients who had undetectable levels of cytokines were excluded from the cytokine ratio analysis. Flow cytometry
Statistical analysis was performed using Prism® (GraphPad, San Diego) software. Supernatant cytokine concentrations were normalized by subtracting the amount of cytokine produced in culture with media alone. The Mann-Whitney Test was used for median comparisons.

Epidemiological and clinical characteristics of the patients with CL are shown in Table 1. The median CD4+ T cell count in the HIV group was higher than in the HIV/Leishmania co-infected group (415, range 108 to 1005 cells/mm³ vs. 203, range 78 to 615 cells/mm³, p=0.05). The median plasma viral load was similar between groups [log₁₀ 3.3 (1.9 to 5.8) copies/ml vs. log₁₀ 4.8 (p=0.3].

PBMCs from HIV-1 infected individuals with CL produced less IFN-γ and IL-13 in response to SLA than those from HIV-1 seronegative patients with CL (Figure 1 and Supplementary Table 1). A significant difference in the median IFN-γ/IL-10 ratio between co-infected individuals (0.2, range 0.1 to 0.4) and those with CL alone (4.7, range 0.3 to 152.5) (p=0.004) was also noted (Figure 1). Moreover, a decreased level of IFN-γ and median IFN-γ/IL-10 ratio were also observed in the plasma of co-infected patients, compared to those with CL alone (Supplementary Table 1).

PBMCs from patients with HIV/Leishmania co-infection produced significantly more IFN-γ (median, 49 vs. 5.6 pg/mL, p=0.02) and IL-10 (median, 227 vs. 0.0 pg/mL, p=0.03) than those with HIV-1 infection alone in response to HIV-1 p24 Ag (Figure 2). Except for one outlier, the IFN-γ/IL-10 ratio was similar in the two groups (Figure 2).

HIV-1 potentiates Leishmania infection in humans, and vice versa (30). Most reports of HIV infection unleashing leishmaniasis as an opportunistic infection have been associated with viscerotopic Leishmania strains(1, 13, 23, 24). However, scattered studies have reported more aggressive manifestations of CL, such as relapsing and diffuse cutaneous disease, in HIV infected patients(15, 19, 22, 25, 26). Mucocutaneous disease may also be more severe in the setting of HIV
The balance between inflammatory and regulatory cytokines creates a controlled immune response that promotes parasite killing but not tissue destruction during CL infection (5, 7). In subjects uninfected with HIV, the levels of IFN-γ and IL-10 produced by immune cells during asymptomatic *Leishmania* infections and those with limited cutaneous pathology are balanced. In contrast, in mucocutaneous leishmaniasis, high levels of IFN-γ, TNF-α and low levels of IL-10 are associated with destruction of the mucous membranes in the nose, mouth and throat cavities and surrounding tissues (14).

In contrast to both visceral leishmaniasis and HIV infection where IFN-γ responses are diminished (10, 12, 17), during CL infection levels of IFN-γ measured at the lesion and from circulating PBMCs remain close to normal (21). This study demonstrates that PBMCs from HIV-1 infected CL subjects produce less IFN-γ in response to SLA compared to HIV-1 seronegative CL subjects. While many cytokine responses are blunted in HIV-1 infection, in this study the IL-10 response was not decreased in the co-infected subjects, resulting in a decreased IFN-γ/IL-10 ratio of cytokine production. Although production of the Th2 cytokine IL-13 was significantly lower in the co-infected group, there was a trend for the IFN-γ/IL-13 ratio to also be decreased, mirroring the IFN-γ/IL-10 ratio (Data not shown). Alterations in the Th1/Th2 cytokine ratios may be a mechanism by which more severe cutaneous disease develops in HIV-1/*Leishmania* co-infection.

The impairment in IFN-γ production appears to be specific to *Leishmania* antigens, since despite lower CD4+ T-cell counts and higher median HIV-1 viral loads in the HIV-1/*Leishmania* co-infected subjects, PBMCs from co-infected patients produced more IFN-γ in response to HIV-1 p24 antigen than PBMCs from subjects only infected with HIV-1. Infection with cutaneous strains of *Leishmania* results in potent IFN-γ responses (27), and this may explain the discrepancy. Other
potential explanations include: cross priming of the HIV specific T-cells by *Leishmania* infection, or differences in T-cell functionality depending upon the phase of immune recovery in antiretroviral treated patients (16, 18, 28-30). Due to the cross sectional nature of our study, we are unable to elucidate the mechanism involved here.

