Microbial Translocation Induces an Intense Proinflammatory Response in Patients With Visceral Leishmaniasis and HIV Type 1 Coinfection

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Background. Leishmania infection is a cofactor in the heightened cellular activation observed in patients with American visceral leishmaniasis and human immunodeficiency virus type 1 (HIV) infection, with or without progression to AIDS (AVL/HIV). Thus, the persistence of a high parasite load despite antileishmanial therapy could be responsible for the continued immune stimulation.

Methods. CD8\(^+\) T cells expressing CD38, parasite load, lipopolysaccharide (LPS), soluble CD14, macrophage migration inhibitory factor (MIF), intestinal fatty acid–binding protein (IFABP), and proinflammatory cytokines (interleukin 1\(\beta\), interleukin 6, interleukin 8, interleukin 17, interferon \(\gamma\), and tumor necrosis factor) were measured in 17 patients with AVL/HIV, 16 with HIV, and 14 healthy subjects (HS).

Results. Lower Leishmania parasitemia was observed after antileishmanial and antiretroviral therapies. However, higher levels of CD38\(^+\) on CD8\(^+\) T cells were observed in both clinical phases of leishmaniasis, compared with HIV cases. AVL/HIV and HIV patients showed higher levels of LPS and IFABP than HS. Proinflammatory cytokine levels were significantly augmented in patients with active coinfection, as well as those with remission of Leishmania infection. LPS levels and Leishmania infection were positively correlated with CD38 expression on CD8\(^+\) T cells and with IL-6 and IL-8 levels.

Conclusions. LPS levels along with the immune consequences of Leishmania infection were associated with elevated cellular activation in coinfected patients. As a consequence, secondary chemoprophylaxis for leishmaniasis or even the use of antiinflammatory drugs or antibiotics may be considered for improving the prognosis of AVL/HIV.

Keywords. visceral leishmaniasis–HIV/AIDS coinfection; microbial translocation; inflammatory cytokines.
enhances the risk of developing AVL in endemic areas and vice versa [1, 3].

The impairment of the immune system caused by HIV and the depletion of specific lymphocyte subsets compromise the immune mechanisms that are involved in parasite control in AVL/HIV patients [4, 5], leading to increased parasite load, drug resistance, and frequent relapses [1, 6, 7]. Similarly, *Leishmania* infection can contribute to faster progression to AIDS by either increasing the plasma viral load [8, 9] or enhancing immune activation through stimulation by parasite antigens [10, 11]. The reduction of CD4+ T lymphocytes counts due to AVL [12, 13] further contributes to the lymphocyte depletion that already occurs in HIV infection [14]. Importantly, low CD4+ T-cell counts (<200 cells/mm³) were maintained during the clinical remission of patients with AVL/HIV, with or without AIDS despite antiretroviral therapy (ART) and satisfactory antileishmanial therapy [15].

Chronic immune activation is a stronger predictor of HIV/AIDS progression than plasma viral load [16, 17]. The cell activation enhances the transcription of the integrated virus, which then infects new targets, increases T-cell proliferation, and results in cell death induced by the activation of both CD4+ and CD8+ T-cell memory populations, resulting in an exhaustion of immune resources [18, 19]. High levels of plasmatic proinflammatory cytokines can also contribute to this activated status [18]. Antigenic stimulation as a consequence of other infections, such as tuberculosis [20], cytomegalovirus infection [21], and hepatitis B and C [22, 23], constitutes another cause of HIV-associated immune activation. Recently, our group reported high levels of cellular activation and low CD4+ T-cell counts in AVL/HIV patients in remission despite anti-Leishmania therapy and undetectable or low viral loads. Leishmania infection was thought to be the cofactor responsible for the enhanced cellular activation observed in patients coinfected with HIV [11], especially those with the visceral form of leishmaniasis. This finding suggests the inefficient control of the parasite after antileishmanial therapy, which may justify the use of secondary prophylaxis [7].

