Chemokines and chemokine receptors coordinate the inflammatory immune response in human cutaneous leishmaniasis

Ana P. Campanelli [a,d], Claudia I. Brodskyn [c,e,f,*], Viviane Boaventura [c], Claire Silva [c], Ana M. Roselino [b], Jackson Costa [c], Ana Cristina Saldanha [c], Luiz Antônio Rodrigues de Freitas [c], Camila Indiani de Oliveira [c], Manoel Barral-Netto [c,e,f], João S. Silva [a], Aldina Barral [c,e,f]

[a] Department of Biochemistry and Immunology, School of Medicine of Ribeirão Preto, University of São Paulo, Brazil
[b] Division of Dermatology, School of Medicine of Ribeirão Preto, University of São Paulo, Brazil
[c] Centro de Pesquisa Gonçalo Moniz, FIOCRUZ-BA, Salvador, Bahia, Brazil
[d] Department of Biological Sciences, Bauru Dental School, University of São Paulo, São Paulo, Brazil
[e] Universidade Federal da Bahia, Salvador, Bahia, Brazil
[f] Instituto de Investigação em Imunologia, Instituto Nacional de Ciência e Tecnologia (INCT), São Paulo, São Paulo, Brazil

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ABSTRACT

Cutaneous leishmaniasis (CL) includes different clinical manifestations displaying diverse intensities of dermal inflammatory infiltrate. Diffuse CL (DCL) cases are hyporesponsive, and lesions show very few lymphocytes and a predominance of macrophages. In contrast, localized CL (LCL) cases are responsive to leishmanial antigen, and lesions exhibit granulocytes and mononuclear cell infiltration in the early phases, changing to a pattern with numerous lymphocytes and macrophages later in the lesion. Therefore, different chemokines may affect the predominance of cell infiltration in distinct clinical manifestations. In lesions from LCL patients, we examined by flow cytometry the presence of different chemokines and their receptors in T cells, and we verified a higher expression of CXCR3 in the early stages of LCL (less than 30 days of infection) and a higher expression of CCR4 in the late stages of disease (more than 60 days of infection). We also observed a higher frequency of T cells producing IL-10 in the late stage of LCL. Using immunohistochemistry, we observed a higher expression of CCL7, CCL17 in lesions from late LCL, as well as CCR4 suggesting a preferential recruitment of regulatory T cells in the late LCL. Comparing lesions from LCL and DCL patients, we observed a higher frequency of CCL7 in DCL lesions. These results point out the importance of the chemokines, defining the different types of cells recruited to the site of the infection, which could be related to the outcome of infection as well as the clinical form observed.

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1. Introduction

Leishmaniasis, caused by protozoan parasites of the genus *Leishmania*, is a major health problem in many regions of the world. *Leishmania* are protozoan parasites that infect human macrophages and cause a wide spectrum of clinical manifestations, including self-healing skin lesions, diffuse cutaneous disease, and mucosal disease (reviewed by Brodskyn et al. [1]). Cutaneous lesions begin at the site of parasite entrance as a small papule, which develops into a nodule that ulcerates in the center. The incubation period ranges from 2 weeks to several months (reviewed by Brodskyn et al. [1]). The most frequent aspect observed in cutaneous leishman-

iasis (CL) cases is a single ulcer with elevated borders and a sharp crater. In the early phase of CL (early CL), lymphoadenopathy can precede the lesion, and we consider early lesions when patients presented lesions until 30 days of infection. In late CL lesions, lymphadenopathy is a rare event, and it is possible to observe a dense infiltration of cells and generally, lesions display more than 30 days of infection [2,3]. Diffuse CL is a rare presentation of human tegumentary leishmaniasis. The presence of multiple and nonulcerated nodules characterizes diffuse CL (DCL), a form that is resistant to a standard antileishmania therapeutic regimen and presents with frequent relapses [3]. In Brazil, there have been approximately 40 cases of DCL studied since 1945 at Universidade Federal do Maranhão (UFMA) and CPqGM (Centro de Pesquisa Gonçalo Moniz [3].

Whereas DCL is characterized by higher numbers of parasites and a lack of cell-mediated immunity to leishmanial antigen, CL leishmaniasis is generally accompanied by strong cellular responses and few parasites in the lesions [4].

Differences in clinical manifestations of the disease are influenced by individual's immune response. Effective cell-mediated host defense implies an induction of protective immune responses and precise migration and localization of effectors cells. For protective immunity, it is essential that appropriate leukocyte subpopulations exhibit suitable receptors on their surface, enabling them to be recruited to the injured tissues [5]. Distinct CL differs not only in severity and course of disease, but also in the composition of cellular infiltrate at the site of infection [6,7]. Diverse composition of dermal cellular infiltration is due to chemokine production and its effects [8,9]. They are characterized by their potent chemotactic activity for leukocytes and are produced by a variety of cells following stimulation by cytokines or microbial products [10].

Chemokines are important in various aspects of pathogenesis and inflammation and probably play an important role in *Leishmania* infections. The parasite itself may be implicated in diverse chemokine induction, as two *L braziliensis* isolates, albeit at similar parasite burdens, induced different pace and/or intensity of chemokine expression, leading to diverse cell recruitment and differential inflammatory responses in mice [11]; these features might ultimately be implicated in disease presentations.

