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MAJOR ARTICLE

Early Cutaneous Leishmaniasis Patients Infected With *Leishmania braziliensis* Express Increased Inflammatory Responses After Antimony Therapy

Rúbia S. Costa,[1] Lucas P. Carvalho,[1,2,3] Taís M. Campos,[1] Andréa S. Magalhães,[1] Sara T. Passos,[1] Albert Schriefer,[1,4] Juliana A. Silva,[1] Ednaldo Lago,[1] Camilla S. Paixão,[1] Paulo Machado,[1,3] Phillip Scott,[4] and Edgar M. Carvalho[1,2,3]

[1]Serviço de Imunologia, Hospital Universitário Professor Edgard Santos, Universidade Federal da Bahia, Salvador, Brazil; [2]Laboratório de Pesquisas Clínicas, Instituto Gonçalo Moniz, Fiocruz, Salvador, Bahia, Brazil; [3]Instituto Nacional de Ciência e Tecnologia em Doenças Tropicais, Universidade Federal da Bahia, Salvador, Brazil; [4]Department of Pathobiology, School of Veterinary Medicine, University of Pennsylvania, Philadelphia

Background. Early cutaneous leishmaniasis (ECL) is characterized by a nonulcerated papular lesion and illness duration less than 30 days. Approximately 4 weeks later, the cutaneous leishmaniasis (CL) ulcers appear. We were surprised to find that failure after antimony therapy (Sb^5) is higher in ECL than CL. We hypothesize that the inflammatory response in ECL patients may increase during Sb^5 therapy, which leads to treatment failure.

Methods. A cohort of 44 ECL patients infected by *Leishmania braziliensis* was established to evaluate the response to Sb^5 and to compare immunologic responses in ECL patients with CL and healthy subjects.

Results. A hierarchical clustering based on cytokine levels showed a weak positive correlation between proinflammatory cytokine levels and those patients that failed Sb^5 treatment. Although Sb^5 therapy decreased interferon-γ and tumor necrosis factor levels in CL patients, we were surprised to find that an increase in these cytokines was observed in ECL patients. Moreover, interleukin (IL)-10 was less able to down-modulate immune responses in ECL.

Conclusions. The enhanced production of proinflammatory cytokines, due in part to the decreased ability of IL-10 to down-modulate immune response during therapy in ECL, promotes the development and persistence of leishmania ulcer despite antimony therapy.

Keywords: early cutaneous leishmaniasis; chemokines; cutaneous leishmaniasis; cytokines; *Leishmania braziliensis*.

Leishmania (*Viannia*) *braziliensis* is the most important causal agent of American tegumentary leishmaniasis (ATL), and cutaneous leishmaniasis (CL) is the most common clinical form of the disease [1]. We have previously shown that the classic ulcerated lesion characteristic of the leishmania ulcer is a relatively late event in the disease [2]. Before an ulcer develops, patients usually present with a large regional lymphadenopathy followed in 1 to 2 weeks by a nonulcerated papular lesion characteristic of early cutaneous leishmaniasis (ECL) [2, 3]. After 2 to 4 weeks, the cutaneous ulcer appears, which characterizes classic CL [3]. Although the Th1 immune response is important to control parasite growth and to prevent *Leishmania* proliferation and dissemination, there is substantial evidence that both $CD4^+$ and $CD8^+$ T-cell activation and production of proinflammatory cytokines are associated with intensity of the inflammatory response and pathology [4–7]. In CL, there is an exacerbated production of tumor necrosis factor (TNF) and interferon (IFN)-γ [8] and a direct correlation between the frequency of CD4 T cells expressing IFN-γ and TNF with ulcer size [8, 9]. There is also a direct correlation between the frequency of $CD8^+$ T cells expressing granzyme B with the intensity of the inflammatory reaction [7]. Moreover, although $CD8^+$ T cells kill *Leishmania*-infected cells, this does not lead to parasite killing [7, 10]. Thus, the exaggerated inflammatory response in CL is due, in part, to parasite persistence with continuous stimulation of the immune system, as well as due to a decreased ability of regulatory cytokines to down-modulate the inflammatory response [11, 12]. The parasite load is higher in ECL than in CL [13], and IFN-γ and TNF levels are higher in CL than in ECL [14]. Moreover, in the late phase of CL, the immune response is more modulated due to regulatory T cells [15, 16]. Thus, it is possible that in the initial phase of the disease, the release of parasite antigens due to drug treatment induce an enhancement of the inflammatory response and consequently more pathology. Alternatively, inflammation and pathology in ECL may be mediated by the innate immune response because inflammatory monocytes are increased in ECL [17], and they are the major source of TNF and metalloproteinases (MMPs) known to be involved with the pathology of CL [17, 18].

