Could the lower frequency of CD8+CD18+CD45RO+ lymphocytes be biomarkers of human VL?

Jorge Clarencio1, Camila I. de Oliveira1, Cecília Favali1, Omar Medina1, Arlene Caldas2, Carlos Henrique Costa3, Dorcas L. Costa3, Cláudia Brodskyn1,4,5, Aldina Barral1,4,5 and Manoel Barral-Netto1,4,5

1Centro de Pesquisas Gonçalo Moniz, Fundação Oswaldo Cruz (FIOCRUZ), Salvador, Brazil
2Universidade Federal do Maranhão, São Luis, Brazil
3Universidade Federal do Piauí, Teresina, Brazil
4Universidade Federal da Bahia, Salvador, Brazil
5Instituto de Investigação em Imunologia, Salvador, Brazil

Keywords: CD18, flow cytometry, Leishmania

Abstract
Toward obtaining a more comprehensive understanding of factors governing activation and/or function during visceral leishmaniasis (VL), we have compared active disease (pre-treatment) versus post-chemotherapy immune response in VL patients by means of ex vivo staining with different cell markers. Our results show that during active disease, the frequency of T cells positive for CD25, CTLA-4 and CD45RO was significantly lower in VL patients compared with healthy controls, whereas cells staining positive for Annexin V and CD95 were significantly higher. In all cases, chemotherapy was able to restore these frequencies to normal levels. Interestingly, significant differences in the frequency of CD18 and in the frequency of CD45RO-positive cells were observed in the CD8+ T cell subset. These two frequencies were also significantly higher in bone marrow when compared with peripheral blood, suggesting a possible compartmentalization of certain CD8+ T cell populations during active disease. Given that CD8+ T cells have been shown to play an essential role in immunity to infection with Leishmania, our data indicate that the lower frequency of CD18+ and CD45RO+ lymphocytes in the bone marrow CD8+ T cell subset may be considered a biomarker of acute VL.

Introduction
Leishmaniasis remains a serious public health problem in several parts of the developing world. The disease presents a wide spectrum of clinical manifestations with either tegumentary or visceral involvement. Visceral leishmaniasis (VL) or kalaazar is a progressive infection with fatal outcome in the absence of treatment. Clinical symptoms of VL may include fever, weight loss, anemia, edema, bleeding episodes and huge hepatosplenomegaly accompanied by a high parasite burden in the spleen and liver. Whereas in the most common forms of tegumentary leishmaniasis parasite growth is controlled and an anti-Leishmania cell-mediated immunity (CMI) is mounted, lack of anti-Leishmania CMI has been considered a hallmark of VL [reviewed in (1)]. However, during active human VL, there is an abundant production of several cytokines (2–4) including IFN-γ and IL-10. This apparent paradox suggests that in-depth studies on differences in cell activation states and, possibly, in molecules involved in cell homing are critical to the comprehension of immunopathogenesis of human VL.

2Data obtained in experimental models of VL suggested that maximal reduction of parasite load in livers of healing mice might be mediated by Leishmania donovani-reactive CD8+ T cells (5). In that study, authors observed that progressive reduction in liver parasite load occurred only when L. donovani-reactive CD8+ T cells entered and persisted in the livers. It was also shown that a successful host defense against L. donovani infection requires both CD4+ and CD8+ T cells, being IFN-γ production the primary role for CD4+ T cells (6). In a re-challenge experiment, it was shown that CD8+ T cells play an important role in host defense as does IL-2, whereas resistance was not affected by treatment with anti-CD4 antibodies (7). Leishmania-specific T cell clones established from asymptomatic VL patients were shown to be CD8+ and able to produce high amounts of IFN-γ (8). In
an experimental model of VL, it was shown that CD8+ T cells display cytotoxic activities against cells expressing parasite antigens as well as an ability to up-regulate the expression of pro-inflammatory cytokines (9). Collectively, these results indicate a prominent role for CD8+ T cells in immunity against *Leishmania* species associated with VL. In parallel, it was shown that re-infection of immune mice with *Leishmania major* elicits a secondary IFN-γ response to which specific CD8+ T cells are essential (10) and that CD8+ T cells are also required for primary immunity in mice infected with *L. major* (11).

Effective prophylactic measurements for VL are hampered by the imprecise comprehension of different aspects of the disease, including its immune regulation. In the present study, we took advantage of studying a group of VL patients during active disease and following cure, aiming at providing some insight in the complex process that leads to development of VL.