In this report, we took advantage of examining different stages of human LCL as well as DCL lesions, and evaluated chemokines and their receptor expression in comparison to the inflammatory responses observed in the lesions. The results indicate that the differential expression of chemokines and chemokine receptors contributes to the differences in the inflammatory patterns observed in skin lesions from distinct human CL presentations, as well as with the outcome of infection.

2. Subjects and methods

2.1. Leishmaniasis patients and controls

Demographical, clinical and laboratory data from the 12 LCL and 5 DCL patients enrolled in this study are shown in Table 1. We considered early LCL as when the patients presented with less than 30 days of infection, and considered late LCL as when patients presented with more than 60 days of infection. The low number of DCL patients in this study is due to the low frequency of this disease in our country [3]. The control group consisted of 10 healthy subjects (seven men and three women; age range, 19–50 years) with a negative result for Montenegro skin test. Biopsy samples were obtained from individuals who underwent plastic surgery. Biopsy samples from nasal polyps were used as positive controls for cytokines and chemokine stains. The Institutional Review Board of Centro de Pesquisa Gonçalo Moniz (FIOCRUZ-Bahia) approved the protocol of this study. Signed informed consent was obtained from all patients.

2.2. Histologic study

Cutaneous punch biopsy samples (4 mm) were immediately fixed in 10% formalin buffered solution. After 18–24 hours of fixation, tissues were embedded in paraffin. Sections 3- to 5-μm thick sections were stained with hematoxylin and eosin (H&E). Histologic sections were examined under an optical microscope to determine the intensity and phenotype of the inflammatory infiltration and the presence of granulomas. Immunohistochemistry using an indirect peroxidase technique was performed in histologic sections to search for *Leishmania* parasites.

2.3. Antibodies

Purified anti-human MCP-1/CCL2, anti-human MCP-3/CCL7, anti-human RANTES/CCL5, anti-human MIP-3 /CCL19, anti-human CCR5, anti-human CCR3, anti-humanCCR2, and anti-human CCR7 antibodies, anti-human TARC/CCL17, anti-human CCR4 (all from Santa Cruz Biotechnology, Sant Cruz, CA) were used to detect chemokines and chemokine receptors in skin. Biotin conjugated horse anti-mouse IgG or donkey anti-goat IgG were obtained from Vector Laboratories (Burlingame, CA). For immunostaining and analysis by flow cytometry, PercP-, PE- and FITC-conjugated antibodies (BD PharMingen, San Diego, CA) against CD3 (UCHT 1), CD4 (RPA-T4), CD8 (RPA-T8), CD14 (M5E2), CD1a (HI149), CXCR3, CCR3, CCR5, CCR4, transforming growth factor (TGF)–β, inteleukin (IL)–10, interferon (IFN)–γ, and respective mouse and rat isotype controls were used.

2.4. Isolation of leukocytes from skin lesions

To characterize leukocytes at the lesion site, biopsy samples from skin lesions were collected and incubated for 1 hour at 37°C in RPMI 1640 containing NaHCO3, penicillin, streptomycin, gentamicin, and 0.28 Wunsch units/ml liberase blendzyme CI (Roche Diagnostics, Indianapolis, IN). Biopsy samples were processed in the presence of 0.05% DNase (Sigma-Aldrich, St. Louis, MO) by use of a Medimachine (BD PharMingen, San Diego, CA), in accordance with the manufacturer's instructions. After processing, cell viability was assessed by Trypan blue exclusion, and cells were filtered through a 50-μm filter and washed before activation and/or immunolabeling.

2.5. In vitro culture

T cells (1×10^5 cells/well) were cultured in 96-well, U-bottom tissue culture plates in the presence of PHA (1 μg/ml). All cultures were performed in RPMI 1640 plus 10% fetal bovine serum (FBS), 2 mmol/L glutamine, 50 U/m penicillin, and 50 μg/mL streptomycin (GIBCO). After 6 hours, T cells were harvested, and the production of cytokines was evaluated by flow cytometry.

2.6. Immunohistochemistry

Fresh frozen tissue samples were obtained from the skin of 17 patients. Fragments of the tissue samples were covered with OCT medium (Ames, Elkhart, IN), snap-frozen in liquid nitrogen, and stored at −190°C until use. Cryostat sections (5 μm) were dried onto glass covered with poly-L-lysine adhesive (Sigma, St. Louis, Mo.) and fixed in cold acetone. The avidin–biotin peroxidase techniques were used for revealing chemokine producing cells on several sections, as previously reported [12].

