MAJOR ARTICLE

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

Joanna R. Santos-Oliveira,[1] Eduardo G. Regis,[2] Carmem B. W. Giacoia-Gripp,[3] Joanna G. Valverde,[6] Priscilla Alexandrino-de-Oliveira,[7] Jose Ângelo L. Lindoso,[8] Hiro Goto,[8] Manoel P. Oliveira-Neto,[4] Jorge O. Guerra,[9] Beatriz Grinsztejn,[4] Selma B. Jerônimo,[6] Mariza G. Morgado,[3] and Alda M. Da-Cruz[1,5]

[1]Laboratório Interdisciplinar de Pesquisas Médicas, [2]Laboratório de Pesquisa sobre o Timo, and [3]Laboratório de Aids e Imunologia Molecular, Instituto Oswaldo Cruz–FIOCRUZ, [4]Instituto de Pesquisa Clínica Evandro Chagas, IPEC-FIOCRUZ, and [5]Disciplina de Parasitologia, Faculdade de Ciências Médicas, Universidade do Estado do Rio de Janeiro; [6]Departamento de Bioquímica, Universidade Federal do Rio Grande do Norte, Natal, [7]Hospital-Dia Prof[a] Esterina Corsini, Hospital Universitário, Universidade Federal do Mato Grosso do Sul, Campo Grande, [8]Instituto de Medicina Tropical de São Paulo, Universidade de São Paulo, and [9]Fundação de Medicina Tropical Dr. Heitor Vieira Dourado, Manaus, Brazil

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 1β, interleukin 6, interleukin 8, interleukin 17, interferon γ, 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.

Coinfection with *Leishmania* organisms and human immunodeficiency virus type 1 (HIV) has been found with increasing frequency in several regions worldwide, mainly because of increasing geographical overlap of these 2 diseases. The first reported cases of visceral leishmaniasis–associated HIV infection were from the Mediterranean basin [1]. Brazil accounts for most of the cases in the Americas, and approximately 1.5%–2% of confirmed American visceral leishmaniasis (AVL) cases reported during 2001–2008 involved HIV coinfection [2, 3], thereby confirming that HIV infection

Received 19 November 2012; accepted 9 January 2013; electronically published 28 March 2013.

Presented in part: XIX International AIDS Conference 2012. Washington, DC, USA, 22-27 July. Oral Poster Discussion and Poster (THPDA0102).

Correspondence: Alda Maria Da-Cruz, MD, PhD, Laboratório Interdisciplinar de Pesquisas Médicas, Instituto Oswaldo Cruz, FIOCRUZ, Av. Brasil 4365, Manguinhos, Rio de Janeiro-RJ, Brazil (alda@ioc.fiocruz.br).

The Journal of Infectious Diseases 2013;208:57–66

DOI: 10.1093/infdis/jit135

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/mm^3) 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 anatomo-functional 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 BDTrue 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 $CD8^{high}$ 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 sensitivity level was 10 pg/mL. sCD14 levels were measured by enzyme-linked immunosorbent assay (ELISA; sCD14 Quantikine; R&D Systems, Minneapolis, MN); the results were expressed as nanograms per milliliter, and the minimum detection limit was 125 pg/mL. IFABP levels were determined by ELISA (Duo Set; R&D Systems). The results were expressed as picograms 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 (AGAGCGTGCCTTGGATTGTG), 3.75 pmol of reverse primer (CGCTGCGTTGATTGCGTTG), and 2.5 pmol of TaqMan probe (FAMT-GCGCACTGCACT GTCGCCCCC-TAMRA). Primers and probes based on kDNA were –AATGGGTGCAGAAAT CCCGTTC (3.75 pmol), CCA CCACCCGGCCCTATTTTAC (3.75 pmol), and FAM-CCCC AGTTTCCCGCCCCGGA-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 10^6–10^{-3} parasites per reaction tube. The isolate used was 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 $CD8^{high}$ T-cells (dependent variable). Absolute $CD4^+$ 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.