Another potential mechanism associated with chronic immune activation is mediated by gut microbial products [24]. The massive depletion of memory CD4+ T cells in mucosal lymphoid tissue during acute HIV infection results in disruption of the anatomofunctional gastrointestinal barrier, enabling the translocation of luminal microbiota into the circulation [25, 26]. Microbial translocation has already been observed in other conditions not related to infectious diseases, such as inflammatory bowel disease [27], graft versus host disease [28], and idiopathic CD4 lymphocytopenia [29]. Mucosal invasion by leishmanial amastigotes and the systemic lymphocyte depletion observed in AVL patients [12, 30, 31] could constitute the immunopathogenic basis for the lipopolysaccharide (LPS)–related activation of lymphocytes and release of proinflammatory cytokines [32]. In this scenario, an overlap in cellular activation due to infections with both HIV and *Leishmania* organisms would enhance the proinflammatory state, thereby worsening the effector response and the subsequent clinical outcome of coinfected patients [32–34].

The mechanism responsible for the maintenance of immune activation during clinical remission is not clear. Considering that *Leishmania* infection has been suggested to be the cofactor responsible for the heightened cellular activation independent of CD4+ T-cell numbers and viral load [11], we hypothesized that the persistence of a high parasitic load even after antileishmanial therapy could be responsible for the continued immune stimulation.

**MATERIALS AND METHODS**

**Study Population**

This cross-sectional study included patients with AVL/HIV; 12 had active disease without previous antileishmanial therapy, and 9 were in the remission phase for at least 6 months after the end of antileishmanial treatment and had no signs or symptoms of active leishmaniasis. Four patients were evaluated in the 2 phases of the disease. The diagnosis of AVL was confirmed by the visualization of amastigotes in Giemsa-stained bone marrow smears. Ten patients with AVL and without HIV infection, who were studied elsewhere [32], were used as controls. All laboratory parameters measured in this study were compared with those of these patients and showed significant differences (Supplementary Table).

Sixteen HIV/AIDS patients without previous leishmaniasis were enrolled in the study and paired with coinfected patients on the basis of viral load. All AVL/HIV and HIV patients received antiretroviral therapy according to Brazilian guidelines for at least 1 year [35, 36]. Fourteen volunteers without leishmaniasis or HIV/AIDS were included as healthy subjects (HS).

**Ethics Statement**

Written informed consent was obtained from all participants. The study was approved by the Fundação Oswaldo Cruz and the IPEC ethical committees.

**Immunologic and Virologic Assessments**

To determine the absolute T-lymphocytes counts, monoclonal antibodies specific for CD4, CD8, and CD3 conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), and PerCP, respectively, were used with a BD True Count reagent kit (BD Biosciences, Franklin Lakes, NJ). Samples were acquired using a FACSCalibur and analyzed with Multiset software (BD). The results were expressed as the number of cells per cubic millimeter.
Plasma HIV RNA levels were quantified using a branched DNA assay (Siemens, Versant HIV RNA 3.0, Tarrytown, NY). The lower limit of detection for this assay was 50 copies/mL.

**Isolation of Peripheral Blood Mononuclear Cells (PBMCs) and Lymphocyte Activation Analysis**

PBMCs were obtained as described elsewhere [11] and labeled with anti-CD8 FITC and anti-CD38 PE monoclonal antibodies (BD Simultest, BD Biosciences, San Jose, CA). Fixed cells were acquired (through at least 10,000 events) on a FACSCalibur and analyzed with CellQuest software (BD Biosciences). The analysis region was established by first gating on the CD3+ T lymphocytes. The results were expressed as the percentage of CD38-positive cells among the total CD8\textsuperscript{high} T cells.

**Quantification of Lipopolysaccharide (LPS), Soluble CD14 (sCD14), and Intestinal Fatty Acid–Binding Protein (IFABP)**

**Plasma Levels**

Plasma samples were stored at −70°C until analysis. The samples were diluted in endotoxin-free water, and LPS levels were quantified using a commercial assay kit (Limulus amebocyte lysate QCL-1000; Cambrex, Milan, Italy). The results were expressed as picograms per milliliter, and the minimum detection level was 10 pg/mL. sCD14 levels were determined by ELISA (Duo Set; R&D Systems, Minneapolis, MN); the results were expressed as picograms per milliliter, and the sensitivity level was 10 pg/mL. sCD14 levels were measured by enzyme-linked immunosorbent assay (ELISA; sCD14 Quantikine; R&D Systems), and the results were expressed as nanograms per milliliter, and the minimum detection limit was 31.2 pg/mL.