Table 1
Patient characteristics

Patient	Age (y)	Gender	Time from lesion	Clinical form	MST	ELISA[a]	*Leishmania* (H&E or PCR)
P1	12	M	15 days	LCL	(+)	(+)	(+)
P2	19	F	15 days	LCL	(+)	(+)	(+)
P3	16	M	60 days	LCL	(+)	(+)	(+)
P4	17	M	15 days	LCL	(+)	(+)	(+)
P5	35	M	20 days	LCL	(−)	(+)	(+)
P6	17	M	60 days	LCL	ND	ND	(+)
P7	52	M	210 days	LCL	(+)	(+)	(+)
P8	58	F	60 days	LCL	(+)	(+)	(+)
P9	14	F	150 days	LCL	(+)	(+)	(+)
P10	23	F	120 days	LCL	(−)	(+)	(+)
P11	17	M	90 days	LCL	(+)	(+)	(+)
P12	40	F	90 days	LCL	(−)	(+)	(+)
P13	28	M	10 years	DCL	(−)	(+)	(+)
P14	19	F	12 years	DCL	(−)	(+)	(+)
P15	28	M	21 years	DCL	(−)	(+)	(+)
P16	38	M	16 years	DCL	(−)	(+)	(+)
P17	21	M	12 years	DCL	(−)	(+)	(+)

M, male; F, female; ND, not done; MST, Montenegro skin test; LCL, localized cutaneous leishmaniasis disseminated; DCL, diffuse cutaneous leishmaniasis; ELISA, enzyme-linked immunosorbent assay; PCR, polymerase chain reaction.
*Histopathology or polymerase chain reaction.

2.7. Flow cytometry analysis

To label the cells for cytometry analysis, skin derived leukocytes were washed and stained for 20 minutes at 4°C with the optimal dilution of each antibody. The leukocytes were washed again and analyzed by flow cytometry (FACScan and CELLQuest software; Becton Dickinson). Multivariate data analysis was performed in the FACSCalibur flow cytometer using CellQuest software (BD Biosciences) by gating the live cells on a side scatter versus forward scatter dot plot or on the population of $CD3^+CD4^+$ or $CD3^+CD8^+$ T cells to assess chemokine receptors expression.

2.8. Intracellular cytokines detection

The intracellular detection of IL-10, TGF-β, and IFN-γ, in leukocytes obtained from lesions of patients was performed in fixed and permeabilized cells using Cytofix/Cytoperm (BD Biosciences). First, the cells were labeled with Abs of cell surface, such as FITC–conjugated anti-CD3. Next, the cells were fixed, permeabilized, stained with PE-labeled anti-human (h) IL-10, PE-labeled anti–hIFN-γ, biotin-labeled anti–hTGF-β, incubated with PE-streptavidin (Invitrogen Life Technologies) or control isotype, and analyzed using a FACS. The absolute numbers of leukocyte/biopsy subsets were calculated through percentage obtained by FACS and the amount of cells were determined in Neubauer chamber.

2.9. Semiquantitative evaluation of sections

Isotype control antibody staining was always tested on the same slide of specific Ab staining and subtracted in the analysis. The number of positively stained cells for each antibody was counted per five consecutive microscopic high-power fields. Results were expressed as the mean of positive cells and as the percentage of positive cells per field, calculated in relation to the total number of inflammatory mononuclear cells.

2.10. Statistical analysis

Results are expressed as mean ± SEM. Statistical analysis was done by nonparametric analysis of variance followed by the Tukey's multiple comparison test (INSTAT software; GraphPad, San Diego, CA), as indicated in the figure legends. Differences were considered significantly different at $p < 0.05$. Analysis was performed using the nonparametric Mann–Whitney test followed by the Bonferroni test.

3. Results

3.1. Characterization of leukocytes in the lesions of LCL and DCL patients

In early CL lesions ($n = 4$, mean of 16 ± 2 days), the inflammatory infiltration was composed of mononuclear cells and polymorphonuclear leukocytes (Fig. 1A), whereas in late CL lesions ($n = 8$, mean of 142.5 ± 60 days) there was a predominance of mononuclear leukocytes (Fig. 1B). In the latter, epithelioid macrophages were mixed with a heavy infiltrate of lymphocytes and macrophages. In contrast, analyses from inflammatory infiltrate in DCL lesions revealed that the inflammatory response was composed of a monotonous infiltrate of vacuolated and heavily parasitized macrophages (Fig. 1C).

