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# Case Report: A Potential Role for Mononuclear Phagocytes in Cutaneous Ulcer Development in Human Immunodeficiency Virus–*Leishmania braziliensis* Coinfection

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*Abstract.* Skin ulcer development in cutaneous leishmaniasis due to *Leishmania braziliensis* infection is associated with a mononuclear cell infiltrate and high levels of tumor necrosis factor (TNF). Herein, we show that despite the absence of *Leishmania*-driven TNF, a cutaneous leishmaniasis patient with acquired immunodeficiency syndrome developed a skin ulcer. The presence of mononuclear phagocytes and high levels of TNF, chemokine (C-C motif) ligand 2 (CCL2), and metalloproteinase-9 in tissue are identified as potential contributors to immunopathology observed in *L. braziliensis*-infected patients.

## INTRODUCTION

Ulcer development in cutaneous leishmaniasis (CL) due to *Leishmania braziliensis* infection is associated with an exaggerated inflammatory response characterized by activated $CD4^+$ T cells and high levels of tumor necrosis factor (TNF).[1,2] Moreover, there is an association between the inflammatory infiltrate and an increased frequency of $CD8^+$ T cells expressing granzyme B.[3–6]

Human immunodeficiency virus (HIV) infection leads to a decrease in $CD4^+$ T cell count, increasing the risk for opportunistic disease progression.[7] Coinfection with HIV and *L. braziliensis* has been reported, and the clinical aspects include large and multiple ulcers as well as mucosal involvement.[8,9] Ulcer development in *L. braziliensis* infection is associated with exaggerated immune response.[1,10] Because HIV patients have decreased numbers of $CD4^+$ T cells, we hypothesize here that the presence of mononuclear phagocyte–derived immunological factors is associated with skin tissue damage in a patient with HIV and *L. braziliensis* coinfection.[11]

Monocytes are circulating cells, precursor of macrophages and dendritic cells, which participate in the immune response to *Leishmania* by several mechanisms, such as secreting soluble factors that recruit or activate leukocytes, presenting antigen, and killing parasites. Circulating monocytes are heterogeneous, and based on the expression of CD14 and CD16, they can be divided into classical ($CD14^+CD16^-$), intermediate ($CD14^+CD16^+$), and nonclassical ($CD14^{dim}CD16^+$) subsets.[12] These subsets differ in their production of inflammatory and regulatory mediators, and the frequency of $CD16^+$ monocytes is often increased in inflammatory conditions.[12] Products secreted by monocytes during CL include TNF, chemokines (CXCL9, CXCL10, and CCL2), and metalloproteinase-9 (MMP-9), and some reports indicate the participation of TNF and monocyte-derived MMP-9 in tissue damage during leishmaniasis.[2,13,14] Herein, we evaluated the immune response from a patient with HIV and *L. braziliensis* coinfection and found mononuclear phagocytes infiltrating the lesion and production of TNF, CCL2, and MMP-9 at lesion site.

## MATERIALS AND METHODS

**Patients.** We evaluated immunological and pathological parameters of an HIV/CL patient and compared it with four CL patients from the same endemic area. All patients had an active cutaneous lesion with no mucosal involvement at the time specimens were collected. Patients with CL without HIV had no past history of leishmaniasis. The immunological evaluation of CL patients without HIV was performed prior to treatment with pentavalent antimony, and the immunological evaluation of the HIV/CL patient was performed at the time of HIV diagnosis prior to antiretroviral therapy (tenofovir, lamivudine, and efavirenz). All participants agreed to participate in the study and signed informed consent. This work was approved by the Ethics Committee Board of the University Hospital of Federal University of Bahia.

**Antigen.** Soluble *Leishmania* antigen (SLA) was prepared from *L. braziliensis* isolated from a patient with CL by sonication, tested for endotoxin using the *Limulus* amebocyte lysate test and used at a concentration of 5 μg/mL.