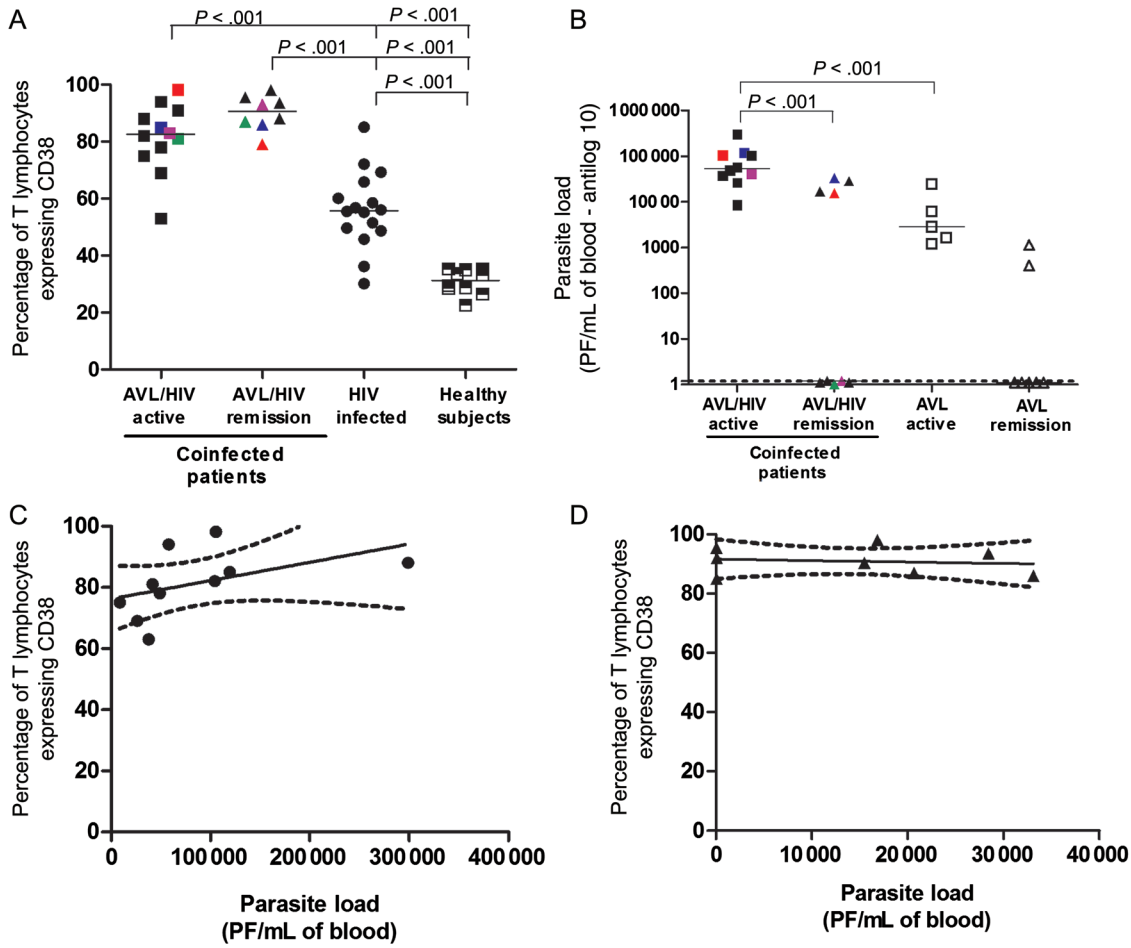

Figure 1. Cellular activation levels and blood quantification of *Leishmania infantum* DNA in patients with American visceral leishmaniasis and human immunodeficiency virus type 1 infection, with or without AIDS (AVL/HIV). *A*, Percentage of $CD38^+$ cells among $CD8^+$ T lymphocytes in AVL/HIV patients during the active phase of AVL (solid squares) and the remission phase of AVL (solid triangles), in HIV patients (solid circles), and in healthy subjects (stained squares). 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. *B*, Real-time polymerase chain reaction (PCR) analysis of the parasite load was performed on blood samples collected from AVL/HIV patients during active disease (solid squares) and during remission (solid triangles). Patients with AVL only were included as controls for both clinical phases of leishmaniasis (active, open squares; remission, open triangles). The results are expressed as the number of parasitic forms (PF) per milliliter of blood. *C*, Correlation between the parasite load and the percentage of $CD38^+$ cells among $CD8^+$ T cells in active AVL/HIV patients. *D*, Correlation between the parasite load and the percentage of $CD38^+$ cells among $CD8^+$ T cells in AVL/HIV patients in remission. Each point represents 1 subject. Horizontal bars indicate the median value. Dashed lines represent the sensitivity of the real-time PCR assay.

