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Decreased memory T-cell response and function in human immunodeficiency virus-infected patients with tegumentary leishmaniasis

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The effects of human immunodeficiency virus (HIV) on the immune response in patients with cutaneous leishmaniasis have not yet been fully delineated. This study quantified and evaluated the function of memory T-cell subsets in response to soluble Leishmania *antigens (SLA) from patients coinfected with HIV and* Leishmania *with tegumentary leishmaniasis (TL). Eight TL/HIV coinfected subjects and 10 HIV seronegative subjects with TL were evaluated. The proliferative response of CD4$^+$ and CD8$^+$ T-cells and naïve, central memory (CM) and effector memory (EM) CD4$^+$ T-cells in response to SLA were quantified using flow cytometry. The median cell division indices for CD4$^+$ and CD8$^+$ T-cells of coinfected patients in response to SLA were significantly lower than those in patients with* Leishmania *monoinfection ($p < 0.05$). The proportions of CM and EM CD4$^+$ T-cells in response to SLA were similar between the coinfected patients and patients with* Leishmania *monoinfection. However, the median CM and EM CD4$^+$ T-cell counts from coinfected patients were significantly lower ($p < 0.05$). The reduction in the lymphoproliferative response to* Leishmania *antigens coincides with the decrease in the absolute numbers of both EM and CM CD4$^+$ T-cells in response to* Leishmania *antigens in patients coinfected with HIV/*Leishmania.

Key words: *Leishmania* - HIV - coinfection - memory CD4$^+$ T-cells

Human immunodeficiency virus (HIV) type 1 is a human retrovirus that infects roughly 34 million people worldwide and is responsible for the majority of the acquired immune deficiency syndrome (AIDS) epidemic. The most severely affected areas are in Sub-Saharan Africa, the Caribbean, Eastern Europe and Central Asia (UNAIDS 2012). *Leishmania* have been recognised as opportunistic pathogens in immunocompromised individuals, including those infected with HIV (Badaro 1997, Cruz et al. 2006). Over the past decades, the HIV epidemic has spread from urban areas to suburban and rural areas, where leishmaniasis is found. In particular, *Leishmania* and HIV infection overlap in several subtropical and tropical regions around the world (Karp & Auwaerter 2007). In northern India, Sudan and Ethiopia, visceral leishmaniasis has been a major scourge in patients infected with HIV (Wolday et al. 2001, Mathur et al. 2006, ter Horst et al. 2008). In Brazil, cutaneous disease is also a common presentation of leishmaniasis in HIV-coinfected patients (Rabello et al. 2003, Carranza-Tamayo et al. 2009, Lindoso et al. 2009). These patients often present with multiple skin ulcers, disseminated lesions and nasopharyngeal and/or genital mucosal damage (Coura et al. 1987, Machado et al. 1992, Da-Cruz et al. 1999, Lindoso et al. 2009). The clinical outcome of *Leishmania* infection is dependent upon the balance of T-helper (Th)1 and Th2 cytokine production during the immune response (Bacellar et al. 2000, Bourreau et al. 2003). In HIV/*Leishmania* coinfected patients with visceral disease, HIV infection alters the Th1/Th2 balance, resulting in an increased Th2 cytokine response while levels of the Th1 cytokines interleukin (IL)-12 and IL-18 are decreased (Nigro et al. 1999, Wolday et al. 2000). This results in more aggressive and relapsing visceral leishmaniasis. The effects of HIV on the immune response in cutaneous leishmaniasis have not yet been fully delineated. Reduced interferon (IFN)-γ production, decreased IFN-γ/IL-10 ratios (Rodrigues et al. 2011) and diminished lymphoproliferative responses to *Leishmania* antigens have been described in HIV-infected patients with the cutaneous form of leishmaniasis (Da-Cruz et al. 1992, 1999). These findings suggest that coinfected HIV/*Leishmania* patients may have a memory T-cell defect. Here, we have quantified and assessed the function of memory T-cell subsets in response to soluble *Leishmania* antigens (SLA) from patients coinfected with HIV and *Leishmania* with tegumentary leishmaniasis (TL).

SUBJECTS, MATERIALS AND METHODS

Eight patients with TL and HIV infection and 10 HIV seronegative subjects with TL were included in the study. The subjects were enrolled from the AIDS clinical centre of the University Hospital Professor Edgard Santos, located in Salvador, Brazil, after informed consent.

