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MAJOR ARTICLE

T-cell and Soluble Co-inhibitory Receptor Expression in Patients With Visceral Leishmaniasis Are Markers of Treatment Response and Clinical Outcome

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Background. Co-inhibitory receptors (immune checkpoints) regulate activated immune cells. Their expression on T cells can limit host defense. We hypothesized that chronic *Leishmania donovani* infection in patients with visceral leishmaniasis (VL) leads to expression of co-inhibitory receptors that could be markers of treatment response and clinical outcome.

Method. A prospective cohort of 21 subjects with VL (7 with HIV coinfection) and 10 controls was established to measure T-cell expression of co-inhibitory receptors (PD-1, Tim-3, LAG-3, CTLA-4, and TIGIT) by flow cytometry in discarded remnants of diagnostic splenic or bone marrow aspirates and peripheral blood collected before and after treatment. Plasma levels of soluble co-inhibitory proteins (sPD-1, sTim-3, sLAG-3, and sCTLA-4) and selected cytokines were determined by immunoassay.

Results. Expression of co-inhibitory receptors in peripheral blood T cells generally reflected findings in spleen and bone marrow aspirates. PD-1 and Tim-3 were upregulated in CD4+ T cells in HIV-negative and HIV-positive subjects with VL compared to controls. CD8+ T cells from HIV-negative subjects with VL displayed a similar pattern. Plasma levels of sPD-1 and sTim-3 were also greater in VL patients than controls. CD8+ and CD4+ T cells coexpressing PD-1 and Tim-3 showed considerable decline with treatment. Mortality in HIV-negative VL patients was associated with increased CD8+ T cells coexpressing Tim-3 and PD-1, triple-positive CD4+ and CD8+ T cells (PD-1^{+}Tim-3^{+}LAG-3^{+}), and elevated sLAG3.

Conclusions. Tim-3 and PD-1 expression on CD4+ and CD8+ T cells, and increased plasma sLAG-3, were markers of treatment response and clinical outcome in patients with VL.

Visceral leishmaniasis (VL), also known as kala-azar, is a neglected tropical disease caused by *Leishmania donovani* and *Leishmania infantum* that primarily affects impoverished populations in sub-Saharan Africa, South Asia, Brazil, and the Mediterranean region [1, 2]. Most people have a subclinical or asymptomatic infection that is controlled by type 1 T cells producing interferon-γ (IFN-γ) to activate macrophages to kill intracellular parasites. Approximately 10% of infected people develop active VL with fever, splenomegaly, cachexia, and pancytopenia that will progress to death if not treated [1, 3]. In these patients, the spleen and bone marrow are heavily parasitized and an effective Th1 immune response fails to develop or is subverted by other immunosuppressive mechanisms [3, 4]. Even with treatment, the mortality rate can be as high as 20%.

During chronic infections and cancer, persistent exposure to antigens can promote expression of co-inhibitory receptors (also referred to as immune checkpoints) on T cells, antigen-presenting cells (APCs), and natural killer (NK) cells to regulate immune cell activation [5, 6]. Expression of co-inhibitory receptors drives T-cell exhaustion and impaired effector function leading to compromised host defense [5, 6]. Programmed cell death 1 (PD-1) regulates activated T cells to by binding PD-L1 and PD-L2 ligands on APCs [7]. Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) suppresses T-cell responses by disrupting B7-CD28 co-stimulation and trans-endocytosis of CD80 and CD86 on APCs [8]. Lymphocyte activation gene 3 (LAG-3) binds to major histocompatibility complex class II molecules to regulate activation [9]. T-cell immunoglobulin and mucin domain-containing molecule 3 (TIM-3) inhibits immune responses by binding galectin-9 [10]. T-cell immunoreceptor with immunoglobulin and immunoreceptor tyrosine-based inhibitory motif domains

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(TIGIT), indirectly inhibits T-cell function by binding CD155 on APCs and modulating interleukin-10 (IL-10) and IL-12 production [11].

In patients with active VL, CD4 and CD8 T cells demonstrate increased expression of immune checkpoints, impaired effector responses, and T-cell exhaustion (reviewed in [12, 13]). Previous studies found increased PD-1 expression in patients with VL in India [14] and Ethiopia [15]. Antibody-mediated blockade of the PD1/PDL-1 axis in *ex vivo* whole blood cultures from Ethiopian patients promoted IFN-γ production [15]. Similarly, *ex vivo* blockade of PD1-PDL1/2 enhanced IFN-γ production by CD4 and CD8 T cells in patients with cutaneous leishmaniasis [16]. Upregulation of co-inhibitory receptors and their ligands were also shown to promote disease in experimental VL, and their blockade improved anti-leishmanial T-cell responses [14, 17, 18].

Treatment of VL relies on chemotherapy with parenteral antimonials, paromomycin, liposomal amphotericin B, oral miltefosine, or a combination of these drugs. Long treatment regimens, high cost, potential severe adverse effects, and the growing reports of clinical failures highlight the need for alternative therapies [19]. Patients who fail to respond to a first-line regimen receive an extended treatment duration or an alternative regimen. Having markers that predict treatment response and clinical outcome would be valuable for optimizing treatment of patients with VL.

METHODS

Patient Consent Statement

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the College of Health Science institutional review boards of Addis Ababa University (protocol code:029/17/DMIP) and University of Gondar (protocol code: V/P/RCS/05/369/2020). All study subjects provided written informed consent before enrollment.