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 1*A*). 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 1*B*). 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 $CD38^+$ cells among $CD8^+$ T cells in the active AVL/HIV patients ($r = 0.60$; $P < .05$; Figure 1*C*). Surprisingly, no correlation was observed in the remission phase, as

high levels of activation were observed despite the lower parasite load (Figure 1*D*). 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 2*A*).

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 2*B* and Supplementary Table). Positive correlation was detected between LPS and sCD14 levels ($r = 0.40$; $P < .05$; Figure 2*C*). 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 2*A* and 2*B*).

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 2*D*). 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 2*E*). 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 2*F*).

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

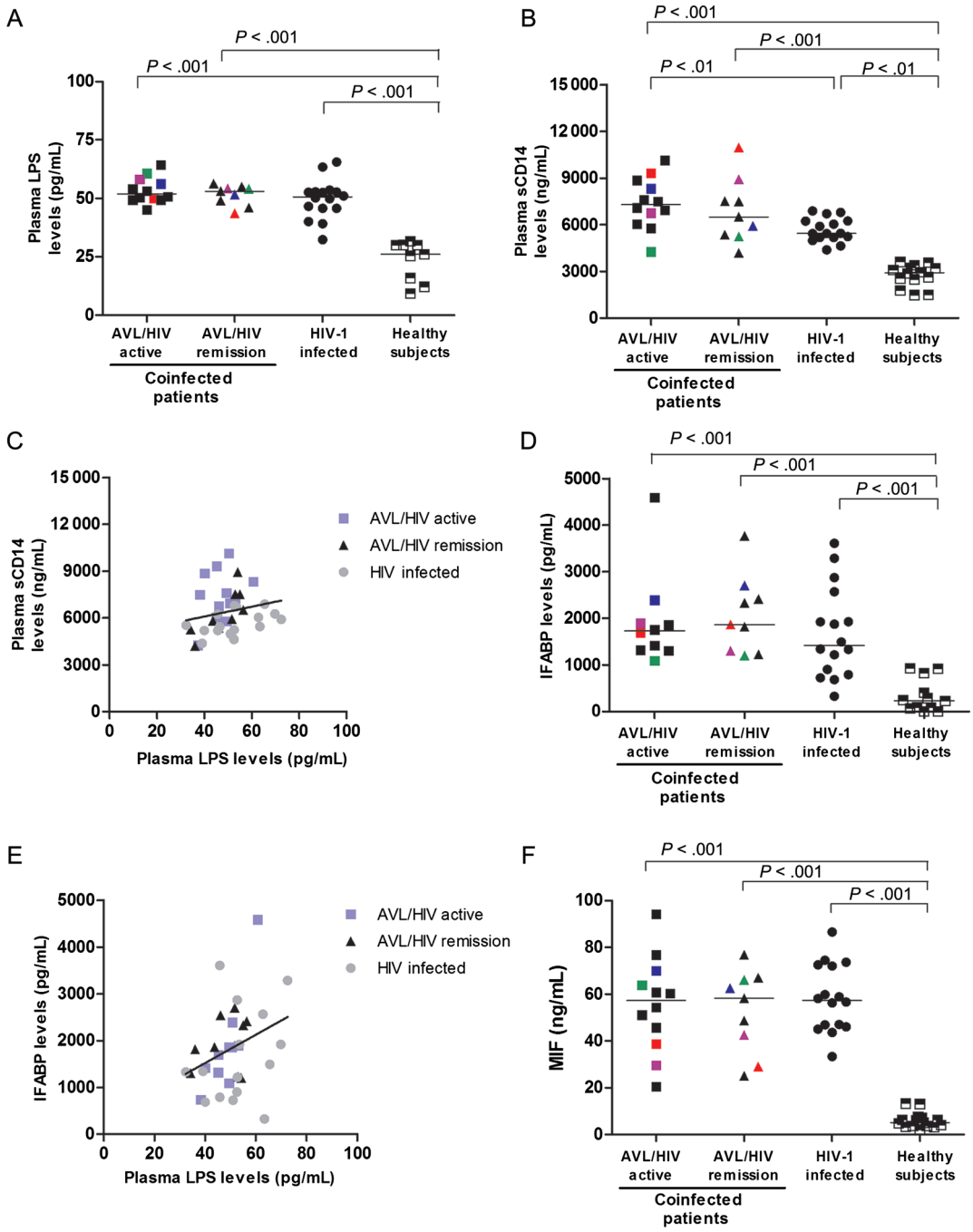

Figure 2. Microbial translocation and gut damage in patients with American visceral leishmaniasis and human immunodeficiency virus type 1 infection, with or without AIDS (AVL/HIV). *A* and *B,* Plasma lipopolysaccharide (LPS) levels (*A*) and soluble CD14 (sCD14) levels (*B*) in AVL/HIV patients during 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.

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

Table 1. Multivariate Analysis of Factors Associated With Cell Activation, Evaluated on the Basis of the Percentage of CD38-Positive Cells Among CD8$^+$ T Lymphocytes, Among Patients Coinfected With *Leishmania (Leishmania) infantum* and Human Immunodeficiency Virus Type 1 (HIV) and Those With HIV Infection Only

	Percentage of CD38$^+$ Cells Among CD8$^+$ T lymphocytes		
Variable	Correlation Coefficient[a]	Standard Error	*P*
CD4$^+$ T-cell count (cells/mm^3)	−0.021	0.014	.875
Viral load (copies/mL)	0.049	0.001	.624
Leishmaniasis (presence or absence)	0.817	5.478	.0001
LPS level (pg/mL)	0.373	0213	.001
sCD14 level (pg/mL)	0.061	0.002	.609
IFABP level (pg/mL)	0.153	0.002	.097
MIF level (ng/mL)	0.124	0.118	.218

Analyses adjusted for CD4$^+$ T-cell count, viral load, LPS level, sCD14 level, MIF level, IFABP level, and *Leishmania infantum* infection. HIV–infected patients were also included in this analysis.