To determine the percentage of different populations of leukocytes present at the *Leishmania* lesions from LCL patients, biopsy samples were collected and leukocytes were isolated after enzymatic digestion. The total numbers of leukocytes isolated were higher in late CL (range, $2–7 \times 10^6$ cells/biopsy) than in early CL ($0.50–2.7 \times 10^6$ cells/biopsy) (Fig. 1(D)). Because of the scarce material obtained from DCL patients, it was not possible to perform, in these samples, flow cytometry analysis. In early CL patients, lymphocytes ($CD3^+$ cells) (ranging from 9×10^4 to $1,4 \times 10^6$ cells/biopsy)
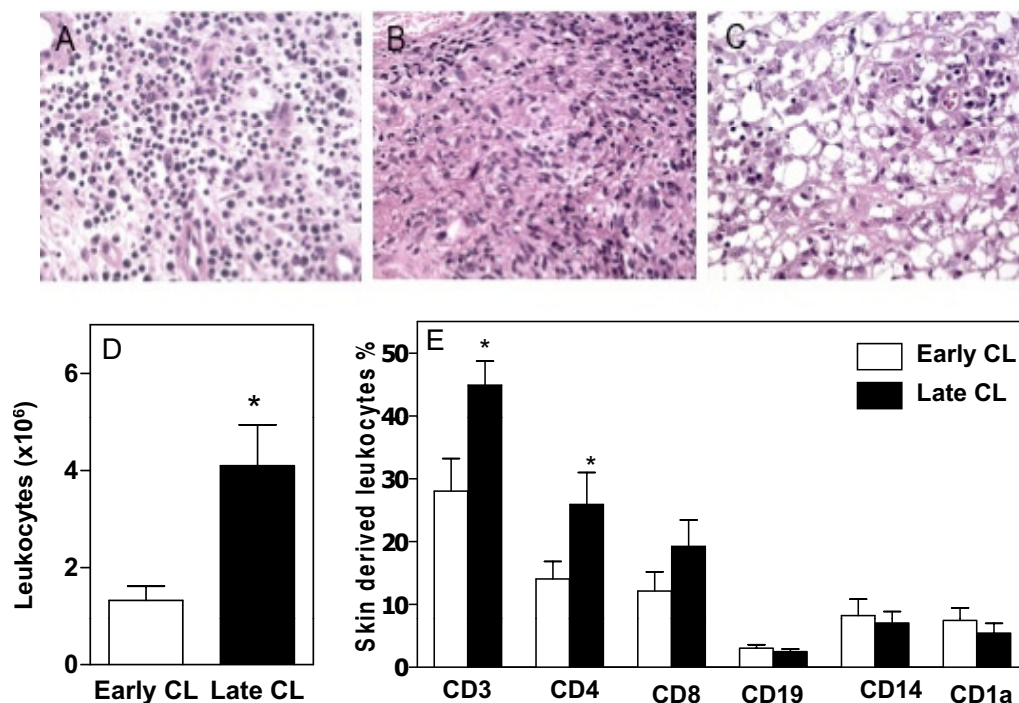
Fig. 1. Inflammatory cells in cutaneous leishmaniasis lesions. (A) Early CL lesion. Inflammatory infiltrate is composed mostly of macrophages mixed with polymorphonuclear leukocytes. H&E, ×200. (B) Late CL lesions. Inflammatory infiltrate is composed of mononuclear leukocytes. Epithelioid macrophages are mixed with a heavy infiltrate of lymphocytes, plasma cells, and macrophages. H&E, ×200. (C) DCL lesions. Inflammatory response consists of a monotonous infiltrate of vacuolated and heavily parasitized macrophages. H&E, ×200. (D and E) Biopsy samples of skin lesions of CL patients were digested with liberase, disrupted, filtered, washed, and the concentration and cell viability determined. In D is shown the total number of skin-derived leukocytes from early CL patients (□) and late CL patients (■). In E is shown the percentage of skin-derived leukocytes that expressed CD3, CD4, CD8, CD19, CD14, and CD1a. Bars show mean ± SEM of five early CL (open bars) and 5 late CL (filled bars) skin biopsy samples tested individually.

represented the main population (11.38–27.18% cells/biopsy), with CD3$^+$CD4$^+$ T cells ranging from 3.25% to 22.43% cells/biopsy (range, 7×10^3 to 6×10^5) and CD3$^+$CD8$^+$ T cells from 4.7% to 26.26% cells/biopsy (range, 9×10^3 to 2×10^5) (Fig. 1E). We also found 7×10^3 to 1×10^5 CD19$^+$ cells/biopsy (range, 1–4.28%), 2×10^4 to 20×10^4 CD14$^+$ cells/biopsy (range, 1.2–19.0%) and $1–3 \times 10^4$ CD1a$^+$ cells/biopsy (range, 0.88–13%) (Fig. 1E). The same pattern of lymphocyte predominance was also observed in late CL patients. Nevertheless, the percentage of skin-derived lymphocytes was significantly higher in late CL biopsy samples ($p < 0,005$), with CD3$^+$CD4$^+$ ranging from 9.96% to 46.15% of cell/biopsy (range, $2–7 \times 10^5$), CD3$^+$/CD8$^+$ from 9.52% to 46% of cells/biopsy (range, $1.5–7.5 \times 10^5$). Concerning B cells (CD19$^+$ cells), our results show that late LCL patients present with a percentage similar to that in early LCL patients (1–3.8% of cell/biopsy), as well as macrophage (CD14$^+$, 2–19.02% of cells/biopsy), and dendritic cells (CD1a$^+$, 0.88–13.07% of cells/biopsy) (Fig. 1E). The number of CD19$^+$ cells ($2–5 \times 10^4$ cell/biopsy), dendritic cells (CD1a$^+$) ($4–8 \times 10^4$ cell/biopsy), and macrophage (CD14$^+$, $2–18 \times 10^4$ cells/biopsy) was also similar to that in early LCL patients.

3.2. Chemokine receptors and cytokines expression on T cells from lesions of LCL patients

We have focused on the profile of chemokine receptors related to the recruitment of Th1 cells and regulatory T cells (CXCR3, CCR5, CCR3, and CCR4) and the main cytokines produced by these cells in early and late CL lesions. Flow cytometry analysis demonstrated the presence of T cells expressing CXCR3, CCR5, CCR3, and CCR4, with a higher prevalence of CXCR3$^+$ T cells in early LCL lesions (Fig. 2A) ($p < 0.05$), whereas CCR4$^+$ T cells predominated in late LCL lesions ($p < 0.05$). In all LCL samples, we observed cells positive for IFN-γ, TGF-β, or IL-10 (Fig. 2). A general analysis of LCL skin lesions indicated no differences in IFN-γ expression. However, a significant higher frequency of IL-10–producing cells as well as TGF-β was also observed in late LCL compared with early LCL (Fig. 2B).

