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Adhesion molecule expression patterns indicate activation and recruitment of CD4+ T cells from the lymph node to the peripheral blood of early cutaneous leishmaniasis patients

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Abstract

Adhesion molecules play a crucial role in cell migration and recruitment. Expression of adhesion molecules that preferentially address cells to inflammatory sites is a critical event in the formation and maintenance of leishmaniasis lesions. In this work, we analyzed the expression of CD11a, CD11b and CD62L, adhesion molecules involved in cell activation and circulation, in CD4+ and CD8+ T cells from peripheral blood and lymph nodes of patients with early cutaneous leishmaniasis. The percentage of expression of CD62L, CD11a and CD11b in total lymphocytes was lower in lymph nodes as compared to peripheral blood. Moreover, differences in adhesion molecule expression between blood and lymph nodes were more striking in CD4+ than CD8+ T cells. Stimulation of PBMC from leishmaniasis patients with soluble Leishmania antigens (SLA) lead to the expansion of CD4+CD62Lhigh cells, CD4+CD11b+ cells and to an increase in the intensity of expression of CD11a in CD4+, but not CD8+ T cells. Our data suggest that early activation events that occur in the lymph nodes of patients recently infected with *Leishmania* lead to changes in T cell adhesion molecule expression, favoring migration to the periphery and increasing the likelihood of further recruitment to lesion sites.

Keywords: Leishmaniasis; Adhesion molecules; Lymph node; Lymphocytes; Peripheral blood

1. Introduction

Leukocyte circulation and recruitment are under the strict control of adhesion molecules expressed by migrating cells and the endothelia [1]. It has been shown that high endothelial venules from secondary lymphoid organs display high levels of PNAd and/or VAP-1, ligands for CD62L [2,3]. Thus, circulating cells that express high levels of CD62L on their surface can be recruited to secondary lymphoid organs through interaction between these adhesion molecules [4,5]. Experiments using animal models have demonstrated that high levels of expression of CD62L is a characteristic of naïve T cells [6], favoring their recruitment to secondary lymphoid organs [7]. Murine T cells that undergo activation in secondary lymphoid organs down-regulate CD62L and up-regulate LFA-1, an adhesion molecule that mediates interaction with activated endothelia, favoring the recruitment of activated T cells to inflamed areas [8,9]. In contrast, human studies of the circulation/recruitment processes are still scarce, and often differ from conclusions obtained using animal studies. As an example, while a high intensity of expression of LFA-1 has also been described primarily as a characteristic of activated T cells [10], at least 50% of the memory/activated human cells are CD62L+ [11].

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The establishment of inflammatory lesions is directly related to the recruitment of cells to highly vascularized connective tissues, and the maintenance of the inflammation depends on the presence of the triggering stimulus and/or recruited activated cells. Human leishmaniasis is a parasitic disease caused by the protozoa *Leishmania*, which leads to the establishment of granulomatous lesions. At least three major clinical forms can be identified in leishmaniasis, with a large spectrum of pathological variations within each form [12]. Despite the clinical outcome, parasites are injected in the host's skin where they gain entrance into phagocytic cells. These cells migrate through the body, and eventually reach the draining lymph nodes, presenting parasite antigens to antigen specific lymphocytes. Cellular interactions within lymph nodes and latter migration to inflammatory sites are crucial events for immunopathology in leishmaniasis.

Previous studies have demonstrated that lymphocytes represent the majority of cells in cutaneous leishmaniasis lesions [13], and that marked lymphadenopathy is a hallmark of early cutaneous leishmaniasis [14]. These data suggest the importance of the communication between the draining lymph node and the lesion, through the peripheral circulation. Since adhesion molecules play an unquestionable role in cell circulation and recruitment, we performed a comparative analysis of adhesion molecule expression between lymphocytes from the blood and draining lymph nodes from the same individual in a group of four early cutaneous leishmaniasis patients and evaluated the effect of specific *in vitro* activation in adhesion molecule expression. Our results provided insights towards understanding the early events of cell activation in leishmaniasis and its effects on adhesion molecule expression and recruitment, which, in turn, likely influence lesion formation.