Participant Enrollment

A prospective cohort of 21 patients suspected to have VL (defined in [20]), with or without HIV coinfection, who were hospitalized at the Leishmaniasis Research and Treatment Center of the University of Gondar were enrolled in the study. Male or nonpregnant female subjects of 18 years of age or older, who had *Leishmania* amastigotes observed on a stained smear of a splenic or bone marrow aspirate, and provided written informed consent were included in the study. Five subjects with stable HIV infection were recruited from the University of Gondar Hospital's antiretroviral treatment clinic, and 5 endemic healthy controls (attendants of VL patients) were enrolled as controls. Controls were matched to VL patients with sex, residence, and ethnicity. All controls were asymptomatic, had no history of VL, and had a negative rk39 serological test result. HIV-negative subjects with VL had diagnostic splenic (n = 8) or bone marrow (n = 5) aspirate samples collected, and HIV-positive subjects with VL patients had splenic aspirates collected (n = 4) as standard of care before initiation of antileishmanial treatment. Patients had a diagnostic bone marrow aspirate performed when splenic aspiration was deemed to have an unacceptable risk of complication (bleeding, severe anemia, jaundice, or platelet count < 40 000/μL). The remnant of the diagnostic aspirate, which would otherwise have been discarded, was used to determine T-cell co-inhibitory receptor expression by flow cytometry. HIV-positive VL patients were treated with a liposomal amphotericin B (AmBisome) infusion and oral miltefosine combination therapy, whereas HIV-negative VL cases were treated with parenteral sodium stibogluconate and paromomycin combination therapy according to the Ethiopian standard of care [21]. Patients were assessed for initial clinical response (absence of fever, improved hematological abnormalities, and a significant regression in the size of the enlarged spleen and liver) on the day after conclusion of treatment. Throughout the manuscript, deceased or "survived" refers to the initial clinical outcome of the hospitalization. Demographic, clinical, and clinical laboratory data from study participants were collected by clinical providers and study staff.

Collection of Diagnostic and Research Specimens

Peripheral blood (n = 21, 10 mL), splenic aspirate (n = 12), and/or bone marrow aspirate (n = 5) were collected from VL patients 1 day befpre initiation of treatment. For research purposes, we used the remnant of the diagnostic splenic (n = 12) or bone marrow aspirates (n = 5) that otherwise would have been discarded and 10 mL peripheral blood collected immediately before treatment (n = 21) or immediately after completion of the final dose of treatment (n = 16). In the 10 control subjects, 10 mL of peripheral blood was collected once. Splenic aspirates, bone marrow aspirates, and blood samples were kept at 4 °C and processed within 1 hour of collection for flow cytometry at the Leishmaniasis Research and Treatment Center. Plasma was separated and immediately frozen at −80 °C.

Parasitological Diagnosis and Determination of *Leishmania* Parasite Load

Parasitological diagnosis of VL was made by microscopic demonstration of *Leishmania* amastigotes in Giemsa-stained aspirates of either spleen or bone marrow. Based on guidelines from the Ethiopian Ministry of Health and World Health Organization [21, 22], parasite load was graded as follows: 6+ = >100 parasites per field, 5+ = 10–100 parasites per field, 4+ = 1–10 parasites per field, 3+ = 1–10 parasites per 10 field, 2+ = 1–10 parasites per 100 field, 1+ = 1–10 parasites per 1000 field, and negative = 0 parasite per 1000 field.

Flow Cytometry

Phenotyping of T cells, NK cells, and NKT cells from the spleen or bone marrow aspirate and peripheral blood was performed using the CytoFLEX LX Flow Cytometer (Beckman Coulter, 21 detectors and 6 lasers) in the University of Gondar flow cytometry laboratory using the following monoclonal antibodies: CD3 PE-Cy7, CD4 FITC, CD56 (NCAM) APC-Cy7, PD-1Alexa Fluor 647, TIGIT-PE (BioLegend), CD8Brilliant Violet 510, CTLA-4PE-Cy5, TIM-3 PE-CF594 (BD Biosciences), and Lag-3PerCP-eFluor 710 (Thermo Fisher Scientific). Flow cytometry staining followed protocols by BioLegend and BD Biosciences. Briefly, 100 µL of the sample (whole blood, splenic aspirate suspension, or bone marrow aspirate) was added to a 5-mL round bottom polystyrene tube and washed with Cell Staining Buffer (CSB, BioLegend). FC block and Zombie UV were added to the pellet and incubated at 4 °C in the dark for 15 minutes. Cells were washed with CSB and monoclonal antibodies added to the pellet at the manufacturer's recommendation concentration and incubated for 30 minutes at 4 °C in the dark. Cells were then washed with CSB, red blood cells lysed in 1 × fix/lyse buffer (BioLegend) at room temperature for 10 minutes in the dark, washed, and the pellet resuspended in 0.5 mL CSB. Samples were immediately acquired on CytoFLEX LX Flow cytometer. Unstained controls were processed with every experiment. Fluorescence overlap was corrected with single-color compensation beads (Ultracomp eBeads, ThermoFisher). FlowJo_v10.8.1 software was used to analyze the data using fluorescence-minus-one controls. The gating strategy for CD4 and CD8 T cells, NK cells, and NKT cells in peripheral blood and splenic and bone marrow aspirates is shown in Supplementary Fig. 1. Co-inhibitory receptor combinations were evaluated using the Boolean gating tool in FlowJo.

Plasma Cytokine and Soluble co-inhibitory Receptor Profiling

Multiplex immunoassays for plasma cytokines (IFN-α, IL-1B, IL-10, TNF-α, IFN-γ, IL-17, and IL-6) using Luminex Discovery Assay (Human Premixed Multi-Analyte Kit, R&D) and soluble checkpoint proteins (Tim-3, LAG-3, PD-1, CTLA-4) using the MILLIPLEX Human Immuno-Oncology Checkpoint protein panel 1 (Millipore, Sigma) were determined in in controls (n = 10), and in VL patients before (n = 21) and after (n = 16) treatment.