3.3. Chemokines and chemokine receptor expression in lesions from LCL and DCL patients

Because we observed a different pattern of cell composition in the lesions of patients with distinct clinical manifestations, we investigated the presence of chemokines responsible for the recruitment of these cells (Figs. 3–5).

First, we analyzed CCL2 expression, a chemokine responsible for the attraction of monocytes. CCL2 expression is similarly expressed in LCL and DCL lesions, although in higher levels when compared with control biopsy results from healthy individuals (Fig. 3). Very low levels (less than 1%) of CCR2 (one of the receptors for CCL2)

were observed in the biopsy samples (Fig. 4). CCR7 and its ligands (CCL19 and CCL21) are essentially involved in homing of various subpopulations of T cells, including regulatory T cells [13]. DCL lesions presented with significantly lower levels of CCL19 than LCL, although expression of CCR7 was similar in the biopsy samples of different clinical manifestations of leishmaniasis investigated (Figs. 3 and 5). Next, we investigated chemokines and chemokine receptors related to Th1 response. LCL showed an increase in the expression of CCL5 compared with the controls, but no significant differences in the expression of this chemokine was found in DCL lesions. However, the expression of its receptor, CCR5, was significantly higher in biopsy samples from LCL patients and almost absent in those from DCL patients. In addition, we analyzed the expression of chemokine that recruit Th2 cells.

CCL7 has been associated with a variety of disease states, including allergic inflammation, presumably because of CCR3 expression on eosinophils and Th2 cells [14]. CCL7 is significantly higher expressed on DCL lesions than LCL lesions; however, the same did not occur with its receptor, CCR3, which is lower expressed in DCL lesions. We found higher expression of CCL17 in LCL lesions (Fig. 3) compared with DCL lesions, although it was also present in control samples ($n = 3$; obtained from women undergoing cosmetic breast surgery), albeit at a lower intensity (data not shown). We observed a higher intensity of this chemokine in immunohistochemical slides in early CL biopsy samples (Fig. 5). CCR4 was also detected in leukocytes from the patients with LCL, and a lower expression of this receptor was observed in DCL lesions, but it was not detected in leukocytes from healthy control subjects.