2. Patients, materials and methods

2.1. Patients

Patients analyzed in this work were from an endemic area for leishmaniasis, Corte de Pedra, which is located near Salvador, BA, Brazil. All patients included in this study were volunteers and treatment was offered to all patients, despite their enrollment in this project. Previous studies have shown that approximately 95% of the patients in this endemic area are infected with *L. braziliensis*. All medical assistance, treatment and clinical form characterization were under the responsibility of Drs. Edgar Carvalho, Paulo Roberto Lima Machado and Aldina Barral. Well-characterized patients with less than 30 days of infection, and the presence of an enlarged draining lymph node adjacent to the lesion site, were selected for blood and lymph node aspirate collection. Patients had not reported previous cases of leishmaniasis, and all presented with a small non-ulcerated isolated lesion at the time of sample collection. Presence of non-ulcerated lesion and lymphadenopathy indicate early stages of infection [14]. All procedures were approved by the Ethical Committee from Federal University of Minas Gerais, as well as by the Ethical Committee at the Hospital Edgar Santos in Salvador, BA, Brazil.

2.2. Preparation of peripheral blood mononuclear cells (PBMC) and lymph node cells

PBMC were obtained by standard Ficoll-Hypaque gradient, as previously described by us [15]. Lymph node cell aspirates and the PBMC were submitted to three washes in RPMI, counted and resuspended to 10^7 cells/ml. PBMC and node cells were then submitted to *ex vivo* analysis of adhesion molecule expression. Adhesion molecule expression analysis was also performed in PBMC after *in vitro* stimulation with soluble *Leishmania* antigen (SLA), at a final concentration of 10 ug/ml for 40 h, as well as in non-stimulated control cultures.

2.3. Analysis of adhesion molecule expression by PBMC and lymph node cells

We analyzed the expression of adhesion molecules CD11a (LFA-1), CD11b (Mac-1), and CD62L (l-selectin) in total lymphocytes and in CD4+ and CD8+ lymphocyte populations, freshly isolated from patients or after culture, using flow cytometry, as previously performed by us [16]. The antibodies used for phenotypic analysis were: anti-CD4-FITC and anti-CD8-FITC, purchased from Pharmingen and PE-labeled anti-CD11a, CD11b, and CD62L, purchased from Caltag. Fluorochrome-labeled isotype controls were added to the staining protocol for each patient. Tukey and Kramer, ANOVA test, from JMP software (SAS) was applied to ascertain statistically significant differences between the means of the groups under comparison.

3. Results

We evaluated the expression of CD62L, CD11a and CD11b in CD4+ and CD8+ lymphocytes from lymph node and blood of patients with early cutaneous leishmaniasis using flow cytometry. Analysis of the percentage of CD4+ or CD8+ T cells and CD19+ B cells did not show any statistically significant differences, comparing blood and lymph node (data not shown), demonstrating that lymphadenopathy was not due to a biased increase of one cell type over the others, but rather, a general expansion of all the lymphocyte sub-populations. *Ex vivo* analysis showed that the frequency of total lymphocytes expressing CD62L, CD11a or CD11b was higher in peripheral blood (52 ± 17, 77 ± 9, 30 ± 8, respectively), as compared to lymph node (10 ± 8, 19 ± 7, 2 ± 0.5, respectively) (Fig. 1A–C, $P < 0.05$). This increased expression of CD62L, CD11a, and CD11b, within the total lymphocyte population in peripheral blood, was reflected within the CD4+ T cell population (82 ± 7 *versus*