**Cytokine Measurement**

A multiplex biometric immunoassay containing fluorescence-labeled microbeads was used for plasma cytokine measurements (Bio-Rad Laboratories, Hercules, CA). The following cytokines were quantified: interferon γ (IFN-γ), tumor necrosis factor (TNF), interleukin 1β (IL-1β), interleukin 6 (IL-6), interleukin 8 (IL-8), interleukin 17 (IL-17), and macrophage inflammatory protein 1β (MIP-1β). Cytokine levels were calculated with Luminex technology (Bio-Plex Workstation; Bio-Rad Laboratories). Data analysis was performed using the software provided by the manufacturer (Bio-Rad Laboratories). A range of 0.51–8000 pg/mL of the recombinant cytokines was used to establish the standard curves and sensitivity of the assay.

Macrophage migration inhibitory factor (MIF) levels were measured by ELISA (Duo Set; R&D Systems), and the results were expressed as picograms per milliliter; the minimum detection level was 31.2 pg/mL.

**Quantification of Leishmania Organisms in Blood Samples by Quantitative Polymerase Chain Reaction (PCR)**

DNA extraction from blood (200 µL) was performed using a DNeasy blood and tissue kit and a spin-column protocol (Qiagen, Valencia, CA) according to the manufacturer’s instructions. DNA concentrations and enrichment relative to protein in all of the samples were determined at 260/280 nm with a spectrophotometer (NanoDrop 2000, Thermo Scientific, Wilmington, DE), and DNA was stored at −70°C until use.

*Leishmania* load was estimated by using a quantitative PCR assay in accordance with previously published protocol [37]. We used major surface protease-associated gene (MAG-1) and kinetoplast DNA (kDNA) as the molecular targets. MAG-1 is specific since it was earlier designed against *mag* gene sequences only known to be present in *Leishmania infantum* [38]. Briefly, specific primers based on MAG-1 consisted of 3.75 pmol of forward primer (AGACGCTGCTTTGGATTGTG), 3.75 pmol of reverse primer (CGCTGGTGTGATTGGTGTG), and 2.5 pmol of TaqMan probe (FAM-GCCACTGACT GTGCCCCCC-TAMRA). Primers and probes based on kDNA were −AATGGGTTCGAAAT CCCGITT(C 3.75 pmol), CCA CACCCCGCCCCTATTCTAC (3.75 pmol), and FAM-CCCC AGTTCCCCGCCCCGA-TAMRA (2.5 pmol).

Absolute quantification of *Leishmania* organisms was determined by comparison with a standard curve constructed using a 10-fold serially diluted sample of *L. infantum* DNA, with 10⁶–10⁻⁷ parasites per reaction tube. The isolate was used from a patient with visceral leishmaniasis from Natal-RN/Brazil and was typed as *L. infantum* (IOC 563). The assay was performed in a 10-µL final volume containing 20 ng of DNA.

Amplification and detection were performed using an ABI Prism 7500 sequence detection system (Applied Biosystems, Carlsbad, CA). Standards, samples, and negative controls were analyzed in triplicate for each run. The cycling parameters were 50°C for 2 minutes, 95°C for 10 minutes, and 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The results were expressed as parasitic forms (PF) per milliliter of blood. The sensitivity of the real-time PCR for MAG-1 was 0.1 parasite/mL.

**Statistical Analysis**

The Mann–Whitney *U* test, Spearman test, and Kruskal–Wallis test were performed using GraphPad Prism software, version 5.0 (GraphPad Prism Inc., San Diego, CA). The values were expressed as medians and interquartile ranges. A multivariate statistical analysis was performed through multiple linear regression (SPSS software, version 9.0) to determine the influence of intervening variables on the percentage of CD38-positive cells among the total population of CD8\textsuperscript{high} T-cells (dependent variable). Absolute CD4\textsuperscript{+} T-cell counts, absolute viral load (presented as copies/milliliter), leishmaniasis (defined as present or absent), and LPS, sCD14, IFABP, and MIF levels were considered as independent variables. The influence of intervening factors, such as LPS level, sCD14 level, viral load, and presence or absence of leishmaniasis, on the levels of proinflammatory cytokines (see dependent variables, described above) was also assessed.
RESULTS

During active disease, the percentage of CD38-positive cells among CD8\(^+\) T-lymphocytes was already elevated in AVL/HIV patients (82.5% [75.7%–90.1%]). In addition, this percentage was as high as that for patients in the remission phase of leishmaniasis (93.5% [87%–97.9%]; Figure 1A). The cellular activation status was higher in the AVL/HIV group, compared with the HIV-AIDS group (55.8% [49%–64%]). As expected, the lowest levels of cellular activation were found in HS (30.7% [28.6%–34%]).