Statistical Analyses

Prism 9 (GraphPad Software, USA) and SPSS version 24 was used to analyze data exported from FlowJo and Luminex assays and for graphic representations. Paired sample *t*-test was performed to compare differences in the expression of checkpoints or plasma cytokine and soluble checkpoints between samples taken before and after anti-leishmanial therapy. Data from before treatment, after treatment, and controls were compared using 1-way analysis of variance. Differences in deceased and surviving subjects with VL were determined using independent *t*-tests. Pearson correlation was used to measure the association between checkpoints and cytokine concentrations in plasma. Significance levels were set at $P < .05$.

RESULTS

Characteristics of Study Subjects

Twenty-one subjects with VL were prospectively enrolled in the cohort. Seven patients with VL were HIV co-infected, whereas the remaining 14 were not. All HIV-negative VL cases had a primary VL, whereas 6 of 7 VL-HIV co-infected cases had relapsed VL. Demographic, hematological, and clinical characteristics of study subjects are shown in Table 1. Subjects with VL were young adults, almost exclusively male, underweight (average body mass index of 16.82), had hepatosplenomegaly and pancytopenia, and had been ill for an average of 9 weeks. Subjects who were HIV co-infected had a high parasite load in the diagnostic splenic or bone marrow aspirate. Three subjects (21%) who were HIV-negative, and 1 HIV-co-infected subject (14.3%) died during treatment.

Co-inhibitory Receptor Expression on Peripheral Blood T Cells, NK Cells, and NKT Cells in VL Patients

We determined the mean percent expression of co-inhibitory receptors (Tim-3, PD-1, CTLA-4, LAG-3, and TIGIT) on T cells, NK cells, and NKT cells in the peripheral blood of healthy controls, and in HIV-negative and HIV-positive subjects with VL collected before treatment (BT) and after treatment (AT) (see the full dataset in Supplementary Table 1). Before treatment, Tim-3 expression on CD8+ and CD4+ T cells was greater in HIV-negative subjects with VL compared to controls ($P < .0001$ and $P = .01$, respectively) (Figure 1*A*; Supplementary Table 2). The mean fluorescence intensity (MFI) of Tim-3 on CD8+ T cells was also increased in HIV-negative subjects with VL (Table 2). Expression of Tim-3 on CD4+ T cells was similarly greater among HIV-positive subjects with VL compared to HIV-infected controls ($P = .038$) (Figure 1*B*; Supplementary Table 2). HIV-negative and HIV-positive subjects with VL demonstrated significant declines in percent expression and MFI of Tim-3 on CD8+ and CD4+ T cells after completion of treatment (Figure 1*A*, *B*; Table 2: Supplementary Table 2). The proportion of PD-1-expressing CD4+ T cells was increased in HIV-positive subjects with VL, but no differences were found in PD-1 expression in HIV-negative subjects with VL and healthy controls (Figure 1*C*; Supplementary Table 2). Nevertheless, there was significant decline of PD-1-expressing CD8+ T cells ($P = .019$) and decreased MFI of PD-1 on CD8+ T cells ($P = .049$) following treatment of HIV-negative VL patients (Figure 1*D*, Table 2). In HIV-positive subjects with VL, PD-1 expression in CD8+ and CD4+ T cells showed no differences between pre- and post-treatment samples but displayed a declining trend with treatment. Expression of LAG-3, TIGIT, and CTLA-4 on CD4 and CD8 T cells, NK cells, and NKT cells showed no difference between subjects with VL and controls and did not decline with treatment (Supplementary Table 2).

Table 1. Demographic, Hematological, and Clinical Characteristics of Study Subjects

Characteristics Mean, (SD) Unless Indicated)	HIV-negative VL, N = 14	HIV-positive VL, N = 7	Healthy Controls, N = 5	HIV Controls, N = 5
Age (y)	24.50 (6.09)	37.29 (5.38)	30.60 (12.90)	41.00 (12.12)
Body mass index	17.43 (2.21)	15.61 (2.55)	19.38 (3.27)	20.44 (1.96)
Male sex (n, %)	13 (92.86%)	7 (100.00%)	4 (80.00%)	5 (100%)
Hemoglobin (g/dL)	8.64 (2.42)	8.73 (1.14)	ND	ND
Duration of illness (d)	71.46 (55.25)	63.00 (37.99)	N/A	N/A
Spleen size (cm below CM)	8.64 (5.72)	13.14 (5.01)	N/A	N/A
Liver size (cm below CM)	4.00 (1.27)	6.50 (2.12)	N/A	N/A
Parasite load (median, IQR)	1.00 (1.00)	6.00 (1.00)	N/A	N/A
Platelet count	57 290 (25 130)	101 570 (16 630)	ND	ND
T, NK, and NKT cell percentages in peripheral blood (mean %, ±SD)				
CD3 T cells	7.68 (5.92)	3.26 (1.76)	3.76 (2.26)	5.07 (3.70)
CD4 T cells	5.03 (4.01)	1.11 (0.87)	2.22 (1.04)	1.65 (1.24)
CD8 T cells	1.42 (1.80)	1.65 (1.07)	0.53 (0.23)	N/D
NK cells	0.91 (0.87)	0.86 (0.54)	1.10 (0.90)	0.97 (0.71)
NKT cells	0.25 (0.28)	0.22 (0.12)	0.23 (0.24)	0.31 (0.33)
Clinical outcomes[a] (n, %)				
Died	3 (21.43%)	1 (14.29%)	N/A	N/A
Survived	11 (78.57%)	5 (71.43%)	N/A	N/A

Abbreviations: CM, costal margin; IQR, interquartile range; N/A, not applicable; N/D, not done; NK, natural killer; VL, viral load.

[a]The clinical outcome of 1 VL-HIV patient who defaulted is unknown.