Abbreviations: IFABP, intestinal fatty acid–binding protein; LPS, lipopolysaccharide; sCD14, soluble CD14; MIF, macrophage migration inhibitory factor.

accordance with similar studies in a cohort of treated coinfected patients in the Mediterranean, thus reinforcing the notion that AVL/HIV patients take longer to achieve *Leishmania* DNA negativity after specific therapy [41]. However, despite successful treatment, *Leishmania* organisms may remain in the bone marrow, and this can explain the frequent episodes of disease reactivation [6, 7, 41]. Herein, although parasite antigens cannot be ruled out as an important factor driving cellular activation in patients with AVL/HIV, 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 and treated AVL/HIV patients, which is consistent with their augmented cellular activation status. Additionally, the positive correlation between LPS and IFABP levels supports the hypothesis that LPS originates in the lumen, as IFABP is released after enterocyte damage [23, 42]. However, LPS levels were similar between coinfected and HIV patients, suggesting that HIV infection itself is such a potent inducer of microbial translocation that LPS levels in visceral leishmaniasis patients harboring this virus are already at a threshold. By consequence, coinfected patients may have reached a plateau where the rate

Table 2. Plasma Proinflammatory Cytokine Levels in the Study Population

Cytokine	Active AVL/HIV (n = 5)	Remission AVL/HIV (n = 7)	HIV Infected	Active AVL (n = 5)	Remission AVL (n = 7)	Healthy Subjects (n = 8)	*P*[a]	*P*[b]	*P*[c]	*P*[d]
IFN-γ	2795 (1344–7104)	2411 (1415–6157)	150 (56–332)	1143 (445–4446)	783 (367–3482)	19 (5.3–145)	.0001	.0001	.126	.05
TNF	490 (284–1503)	374 (302–1207)	165 (86–200)	143.5 (37.7–555)	100 (57.3–588.5)	2 (1.5–8.5)	.0001	.001	.05	.07
IL-6	536 (61–6128)	947 (276–9068)	81 (45–110)	416 (211–557)	49 (30–115)	1 (0.3–2.5)	.0001	.006	.47	.04
IL-8	2447 (1050–10 844)	3244 (2439–17 999)	170 (130–220)	8000 (1887–13 578)	4325 (427–8988)	2.5 (1.5–3.0)	.01	.006	.536	.445
IL-1β	16 (3.5–209)	112.5 (17.1–351)	15 (10–20)	9.7 (4–226)	7.5 (2–138)	0.5 (0.4–0.62)	.01	.0001	.958	.05
IL-17	231 (118–417)	256 (161–500)	65.2 (46–90)	288 (102–398)	193.5 (131–626.6)	2 (2–16)	.0008	.0006	.874	.837
MIP-1β	2157 (430–3928)	546 (441–923)	243 (165–318)	730.3 (418–3363)	571.5 (426–1111)	14.3 (0.1–50.2)	.008	.008	.924	.571

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.