**Materials and methods**

**Study population**

VL patients (*n* = 18) were recruited in São Luiz do Maranhão (Maranhão, Brazil) and Teresina (Piauí, Brazil), an area endemic for *Leishmania chagasi* infection. Informed consent was obtained from patients or their guardians and all procedures were approved by the local Ethics Committee (CEP/HUPES–UFBA and HUPD–UFMA) and were conducted following recommendations outlined in the Helsinki Declaration. All patients were subjected to a complete physical examination as well as to clinical and laboratory evaluations. Diagnosis was confirmed by identification of the amastigote forms of *Leishmania* sp. in Giemsa-stained smears of bone marrow aspirates. Clinical characteristics of the patients (11 males and seven females, age range 15–46 years old) are described.
All patients received N-methylglucantime antimonite (Glucantime/C226) as the drug of first choice at the dose of 20 mg Sb/C05 kg/C01 day/C01 (maximum dose of 810 mg day/C01), intravenously, over a period of 20–30 days. No patients in this series exhibited marked anemia (3+/4+ and/or hemoglobin ≤5 g dl/C01), hemorrhagic phenomena, severe diarrhea or a rigorously impaired nutritional status. All patients underwent clinical follow-up every 10 days during treatment and every month, for up to 3 months, after treatment. Clinical assessment consisted of recording the symptoms reported by the patient and complete clinical examination including measurement of spleen, liver size and weight. Healthy individuals (n = 14) (eight males and six females, age range 20–45 years old) were from the same endemic area and were in the same age range as VL patients. Of note, there was a significant increase in total leukocyte numbers observed in VL patients when we compared pre- post-treatment (2448 ± 775 versus 4527 ± 1506; mean ± SD, P = 0.008). In terms of lymphocytes, there was an increase in total lymphocyte count observed for VL patients pre- post-treatment (1022 ± 373 versus 1661 ± 801; mean ± SD; P = 0.064) but this increase was not significant. Due to the neutropenia and lymphopenia that are characteristic of VL caused by L. chagasi (12), experiments could not be performed with all patients.
at all times. Controls did not show any sign of infection at the time of the study and had negative anti-Leishmania serology and delayed-type hypersensitivity. Informed consent was obtained from all individuals enrolled.

**Parasite and antigen**

*Leishmania chagasi* (MHOM/BR00/MER/STRAIN2) was used for antigen preparation. Details concerning isolation and characterization of this parasite strain have been described elsewhere (13). Parasites were cultivated in Schneider’s medium (Sigma–Aldrich, São Paulo, Brazil) supplemented with 5% FCS and Gentamycin (50 μg ml⁻¹) (all from Invitrogen, São Paulo, Brazil). soluble Leishmania antigen (SLA) was prepared as described previously (14).

**Isolation of PBMC and cell culture**

PBMC from VL patients and endemic controls were obtained by passage over a Ficoll Hypaque gradient (Sigma–Aldrich). PBMC were stained directly for ex vivo detection of CD25, CD152 (CTLA-4), CD18, CD45RO and CD95, CD4 and CD8 by flow cytometry. Alternatively, PBMC were washed three times and re-suspended at a concentration of 3 × 10⁶ ml⁻¹ in RPMI 1640 medium supplemented with 2 mM L-glutamine, penicillin (100 U ml⁻¹), streptomycin (100 μg ml⁻¹) (all from Invitrogen) and 10% human AB serum (Sigma–Aldrich). Cells were plated in 48-well plates (Corning Incorporated Life Sciences, Lowell, MA, USA) and incubated at 37°C, 5% CO₂ in the presence or absence of SLA (4 μg ml⁻¹) for 2 days. Cells were then collected and stained for detection of CD25, CD4 and CD8 or for CD4 and CD8 detection and Annexin V binding by flow cytometry. Alternatively, cells were plated in 24-well tissue culture plates at a concentration of 5 × 10⁶ cells ml⁻¹ and supernatants were harvested at 24, 48 and 96 h following incubation at 37°C, 5% CO₂.

**Flow cytometry**

Cells were stained ex vivo, simultaneously, with anti-human surface CD4 (RPA-T4) and CD8 (RPA-T8) (both from BD Biosciences, San Jose, CA, USA) conjugated to PE and PerCP, respectively, and anti-human CD25 (FTR-D4), anti-human CTLA-4 (CFRT-32), anti-human CD18 (GYU-09), anti-human CD45RO (XZD-65), Annexin V or anti-human CD95 (FTY-00) conjugated to FITC (all from BD Biosciences). Isotype controls were used as appropriate. For each sample, 20 000 events were analyzed using CELLQuest™ software and a FACSort® flow cytometer (Becton Dickinson Immunocytometry).

**Statistical analysis**

The data were presented individually for each patient or healthy individual tested. The significance of the results was calculated by one-way analysis of variance followed by Bonferroni’s
post-test using Prism (Graph Pad Software, San Diego, CA, USA) and a *P*-value < 0.05 was considered significant.