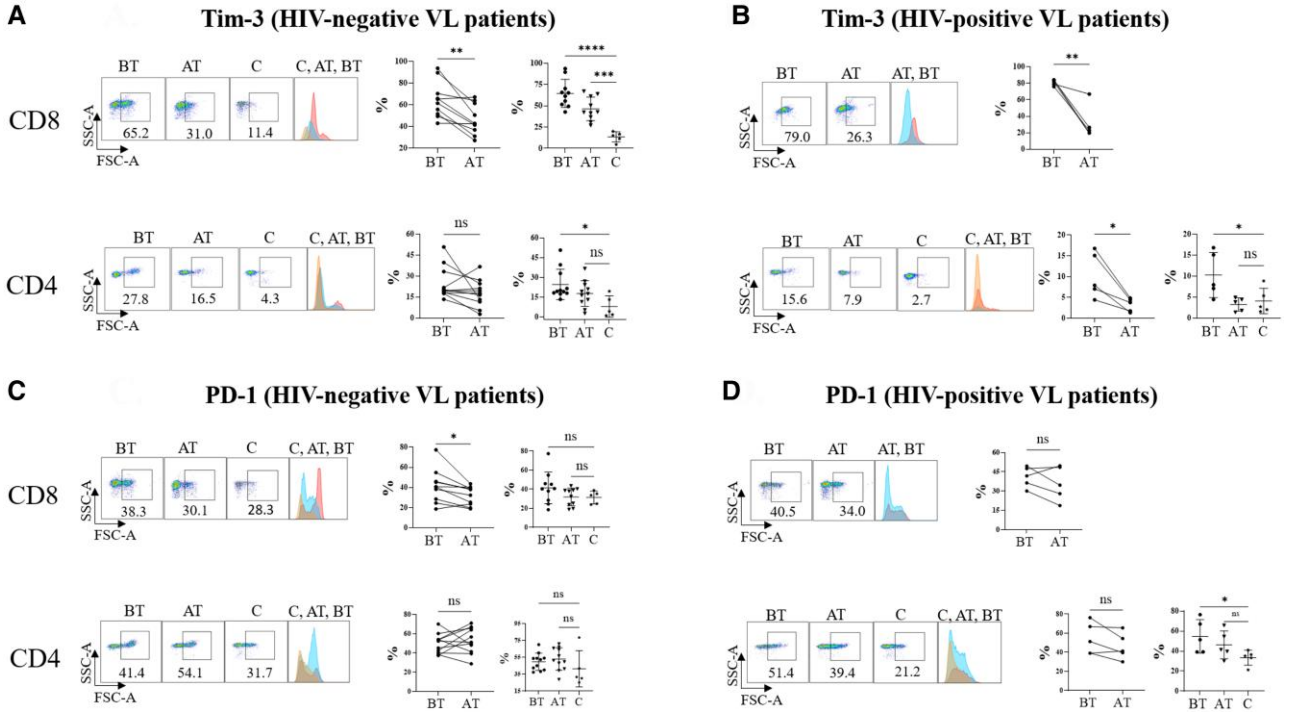

Figure 1. Co-inhibitory receptor expression on peripheral blood CD4 and CD8 T cells in subjects with VL. Flow cytometry dot plots, histogram, and scatter plot presentations of Tim-3 (A and B) and PD-1 (C and D) in HIV-negative (A and C) and HIV-positive (B and D) subjects with VL before (BT) and after (AT) treatment and controls (C). Paired BT and AT samples are shown with line graphs. Comparison of co-inhibitory receptor expression on CD8+ T cells from HIV-positive VL patients with HIV patient controls was not possible because of insufficient anti-CD8 antibodies. ns: $P > .05$; *$P < .05$; **$P < .01$; ***$P < .001$; ****$P < .0001$.

Table 2. MFI of T-cell Co-inhibitory Receptor Expression in Peripheral Blood of VL Patients and Controls

	HIV-negative VL Patients				HIV-positive VL Patients			
	MFI of checkpoint receptors (SD)				MFI of checkpoint receptors (SD)			
	BT, n = 11	AT, n = 11	CON, n = 5	*P* value[a]	BT, n = 5	AT, n = 5	CON, n = 5	*P* value[a]
CD4 + Tim-3+	4153 (1009)	2879 (785)	3277 (898)	BT versus AT: **.006** BT versus CON: .119 AT versus CON: .383	2693 (405)	2727 (282)	3090 (632)	BT versus AT: .872 BT versus CON: .271 AT versus CON: .275
CD8 + Tim-3+	3809 (804)	3118 (710)	2551 (730)	BT versus AT: **.037** BT versus CON: **.012** AT versus CON: .172	2574 (236)	2330 (241)	ND	BT versus AT: **.049** BT versus CON: N/A AT versus CON: N/A
CD4 + PD-1+	7955 (2051)	5376 (1430)	4750 (1123)	BT versus AT: .002 BT versus CON: .006 AT versus CON: .404	7564 (2045)	5468 (862)	4281 (184)	BT versus AT: **.030** BT versus CON: **.007** AT versus CON: **.017**
CD8 + PD-1+	6794 (2130)	5297 (712)	4316 (639)	BT versus AT: **.049** BT versus CON: **.026** AT versus CON: **.022**	5482 (860)	4527 (486)	ND	BT versus AT: .120 BT versus CON: N/A AT versus CON: N/A
CD4 + LAG-3+	13 759 (1328)	19 490 (13 226)	13 434 (1252)	BT versus AT: .195 BT versus CON: .652 AT versus CON: .333	17 566 (5976)	22 738 (13 031)	13 837 (731)	BT versus AT: .349 BT versus CON: .261 AT versus CON: .220
CD8 + LAG-3+	15 565 (4598)	15 053 (2873)	14 308 (1496)	BT versus AT: .841 BT versus CON: .777 AT versus CON: .639	13 036 (1372)	16 096 (35 378)	ND	BT versus AT: .502 BT versus CON: N/A AT versus CON: N/A

Abbreviations: AT, after treatment; BT, before treatment; CON, control; MFI, Mean Fluorescence Intensity; N/A, not applicable; ND, not done; SD, standard deviation.