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

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

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.

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

Acknowledgments. We thank Dr P. Bozza, for helpful discussions about cytokines results; E. F. Assis, for laboratorial assistance with the multiplex assay; F. Freire Neto, for his kind contribution to parasite load

analysis by real-time PCR; and Dr F. Carvalho-Costa, for helpful discussions about multivariate analysis and assistance with statistical analysis.

Financial support. This work was supported by the Programa Nacional de DST/AIDS, Ministério da Saúde (grant ED00095/2007) and Instituto Oswaldo Cruz (internal funds). J. R. S. O. received a fellowship from FAPERJ (FAPERJ nota 10). A. M. C. is a researcher fellowship from CNPq and FAPERJ.

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.

References

1. Alvar J, Aparicio P, Aseffa A, et al. The relationship between leishmaniasis and AIDS: the second 10 years. Clin Microbiol Rev **2008**; 21:334–59.
2. Rabello A, Orsini M, Disch J. *Leishmania*/HIV co-infection in Brazil: an appraisal. Ann Trop Med Parasitol **2003**; 97(Suppl 1):17–28.
3. Sousa-Gomes ML, Maia-Elkhoury AN, Pelissari DM, et al. Co-infection *Leishmania*/HIV in Brazil: epidemiological, clinical and laboratorial aspects. Epidemiol Serv Saúde **2011**; 20:519–26.
4. Wolday D, Akuffo H, Fessahaye G, et al. Live and killed human immunodeficiency virus type-1 increases the intracellular growth of *Leishmania donovani* in monocyte-derived cells. Scand J Infect Dis **1998**; 30:29–34.
5. Barreto-de-Souza V, Pacheco GJ, Silva AR, et al. Increased *Leishmania* replication in HIV-1-infected macrophages is mediated by tat protein through cyclooxygenase-2 expression and prostaglandin E2 synthesis. J Infect Dis **2006**; 194:846–54.
6. Casado JL, Lopez-Velez R, Pintado V, et al. Relapsing visceral leishmaniasis in HIV-infected patients undergoing successful protease inhibitor therapy. Eur J Clin Microbiol Infect Dis **2001**; 20:202–5.
7. Cota GF, de Sousa MR, Rabello A. Predictors of visceral leishmaniasis relapse in HIV-infected patients: a systematic review. PLoS Negl Trop Dis **2011**; 5:e1153.
8. Zhao C, Papadopoulou B, Tremblay MJ. *Leishmania infantum* enhances human immunodeficiency virus type-1 replication in primary human macrophages through a complex cytokine network. Clin Immunol **2004**; 113:81–8.
9. Mock DJ, Hollenbaugh JA, Daddacha W, et al. Induces survival, proliferation and elevated cellular dNTP levels in human monocytes promoting acceleration of HIV co-infection. PLoS Pathog **2012**; 8:e1002635.
10. Bernier R, Barbeau B, Tremblay MJ, et al. The lipophosphoglycan of *Leishmania donovani* up-regulates HIV-1 transcription in T cells through the nuclear factor-kappa B elements. J Immunol **1998**; 160:2881–8.
11. Santos-Oliveira JR, Giacoia-Gripp CB, Alexandrino-de-Oliveira P, et al. High levels of T lymphocyte activation in *Leishmania*-HIV-1 co-infected individuals despite low HIV viral load. BMC Infect Dis **2010**; 10:35863.
12. Saha S, Mondal S, Banerjee A, et al. Immune responses in kala-azar. Indian J Med Re **2006**; 123:245–66.
13. Goto H, Prianti MG. Immunoactivation and immunopathogeny during active visceral leishmaniasis. Rev Inst Med Trop São Paulo **2009**; 51:241–6.
14. Sinha PK, Bimal S, Singh SK, et al. Pre- & post-treatment evaluation of immunological features in Indian visceral leishmaniasis (VL) patients with HIV co-infection. Indian J Med Res **2006**; 123:197–202.
15. Alexandrino-de-Oliveira P, Santos-Oliveira JR, Dorval ME, et al. HIV/AIDS-associated visceral leishmaniasis in patients from an endemic area in Central-West Brazil. Mem Inst Oswaldo Cruz **2010**; 105:692–7.
16. Liu Z, Cumberland WG, Hultin LE, et al. Elevated CD38 antigen expression on $CD8^+$ T cells is a stronger marker for the risk of chronic HIV disease progression to AIDS and death in the Multicenter AIDS Cohort Study than $CD4^+$ cell count, soluble immune activation markers, or combinations of HLA-DR and CD38 expression. J Acquir Immune Defic Syndr Hum Retrovirol **1997**; 16:83–92.