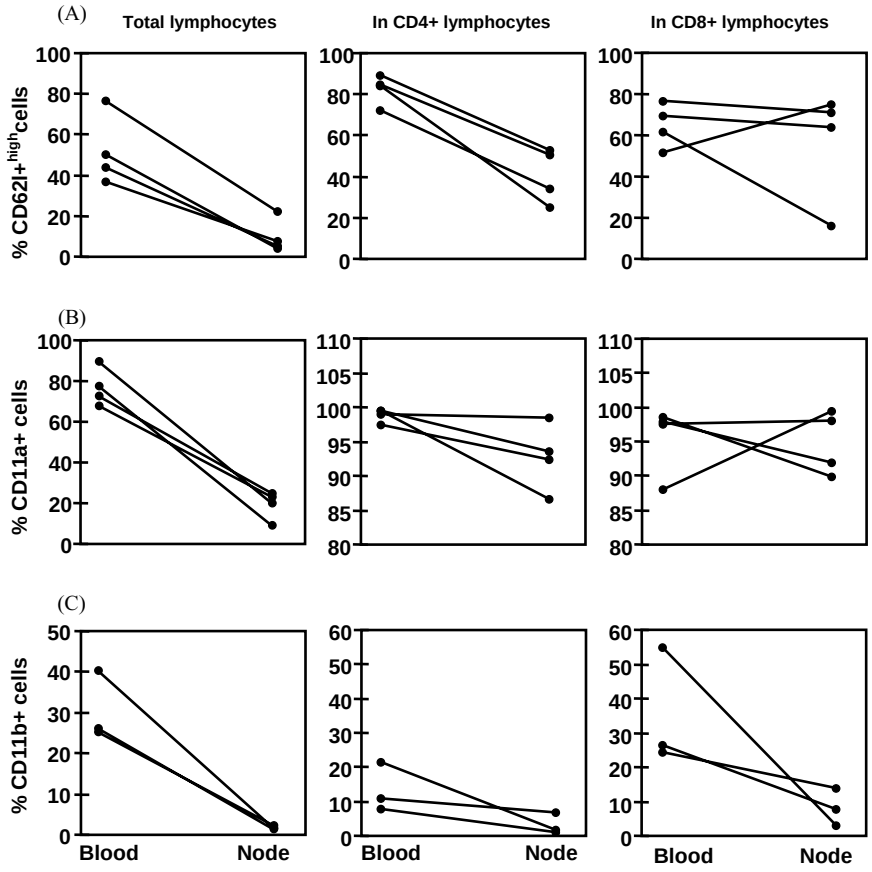

Fig. 1. Percentage of expression of CD62L (A), CD11a (B) and CD11b (C) in total lymphocytes as well as CD4+ and CD8+ sub-populations from peripheral blood and lymph nodes of patients with early cutaneous leishmaniasis.

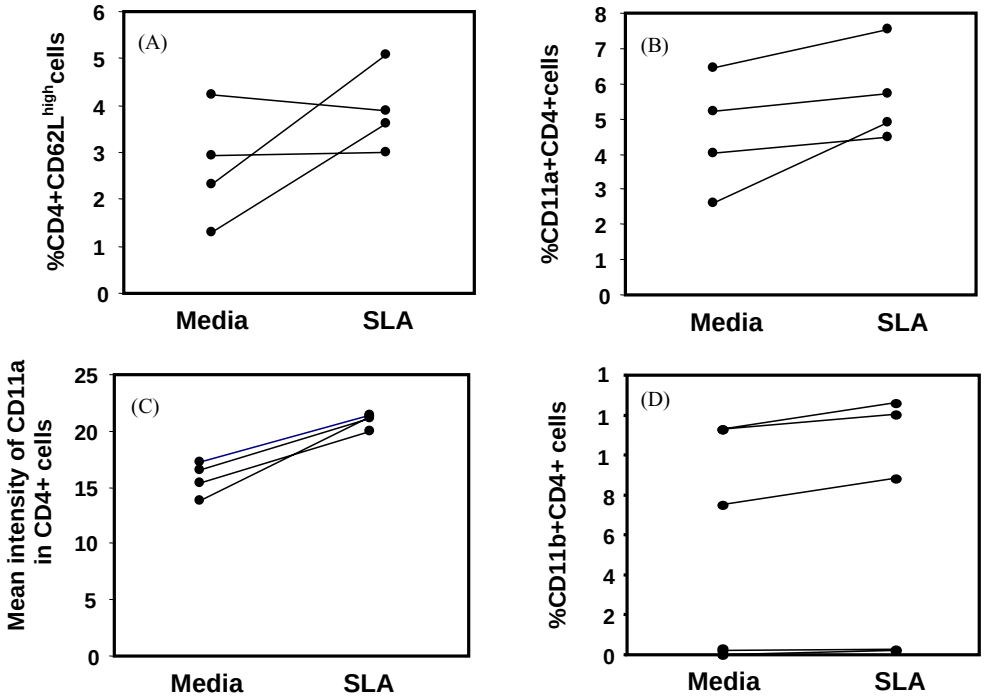

Fig. 2. Effect of in vitro stimulation of PBMC from early cutaneous leishmaniasis.