**Peripheral blood assay.** Peripheral blood mononuclear cells (PBMCs) were obtained from heparinized venous blood layered over a Ficoll-Hypaque gradient (GE Healthcare, Piscataway, NJ), then washed and resuspended in RPMI 1640 media, supplemented with 10% heat inactivated AB serum (Sigma-Aldrich, St. Louis, MO). Cells were cultured at 37°C, 5% $CO_2$ in presence of SLA (5 μg/mL), and after 72 hours, cytokines (interferon gamma [IFN-γ] and TNF), chemokines (CXCL9, CXCL10), and MMP-9 levels were determined by enzyme-linked immune sorbent assay (ELISA) (R and D Systems) on culture supernatants. For flow cytometry, cells were stained as described in the section Immunohistochemistry.

**Immunohistochemistry.** Tissues obtained from lesion biopsies of HIV/CL and CL patients were fixed in buffered formaldehyde and embedded in paraffin. Deparaffinization (5-μm thick sections), antigen retrieval, and dehydration were performed using Trilogy™ 1:100 (Cell Marque, Rocklin, CA) at 96°C. Immunohistochemistry reactions were performed after blockage of peroxidase activity with 3% hydrogen peroxide for 5 minutes and proteins with Protein Block Serum-Free

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(DAKO, Carpinteria, CA) for 15 minutes. The slides were incubated at 25°C with the respective antibodies and dilutions: anti-TNF alpha, 1:70 (MCA1385; AbD Serotec, Raleigh, NC); anti-MMP-9, 1:25 (MA12894; Thermo Scientific, Marietta, OH); anti-CXCL9 1:100 (AHP1864; AbD Serotec); anti-CCL2 1:25 (MCA5981GA; AbD Serotec, Raleigh, NC); and anti-CXCL10 1:10 (AHP782; AbD Serotec). A Mouse and Rabbit Peroxidase Kit/HRP (DBS KP500) was used to perform the reaction according to the manufacturer's recommendations. All of the slides were counterstained with Harris hematoxylin, dehydrated and mounted with Canada balsam and glass coverslips.

For image capture, an optical microscope (Olympus BX51, Waltham, MA) attached to a digital camera system (Olympus Q5) was used, and Image-Pro Plus (Media Cybernetics, Rockville, MD) was used for image analysis. Slides were photographed (five randomized fields from each section) at 40× and then stained with respective antibodies. In each field, the number of positive cells was quantified using the selection feature and semiautomatic counter of 1.48v ImageJ software (National Institutes of Health, Bethesda, MD). The positive cells were defined by the identification of the amplified molecules that reacted with the chromogenic substrate, DAB (eBioscience, San Diego, CA). In all reactions, a preselected pattern section was used as positive control and a section that had not been incubated with the primary antibody, for the negative control.

**Flow cytometry.** For flow cytometry, PBMC were obtained, stained with fluorochrome-conjugated antibodies for surface markers (CD4, CD8, CD14, and CD16 [BD-Bioscience, San Jose, CA]), and fixed using 2% formaldehyde. Samples were acquired (200,000 events) on a FACScanto II flow cytometer (BD Pharmingen, San Jose, CA), and analysis was performed using FlowJo software (Tree Star, Ashland, OR). The cells were gated based on the live cell gate and then gated on monocyte population based on size and complexity.