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Clinical and demographic characteristics of the HIV/*Leishmania* coinfected subjects are presented in Table I. All *Leishmania* monoinfected patients had active cutaneous leishmaniasis at the time of inclusion in the study. Six of these patients had cutaneous leishmaniasis, one had disseminated cutaneous leishmaniasis and three had mucosal leishmaniasis.

Peripheral blood mononuclear cells (PBMCs) were isolated by passage over a Ficoll-Hypaque gradient (Amersham Biosciences, Piscataway, NJ, USA). PBMCs (3 x 10^6 cells) were resuspended in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 2 mM L-glutamine, penicillin (100 U/mL), streptomycin (100 mg/mL) (GIBCO) and 5% heat-inactivated human AB serum. The cells were stimulated with SLA obtained from the *Leishmania chagasi* (MHOM/BR2000/Merivaldo2) strain [isolated from a patient with visceral leishmaniasis (Baleeiro et al. 2006)] or phytohemagglutinin (PHA) (Sigma-Aldrich). Control cultures were also established with medium alone.

To evaluate the proliferative response of T-cell subsets to SLA, 1.5 x 10^6 PBMC/mL were labelled with carboxyfluorescein succinimidyl ester (Invitrogen, Eugene, OR, USA) as described by the manufacturer. PBMCs were cultured in 12 × 75-mm tissue culture tubes for five days at 37ºC in a humidified 5% CO_2 atmosphere. Then, the cells were washed with phosphate-buffered saline (PBS)/bovine serum albumin and surface stained with anti-CD4-phycoerythrin (PE) (Becton Dickinson, CA, USA) and anti-CD8-PE-Cy5 (eBioscience, CA, USA) for 30 min at 4ºC. After fixation in 1% formaldehyde, the cells were acquired by FACSAria (Becton Dickinson) and analysis was performed using FlowJo software version 7.6 (Tree Star Inc, Ashland, OR, USA). At least 30,000 events were analysed per sample. The proliferation intensity was determined through the percentage of the cell division index. The threshold to define positive proliferative response was a cell division index above 0.06 for $CD4^+$ T-cells and above 0.09 for the $CD8^+$ T-cell subset as described previously (Pinto et al. 2011).

Alternatively, to determine the frequency of memory $CD4^+$ T-cells, 1.5 x 10^6 PBMCs were cultured with SLA, PHA or PBS for 18 h in supplemented RPMI-1640 medium in the presence of 0.5 µg of purified anti-CD28 antibody at 37ºC in a humidified 5% CO2 atmosphere. Then, the cells were stained with anti-CD4-(fluorescein isothiocyanate), anti-CD45RA-PE, anti-CD62L-PE-Cy5 and anti-CCR7-PE-Cy7 antibodies (Becton Dickinson). Cells were acquired by FACSAria and analysed using the Flowjo software. Naïve cells were defined as $CD4^+CD45RA^+CD62L^+CCR7^+$, central memory (CM) cells were defined as $CD4^+CD45RA\text{-}CD62L^+CCR7^+$ and effector memory (EM) cells were defined as $CD4^+CD45RA\text{-}CD62L\text{-}CCR7\text{-}$. Absolute naïve, CM and EM lymphocyte subpopulation counts were obtained by multiplying the subpopulation percentage by the absolute lymphocyte count.

The data are expressed as median and interquartile range (IQR). Statistical analysis was performed using GraphPad Prism 5.02 (La Jolla, CA, USA). The Mann-Whitney *U* test was used for group comparisons and the Wilcoxon test was used for the analyses of cell count and cell proportion in the presence and absence of antigen.

Institutional review board approvals were obtained from Brazilian National Ethical Commission, Oswaldo Cruz Foundation and the University of California San Diego Human Research Protection Program.

RESULTS

The median $CD4^+$ T-cell count from HIV/*Leishmania* coinfected patients (241 cells/mm^3, IQR 85-453) was significantly lower compared with that of *Leishmania* monoinfected patients (784 cells/mm^3, IQR 599-1477) ($p = 0.004$). The median HIV viral load of HIV/*Leishmania* coinfected patients was 4.6 log copies/mL (IQR 1.9-5.2). The sex ratio and median age were similar in both groups.