41 ± 13; 99 ± 1 *versus* 93 ± 4; 13 ± 7 *versus* 3 ± 3 for blood *versus* node and for each adhesion molecule, respectively) (Fig. 1A–C, $P < 0.05$). Although we also observed individual differences at the level of adhesion molecule expression by CD8+ T cells between the lymph node and peripheral blood, the comparison of the means did not show statistical significant differences.

To evaluate the effects of the antigen specific cellular response on the expression of these adhesion molecules, PBMC were cultured with SLA and analyzed for the expression of CD62L, CD11a, and CD11b in the CD4 + T cell subset following 40 h. As shown in Fig. 2, CD4+ T cells expressed a higher frequency of CD62L (Fig. 2A) following culture with SLA as compared to the media control (27 ± 11 *versus* 39 ± 8, respectively). While the percent of CD4+ T cells expressing CD11a did not increase with SLA stimulation (Fig. 2B), the mean intensity of expression of this molecule increased dramatically (Fig. 2C) following stimulation as compared to the media control (157 ± 15 *versus* 210 ± 6, respectively). Lastly, there was no statistically significant difference in the expression of CD11b following SLA stimulation (Fig. 2D).

4. Discussion

Early T cell activation is a critical event in the initiation of the immune response to *Leishmania* infection. It has been demonstrated that activation of T cells leads to changes in adhesion molecule expression [10,11], influencing cell migration and recruitment. In this work, we analyzed adhesion molecule expression in cells from the draining lymph node of individuals recently infected by *Leishmania*, as well as from the peripheral blood of the same patients. We studied the expression of CD62L, CD11a and CD11b in cells freshly isolated from the patients, as well as in PBMC submitted to culture in the presence of SLA. Our results demonstrated that cells expressing these activation induced, adhesion molecules are more frequently found in the peripheral blood of the patients, likely reflecting their migration to the blood after activation in the draining lymph nodes. Comparative analysis between two compartments of the same patient at early stages of infection provided insights towards activation mechanisms involved with lesion formation in human leishmaniasis.

CD62L is an adhesion molecule of the selectin family, widely expressed by lymphocytes, that recognizes mucin-like glycoproteins in the endothelia. While CD62L seems to play an important role in addressing human T cells to lymphnodes [2,3], at least half of human activated T cells express CD62L [11]. We evaluated the expression of this molecule by lymphocytes from blood and lymph node of patients with early cutaneous leishmaniasis, determining the frequency of cells expressing high levels of CD62L (CD62Lhigh) in both compartments. We observed a lower percentage of CD62Lhigh lymphocytes in lymph node as compared to blood in all patients analyzed (Fig. 1A). This decreased percentage of CD62Lhigh lymphocytes in lymph nodes was a consequence of a decrease in the CD4+ CD62Lhigh T cells in all patients. Only one out of four patients displayed a lower percentage of CD8+ CD62Lhigh cells in lymph node as compared to blood (Fig. 1A). The lower percentage of CD4+ CD62Lhigh cells in lymph nodes as compared to blood may reflect a down regulation of this molecule in CD4+ T cells, due to early activation events taking place in the lymph node of patients with initial cutaneous leishmaniasis. Alternatively, the higher percentage of CD4+ CD62Lhigh cells in the peripheral circulation may represent the expansion of activated/memory cells in patients with early cutaneous leishmaniasis.

To determine whether stimulation with *Leishmania* antigens would cause a down regulation of CD62L in CD4+ T cells, we cultured PBMC from patients with early cutaneous leishmaniasis with SLA. While the percentage of CD4+ CD62Lhigh did not change much in two patients, it was twice as high in stimulated, *versus* non-stimulated, cultures from the other two patients (Fig. 2A). Importantly, we did not observe a significant decrease in CD62Lhigh cells in any of the stimulated cultures. Thus, activation leads to expansion of specific CD4+ CD62Lhigh cells previously activated *in vivo*, rather than causing a decrease in expression of CD62L. Based on this, it is likely that the higher percentage of CD4+ CD62Lhigh cells that we observed in the peripheral blood of the patients as compared to lymph node, is due to an expansion of this population in the nodes, followed by migration to the blood. Moreover, it has been shown that CD62L plays an important role in the homing of human T cells to inflammatory sites [17]. Thus, it is possible that this phenotype may participate in the establishment of lesion in early cutaneous patients.