**HIV diagnosis.** HIV diagnosis was performed using the ELISA kit HIV-1.2.O (DiaSorin, Saluggia, Italy).

## RESULTS

**Case report.** In October 2011, a 31-year-old woman living in a *L. braziliensis* transmission area was admitted at the health post of Corte de Pedra, a reference center for American tegumentary leishmaniasis management, in Bahia state, northeastern Brazil. The patient had a 12 × 35-mm ulcer at the lower back region with 30 days of evolution. Montenegro's skin test (delayed type hypersensitivity test for *Leishmania*) was positive with induration of 10 × 12 mm. The patient was started on intravenous pentavalent antimony (20 mg/kg of body weight, daily for 20 days). In January 2012, the patient was readmitted to the heath post with reactivation (20 × 50 mm) of the original lesion, and a new series of pentavalent antimony was administered for 30 days. In May 2012, the patient presented partial healing of the lesion, and a third series of pentavalent antimony was administered for 20 days. In August 2012, the patient returned to the health post presenting with an active lower back lesion with secondary infection and new oral mucosal lesions. During this visit, skin biopsy from the primary lesion was performed, which showed *Leishmania* amastigote forms characterized as *L. braziliensis* species, detected by polymerase chain reaction.[15] The patient was referred to the Federal University Hospital at Salvador, Bahia, and treated with amphotericin B starting on September 2012 through January 2013. After she had accumulated a dose of 975 mg of amphotericin B, the ulcer was partially healed, and in March 2013, she was discharged from the hospital with complete cicatrization of the lesion after reaching a total dose of 1,500 mg of amphotericin B. In August 2013, the patient returned to the health post of Corte de Pedra with a history of treatment of indeterminate meningitis, presenting oral lesions and reactivation of the previous lower back ulcer. The patient was referred again to the Federal University Hospital at Salvador where HIV infection was diagnosed. Blood was collected and a biopsy was performed for immunological studies. The patient had a viral load of 89,296 copies/mL, $CD4^+$ cell counts of 105 cells/$mm^3$, and $CD8^+$ cell counts of 422 cells/$mm^3$. Antiretroviral therapy with tenofovir, lamivudine, and efavirenz was started. Also, the reactivation of cutaneous leishmaniasis was treated with liposomal amphotericin B (200 mg/day) associated with pentoxifylline (400 mg/12 hours) that was administered for 13 days. In November 2013, the patient returned for follow-up, and total scar formation was observed in the lesion. At this time the patient presented with undetected viral load, CD4 and CD8 cell counts of 171 and 409 cells/$mm^3$, respectively.

**Immunological results.** We evaluated immunological parameters from the HIV/CL and four CL patients from the same *L. braziliensis* transmission area. The frequency of monocyte subsets, $CD4^+$ and $CD8^+$ T cells was assessed by flow cytometry and is represented in Supplemental Table 1. The frequency of monocyte subsets was comparable between CL and HIV/CL individuals, and the HIV/CL patient had lower CD4/CD8 T cells ratio when compared with CL individuals (Supplemental Table 1).

To define the factors involved in parasite killing, tissue damage, and mononuclear phagocyte cell recruitment, we stimulated PBMCs from HIV/CL and CL patients with SLA for 72 hours and determined the levels of IFN-γ, TNF, IL-10, CXCL-9 and CXCL-10, and MMP-9 in culture supernatants by ELISA (Table 1). As expected, CL patients produced high levels of IFN-γ and TNF, and no IFN-γ was observed in the HIV/CL patient (Table 1). Unexpectedly, PBMC from the HIV/CL patient did not produce TNF in response to SLA (Table 1). Because the histopathology showed mononuclear phagocytes infiltrating the lesion obtained from the HIV/CL patient, and soluble factors derived from these cells can be associated with tissue damage, we decided to determine the production of chemokines (CXCL-9 and CXCL-10) involved

TABLE 1
Immune response from participants

| | CL (*N* = 4) | | HIV/CL | |
|---|---|---|---|---|
| | Media (pg/mL) | SLA (pg/mL) | Media (pg/mL) | SLA (pg/mL) |
| IFN-γ | 0 | 4,750 ± 2,848 | 2 | 12 |
| TNF | 0 | 1,359 ± 1,616 | 0 | 0 |
| IL-10 | 1 ± 2 | 118 ± 100 | 4 | 0 |
| CXCL-9 | 0 | 2,879 ± 1,642 | 0 | 3,000 |
| CXCL-10 | 0 | 5,216 ± 4,623 | 0 | 5,500 |
| MMP-9 | 912 ± 961 | 6,118 ± 3,240 | 1,503 | 6,555 |

CL = cutaneous leishmaniasis; CXCL = chemokine ligand; ELISA = enzyme-linked immune sorbent assay; HIV = human immunodeficiency virus; IFN = interferon; IL = interleukin; MMP = metalloproteinase; SLA = soluble *Leishmania* antigen; TNF = tumor necrosis factor.
Peripheral blood mononuclear cells were cultured for 72 hours in absence (media) or presence of SLA (5 μg/mL) and proteins measured by ELISA.