The median cell division indices in response to SLA for $CD4^+$ (0.06, IQR 0.05-0.1) and $CD8^+$ T-cells (0.05, IQR 0.01-0.08) from HIV/*Leishmania* coinfected patients were significantly lower than those from *Leishmania* monoinfected patients ($CD4^+$ T-cell: 0.18, IQR 0.15-0.26;

TABLE I

Clinical and epidemiological characteristics of human immunodeficiency virus (HIV)/*Leishmania* coinfected patients

Coinfected group	Gender	Age (year)	$CD4^+$ T-cell count (cell/mm^3)	Viral load (log copies/mL)	ART use	Leishmaniasis form
TL/HIV 03	F	42	93	5.4	Unknown	Mucocutaneous active
TL/HIV 05	F	22	291	1.9	Unknown	Cutaneous active
TL/HIV 06	M	36	165	5.3	Unknown	Cutaneous treated
TL/HIV 08	F	62	415	2.7	Yes	Disseminated cutaneous treated
TL/HIV 09	M	59	78	5.0	Yes	Cutaneous active
TL/HIV 12	M	36	615	1.9	Yes	Cutaneous active
TL/HIV 13	M	36	104	5.0	No	Cutaneous active treated
TL/HIV 14	M	25	241	4.6	Yes	Mucocutaneous active

ART: antiretroviral treatment; F: female; M: male; TL: tegumentary leishmaniasis.

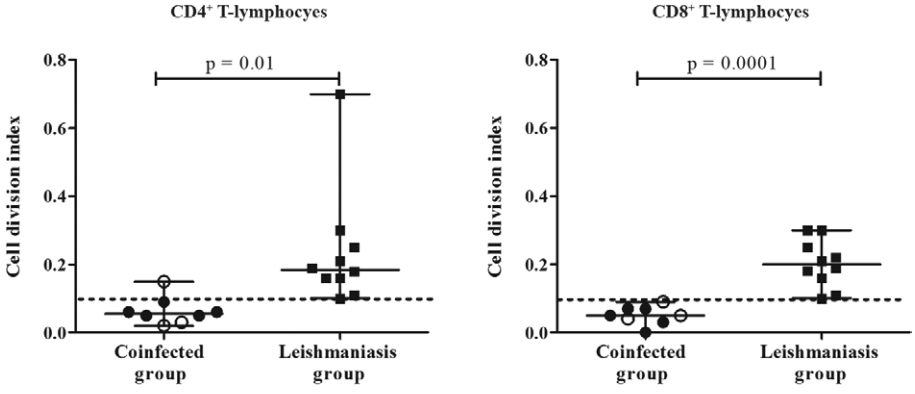
$CD8^+$ T-cell: 0.2, IQR 0.15-0.26) ($p < 0.05$) (Figure). A lymphoproliferative response to SLA was detected in only one coinfected patient (TL/HIV 06) (12%) who had a previously treated cutaneous leishmaniasis infection. In contrast, $CD4^+$ and $CD8^+$ T-cell subsets from all *Leishmania* monoinfected patients proliferated in response to SLA. The $CD4^+$ and $CD8^+$ T-cell subsets from both HIV/*Leishmania* coinfected and *Leishmania* monoinfected patients all proliferated in response to PHA.

The proportions of naïve, CM and EM $CD4^+$ T-cells obtained after stimulation with SLA were similar between the HIV/*Leishmania* coinfected and *Leishmania* monoinfected groups (Table II). However, the median absolute counts of naïve, CM and EM $CD4^+$ T-cells from HIV/*Leishmania* coinfected patients were significantly lower compared with those of *Leishmania* monoinfected patients ($p < 0.05$). Following SLA stimulation, a statistically significant increase in the median absolute counts of EM $CD4^+$ T-cells was observed in the cultures from *Leishmania* monoinfected patients ($p = 0.005$). In contrast, there was no change in the median absolute counts of EM $CD4^+$ T-cells of HIV/*Leishmania* coinfected patients in the presence of SLA and a statistically significant decrease in the CM $CD4^+$ T-cell count was observed after culture with SLA ($p = 0.02$). However, there was a statistically significant increase in the absolute number of EM $CD4^+$ T-cells from both HIV/*Leishmania* coinfected and *Leishmania* monoinfected groups following PHA stimulation.