The intensity of expression of CD11a (LFA-1, leukocyte function antigen-1) by T cells has been correlated with activation [10]. We analyzed the percentage of cells expressing CD11a and, as a parameter to access activation, the intensity of CD11a expression in CD4+ and CD8+ T cells from blood and lymph node of patients with early cutaneous leishmaniasis was determined. We observed that the percentages of lymphocytes expressing CD11a in lymph nodes were significantly lower than in peripheral blood (Fig. 1B). Analysis of CD11a expression by CD4+ T cells showed that, in general, there is a decreased percentage of CD4+ CD11a + cells in lymph node as compared to blood, although one patient displayed very similar percentages of CD4+ CD11a + cells in the lymph node as compared to blood (Fig. 1B). Again, the higher percentage of CD4+ CD11a + cells observed in blood as compared to lymph nodes may indicate recruitment of activated CD4+ T cells from the node to the periphery. However, this general decrease in the percentage of CD4+ CD11a + cells in the node was not great enough to account for the very low percentage of total lymphocytes expressing CD11a in lymph node as compared to

blood. CD8+ CD11a + T cells, displayed a heterogeneous distribution among patients (Fig. 1B). Thus, the decrease in the percentage CD11a expression in total lymphocytes is partially due to a decreased percentage of T cells expressing CD11a, but other lymphocytes must also be involved.

Analysis of the average intensity of expression of CD11a in CD4+ or CD8+ T cells as a parameter for cell activation did not change significantly when comparing between the two compartments (data not shown). However, *in vitro* stimulation of cutaneous patient PBMC with SLA led to a higher intensity of expression of CD11a (Fig. 2C), indicating activation.

CD11b (Mac-1) is another leukointegrin that shares a chain (CD18) with CD11a and also recognizes the same ligands: constitutive ICAM-2 and inducible ICAM-1 [18]. While cells from the monocytic lineage predominantly express CD11b, a very low percentage of T cells express this molecule. It has been shown that cell activation induces an increase in CD11b expression by T cells [19]. Again, analysis of CD11b expression showed a lower percentage of this molecule in lymph node as compared to blood cells from leishmania patients (Fig. 1C). This decrease was observed in both CD4+ and CD8+ T cells, although decreased expression of CD11b in other populations, such as B cells, seems to contribute to the lower percentage of CD11b expression in total lymphocytes. Thus, the elevated frequency of lymphocytes expressing CD11b in peripheral blood as compared to lymph node, may also be an indicative of recruitment of activated cells to the periphery.

It is noteworthy to mention the difficulty in obtaining lymph node material from leishmaniasis patients and the limitations concerning the amount of material obtained. Lymph node material was collected due to the need, in some patients, of confirmatory parasitological diagnosis. Thus, upon need, this material was collected and part used in our study. This explains why these analyses were made with four individuals. Although four patients were analyzed, our study demonstrates, for the first time, differences in adhesion molecule expression between two compartments in the same individual allowing for a precise comparison at the level of each individual. Moreover, the changes that we observed were very consistent when comparing among patients and supported by appropriate statistical analysis of the mean results. Taken together, our data suggest that activated T cells are predominantly found in the peripheral blood of early cutaneous leishmaniasis patients, as compared to lymph node, as shown by the higher percentages of circulating T cells expressing CD62Lhigh, CD11a and CD11b. It is likely that activated T cells that have undergone phenotypic changes in the lymph node due to infection-related activation, gained access to the circulation where they are available for recruitment to lesion sites. The fact that these cells are accumulated in the periphery may be related to the small initial lesions at the time of blood collection. Moreover, our data suggest that CD4+ T cells are preferentially activated in early stages of leishmaniasis, since the observed *ex vivo* changes in adhesion molecule expression in total lymphocytes were consistently observed within the CD4+ T cell subpopulation. This initial activation of CD4+ T cells could favor their recruitment to lesion sites. Analysis of other parameters such as activation markers, cytokine and chemokine production by lymph node cells is currently being performed by others in our group. Further studies concerning the lesion composition in these patients will be performed by us, in order to determine the importance of these adhesion molecule expression patterns in the recruitment to lesion sites, providing insights towards the mechanisms of lesion formation in human leishmaniasis.

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