**Results**

**Outcome of CD8 and CD4 T lymphocyte phenotypic markers and PBMC functions as assessed by SLA-dependent cytokine release in VL patients, pre- and post-glucantime-based therapy**

During active disease, cells from VL patients displayed a 5-fold decrease in *ex vivo* frequency of CD25+ T cells (4.2–0.8% in CD4+ T cells and 2.1–0.5% in CD8+ T cells) (Fig. 1A and B). Following chemotherapy, the frequency of CD25+ cells returned to levels observed in healthy controls: 4.2% in CD4+ T cells and 2.5% in CD8+ T cells (Fig. 1A and B, respectively). PBMC from VL patients failed to proliferate *in vitro* in response to SLA, which can be associated with a lower frequency of CD25+ cells at the same time (data not shown). As shown in Fig. 2, the frequency of CD25+ T cells during active disease was significantly increased after SLA stimulation. In treated VL patients, frequency of CD25+ cells was higher than that observed prior to treatment and was significantly up-regulated after SLA stimulation, for both CD4+ and CD8+ T cells (Fig. 2A and B, respectively). Importantly, SLA stimulation led to an increase in IFN-γ production; this increase, however, was significant only in samples obtained post-treatment (Fig. 3A). On the contrary, cells obtained prior to treatment, when stimulated with SLA, significantly up-regulated IL-10 production (Fig. 3B). We did not detect significant changes in tumor necrosis factor (TNF)-α production when comparing pre- and post-treatment cultures (Fig. 3C). Overall, IFN-γ, IL-10 and TNF-α production by healthy individuals remained unchanged, irrespective of SLA stimulation (Fig. 3). During active VL, the frequency of CTLA-4+ T cells was significantly lower in VL patients and increased to levels observed in healthy individuals following treatment, in both CD4+ and CD8+ T cells (Fig. 4A and B, respectively). Moreover, changes in the frequency of CD25+ and CTLA-4+ T cells in VL patients’ cells were also paralleled in the expression of these markers as shown by analysis of the mean fluorescence intensity (MFI) of CD25 and CTLA-4 (data not shown). We did not detect any significant differences in the frequency of CD28+ T cells in VL patients before and after treatment (data not shown).

**Annexin V binding among the CD95+, CD4+ and CD8+ lymphocyte population in PBMC obtained from VL patients, pre- and post-glucantime-based therapy**

During active disease, the percentage of *ex vivo* phosphatidylserine exposure as measured by Annexin V binding was significantly higher in VL patients, in both CD4+ and CD8+ T cells and returned to normal levels after cure (Fig. 5A and B). When cells were stimulated with SLA, Annexin V binding did not change in CD4+ T cells (Fig. 4C); however, in SLA-stimulated CD8+ T cells, Annexin V binding was significantly lower (Fig. 4D), indicating that antigen stimulation is able to significantly enrich for SLA-reactive CD8+ T cells. Since apoptosis can be induced by binding of CD95-CD95L, we then investigated whether the frequency of CD95+ T cells was altered. As observed earlier, the frequency of CD95+ T cells was significantly higher during active disease and returned to levels observed in healthy individuals following treatment, in both CD4+ and CD8+ T cells (Fig. 6A and B, respectively). These data suggest that higher percentage of Annexin V+ T cells prior to treatment may be associated with increased apoptosis by expression of CD95 by these same T cells.

**In VL patients, is the lack of the CD8+CD18+ T lymphocyte subset a marker of their re-distribution in tissues such as the bone marrow?**

It has been shown that the levels of *ex vivo* CD45RO expression can be used to predict the intensity of the T cell response to *Leishmania* parasites for both IFN-γ and IL-10 production (15). The frequency of CD45RO+ T cells, during active VL, was also significantly lower in both CD4+ and CD8+ T cells (Fig. 7A and B), when compared with healthy individuals. We then examined the frequency of CD45RO+ T cells in the bone marrow, which was significantly higher.
when compared with peripheral blood, in both CD4+ and
CD8+ T cells (Fig. 7C and D). Last, we also probed for the
frequency of CD18+ T cells. In CD4+ T cells, we did not de-
tect any significant differences comparing active disease
and post-treatment (Fig. 8A), differently from what was ob-
served with CD8+ T cells (Fig. 8B). On the contrary to what
we observed with CD45RO+ cells, the percentage of
CD4+CD18+ T cells was significantly lower in the bone mar-
row (Fig. 7C). Surprisingly, the percentage of CD8+CD18+
T cells was significantly higher in bone marrow, indicating,
again, compartmentalization of certain CD8+ T cell subsets
during active VL.

Discussion
In the present study, we have compared the phenotype of
VL patients’ CD4+ and CD8+ T cells obtained during active
VL and after chemotherapy. Overall, alterations during active
disease were marked in both cell populations and returned
to normal levels after treatment. Of note, there was a signifi-
cant decrease in the frequency of ex vivo CD18+CD8+ T
cells during active disease. The frequency of CD18+
T cells was also significantly elevated in the bone marrow,
a finding not observed for CD4+ T cells.