[a]P value from comparison of BT and AT, BT and CON, and AT and CON determined by one-way ANOVA.

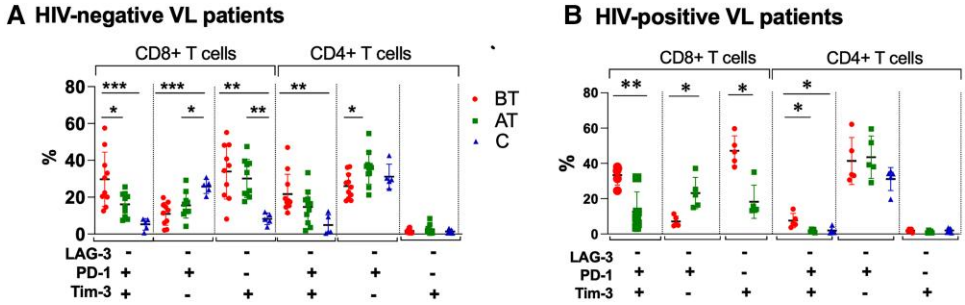

Figure 2. Combined expression of co-inhibitory receptors on peripheral blood CD4+ and CD8+ T cells of subjects with VL. Scatter plot representation for the combined expression of Tim-3, PD-1, and LAG-3 on CD8 + and CD4+ T cells of HIV-negative subjects with VL (*A*) and HIV-positive subjects with VL (*B*) and their controls. Data are presented as percentage of CD4 and CD8 T cells that express the co-inhibitory receptor relative to total CD4 and CD8 T cells. Note that LAG-3$^+$PD-1$^+$Tim-3$^+$ T cells are not presented because the percent of cells was negligible (full dataset available in Supplementary Table 1 and Supplementary Table 2).

Combined Expression of Immune Checkpoints on Peripheral Blood CD4+ and CD8+ T Cells of Subjects With VL

We used Boolean combination gating to find higher combined expression of PD-1 and Tim-3 (PD-1$^+$Tim-3$^+$LAG-3$^-$) on CD8+ T cells ($P = .003$) and CD4+ T cells ($P = .006$) of HIV-negative VL patients compared to endemic healthy controls (Supplementary Table 3). CD8+ T cells, but not CD4+ T cells, that exclusively expressed Tim-3 (PD-1$^-$Tim-3$^+$ LAG-3$^-$) were also increased in HIV-negative patients with VL ($P = .002$). Conversely, there was no increase in solo PD-1 or Lag-3 expression on CD4+ and CD8+ T cells in HIV-negative subjects with VL. We were unable to compare co-inhibitory receptor expression on CD8+ T cells from HIV-positive subjects with VL because we did not have data from HIV-positive controls. However, the proportion of CD4+ T cells with combined expression of PD-1 and Tim-3 (PD-1$^+$Tim-3$^+$LAG-3$^-$) was greater in HIV-positive subjects with VL than in controls. Solo expression of Tim-3, PD-1, and LAG-3 on CD4+ T cells showed no significant differences between HIV-positive subjects with VL and controls. CD8+ and CD4+ T cells coexpressing PD-1 and Tim-3 (PD-1$^+$Tim-3$^+$LAG-3$^-$) declined with treatment in both HIV-negative and HIV-positive subjects with VL (Figure 2*A*, *B*, and Supplementary Table 3).

Immune Checkpoint Inhibitor Expression on T Cells as a Marker of Clinical Outcome in Subjects With VL

There were no differences in the overall percent of T cells, NK cells, and NKT cells populations, in subjects with VL who died before completion of treatment compared to those who survived (Supplementary Table 4). However, the percent CD8+ T cells that expressed PD-1 ($P = .025$) or LAG-3 ($P = .007$) was increased in HIV-negative subjects with VL who died compared to those who survived (Figure 3*A* and 3*B*). LAG-3+ CD4+ T cells were marginally increased ($P = .052$) in deceased compared to surviving HIV-negative subjects with VL. No significant differences in Tim-3, CTLA-4, and TIGIT expression on CD4+ and CD8+ T cells were found between deceased and surviving HIV-negative subjects with VL (Supplementary Table 4). Coexpression of PD-1 and Tim-3 (PD-1$^+$Tim-3$^+$LAG-3$^-$) on CD8+ T cells was significantly greater in deceased compared to surviving HIV-negative subjects with VL ($P = .039$) (Figure 3*C*). Deceased HIV-negative subjects with VL also had an increased proportion of CD8+ ($P = .007$) and CD4+ T cells ($P = .014$) coexpressing the 3 co-inhibitory receptors (PD-1$^+$Tim-3$^+$LAG-3$^+$) compared to subjects who survived (Figure 3*C*). CD4+ and CD8+ T cells exclusively expressing LAG-3, PD-1, or Tim-3 were not increased in deceased versus surviving HIV-negative VL patients (Figure 3*C* and Supplementary Table 4). Collectively, these data suggest that c-expression of 2 or more co-inhibitory receptors on T cells is a marker of VL morbidity and mortality. We were unable to compare deceased versus surviving HIV-VL coinfected patients because there was only 1 death in this cohort.