In infectious diseases, early therapy is associated with a high rate of cure and fast healing time. Unexpectedly, however, ECL patients have a much higher rate of failure to antimony therapy (Sb^5) than patients with CL [14, 19]. In CL, Sb^5 therapy

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Correspondence: E. M. Carvalho, MD. Serviço de Imunologia, Hospital Universitário Prof. Edgard Santos, Universidade Federal da Bahia. Rua João das Botas, S/N, 5º andar, Canela, Salvador, Bahia, Brazil, 40170-110 (imuno@ufba.br).

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is followed by a decrease in IFN-γ and TNF production [20], but whether this is the case in ECL is unknown. By studying a cohort of ECL patients and comparing them with CL patients, we found that an enhanced immune response occurs in ECL in spite of treatment. We hypothesize that, in ECL, either increased parasite killing and antigen release fuels the inflammatory response or that independent of chemotherapy a natural evolution of the immunologic response occurs, increasing inflammation and leading to ulcer development despite therapy.

MATERIAL AND METHODS

Experimental Design

This is a mixed study with a cohort component in which we attempt to correlate immunologic responses before and during therapy with cure of failure to Sb^5 therapy in patients with ECL, and it is a cross-sectional study comparing the immunologic response of these individuals with CL patients and healthy subjects (HS).

Study Population

The 44 patients with ECL as well the healthy controls were recruited from March 2011 to July 2015, and the 17 ECL and 14 CL patients were recruited from September of 2014 to July 2015. All ECL and CL patients were from the Health Post of Corte de Pedra, Tancredo Neves-Brazil, a well known area of *L braziliensis* transmission. The HS lived in an area without evidence of *L braziliensis* infection. Early cutaneous leishmaniasis was defined as a patient with a papular lesion and enlargement of a regional lymph node or only lymphadenopathy with history of illness duration less than 30 days and detection of *L braziliensis* deoxyribonucleic acid by polymerase chain reaction (PCR), as previously described [21]. The sample size was calculated based on our previous data of TNF production in ECL who failed or were cured after antimony therapy [14]. Patients with CL had 1 well limited ulcer and illness duration between 30 to 75 days, and diagnosis was confirmed by PCR [21]. The age, gender, number of lesions, and localization of the lesions were similar in ECL and CL patients. However, illness duration and the size of the major lesion were greater in CL than in ECL patients. Immunologic studies in ECL and CL were performed before and 15 days after initiation of Sb^5. The present study was approved by Federal University of Bahia Institutional Review Board (12/25) and the National Commission of Ethics in Research (612.907), and all patients signed an informed consent.

Treatment of Leishmaniasis

All patients were treated with intravenous glucantime (Sanofi Aventis) in a dose of 20 mg/kg of weight for 20 days. Patients were followed with an interval of 15 days up to 90 days. Cure was defined as a complete healing of the ulcer and re-epithelization of the skin in the absence of raised borders. Failure was defined as persistence of active ulcer or scar of the lesion with persistence of raised borders on day 90. Those who were not cured at day 90 received a second course of Sb^5 and were followed every 30 days for up to 6 months. Patients who failed 2 courses of antimony received amphotericin B.