The most important alteration in unresponsive T cells is
their incapacity to produce IL-2 and, in consequence, inca-
pability to proliferate and differentiate into effector cells fol-
lowing antigen encounter [reviewed in (16)]. Indeed, ex vivo
staining of VL patients’ T cells showed lower frequency of
CD25+ T cells. However, in vitro stimulation of VL patients’
PBMC with SLA significantly up-regulated the frequency of
CD25+ T cells. The role of cells that constitutively express
CD25 and the transcriptional regulator Foxp3, or regulatory
T cells (Tregs), has been recently addressed in leishmania-
isis. In cutaneous leishmaniasis, IL-10 production by naturally
occurring Tregs (CD4+CD25+Foxp3+) is crucial for persis-
tence of L. major (11). In human VL, it was shown that
CD4+CD25+ (Foxp3high) were not a major source of IL-10
during active disease (17), implicating other cell subtypes in
the immunosupression phenomenon, characteristic of
human VL.

CD18 is required for optimal development and function of
CD4+CD25+ T cells, since the absence of CD18 led to di-
minished Treg numbers (18) and to impaired cell–cell con-
tact between Tregs (CD25+CD4+CD127−) and dendritic
cells (19). In the present study, the frequency of
CD4+CD18+ T cells was similar among controls, VL patients
and cured VL individuals, although the frequency of
CD4+CD18+ T cells was higher in peripheral blood than in bone marrow. Regarding the CD8+ subset, the frequency of CD18+ T cells was significantly lower during active disease and higher in the bone marrow. CD18 mediates adhesion to extracellular matrix components and to other cell types. Therefore, we can suggest that the higher frequency of CD8+CD18+ T cells observed in the bone marrow may reflect a selective retention at this site by means of a more pronounced interaction with the extracellular matrix features of the bone marrow. Indeed, MFI of CD18 expression on CD8+ T cells was significantly higher in the bone marrow when compared with peripheral blood (88.6 ± 2.9 versus 82.5 ± 3.1, respectively, mean ± SD). Consequently, the under-representation of this particular CD8+ subset in the periphery can reflect in an impaired immune response. It remains to be determined how this finding may be related to the elevated cytokine plasma levels observed in VL (2, 3).

CD18 deficiency results in L. major dissemination and an incomplete Th1 immune response (20) and, conversely, VL asymptomatic dogs exhibit well-organized liver granulomas with cells expressing CD44 and CD18 (21). A subset of CD8+CD18^{high} T cells that up-regulate IL-12R expands in cancer patients receiving IL-12 and the activation of such cells results in IFN-γ production as well as in enhanced cytolytic activity (22). It would be interesting to determine whether the CD8+CD18+ subset from VL patients is able to display cytolytic activity following stimulation with parasite antigen. CD8+ T cells have been shown to contribute to parasite restrain in vivo as well as to the formation of hepatic granulomas in murine VL (6, 23). It was found that antigen-specific cytotoxic clones develop in the spleen of Leishmania infantum-infected BALB/c mice, show an activated phenotype and become susceptible to apoptotic cell death late in the course of the disease (9).

Our results on the frequency of CD45RO+ T cells reinforce the finding that levels of circulating memory T cells are reduced in VL patients (24, 25). We can speculate that this decreased frequency of CD45RO+ lymphocytes during VL reflects selective migration/retention in infected organs as observed herein for the bone marrow compartment. Of note, MFI of CD45RO expression in the bone marrow was significantly higher in both CD4+ and CD8+ T cells (109.7 ± 3.7 and 97.5 ± 4.4, respectively, mean ± SD) when compared with MFI values observed in peripheral blood (102.3 ± 2.7 and 93.4 ± 2.8, respectively, mean ± SD). In animal models of VL, the spleen and the bone marrow can become sites of chronic infection, where...
parasites might survive during the life of the animal (26, 27). Therefore, the identification of specific cell populations that are involved in disease control in the bone marrow, for instance, may allow the development of more targeted control strategies. In parallel, our study also reinforced that immunological data obtained from peripheral blood do not, in the case of CD45RO and CD18, accurately reflect the immune response occurring at the infection site.

Funding
Conselho Nacional de Desenvolvimento Científico e Tecnológico fellowship to C.F.

Acknowledgements
C.I.O., J.M.C., C.B., A.B. and M.B.N. are senior investigators from Conselho Nacional de Desenvolvimento Científico e Tecnológico and Instituto de Investigação em Imunologia (iii). We would like to thank Juqueline Cristal and Jorge Tolentino for technical assistance.

Abbreviations
CMI cell-mediated immunity
MFI mean fluorescence intensity
SLA soluble Leishmania antigen
Treg regulatory T cell
VL visceral leishmaniasis
TNF tumor necrosis factor

References