The next step was to investigate whether the elevated parasite load was responsible for the maintenance of immune stimulation. AVL/HIV patients had a much higher parasite load, as determined by MAG-1 quantification, during active disease (53 356 PF/mL [34 913–108 786 PF/mL]), compared with coinfected patients in the remission phase (1.2 PF/mL [1–22 640 PF/mL]; \(P < .05\); Figure 1B). These results indicate that antileishmanial therapy was successful in reducing the number of circulating parasites. As expected, during active disease, the parasite levels in the coinfected patients were higher than those in the AVL-only patients (active, 2856 PF/mL [1440–15 350 PF/mL]). The parasite load was positively correlated with the percentage of CD38-positive cells among CD8\(^+\) T cells in the active AVL/HIV patients (\(r = 0.60\); \(P < .05\); Figure 1C). Surprisingly, no correlation was observed in the remission phase, as
high levels of activation were observed despite the lower parasite load (Figure 1D). Parasite load was also quantified with primers targeting kDNA minicircles [37], and the results were similar to those for MAG-1. Active AVL/HIV patients showed the highest kDNA copy numbers, whereas for patients in the remission phase, they were significantly lower (active, 132 000 PF/mL [25 000–288 000 PF/mL]; remission, 178 PF/mL [62–18 000 PF/mL]). Thus, we suggest that having leishmaniasis with its associated pathologic damage is a background factor for the observed enhanced cellular activation and that such activation may not be directly associated with the presence of the parasite itself.

Because the majority of AVL/HIV patients had undetectable or low viral loads and reduced parasitemia (Supplementary Table) after therapy, we further investigated whether LPS could be influencing T-cell activation [24, 32]. Patients with active AVL/HIV had much higher median levels of LPS (52 pg/mL [49.5–57.7 pg/mL] than HS (26 pg/mL [14–29 pg/mL]; P < .001). However, the levels were similar to those observed in HIV patients (50.6 pg/mL [45.7–53 pg/mL]; Figure 2A).

Enhanced circulating levels of sCD14 were observed in active AVL/HIV patients, compared with HIV patients. The difference was more pronounced when coinfected patients were compared with HS (Figure 2B and Supplementary Table). Positive correlation was detected between LPS and sCD14 levels (r = 0.40; P < .05; Figure 2C). Interestingly, LPS and sCD14 levels were still elevated during the remission phase, and the levels were similar to those observed in active AVL/HIV patients (Figure 2A and 2B).

Considering that gut damage has been implicated in the increased intestinal permeability and microbial translocation into the circulation [25, 26, 39, 40], we evaluated whether the Leishmania–HIV association could lead to enhanced enterocyte damage. AVL/HIV showed augmented IFABP levels, but these levels were not different from those of HIV patients (Figure 2D).

All of the groups presented significantly higher IFABP levels than HS (P < .001). On examination of all patients infected with HIV, a positive correlation was verified between IFABP and LPS levels (r = 0.40; P < .05), suggesting the occurrence of intestinal damage (Figure 2E). To some extent, these results indicate that tissue damage is an indirect key factor in the ongoing T-cell activation observed in patients with AVL/HIV, even after receipt of antileishmanial therapy.

MIF is a proinflammatory cytokine that is released in response to many stimuli, including endotoxemia (such as due to high LPS levels) [49]. In comparison with HS, AVL/HIV (active and remission phase), and HIV-AIDS patients showed significantly higher levels of MIF (P < .001), although no significant difference was found between coinfected patients and HIV patients (Figure 2F).

To determine which factors were associated with the high activation levels observed in the coinfected patients, a multivariate statistical analysis was performed. The model showed that the presence of Leishmania infection influenced T-cell activation (P < .001). Additionally, we observed a significant positive correlation between LPS and CD38 on CD8+ T-lymphocytes, after adjustment for CD4+ T-cell count, HIV viremia, and sCD14, IFABP, and MIF levels (P < .001; Table 1). Leishmania load was not included as a variable in the model because it was not present in all 3 patient groups evaluated (Table 1).