Soluble *Leishmania* Antigen Preparation

The soluble *Leishmania* antigen (SLA) was prepared from an *L braziliensis* isolated (MHOM/BR/2001) from a patient with CL, by sonication and centrifugation, as previously described [22]. It was tested for endotoxin using the Limulus amebocyte lysate test and used at a concentration of 5 μg/mL.

Determination of Cytokines, Chemokines, and Metalloproteinase-9 for Enzyme-Linked Immunosorbent Assay

Peripheral blood mononuclear cells (PBMCs) were obtained from heparinized venous blood by density gradient centrifugation, washed, and resuspended in Roswell Park Memorial Institute 1640 media. Cells (3×10^6) were cultured at 37°C, at 5% CO_2, in the presence of SLA (5 μg/mL) or only with medium for 72 hours. The levels of cytokines (interleukin [IL]-10, TNF, IFN-γ, IL-1β), chemokines (CXCL-9, CXCL-10), and MMP-9 were measured by enzyme-linked immunosorbent assay (DuoSet; R&D Systems). To determine whether IL-10 down-modulates the cytokine production, recombinant human IL-10 (R&D Systems), at 100 ng/mL plus SLA (5 μg/mL), was also added to PBMC cultures from patients with ECL. To determine whether CXCL-9 and CXCL-10 production was independent of IFN-γ, monoclonal antibody anti-IFN-γ (R&D Systems), at 5 μg/mL plus SLA (5 μg/mL), was added to PBMC cultures from ECL.

Statistical Analysis

The data were analyzed using nonparametric tests. To analyze PBMC cultures in different conditions within the same group of individuals, the Wilcoxon test was used, and to detect differences between groups, the Mann-Whitney *U* test was used. All of the results were expressed by median values and were analyzed considering a value of $P < .05$ (statistically significant). The analysis of hierarchical clustering was realized by the platform GenePattern (Broad Institute).

RESULTS

Demographic and Clinical Features of Early Cutaneous Leishmaniasis Patients Regarding the Response to Antimony

The demographic and clinical features of 44 patients with ECL classified in 2 groups according to the response to Sb^5 are shown on Table 1. Of 44 patients with ECL, 12 (27%) were cured at day 90 and 32 (73%) failed to Sb^5 therapy. There was no difference between the groups regarding age, gender, number and size of the lesion at admission, as well as size of lymphadenopathy. The size of the lesion on day 30 after initiated therapy was higher in the group of patients who failed. We also but did not find any difference among CXCL-9, CXCL-10, TNF, IFN-γ, and IL-10

Table 1. Baseline Evaluation of Demographic and Clinical Features of Early Cutaneous Leishmaniasis Patients According to the Response to Antimony Therapy

Demographic and Clinical Finding	Treatment Outcome		
	Cure (N = 12)	Failure (N = 32)	($P < .05$)
Age (years; M ± SD)	32 ± 7.9	29 ± 6.6	$P > .05$[a]
F/M (%)	37:67	44:56	$P > .05$[b]
Number of lesion	1 ± 1	1 ± 1.2	$P > .05$[c]
Size of the lesion at admission (mm)	7.3 ± 3.2	8.1 ± 2.8	$P > .05$[c]
Major size of the lesion in day 30	11 ± 3.8	15 ± 4.9	$P = .0021$[c]
Size of lymphadenopathy (mm; M ± SD)	34 ± 19.3	35 ± 12.1	$P > .05$[c]

Abbreviations: M, mean; SD, standard deviation.

[a]Student's *t* test.

[b]Fisher's exact test.

[c]Non-parametric test, Mann-Whitney *U*.

levels determined before and during therapy with response or failure to treatment (see Supplementary Figure 1).