DISCUSSION

This study demonstrates that a reduction in the lymphoproliferative response to *Leishmania* antigens coincides with a decrease in the absolute numbers of both EM and CM $CD4^+$ T-cells in response to *Leishmania* antigens in patients coinfected with HIV/*Leishmania*. Although the proportion of memory $CD4^+$ T-cells subsets was similar between monoinfected *Leishmania* and coinfected HIV/*Leishmania* patients, the median absolute count of EM lymphocytes in coinfected patients was four-fold lower compared with that of *Leishmania* monoinfected patients. Moreover, following *Leishmania* antigen stimulus, the EM $CD4^+$ T-cell count of HIV/*Leishmania* coinfected patients remained below 10 cells/mm^3, whereas we observed a three-fold increase in the EM $CD4^+$ T-cell count in the *Leishmania* monoinfected patients. The low number of EM $CD4^+$ T-cells in HIV/*Leishmania* coinfected patients might explain the reduced IFN-γ production in response to *Leishmania* antigens observed in coinfected subjects as well as the atypical parasitic spread, frequent relapse and treatment failure observed in these patients (Da-Cruz et al. 1992, Cruz et al. 2006, Lindoso et al. 2009). Although memory and effector $CD8^+$ T-cell profiles were not evaluated in our study, $CD8^+$ T-cells from all HIV/*Leishmania* coinfected patients were unable to proliferate in response to SLA. Previous work has demonstrated the protective role of $CD8^+$ T-cells in *Leishmania* infection, with healing of lesions associated with high IFN-γ production and an increase in the number of $CD8^+$ T-cells (Da-Cruz et al. 1994, Muller et al. 1994).

The absence of a lymphoproliferative response to *Leishmania* antigens in both the $CD4^+$ and $CD8^+$ T-cell subsets examined in our study suggests impairment of the cellular immune response directed at the parasitic infection. Although the HIV/*Leishmania* coinfected group was heterogeneous with respect to clinical presentation of leishmaniasis (cutaneous vs. mucosal) and stage of treatment (active vs. treated), a significant lack of immune response to *Leishmania* antigens was observed



Proliferative response to *Leishmania* antigens of $CD4^+$ and $CD8^+$ T-cells using flow cytometry from human immunodeficiency virus (HIV)/*Leishmania* coinfected patients with active tegumentary leishmaniasis (TL) (●) and *Leishmania* monoinfected patients (■). Open circles (○) indicate HIV/*Leishmania* coinfected patients with treated TL. Medians are indicated by horizontal bars. Mann Whitney *U* test was used to compare groups. Significant differences were found between groups ($p < 0.05$). A: cell division index of $CD4^+$ T-cells. The dashed line represents the threshold to define positive proliferative response (cell division index > 0.06); B: cell division index of $CD8^+$ T-cells. The dashed line represents the threshold to define positive proliferative response (cell division index > 0.09). Median cell division index without stimulus (medium): HIV/*Leishmania* co-infected patients 0.02 [interquartile range (IQR) 0.01-0.03] and 0.02 (IQR 0.0-0.04), *Leishmania* monoinfected patients 0.03 (IQR 0.0-0.07) for $CD4^+$ and $CD8^+$ T-cell subsets, respectively. Median cell division index following phytohaemagglutinin stimulation: HIV/*Leishmania* co-infected patients 0.7 (IQR 0.2-1.3) and 1 (IQR 0.6-1.4), *Leishmania* only infected patients 0.3 (IQR 0.1-0.5) and 0.2 (IQR 0.1-0.4) for $CD4^+$ and $CD8^+$ T-cell subsets, respectively.

across this group of subjects. Only one HIV/*Leishmania* coinfected patient exhibited a proliferative response to *Leishmania* antigens. This patient was successfully treated for TL before his inclusion in the study and his lesions were in remission. However, the two other coinfected individuals with treated TL did not develop a proliferative response. Additionally, we found that a decrease in the number of CM $CD4^+$ T-cells occurred following SLA stimulation in all cultures of coinfected patients and EM $CD4^+$ T-cell counts of these patients did not change regardless of presentation of leishmaniasis or stage of treatment. Previous work has shown that the restoration of $CD4^+$ T-cell-specific responses to antigens in HIV-infected patients undergoing antiretroviral treatment (ART) requires a sustained increase of at least 60 $CD4^+$ T-cells/μL compared with baseline levels and a reduced viral load (Li et al. 1998). In addition to the quantitative immune defect observed in HIV infection, the quality of the immune response (i.e., degree of immune memory impairment) may also be important in the host response to other pathogens such as *Leishmania*.