4. Discussion

After infection with *Leishmania* in the skin, a local inflammatory process is initiated; this involves the accumulation of leukocytes at the site of parasite delivery. The composition of the cell populations recruited in this early phase of the infection seems to be essential for defining the outcome of the disease. During this process, members of the chemokine family have a fundamental role in attracting specific subsets of leukocytes to the site of infection [8,9]. Possibly the evolution of early CL to late CL can be related to the differences in chemokines and chemokine receptor expression, which would influence the recruitment of cells able to destroy the parasite and would allow a better outcome of the disease. However, this can only be hypothesized for CL, which, in the present study, was studied at two time points: early and late. Importantly, this is not the case for DCL. Patients who are diagnosed with this clinical manifestation of DCL do not display an early phase. All patients present with a very long course of disease, and this is considered a severe form of cutaneous leishmaniasis. Therefore, the lack of che-
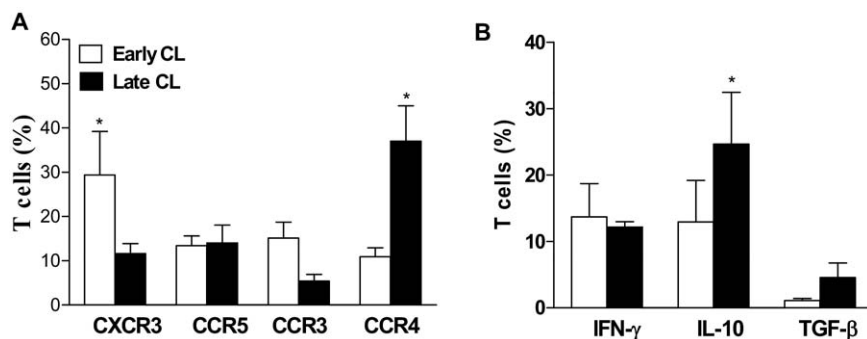
Fig. 2. Chemokine receptors and cytokines involved in human inflamed skin from CL patients. Biopsy samples of skin lesions of patients with leishmaniasis were digested with liberase, disrupted, filtered, washed, and the concentration and cell viability determined. (A) Percentage of skin-derived leukocytes from early CL patients (open bars, $n = 5$) and late CL patients (filled bars, $n = 5$) that expressed CXCR3, CCR5, CCR3, and CCR4. Results are mean ± SEM of data from donor patients tested individually. (B) Skin-derived cells were cultured for 6 hours and the intracellular cytokine assessed by flow cytometry. Bars show percentage of skin-derived leukocytes positive for IFN-γ, IL-10, or TGF-β after cultures (6 hours) performed with PHA. *$p < 0.05$.
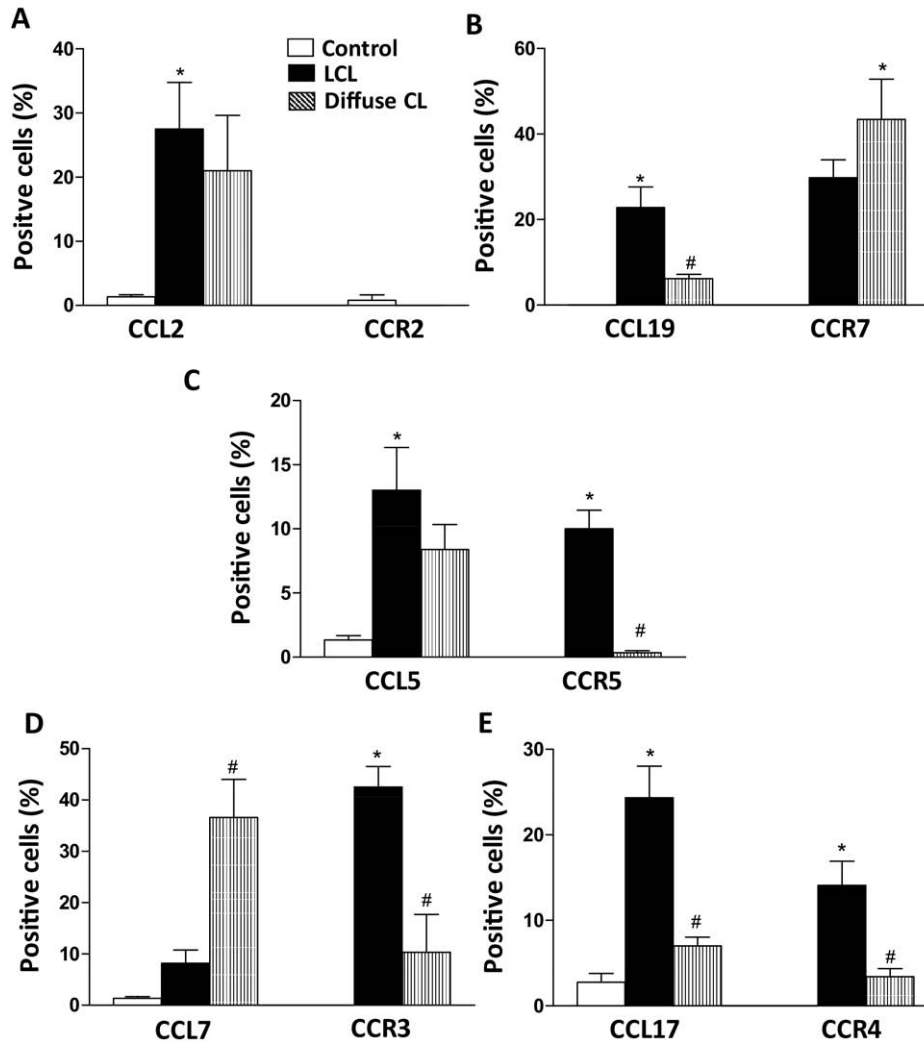
Fig. 3. Positive cells to chemokines and chemokine receptors in skin lesions of patients with leishmaniasis. Skin sections obtained from biopsy samples of patients with leishmaniasis and control samples were fixed with acetone and the expression of CCL2 and CCR2 (A), CCL7 and CCR3 (B), CCL5 and CCR5 (C), CCL17 and CCR4 (D), and CCL19 and CCR7 (E) investigated by immunohistochemistry, using specific mAb against the chemokines and their receptors followed by biotinylated antimouse IgG and avidin–biotin peroxidase Abs. Slides were then developed with DAB (brown color) and counterstained with hematoxylin. The number of positively stained cells for each antibody was counted per five consecutive microscopic high-power fields. Results were expressed as the mean of positive cells and as the percentage of positive cells per field, calculated in relation to the total number of inflammatory mononuclear cells. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ between patient groups and controls.

mokine/chemokine receptors in this form of disease could contribute to a failure to recruit certain cells important for the resolution of the disease.

Analyzing the T cells present in the early and late stages of the disease, we observed that in the late phase there is a predominance of CCR4 T cells and IL-10–producing cells, in agreement with the recruitment of Treg cell [15,16]. Recently, it was shown a higher expression of CXCR3 and CCR4 in the recruitment of regulatory T cells in the inflamed human liver. The authors proposed that CXCR3 mediates the recruitment of Treg cells via hepatic sinusoidal endothelium and CCR4 ligand recruit Treg cells to sites of inflammation in patients with chronic hepatitis. In our results, we found an increase in CXCR3 in the early phase of the disease and CCR4 in the late stage of LCL, as well as IL-10–producing cells and CCL17, in agreement with a regulatory role for T cells in the late stages.

CCL2, or monocyte chemoattractant protein–1 (MCP-1), is a highly specific chemotactic factor for macrophages and T-lymphocytes [17,18] and was increased in LCL lesions compared with control biopsy samples; but no differences were found between LCL and DCL lesions. Concerning CCL5 expression, no differences were ob-

served between LCL and DCL, although the expression of CCR5 was almost absent in DCL lesions, which could explain the lack of neutrophils in these lesions in contrast to LCL patient lesions, mainly in the patients in the early phase of the disease. CCR5 is present, mainly in Th1 cells, and this mechanism could be responsible for IFN-γ–producing cells recruited to the areas of biopsy samples in early and late LCL [19,20].