Hierarchical Clustering of Cytokines and Chemokines Production in Patients With Early Cutaneous Leishmaniasis

Because we did not find an association between the production of 1 specific chemokine or cytokine with response or failure to therapy, a hierarchical clustering taking into account the production of these molecules in each patient who failed or was cured was performed. The analysis was conducted based on the production of chemokines and cytokines by PBMCs stimulated with SLA. The result of the clustering dendrogram is shown in Figure 1. Group 1 represents the low producers of cytokines, and group II represents the high producers of cytokines. After determination of the clinical outcome of these on cure (C) or failure (F), the relative risk for failure to treatment (RR) in each group was determined. Early cutaneous leishmaniasis patients high producers of cytokines (Group II) were 1.5-fold more likely to fail therapy compared with Group 1.

Production of Chemokines, Cytokines, and Metalloproteinase-9 in the Early Cutaneous Leishmaniasis

To determine the profile of the immune response early in infection, the production of cytokines and MMP-9 in cultures without stimulus or stimulated with SLA was compared between ECL and HS. Although there was very little production of cytokines and no difference between the 2 groups in unstimulated cultures, patients with ECL produce more CXCL-9, CXCL-10, IFN-γ, TNF, IL-10, and IL-1β than HS in cultures stimulated with SLA (Figure 2A–C). We had previously shown that TNF and IFN-γ are higher in CL than in ECL [14]. In this study, we observe that IFN-γ, TNF, CXCL-9, CXCL-10, and IL-10 levels were higher in CL than in ECL (see Supplementary Figure 2).

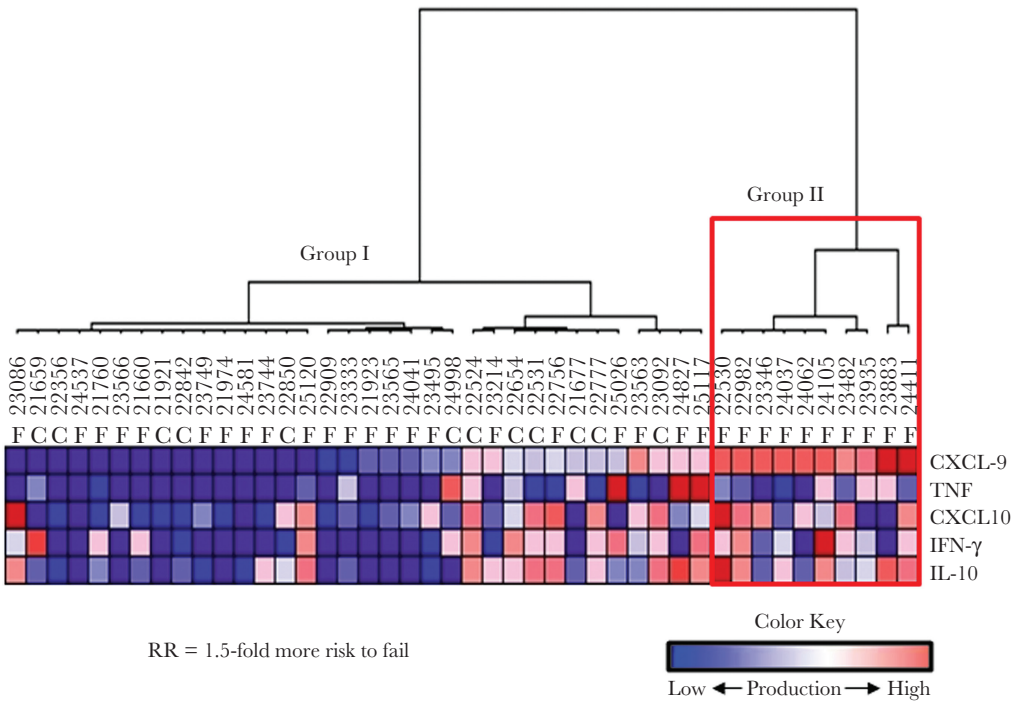

Figure 1. Hierarchical clustering of healing and failure groups represented by the production of cytokines and chemokines. Peripheral blood mononuclear cells of patients with early cutaneous leishmaniasis (ECL) before treatment with glucantime (N = 44) were stimulated with soluble *Leishmania* antigen (5 μg/mL). The columns represent the ratios of the relative production of cytokine in each ECL patient who fail (F) or cure (C), and the lines are the results of individual production of each cytokine and chemokine. Blue indicates low production and red indicates high production. The analysis hierarchical clustering was realized by Plataform GenePartten from Broad Institute by the method of similarity. The relative risk (RR) was calculated from the program MedCalc easy-to-use statistical softwere. RR = 1.5-fold more risk to fail, $P = .006$.