Plasma Soluble Immune Checkpoint Proteins are Associated With Cellular Expression and a Marker of Clinical Outcome in Patients With VL

Immune checkpoint proteins can be released from the cell surface as soluble proteins [23]. In agreement with cellular expression, we found increased soluble Tim-3 (sTim-3) in the plasma of HIV-negative ($P < .0001$) and HIV co-infected ($P = .017$) subjects with VL compared to controls (Figure 4; Supplementary Table 5). Following treatment, sTim-3 declined sharply in both HIV-positive and HIV-negative subjects with VL ($P = .008$ and $P = .029$, respectively). HIV-negative and HIV-positive subjects with VL also displayed elevated plasma levels of sPD-1 relative to controls ($P = .008$ and $P = .0006$, respectively), which decreased after treatment ($P = .009$ and $P = .044$, respectively) (Figure 4; Supplementary Table 5). We did not observe differences in sLAG-3 and sCTLA-4 between VL patients and controls, and we did not measure the plasma concentration of sTIGIT. However, plasma sLAG-3 was greater in deceased compared to surviving HIV-negative subjects with VL ($P = .008$) (Supplementary Table 4), in agreement with the increased CD8+ Tcell expression of LAG-3 in patients who died. No significant differences in

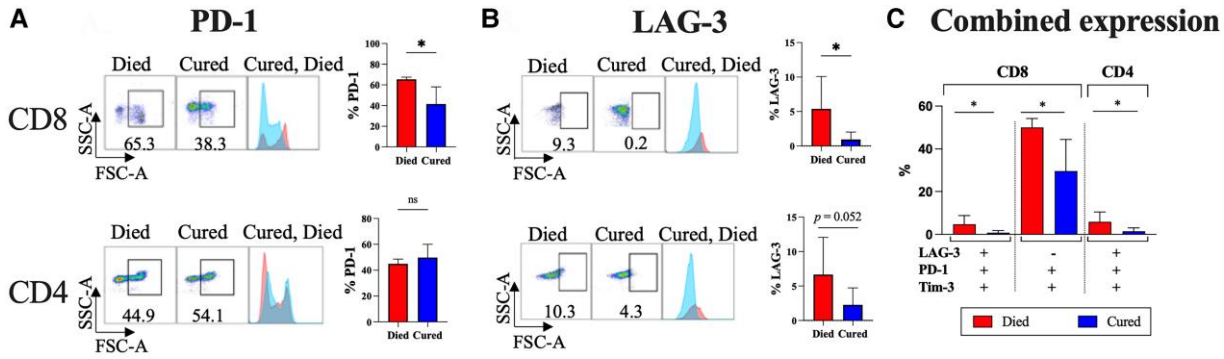

Figure 3. T-cell co-inhibitory receptor expression is associated with clinical outcomes in subjects with VL. Dot plot, histogram, and scatter dot plot representations for expression of PD-1 (*A*) and LAG-3 (*B*) on peripheral blood CD8+ and CD4+ T cells at baseline (pretreatment) among deceased and surviving HIV-negative subjects with VL. *C*, Column graph displaying combined expression of Tim-3, PD-1, and LAG-3 on CD8+ and CD4+ T cells that were significantly different between deceased and surviving HIV-negative VL patients. ns: $P > .05$, $^{*}P < .05$.

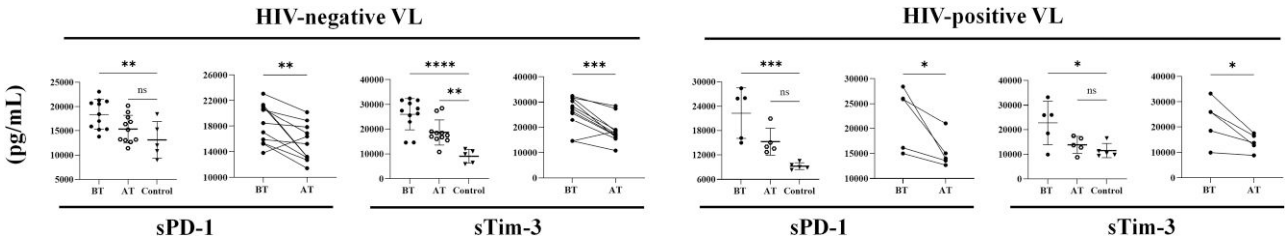

Figure 4. Plasma concentration of soluble co-inhibitory receptors in subjects with VL. Concentrations (pg/mL) of sPD1 and sTim-3 were determined in plasma from HIV-negative and HIV-positive VL subjects before (BT) and after (AT) treatment and in controls by immunoassay. Data are shown as scatter plots of BT, AT, and control groups with the median and interquartile range, and by line graphs of paired BT and AT samples. ns: not significant, $P > .05$, $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$, $^{****}P < .0001$.

sTim-3, sPD-1, and sCTLA-4 were observed between deceased and surviving HIV-negative subjects with VL. We observed a significant positive correlation between the level of sPD-1 in plasma and expression of PD-1 ($r = 0.5$, $P = .045$) and Tim-3 ($r = 0.6$, $P = .026$) on CD8+ T cells (Figure 5*A*). However, plasma sLAG-3 and s-Tim-3 did not correlate with cellular co-inhibitory receptor expression (Figure 5*A* and 5*B*).

Comparison of Cellular Expression of Immune Inhibitory Checkpoints in Peripheral Blood and Splenic or Bone Marrow Aspirates From VL Patients