CCR4 receptor on T lymphocytes and its TARC/CCL17 are implicated in lymphocyte–endothelial interactions during lymphocyte recruitment to normal and inflamed cutaneous sites. Immunohistochemistry suggests that TARC/CCL17 is constitutively expressed and hyperinducible on cutaneous venules and some other systemic venules. Because CCR4 is predominantly expressed on the Th2 type [21], it is suggested that TARC/ CCL17 is involved in Th2-polarized diseases. In our study, we observed that the levels of TARC/CCL17 are higher in LCL lesions compared with DCL. Results from our group have shown a low frequency of CD4+ T cells producing IL-4 in LCL lesions (results not published), but a higher frequency of CD4 T cells producing IFN-γ and IL-10.

An important finding was the expression pattern of CCL7 that was more prominent in the DCL lesions. CCL7 is among the most
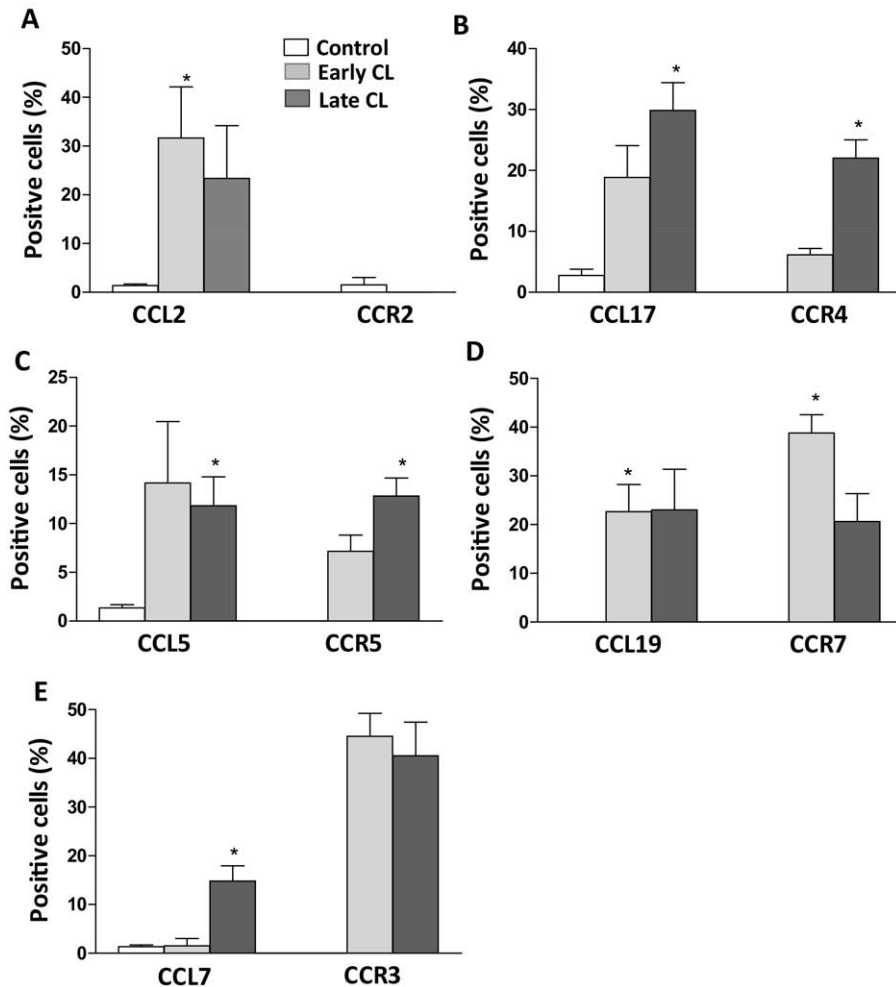
Fig. 4. Expression of chemokines and chemokine receptors in early and late CL lesions. Biopsy samples from patients with localized cutaneous leishmaniasis (LCL) were assayed as described above, separated at different stages of human LCL (early CL and late CL), and evaluated for chemokines and chemokine receptor expression.

pleiotropic chemokines since it recruits all major leukocyte classes (monocytes, eosinophils, basophils, NK cells, T lymphocytes, and neutrophils) by binding to at least three different chemokine receptors (CCR1, CCR2, and CCR3) [22]. Recently, Katzman and Fowell [23] showed that the tissue-specific accumulation of effector T cells can be subverted by the pathogen at the infection site. Using the *L major* murine model of dermal infection, the authors observed a restricted chemokine profile at the infection site, expression of Th2-cell–attracting CCL7, but not Th1-cell–attracting chemokines. This result is very important, because it demonstrates a dichotomy between LN and site responses. Although in DCL lesions the number of T cells is scarce, we hypothesize that CLL7 in the lesions could attract Th2 cells. In fact, during active disease, PBMC from patients did not express mRNA for IFN-γ while expressing mRNA for IL-2, IL-4, and IL-10, pointing out the role of Th2 cells in this disease [24].