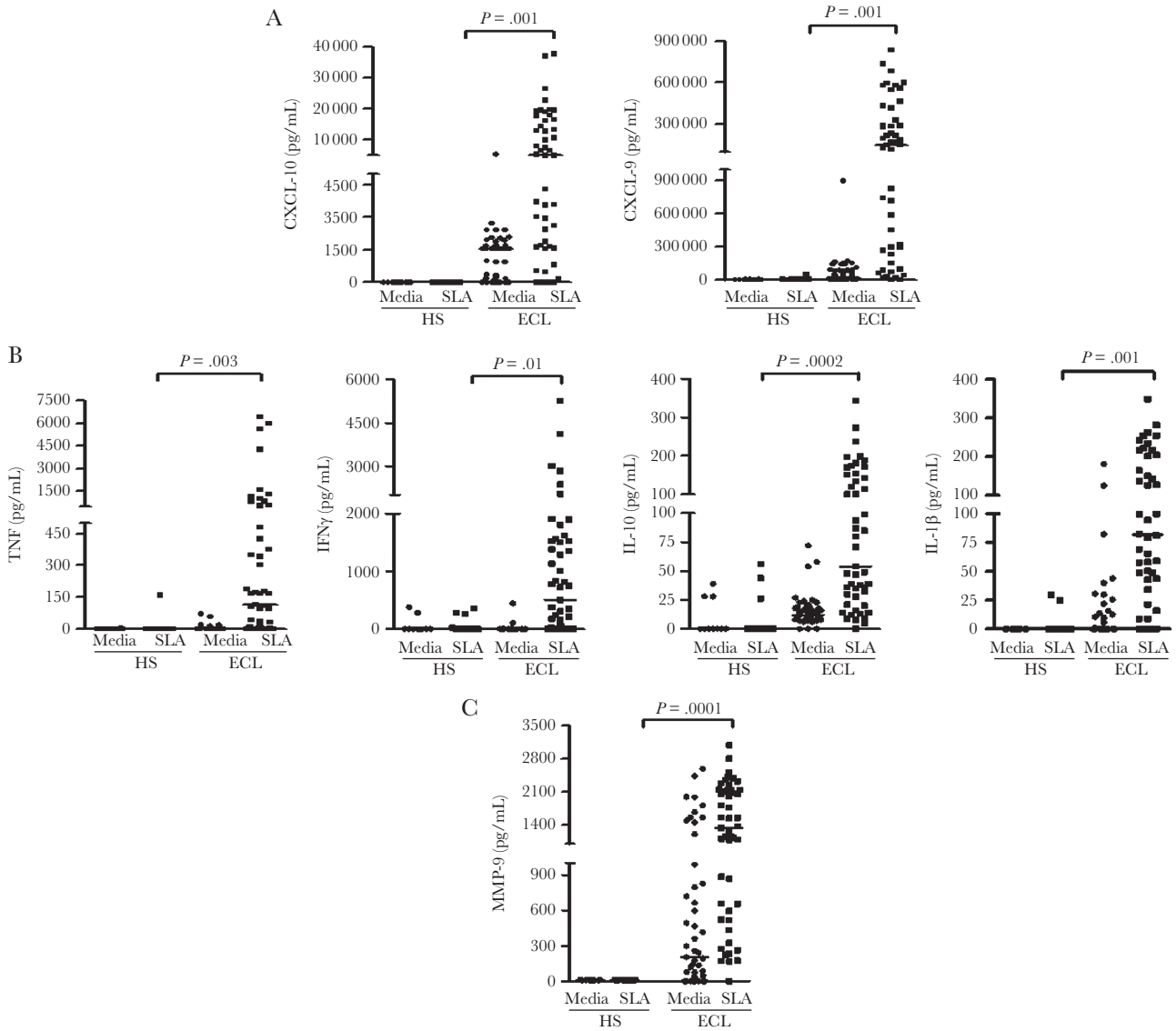

Figure 2. Production of chemokines, cytokines and metalloproteinase (MMP)-9 in ECL. Peripheral blood mononuclear cells of healthy subjects (HS) and patients with early cutaneous leishmaniasis (ECL) before treatment with glucantime (N = 44), and HS (N = 10) were kept unstimulated or stimulated with soluble *Leishmania* antigen ([SLA] 5 μg/mL). The chemokines (A), cytokines (B), and MMP-9 (C) levels were determinated by enzyme-linked immunosorbent assay. Statistical analysis was performed by Mann-Whitney *U* test. ($P < .05$). Abbreviations: IFN, interferon; IL, interleukin; TNF, tumor necrosis factor.

Correlation Between Chemokine and Cytokine Production in Patients With Early Cutaneous Leishmaniasis

The chemokines CXCL-9 and CXCL-10 are associated with IFN-γ production and recruit of monocytes and T cells to the inflammatory tissues [23]. They are also highly expressed in ECL [24]. To evaluate whether the production of these chemokines was related to the IFN-γ production or due to activation of innate cells, a correlation between IFN-γ and these chemokines was evaluated. In this study, we show a weak correlation between CXCL-9 and CXCL-10 ($P = .02$, r = 0.34; Figure 3A), IFN-γ and CXCL-9 ($P = .01$, r = 0.35; Figure 3B), and IFN-γ and IL-1β ($P = .01$, r = 0.34; Figure 3C) and a moderate correlation between IFN-γ and CXCL-10 ($P = .01$, r = 0.45; Figure 3D). The weak correlation between IFN-γ and proinflammatory chemokine and IL-1β and the importance of monocytes in the pathogenesis of ECL [25] indicates that cells and molecules related to the innate immune response play an important role in the secretion of chemokines in ECL.

Correlation Among CXCL-9, CXCL-10, Tumor Necrosis Factor, and Interferon-Gamma With Interleukin-10 in Patients With Early Cutaneous Leishmaniasis

Interleukin-10 is an important regulatory cytokine, and, in CL, a direct correlation between proinflammatory cytokines and IL-10 is observed [26]. The correlation among CXCL-9, CXCL-10, TNF, and IFN-γ production with IL-10 in ECL patients is shown in Figure 4. There was a moderate direct correlation between production of CXCL-9 and IL-10 ($P = .0001$, r = 0.55;