In HIV seronegative patients, PBMCs from subjects with mucocutaneous leishmaniasis produce higher levels of TNF-α than subjects with cutaneous disease (5). HIV-infected individuals have higher levels of circulating TNF-α than uninfected subjects, and this may contribute to the more severe mucosal disease seen in co-infection (3). Although we observed a slight trend towards higher levels of TNF-α in response to SLA in the HIV/*Leishmania* co-infected group compared to the subjects with CL alone, the TNF-α/IL-10 ratio was similar in both groups. Although we speculate that an altered TNF-α/IL-10 ratio in co-infected individuals may contribute to the increased dermatopathology seen in these individuals, our cohort did not have enough power to detect this effect.

In summary, low IFN-γ production and the altered ratio of inflammatory and regulatory cytokines creates a milieu that supports replication of the *Leishmania* parasites. These alterations may explain how disseminated cutaneous infection and visceralization of species typically causing only cutaneous disease may occur in the setting of HIV and *Leishmania* co-infection.

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Figure Legends

Figure 1. Graphs demonstrating IFN-γ, TNF-α, IL-4, IL-10 production by PBMCs from HIV/Leishmania co-infected (n=8) and Leishmania mono-infected (n=9) patients; and IL-13 production by PBMCs from HIV/Leishmania co-infected (n=6) and Leishmania mono-infected (n=5) patients. PBMC were stimulated in vitro with soluble Leishmania antigen (SLA) and supernatants were collected after 48 hours. In the last two panels the ratio of IFN-γ/IL-10 and TNF-α/IL-10 cytokine production between the HIV/Leishmania co-infected (n=6) and Leishmania mono-infected (n=8 and 7, respectively) patients is demonstrated. Patients who had undetectable levels of cytokines (HIV/Leish3 and HIV/Leish9) were excluded of the cytokine ratio analysis. Median values are indicated by horizontal bars. Mann-Whitney test was used to compare groups. For the individual cytokines, significant differences were found between groups for IFN-γ and IL-13 (p=0.004 and p=0.03, respectively). A significant difference was also found for the IFN-γ/IL-10 ratio (p=0.004), but not the TNF-α/IL-10 ratio.

Figure 2. Graphs demonstrating IFN-γ, TNF-α, IL-4, and IL-10 production by PBMC from HIV infected (n=21), and HIV/Leishmania co-infected (n=8) patients. PBMC were stimulated in vitro with HIV p24 protein and supernatants were collected after 48 hours. In the last panel the ratio of IFN-γ/IL-10 cytokine production between the HIV/Leishmania co-infected (n=7) and HIV mono-infected (n=7) patients is demonstrated. Medians are indicated by horizontal bars. Mann Whitney test was used to compare groups. For the individual cytokines, significant differences between groups were found for IFN-γ and IL-10 (p=0.02, and p=0.03 respectively). A significant difference was not found between the groups for the IFN-γ/IL-10 ratio (p=0.07).
Table 1. Clinical and epidemiological characteristics of the *Leishmania*/HIV co-infected and *Leishmania* patients

<table>
<thead>
<tr>
<th>Patients ID</th>
<th>Age (yr)</th>
<th>Gender</th>
<th>Clinical status</th>
<th>T CD4+ Lymphocytes (cells/mm³)</th>
<th>Viral Load (log₁₀ copies/ml)</th>
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<tr>
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<td>F</td>
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<tr>
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<tr>
<td>Median</td>
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<td>(78-615)</td>
<td>(1.9-5.4)</td>
</tr>
</tbody>
</table>

Leish24 69 M Cutaneous mucosa active
Leish28 37 M Cutaneous active
Leish31 20 M Cutaneous active
Leish32 22 F Cutaneous active
Leish33 66 M Cutaneous active
Leish35 63 M Cutaneous active
Leish36 27 M Cutaneous mucosal active
Leish37 76 M Cutaneous active
Leish38 38 F Cutaneous mucosal active

Median 38

Range (20-76)

HIV infected group (n=22) CD4+ T cell subset: median 415 cell/mm³ (range 108 to 1005); viral load: median log₁₀ 3.3 copies/ml (range 1.9 to 5.8) Age: median 39 years (24 to 72); 62% of male* Differences were considered significant when *P* was 0.05 (Mann-Whitney U test).