Similar to T-cell activation, the plasma levels of proinflammatory cytokines were also highly elevated in coinfected patients, compared with those in HIV patients and HS (Table 2). Coinfected patients showed significantly higher median serum levels of all of the inflammatory cytokines tested, compared with levels in HIV patients or AVL-only patients (Table 2).

The factors underlying this cytokine storm in coinfected patients were also analyzed (Table 3). For such an analysis, dependent variables (cytokines) and independent variables (viral load, LPS level, sCD14 level, and Leishmania infection) were considered. LPS levels were positively correlated with IL-6 and IL-8 levels (Table 3). Soluble CD14 levels were positively correlated with IL-8 level and negatively correlated with MIP-1β level. Moreover, Leishmania infection was positively correlated with levels of TNF, IL-6, IL-8, IL-17, and MIP-1β. Taken together, these data suggest that an activated and inflammatory state can indeed be observed in AVL/HIV patients.

**DISCUSSION**

A previous study demonstrated that AVL/HIV patients have enhanced cellular activation levels, even during ART and after antileishmanial treatment. Leishmania infection was the cofactor associated with the percentage of CD38 expression among CD8+ T-lymphocytes, independent of CD4+ T-cell counts and viral load [11]. In the present study, we first investigated whether the persistence of Leishmania parasites after therapy could explain the activated phenotype observed in treated coinfected patients.

We found that both active AVL/HIV patients and those in remission had a high percentage of CD38 expression among CD8+ T lymphocytes, indicating that coinfected patients have an increased cellular activation status independent of the clinical phase of leishmaniasis, ART use, or even receipt of antileishmanial treatment. Interestingly, the majority of AVL/HIV patients in remission showed lower parasite loads than patients in the active phase of AVL. Blood parasite clearance in treated coinfected patients appeared to be as effective as that in treated AVL-only patients, as similar median numbers of estimated promastigote forms were detected between the 2 groups of patients (MAG-1 copy numbers were undetectable in 5 AVL/HIV only patients and 3 AVL only patients). On first glance, this...
result suggests that antileishmanial therapy was successful in reducing the parasite load, despite HIV infection and poor immune reconstitution (Supplementary Table). However, the patients in remission did not completely clear *Leishmania* kDNA from the blood, with kDNA remaining detectable even after 6 months of antileishmanial treatment. This result is in

**Figure 2.** Microbial translocation and gut damage in patients with American visceral leishmaniasis and human immunodeficiency virus type 1 infection, with or without AIDS (AVL/HIV). A and B, Plasma lipopolysaccharide (LPS) levels (A) and soluble CD14 (sCD14) levels (B) in AVL/HIV patients during active disease and remission, in HIV patients, and in healthy subjects. C, Correlation between plasma LPS and sCD14 levels in AVL/HIV and HIV patients. D, Plasma levels of intestinal fatty acid–binding protein (IFABP). E, Correlation between plasma LPS and IFABP levels in AVL/HIV and HIV patients. F, Plasma levels of macrophage migration inhibitory factor (MIF). Each point represents 1 subject. The horizontal bars indicate the median value. In panels A, B, D, and F, the Wilcoxon matched-pairs *t* test was used for 4 individuals evaluated before and after antileishmanial therapy. Data for each of these patients are identified by a different color.
Virus Type 1 (HIV) and Those With HIV Infection Only

level, IFABP level, and Leishmania (Leishmania) infantum reactivation [6, 7, 41]. Herein, although parasite antigens cannot be ruled out as an important factor driving cellular activation in AVL/HIV patients, we did not observe a positive correlation between parasite load and the percentage of CD38 expression among CD8+ T-cells in coinfected patients in remission. Such a correlation was only observed for active AVL/HIV patients. In addition to leishmanial antigens [13], LPS has been implicated in T-cell activation in visceral leishmaniasis patients [32]. Considering that LPS also plays an important role in HIV pathogenesis [24], we expected that this microbial product would be a relevant factor in maintaining the activation status in coinfected patients. Increased LPS levels were found in both active AVL/HIV and Leishmania AVL-infected patients, thus reinforcing the notion that LPS originates in the lumen, as IFABP is released after enterocyte damage [23, 42]. However, LPS levels were similar in concert with similar studies in a cohort of treated coinfected patients [10].