Studies in both mice and humans have demonstrated multiple $CD4^+$ T-cell phenotypes and a broad spectrum of functions have been attributed to these cells in different viral infections (Chen et al. 2001, de Rosa et al. 2001, Roman et al. 2002). Memory T-cells are divided into subpopulations based on the expression of CCR7, a chemokine receptor involved in homing to secondary lymphoid organs and CD45RA, a cell surface molecule involved in T-cell activation. T-cells expressing CD45RA and CCR7 are termed naïve, whereas those with downregulated CD45RA are termed CM cells and produce IL-2. Memory T-cells lacking expression of CCR7 and CD45RA are termed EM T-cells and produce IFN-γ (Sallusto et al. 1999). The functional and phenotypic heterogeneity of memory T-cells are influenced by variations in antigen exposure and persistence. A dominant single IL-2 and/or IL-2/IFN-γ CD4 T-cell response is normally associated with clearance of antigen, whereas a dominant single IFN-γ CD4 T-cell response is associated with antigen persistence and high antigen levels (Harari et al. 2005). Effector $CD4^+$ T-cells are lost in the absence of parasites, whereas CM $CD4^+$ T-cells are maintained. Upon secondary infection, these CM T-cells become effector T-cells and mediate protection (Zaph et al. 2004). In our study, a decrease in the number of CM $CD4^+$ T-cells was observed in the coinfected group following stimulation with SLA. It is possible that during coinfection with HIV and *Leishmania*, the immune activation caused by both HIV and *Leishmania* infection induces increased apoptosis of parasite-specific memory $CD4^+$ T-lymphocytes (Wolday et al. 1999a, b). The decreased numbers of CM cells and EM cells would reduce the ability of the immune system to develop an adequate systemic response to control the *Leishmania* infection. However, the overall hyporesponsiveness of the cells to *Leishmania* antigen stimulation could also suggest that a qualitative defect may be contributing to this phenomenon. Thus, although an impaired memory response is known to be related to the depletion of $CD4^+$ T-cells *via* mechanisms related directly or indirectly to HIV (systemic activation and replicative senescence), a defective functional memory response may also be playing a role (Moro-Garcia et al. 2012). Interestingly however, we found that our coinfected cells responded well to PHA, suggesting that the immunodeficiency was parasite-specific.

Viral suppression by ART may be a contributing factor and in this study, several of our subjects were not receiving ART. In the future, we will examine how the

TABLE II

Quantification of naïve, central (CM) and effector (EM) memory $CD4^+$ T-cell subsets in response to soluble *Leishmania* antigens (SLA) and to phytohaemagglutinin (PHA) from of human immunodeficiency virus (HIV)/*Leishmania* coinfected and *Leishmania* monoinfected groups

	Medium			SLA			PHA		
$CD4^+$ T-cell subset	Coinfected	Monoinfected	p	Coinfected	Monoinfected	p	Coinfected	Monoinfected	p
Naïve									
n (%)	30 (17-37)	43 (18-80)	0.8	39 (17-64)	34 (15-57)	0.7	2 (1-4)[b]	4 (2-9)[b]	0.5
n (cell/mm^3)	9 (3-14)	17 (5-65)	0.02	3 (2-11)	42 (25-106)	0.03	2 (0-6)[b]	13 (4-28)	0.02
CM									
n (%)	23 (8-31)	24 (6-32)	0.6	11 (4-25)	8 (2-23)[a]	0.9	1 (0-4)[b]	2 (1-3)	0.3
n (cell/mm^3)	55 (31-105)	49 (19-103)	0.001	3 (1-5)[a]	46 (25-119)	0.8	1 (0-3)[b]	8 (2-16)[b]	0.02
EM									
n (%)	12 (11-29)	9 (6-15)	0.2	21 (11-43)	17 (14-23)[a]	0.07	35 (20-40)[b]	30 (25-38)[b]	0.8
n (cell/mm^3)	8 (5-11)	36 (12-63)	0.0009	6 (2-9)	122 (69-143)[a]	0.0001	27 (11-58)[b]	175 (105-236)[b]	0.0005

Wilcoxon test was used to compare cells cultured with medium and SLA (*a*) and cells cultured with medium and PHA (*b*) for each group. Data are presented as median (interquartile range). p: Mann-Whitney *U* test was used to compare HIV/*Leishmania* coinfected and *Leishmaniasis* monoinfected groups.

immune response to *Leishmania* is reconstituted under ART. Although the impact of HIV-1 on host cellular immune function clearly plays an important role in facilitating *Leishmania* infection in dually infected individuals, there is increasing evidence that other mechanisms for the interaction of these two pathogens might also be involved. Particularly, the immunological mechanisms involved in the pathogenesis of mucocutaneous lesions in HIV-infected individuals remain unclear. We hypothesise that innate immunity to *Leishmania* is also compromised in coinfected patients, resulting in the more severe pathogenesis of mucocutaneous lesions observed in coinfected patients. Future work will be required to evaluate the effect of coinfection on innate immunity.

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