We compared the mean percent expression of each immune checkpoint in peripheral blood with paired splenic (n = 8) or bone marrow aspirates (n = 5) in subjects with VL. In HIV-negative patients with VL, there were no differences in expression of any immune checkpoints in paired peripheral blood and spleen samples; however, co-inhibitory receptor expression in bone marrow aspirates tended to be higher than in paired peripheral blood samples, except that Tim-3^{+} CD8+ T cells were more frequent in blood samples ($P = .026$) (Supplementary Table 6). In HIV-positive subjects with VL, there was also a higher percent expression of co-inhibitory receptors on CD8+ and CD4+ T cells in the spleen relative to peripheral blood, again with the exception that Tim-3^{+} CD8+ T cells were greater in peripheral blood ($P = .018$) (Supplementary Table 6). There were no significant differences in combined expression of PD-1, Tim-3, and LAG-3 on CD4+ and CD8+ T cells between peripheral blood and paired splenic aspirate samples of HIV-negative patients with VL (Supplementary Table 6). In HIV-negative subjects with VL who had bone marrow aspiration (subjects with severe disease and greater mortality), peripheral blood contained more CD8+ T cells with combined expression of Tim-3 and PD-1 (PD-1^{+}Tim-3^{+}LAG-3^{-}) than the paired bone marrow samples ($P = .046$). Given the association of CD8+ T cells that coexpress Tim-3 and PD-1 with morbidity and mortality in HIV-negative VL patients (Figure 2*C*, 3*D*), the greater abundance of CD8+ T cells coexpressing Tim-3 and PD-1 in the peripheral blood relative to bone marrow may suggest the expansion of CD8+ T cells with exhausted phenotype into the circulation in patients with severe disease and poor outcome. In HIV-positive VL patients, the proportion of PD-1^{+}Tim-3^{+}LAG-3^{-} CD8+ T cells in the peripheral blood was also marginally higher ($P = .066$) than in the paired splenic aspirate samples.

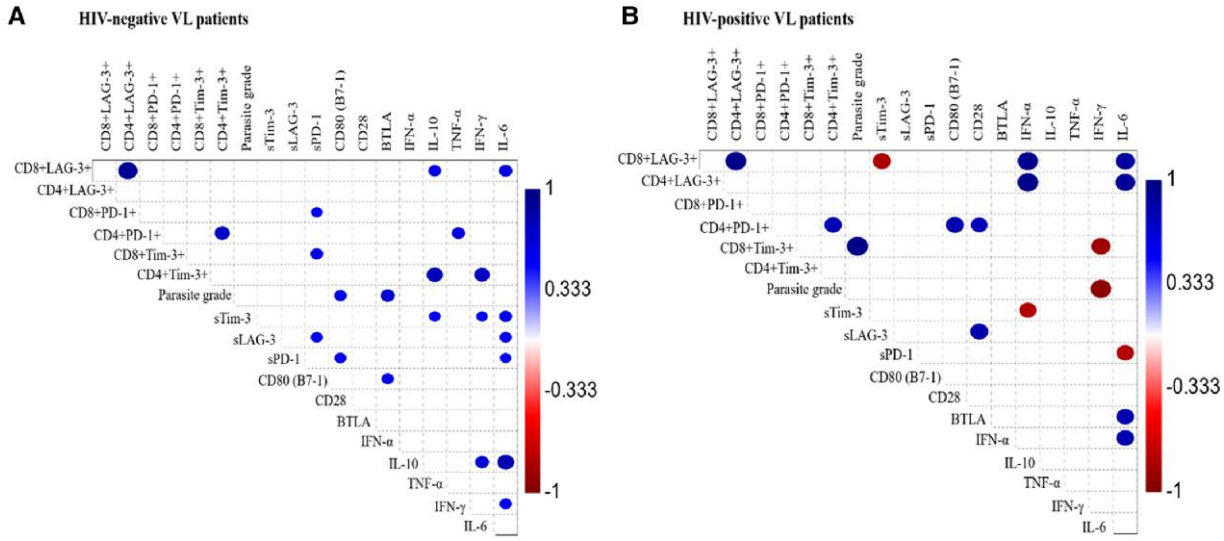

Figure 5. Correlation between T-cell co-inhibitory receptor expression, plasma soluble co-inhibitory receptors and cytokines, and parasite grade. *A*, HIV-negative and (*B*) HIV-positive subjects with VL. Determined by Pearson correlation. Empty cells indicate absence of correlation. Dots represent significant correlations ($P < .05$), with a larger dot representing a smaller *P* value. The color of dots corresponds to the correlation coefficient value as presented in the color scale at the side of each graphic.

Relationship of co-inhibitory Receptors With Plasma Cytokine Levels and Parasite Load in Subjects With VL

Compared to endemic healthy controls, HIV-negative subjects with VL showed increased plasma concentrations of IFN-γ, IL-6, TNF-α, and IL-10, all of which decreased significantly with treatment (Supplementary Table 5). In HIV-positive subjects with VL, IFN-γ, IL-10, and TNF were increased compared to HIV-positive controls, and IFN-γ and IL-10 decreased significantly with treatment. IL-6 was higher in deceased versus surviving HIV-negative subjects with VL ($P = .024$). Deceased HIV-negative subjects with VL had insignificantly higher plasma levels of IFN-γ and IL-10 compared to subjects who survived (Supplementary Table 4). Pearson correlation analysis revealed that sTim-3, sLAG-3, and sPD-1 showed significant positive correlations with IL-6, as did IL-10 ($r = 0.7903$, $P = .0008$) and IFN-γ ($r = 0.5709$, $P = .033$) in HIV-negative subjects with VL (Figure 5*A*). In HIV-positive subjects with VL, sPD-1 was negatively correlated with IL-6 ($r = -0.7961$, $P = .032$). Likewise, a negative correlation was observed between sTim-3 and IFN-α of HIV-coinfected subjects with VL ($r = -0.7828$, $P = .037$). In HIV-negative subjects with VL, both cellular expression and soluble forms of Tim-3, PD-1, and LAG-3 showed no significant correlation with *Leishmania* parasite grade. In HIV-positive subjects with VL, however, there was strong positive correlation between parasite load and Tim-3+ CD8+ T cells ($r = 0.96$, $P < .001$), and negative correlation with plasma IFN-γ ($r = -0.935$, $P = .002$) (Figure 5*B*).

DISCUSSION

In this prospective cohort study, we found that VL in both HIV-negative and HIV-positive patients was accompanied by an increased proportion of CD4+ and CD8+ T cells that expressed PD-1 and Tim-3, and these markers declined with treatment. Plasma levels of soluble PD-1 (sPD-1) and sTim-3 were also elevated in subjects with VL and responded to treatment. Although it is not surprising that expression of these pathological receptors declined with clinical improvement, the significant reduction at the completion of the 17-day treatment regimen suggests that they could be used as markers of early treatment response and could help identify patients at risk for poor clinical outcomes.