Table 2. Plasma Proinflammatory Cytokine Levels in the Study Population

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Active AVL/HIV (n = 5)</th>
<th>Remission AVL/HIV (n = 7)</th>
<th>HIV Infected</th>
<th>Active AVL (n = 5)</th>
<th>Remission AVL (n = 7)</th>
<th>Healthy Subjects (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ</td>
<td>2795 (1344–7104)</td>
<td>2411 (1415-6157)</td>
<td>150 (66-332)</td>
<td>1143 (445–4446)</td>
<td>783 (667–3482)</td>
<td>19 (5.3–145)</td>
</tr>
<tr>
<td>TNF</td>
<td>490 (284–1503)</td>
<td>374 (302–1207)</td>
<td>165 (66-200)</td>
<td>143.5 (37.7–555)</td>
<td>100 (57.3–588.5)</td>
<td>2 (1.5–8.5)</td>
</tr>
<tr>
<td>IL-6</td>
<td>536 (61-6128)</td>
<td>947 (276–9068)</td>
<td>81 (45–110)</td>
<td>416 (211–557)</td>
<td>40 (30–119)</td>
<td>1 (0.3–2.5)</td>
</tr>
<tr>
<td>IL-8</td>
<td>2447 (1050–10 844)</td>
<td>3244 (2439–17 999)</td>
<td>170 (130–220)</td>
<td>8000 (1887–13578)</td>
<td>4325 (427–8988)</td>
<td>2.5 (1.5–3.0)</td>
</tr>
<tr>
<td>IL-1β</td>
<td>16 (3.5–209)</td>
<td>112.5 (17.1–351)</td>
<td>15 (10–20)</td>
<td>9.7 (4–226)</td>
<td>7.5 (2–138)</td>
<td>0.5 (0.4–6.2)</td>
</tr>
<tr>
<td>IL-17</td>
<td>231 (118–417)</td>
<td>256 (161–500)</td>
<td>65.2 (46–90)</td>
<td>288 (102–398)</td>
<td>193.5 (131–626.6)</td>
<td>2 (2–16)</td>
</tr>
<tr>
<td>MIP-1β</td>
<td>2157 (430–3928)</td>
<td>546 (441–923)</td>
<td>243 (165–318)</td>
<td>730.3 (418–3363)</td>
<td>571.5 (426–1111)</td>
<td>14.3 (0.1–50.2)</td>
</tr>
</tbody>
</table>

Data are median pg/mL (interquartile range). Abbreviations: AVL, American visceral leishmaniasis; AVL/HIV, American visceral leishmaniasis/human immunodeficiency virus type 1 coinfection; IL-1β, interleukin 1β; IL-6, interleukin 6; IL-8, interleukin 8; IL-17, interleukin 17; MIP-1β, macrophage inflammatory protein-1β; TNF, tumor necrosis factor.

a Active AVL/HIV vs HIV-infected patients.
b Remission AVL/HIV vs HIV-infected patients.
c AVL/HIV vs AVL-only patients during the active phase.
d AVL/HIV vs AVL-only patients during the remission phase.
of translocation or even the rate of LPS clearance could not be augmented any more.

After adjustment for confounding variables, LPS levels and *Leishmania* infection were the factors significantly associated with T-cell activation. In contrast, parasite load was not correlated with the high percentage of CD38 expression among CD8+ T-lymphocytes in a bivariate analysis, most likely because the parasite burden was lower after therapy in comparison with burden in patients with active disease. No association was detected even when coinfected patients were matched by viral load in a multivariate analysis (data not shown). Thus, we reasoned that the variable “*Leishmania* infection” discriminated the presence or absence of the disease (visceral leishmaniasis) together with its immunopathologic disorders.