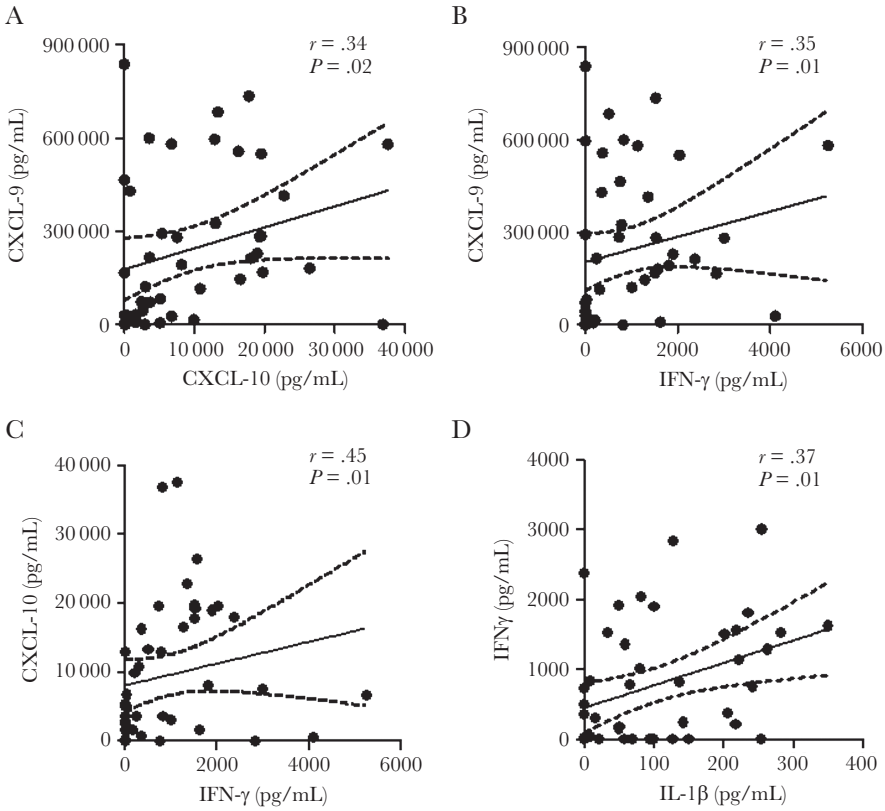

Figure 3. Correlation between production of chemokines and cytokines in early cutaneous leishmaniasis (ECL). The CXCL-9, CXCL-10, interferon (IFN)-γ, and interleukin (IL)-1β levels were obtained from cell cultures stimulated with soluble *Leishmania* antigen (5 μg/mL) of 44 ECL patients before treatment with glucantime using enzyme-linked immunosorbent assay technique. The correlation between levels of CXCL-9 and CXCL-10 (A), CXCL-9 and IFN-γ (B), CXCL-10 and IFN-γ (C), and IFN-γ and IL-1β (D). Data were analyzed by the Spearman correlation test. Statistical significance, $P < .05$.

Figure 4A), CXCL-10 and IL-10 ($P = .0001$, r = 0.54; Figure 4B), and TNF and IL-10 ($P = .002$, r = 0.44; Figure 4C) and between IFN-γ and IL-10 ($P = .001$, r = 0.48; Figure 4D) in patients with ECL.

Influence of Neutralization of Interferon-Gamma and Exogenous Addition of Interleukin-10 in the Immune Response in Patients With Early Cutaneous Leishmaniasis

It is known that IL-10 has inhibitory effects on monocyte function and down-modulates the Th1 immune response [27, 28]. Similar to what we have previously shown in CL [11], in the present study, the addition of IL-10 to SLA-stimulated PBMC culture of ECL decreased the TNF, IFN-γ, and CXCL-9 levels (see Supplementary Figure 3). However, when we evaluated the ability of IL-10 to down-modulate CXCL-10 production, we observed an opposite effect of this cytokine in the 2 different stages of the disease (Figure 5). Although the CXCL-10 levels decreased from 1348 to 625 pg/mL ($P = .01$) in the presence of recombinant IL-10 in CL, exogenous addition of IL-10 enhanced the production of CXCL-10 from 175 to 785 pg/mL ($P = .009$) in ECL (Figure 5A). Interferon-γ is the main cytokine that activates macrophages [14]. It is known that neutralization of IFN-γ decreases TNF production in CL [29]. In this study, anti-IFN-γ was added to cell culture of ECL and CL patients. Neutralization of IFN-γ significantly decreased the production of CXCL-9 in both ECL and CL patients (see Supplementary Figure 4). However, an opposite effect of IL-10 was observed in CXCL-10 production. Although neutralization of IFN-γ decreased CXCL-10 in CL from 900 to 425 pg/mL ($P = .01$), it enhanced the production of this chemokine in ECL from to 379 to 929 pg/mL ($P = .02$) (Figure 5B).