17. Giorgi JV, Hultin LE, McKeating JA, et al. Shorter survival in advanced human immunodeficiency virus type 1 infection is more closely associated with T lymphocyte activation than with plasma virus burden or virus chemokine coreceptor usage. J Infect Dis **1999**; 179:859–70.
18. Appay V, Sauce D. Immune activation and inflammation in HIV-1 infection: causes and consequences. J Pathol **2008**; 214:231–41.
19. Douek DC, Roederer M, Koup RA. Emerging concepts in the immunopathogenesis of AIDS. Annu Rev Med **2009**; 60:471–84.
20. Rodrigues DS, Cunha RM, Kallas EG, et al. Distribution of naive/effector $CD4^+$ T lymphocytes and expression of CD38 on $CD8^+$ T lymphocytes in AIDS patients with tuberculosis. Braz J Infect Dis **2003**; 7:161–5.
21. Doisne JM, Urrutia A, Lacabaratz-Porret C, et al. $CD8^+$ T cells specific for EBV, cytomegalovirus, and influenza virus are activated during primary HIV infection. J Immunol **2004**; 173:2410–8.
22. Gonzalez VD, Falconer K, Blom KG, et al. High levels of chronic immune activation in the T-cell compartments of patients coinfected with hepatitis C virus and human immunodeficiency virus type 1 and on highly active antiretroviral therapy are reverted by alpha interferon and ribavirin treatment. J Virol **2009**; 83:11407–11.
23. Sandler NG, Koh C, Roque A, et al. Host response to translocated microbial products predicts outcomes of patients with HBV or HCV infection. Gastroenterology **2011**; 141:1220–30, 1230.e1–3.
24. Brenchley JM, Price DA, Schacker TW, et al. Microbial translocation is a cause of systemic immune activation in chronic HIV infection. Nat Med **2006**; 12:1365–71.
25. Mehandru S, Poles MA, Tenner-Racz K, et al. Primary HIV-1 infection is associated with preferential depletion of $CD4^+$ T lymphocytes from effector sites in the gastrointestinal tract. J Exp Med **2004**; 200:761–70.
26. Brenchley JM, Schacker TW, Ruff LE, et al. $CD4^+$ T cell depletion during all stages of HIV disease occurs predominantly in the gastrointestinal tract. J Exp Med **2004**; 200:749–59.
27. Caradonna L, Amati L, Magrone T, et al. Enteric bacteria, lipopolysaccharides and related cytokines in inflammatory bowel disease: biological and clinical significance. J Endotoxin Res **2000**; 6:205–14.
28. Cooke KR, Olkiewicz K, Erickson N, et al. The role of endotoxin and the innate immune response in the pathophysiology of acute graft versus host disease. J Endotoxin Res **2002**; 8:441–8.
29. Lee PI, Ciccone EJ, Read SW, et al. Evidence for translocation of microbial products in patients with idiopathic CD4 lymphocytopenia. J Infect Dis **2009**; 199:1664–70.
30. Baba CS, Makharia GK, Mathur P, et al. Chronic diarrhea and malabsorption caused by *Leishmania donovani*. Indian J Gastroenterol **2006**; 25:309–10.
31. Hicks L, Kant P, Tay PH, et al. Visceral leishmaniasis presenting with intestinal failure: a case report and literature review. Eur J Gastroenterol Hepatol **2009**; 21:117–22.
32. Santos-Oliveira JR, Regis EG, Leal CR, et al. Evidence that lipopolisaccharide may contribute to the cytokine storm and cellular activation in patients with visceral leishmaniasis. PLoS Negl Trop Dis **2011**; 5: e1198.
33. Peruhype-Magalhães V, Martins-Filho OA, Prata A, et al. Mixed inflammatory/regulatory cytokine profile marked by simultaneous raise of interferon-gamma and interleukin-10 and low frequency of tumour necrosis factor-alpha(+) monocytes are hallmarks of active human visceral leishmaniasis due to *Leishmania chagasi* infection. Clin Exp Immunol **2006**; 146:124–32.
34. Nylén S, Maurya R, Eidsmo L, et al. Splenic accumulation of IL-10 mRNA in T cells distinct from $CD4^+CD25^+$ (Foxp3) regulatory T cells in human visceral leishmaniasis. J Exp Med **2007**; 204:805–17.
35. Brasil, Ministério da Saúde, Secretaria de Vigilância em Saúde, Departamento de Vigilância Epidemiológica. Manual de vigilância e controle da leishmaniose visceral. Brasília: Ministério da Sáude, **2006**. http://portal.saude.gov.br/portal/arquivos/pdf/manual_leish_visceral2006.pdf. Accessed November 2012.