Macrophages/monocytes and dendritic cells, in addition to being the host cells harboring *Leishmania* amastigotes, are also potent producers of proinflammatory cytokines upon Toll-like receptor 4 activation by LPS [43]. In our study, we demonstrated an enhanced inflammatory cytokine milieu in AVL/HIV patients that was independent of the clinical phase of leishmaniasis, as serum levels were still elevated after antileishmanial treatment. In addition, these levels were much higher than those observed in HIV [44] or AVL-only patients [32–34]. The data suggest that although LPS levels had reached a plateau, the immunostimulatory functions of LPS-induced cytokines or even the remaining leishmanial antigens may contribute to the complex pathologic environment found in these patients. In accordance with our results, a previous study also described higher levels of TNF and IFN-γ, which remained elevated 5 months after antileishmanial treatment and ART use [45]. Thus, as proposed for HIV-positive patients [46], the augmented TNF levels observed in our coinfected patients could contribute to microbial translocation by disrupting mucosal barrier integrity. Moreover, the presence of *Leishmania* organisms in the mucosal gut [47, 48] and the frequent diarrheal episodes in AVL/HIV patients can be key factors in aggravating intestinal permeability. Our results indicate that the cytokine network induced by the *Leishmania*/HIV association differs from monoinfections and suggest an in vivo synergistic effect in coinfected patients.

An analysis of the factors that could be associated with this cytokine storm revealed that LPS was positively correlated with levels of IL-6 and IL-8, which were most likely produced by LPS-stimulated innate cells. In coinfected patients, *Leishmania* infection was also positively correlated with not only IL-6 and IL-8 levels but also with TNF, IL-17, and MIP-1β levels. Thus, AVL/HIV coinfection results in much higher levels of plasma inflammatory cytokines, which in turn can contribute to the vicious cycle of persistent immune activation [18, 19, 40].

In summary, the results suggest that LPS levels, along with the immune consequences of leishmaniasis (“*Leishmania* infection” variable), are associated with high levels of cellular activation in coinfected patients. However, these cofactors appear to contribute to cellular activation by enhancing the plasma cytokine storm, not via the parasite burden, as low levels of *Leishmania* DNA were observed after specific treatment and ART use.

Taken together, we speculate that the translocation of microbial products may have more severe consequences in AVL/HIV patients, resulting in enhanced inflammatory cytokines status, which may promote and refuel cellular activation. The consequences of persistent T-cell activation on immune reconstitution, culminating in the exhaustion of immune resources in coinfected patients, need to be understood. Finally, it raises the issue that the therapeutic strategies currently used could not be sufficient to address all the pathogenic consequences of *Leishmania*-HIV coinfection. Our results may offer a rational basis for the introduction of secondary chemoprophylaxis for leishmaniasis or even for the use of antiinflammatory drugs or antibiotics against gram-negative bacteria.

### 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

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#### Table 3. Multivariate Analysis of the Association Between Proinflammatory Cytokine Levels and Viral Load, Lipopolysaccharide (LPS) Level, Soluble CD14 (sCD14) Level, and Leishmania Infection Among Patients Coinfected With *Leishmania* (*Leishmania*) *infantum* and Human Immunodeficiency Virus Type 1 (HIV) and Those With HIV Infection Only

<table>
<thead>
<tr>
<th>Variable</th>
<th>LPS Level, P</th>
<th>Viral Load, P</th>
<th>sCD14 Level, P</th>
<th>Leishmania Infection, P</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF level</td>
<td>.296</td>
<td>.709</td>
<td>.980</td>
<td>.003</td>
</tr>
<tr>
<td>IL-1β level</td>
<td>.685</td>
<td>.766</td>
<td>.979</td>
<td>.122</td>
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<tr>
<td>IL-6 level</td>
<td>.018</td>
<td>.849</td>
<td>.527</td>
<td>.005</td>
</tr>
<tr>
<td>IL-8 level</td>
<td>.008</td>
<td>.968</td>
<td>.019</td>
<td>.001</td>
</tr>
<tr>
<td>IL-17 level</td>
<td>.939</td>
<td>.856</td>
<td>.246</td>
<td>.001</td>
</tr>
<tr>
<td>MIP-1β level</td>
<td>.968</td>
<td>.563</td>
<td>.011</td>
<td>.001</td>
</tr>
<tr>
<td>MIF level</td>
<td>.141</td>
<td>.399</td>
<td>.877</td>
<td>.969</td>
</tr>
</tbody>
</table>

*Abbreviations: IL-1β, interleukin 1β; IL-6, interleukin 6; IL-8, interleukin 8; IL-17, interleukin 17; LPS, lipopolysaccharide; MIF, macrophage migration inhibitory factor; MIP-1β, macrophage inflammatory protein 1β; TNF, tumor necrosis factor.*
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