The current study also showed that CCR7 and CCL19 are expressed in high levels at LCL lesions. CCL19 is produced in lymphoid tissues and at sites of inflammation, it binds exclusively to CCR7 on activated T and B cells and mature dendritic cells, and induces the chemotaxis of these cells [25]. Thus, it is reasonable to speculate that CCL19 expression observed in our study may influence trafficking of dendritic cells and T cells after *L braziliensis* and *L amazonensis* infection. However, it is important to note that this chemokine was expressed in minor intensity in DCL lesions. The differences between CCL19 expressions could be correlated with

the composition of inflammatory infiltrate at the site of *L braziliensis* and *L amazonensis* infection.

The pattern of chemokines observed in early and late lesions could be related to the cytokine pattern evaluated. In fact, CCL5, CCR5, and CXCR3 present in tissues could favor a predominant Th1 cell migration as seen in rheumatoid arthritis [26], and the resistance to the infection could be related to the induction of these chemokines and IFN-γ production, although no differences were observed in the production of this cytokine in the early or late stages of LCL. In contrast, CCL17 expression in late LCL patients could result in attraction of cells CCR3+ and CCR4+ for the inflammatory focus leading to IL-10 and TGF-β production and to the control of the inflammatory reaction. In agreement with this possibility, recently we showed that functional CD4+CD25+ T cells accumulate in sites of *Leishmania* infection in human beings, and those cells are able to produce large amounts of IL-10; in addition, TGF-β can contribute to the local control of effector T cell functions and/or disease outcome [15].

This study has demonstrated the expression of chemokines and chemokine receptors in leishmaniasis skin lesions; it is thus notable because chemokines are involved in Th1/Th2 lymphocyte differentiation [26,27] as well as an immunomodulatory role, recruiting regulatory T cells to the site of infection and precluding an excessive inflammatory response. Whether the expression of different chemokines may influence clinical manifestation of leishmaniasis rather than leukocyte recruitment to inflammatory sites requires further investigation. New approaches will aid in the elu-
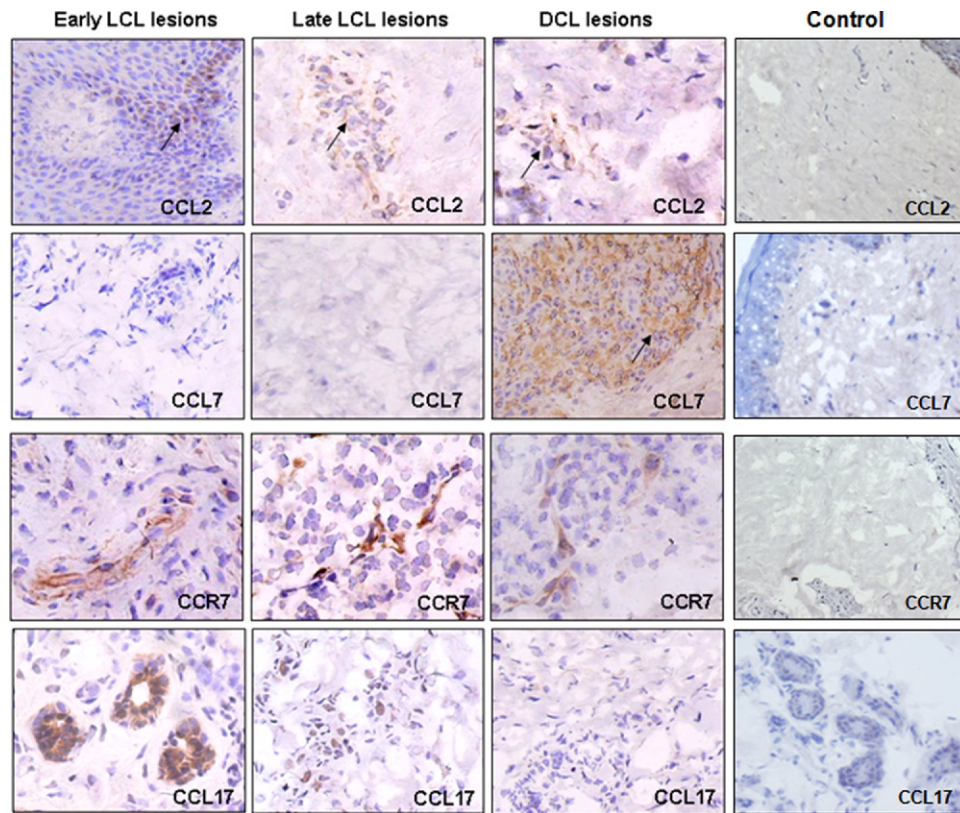
Fig. 5. Expression of chemokines and chemokine receptors in lesions from CL patients. Skin sections obtained from biopsy samples of patients with leishmaniasis and control samples were fixed with acetone, and expression of CCL2, CCL7, CCL17, and CCR7 was investigated by immunohistochemistry. Slides were stained with mouse antihuman CCL2, antihuman CCL7, antihuman CCL17, and antihuman CCR7 mAb, followed by biotinylated antimouse IgG and avidin–biotin peroxidase. Slides were then developed with DAB (brown color) and counterstained with hematoxylin. Experiment shown is representative of results obtained with 10 CL patients. Original magnification, ×400.

cidation of the precise mechanisms and role of chemokines in leishmaniasis pathology.

Acknowledgments

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