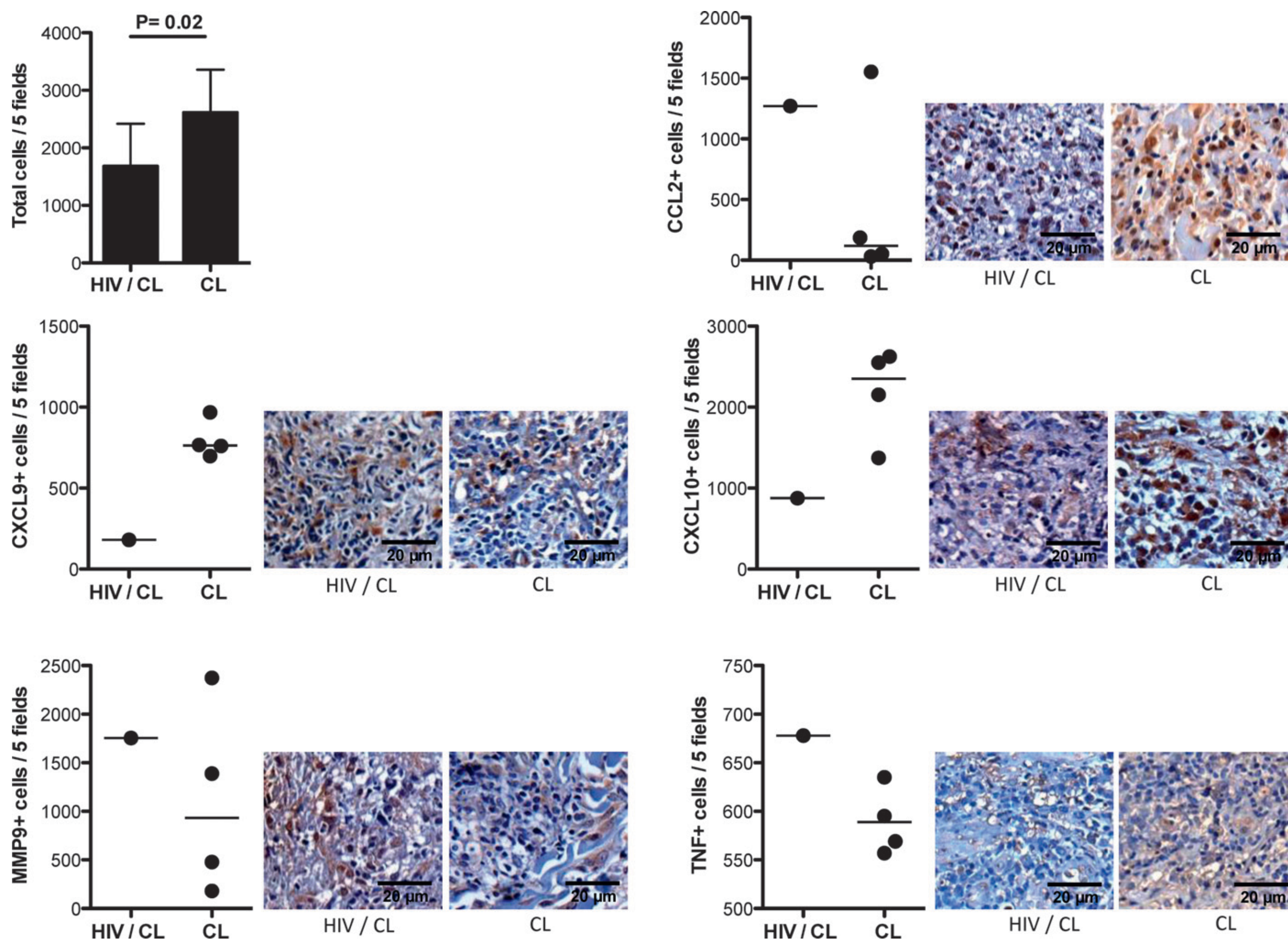


FIGURE 1. Immunohistochemistry for chemokines (CCL2, CXCL9, and CXCL10), metalloproteinase (MMP-9), and tumor necrosis factor (TNF). Immunohistochemistry was performed on lesion biopsies.