36. Brasil, Ministério da Saúde, Departamento de DST, Aids e hepatites virais. Protocolo Clínico e diretrizes Terapêuticas para adultos vivendo com HIV/AIDS. http://www.aids.gov.br/sites/default/files/anexos/publicacao/2013/52934/_p_vers_atilde_o_preliminar_do_protocolo_cl_iacute_26118.pdf. Accessed April 2013.
37. Weirather JL, Jeronimo SM, Gautam S, et al. Serial quantitative PCR assay for detection, species discrimination, and quantification of *Leishmania* spp. in human samples. J Clin Microbiol **2011**; 49:3892–904.
38. McCoy JJ, Beetham JK, Ochs DE, et al. Regulatory sequences and a novel gene in the msp (GP63) gene cluster of *Leishmania chagasi*. Mol Biochem Parasitol **1998**; 95:251–65.
39. Estes JD, Harris LD, Klatt NR, et al. Damaged intestinal epithelial integrity linked to microbial translocation in pathogenic simian immunodeficiency virus infections. PLoS Pathog **2010**; 6:e1001052.
40. Brenchley JM, Douek DC. Microbial translocation across the GI tract. Annu Rev Immunol **2012**; 30:149–73.
41. Antinori S, Calattini S, Longhi E, et al. Clinical use of polymerase chain reaction performed on peripheral blood and bone marrow samples for the diagnosis and monitoring of visceral leishmaniasis in HIV-infected and HIV-uninfected patients: a single-center, 8-year experience in Italy and review of the literature. Clin Infect Dis **2007**; 44:1602–10.
42. Lieberman JM, Sacchettini J, Marks C, et al. Human intestinal fatty acid binding protein: report of an assay with studies in normal volunteers and intestinal ischemia. Surgery **1997**; 121:335–42.
43. Miller SI, Ernst RK, Bader MW. LPS, TLR4 and infectious disease diversity. Nat Rev Microbiol **2005**; 3:36–46.
44. Connolly NC, Riddler SA, Rinaldo CR. Proinflammatory cytokines in HIV disease - a review and rationale for new therapeutic approaches. AIDS Rev **2005**; 7:168–80.
45. Medrano FJ, Rey C, Leal M, et al. Dynamics of serum cytokines in patients with visceral leishmaniasis and HIV-1 co-infection. Clin Exp Immunol **1998**; 114:403–7.
46. Nazli A, Chan O, Dobson-Belaire WN, et al. Exposure to HIV-1 directly impairs mucosal epithelial barrier integrity allowing microbial translocation. PLoS Pathog **2010**; 6:e1000852.
47. Balkhair A, Ben Abid F. Gastric and cutaneous dissemination of visceral leishmaniasis in a patient with advanced HIV. Int J Infect Dis **2008**; 12:111–3.
48. Gómez Senent S, Adan Merino L, Mora Sanz P. Kala azar with gastric involvement. Gastroenterol Hepatol **2009**; 32:176–7.
49. Calandra T, Roger T. Macrophage migration inhibitory factor: a regulator of innate immunity. Nat Rev Immunol **2003**; 3:791–800.