The PD-1/PD-L1 axis has been identified as a regulator of anti-leishmanial immunity in patients with VL and in representative animal models [16, 17, 24]. Increased expression of Tim-3 on CD8+ T cells was also found in VL patients [25]. Tim-3 impedes anti-VL immune response by downregulating the activation and maturation of dendritic cells [26]. We also found increased combined expression of PD-1 and Tim-3 in subjects with VL, which is characteristic of exhausted T cells, and was reported in patients with cutaneous leishmaniasis [16]. Mortality in HIV-negative subjects with VL was associated with coexpression of PD-1 and Tim-3 on CD8+ T cells, but not with their solo expression. Thus, severe VL may be driven by the combined expression of PD-1 and Tim-3. Coexpression is not surprising because transcription of multiple co-inhibitory receptors was found to be cooperatively driven by a common immunoregulatory pathway [27]. Consistent with findings in other chronic infections [28], subjects with VL expressed LAG-3 in a lower proportion of T cells

compared to PD-1 and Tim-3, and LAG-3 expression was no different than controls. LAG-3 may be retained intracellularly or rapidly shed from the cell surface [29]. However, in HIV-negative subjects with VL who died before treatment was completed, we found a greater proportion of LAG-3+ PD-1+ Tim-3+ triple-positive CD4+ and CD8+ T cells compared to those who survived, suggesting that LAG-3 may have an additive effect when expressed concurrently with other co-inhibitory receptors. Although high T-cell CTLA-4 expression was previously reported in VL, its blockade did not decrease in parasite load [14]. Our study found no difference in CTLA-4 expression in peripheral blood T cells from subjects with VL and controls.

Expression of T cell co-inhibitory receptors over the course of disease, treatment, and recovery, and their potential as markers for poor clinical outcome had not been previously investigated. In northwest Ethiopia, where this study was conducted, reported mortality rates ranged from 3% to 40% [20, 30–32], consistent with the 20% early mortality in this study. Risk factors for poor treatment outcomes include malnutrition, severe anemia, liver dysfunction, edema, older age, signs of sepsis, high parasite burden, and co-infection with HIV [20, 33, 34]. Only malnutrition (low body mass index) and HIV co-infection were present in our cohort. The potential for immune checkpoint expression to identify patients at higher risk of a poor treatment outcome, as shown here, would be valuable in optimizing clinical care. However, flow cytometry is expensive and not available in most settings where patients with VL are seen. Consistent with high T-cell expression of PD-1 and Tim-3, we observed elevated plasma sPD-1 and sTim-3, measured by simple immunoassay, in both HIV-negative and HIV-positive patients with VL. Parallel to the finding in T cells, plasma sLAG-3 was greater in deceased compared to surviving subjects with VL. Collectively, these findings suggest that soluble immune checkpoints could be useful markers of poor clinical outcomes in VL patients.

Host resistance to leishmaniasis is related to production of IFN-γ by CD4+ T cells, CD8+ T cells, and NK cells which activate macrophages to produce nitric oxide and kill the parasite [3, 4]. Despite IFN-γ production being a marker of protection during leishmaniasis, we and others demonstrated high levels of IFN-γ in the bloodstream of patients with active VL [35]. Our findings in this study suggest that IFN-γ production in patients with VL is part of a continuum of T-cell activation that ultimately leads to exhaustion. On the other hand the anti-inflammatory cytokine IL-10 is increased during VL and downregulates the production of IFN-γ and impairs *Leishmania* killing [36]. In this study, we observed an increase in IL-10 irrespective of the level of IFN-γ and parasite burden.

This study has limitations. First, the small sample size requires confirmation of these findings in a larger study. Second, our evaluation of the association of immune checkpoints with clinical outcome was limited to early mortality and posttreatment survival. Studies with rigorous scoring of disease severity and longitudinal follow-up are needed to determine if immune checkpoints can be used as markers of durable cure or relapse because persistently elevated levels could prompt an extended or alternate treatment regimen. Third, we did not evaluate the impact of the inhibitory receptor expression on T-cell effector function and parasite control. A mechanistic understanding of the functional role of these co-inhibitory receptors in human VL is critical. We posit that the immune checkpoint(s) that have greater suppression of effector function would be the best markers for disease severity and clinical outcomes.

In conclusion, we found increased expression of PD-1 and Tim-3 on CD4+ and CD8+ T cells, and their plasma soluble forms in patients with VL, which declined with treatment. Mortality in HIV-negative subjects with VL was associated with increased CD8+ T cells coexpressing Tim-3 and PD-1, triple-positive CD4+ and CD8+ T cells (PD-1^{+}Tim-3^{+}LAG-3^{+}), and elevated sLAG3. These data suggest that T-cell co-inhibitory receptor expression may be clinically useful in identifying patients at risk for poor treatment response or clinical outcome, which could prompt a change in type or duration of therapy.

Supplementary Data

Supplementary materials are available at *Open Forum Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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Author Contributions. M.A., T.A., and P.C.M. conceptualized the study and designed the methodology. M.A. and Y.O. conducted the experiments. M.A., Y.O., H.F., D.A., T.M., R.H., B.L.T., A.H., T.A., and P.C.M. contributed to data curation and formal analysis. M.A. wrote the original draft of the manuscript. Y.O., H.F., D.A., T.M., R.H., B.L.T., A.H., T.A., and P.C.M. reviewed and edited the manuscript. P.C.M. secured the funding. T.A. and P.C.M. supervised the study. All authors critically reviewed and subsequently approved the final version.

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Data Availability. The data presented in this study are available in the article and supplementary tables.

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