in mononuclear cells recruitment, and MMP-9, which has been shown to contribute to tissue damage in tegumentary leishmaniasis.[13] The levels of CXCL-9, CXCL-10 and MMP-9 were as high in the HIV/CL patient as those observed in CL individuals (Table 1). Dissociation between peripheral blood and skin immune response has been demonstrated.[16] Therefore, we decided to investigate whether inflammatory soluble factors secreted by mononuclear phagocytes would be present at lesion site (Figure 1). We found high amounts of CCL2, MMP-9, and TNF in the lesion from HIV/CL patient (Figure 1). Altogether, these results suggest that *L. braziliensis* parasite products may activate mononuclear phagocytes to produce inflammatory mediators in the absence of cell-mediated immune response. Future functional studies must be performed to test this hypothesis.

## DISCUSSION

HIV and CL due to *L. braziliensis* infection are diseases with contrasting immune responses. Although HIV causes immunosuppression with a decrease in $CD4^+$ T cell numbers, CL patients develop strong $CD4^+$ T cell response with high levels of IFN-γ and TNF. HIV and *L. braziliensis* coinfection is unusual, mainly due to the low prevalence of HIV-infected individuals living in *L. braziliensis* transmission regions.[17] A previous immunology study in HIV/CL patients has revealed lower levels of IFN-γ and IL-13 and low ratios of IFN-γ to IL-10 in response to stimulation with SLA when compared with CL-monoinfected patients.[18] In this report, we evaluated the immune response of a HIV/CL patient with low $CD4^+$ T cell counts, which, not surprisingly, did not produce IFN-γ in response to SLA.

Several studies indicate that the inflammatory response is the main factor driving skin ulcer development in CL, and a major role has been attributed to high levels of TNF observed in these individuals.[19,20] TNF can be detected in very early phases of *L. braziliensis* infection, and the levels increase as mononuclear cells infiltrate the lesion.[21] Interestingly, although in this study, we could not detect TNF in PBMC culture of the HIV/CL patient, staining for TNF was observed in the ulcer from this individual. These data suggest that T cells may not be the source for TNF production in peripheral blood and lesions during CL. We have previously shown that TNF is produced by $CD4^+$, $CD8^+$, and mononuclear phagocytes during CL.[10] Thus, since cell-mediated immune responses were decreased in the HIV/CL patient, we suggest here that mononuclear phagocytes are the main source of TNF in this individual. However, the contribution of lymphocytes to TNF

production and tissue damage cannot be ruled out because these cells, while in low amounts, were also present in lesion of the HIV/CL patient.

Monocytes may contribute to tissue damage by secreting inflammatory cytokines, chemokines, and MMPs. CXCL-9 and CXCL-10 are monocyte-derived chemokines that recruit lymphocytes. Although the levels of these chemokines were increased in cultures from CL and HIV/CL patients, low staining of CXCL-9 and CXCL-10 was found at the lesion site from the HIV/CL patient. Both CXCL-9 and CXCL-10 can be induced by IFN-γ, however, the HIV/CL patient did not produce IFN-γ, indicating other pathway involved in the induction of these chemokines. In fact, *L. braziliensis*-infected macrophages produce high levels of CXCL-9 and CXCL-10 in the absence of IFN-γ, indicating that factors from parasite that is also present in SLA may activate monocytes to produce these chemokines.[22] Our data show discordance between the peripheral blood and lesion site immune response. Dissociation between peripheral blood and peripheral tissue has also been observed in the immune response to purified protein derivative in healthy individuals.[16]

Degradation of extracellular matrix by MMPs contributes to cellular infiltration and tissue damage. MMP-9 degrades collagen type IV, the main component of basal membrane, present in skin, and participates in tissue destruction in inflammatory skin diseases such as psoriasis and dermatitis.[23,24] It has been reported that MMP-9 is produced by *L. braziliensis*-infected macrophages and is present in lesions of the inflammatory clinical form of *Leishmania* infection, mucosal leishmaniasis.[13] Here we showed that MMP-9 is present in lesions from the HIV/CL patient and CL individuals, suggesting that the elevated production of this enzyme may be contributing to tissue damage in both cases. Our results support the hypothesis of a major role for mononuclear phagocyte in induction of tissue damage in CL patients.

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