Cytokines and Chemokines Production Before and During Antimony Therapy in Early Cutaneous Leishmaniasis and Cutaneous Leishmaniasis Patients

The production of CXCL-9, CXCL-10, TNF, and IFN-γ on day 0 and day 15 of Sb^5 therapy in ECL and CL are shown in Figure 6A and B, respectively. In both ECL and CL patients, there was an increase of CXCL-9 and CXCL-10 on day 15 of therapy. However, we found a remarkable difference between CL and ECL patients in IFN-γ and TNF production during therapy. Although the median levels of IFN-γ in CL before and during therapy drop from 2601 to 1428 pg/mL ($P < .05$) and TNF drop from 530 to 299 pg/mL ($P < .05$), both IFN-γ and TNF levels enhanced during Sb^5 therapy in ECL. The IFN-γ levels and TNF before and during therapy in ECL were 240 and 700 pg/mL and 166 and 456 pg/mL, respectively ($P < .05$).

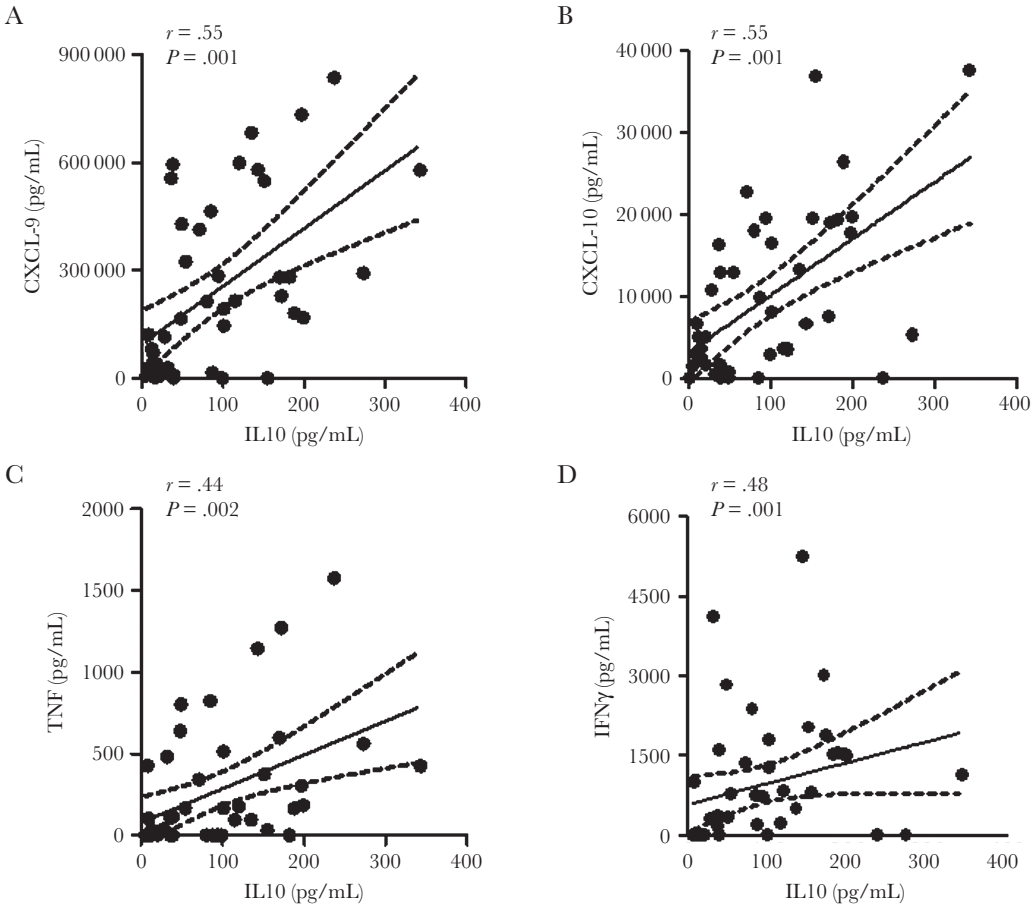

Figure 4. Correlation between production of CXCL-9, CXCL-10, tumor necrosis factor, and interferon (IFN)-γ with interleukin (IL)-10. The data were obtained from cell cultures stimulated with soluble *Leishmania* antigen ([SLA] 5 μg/mL) of 44 early cutaneous leishmaniasis patients before treatment with glucantime using enzyme-linked immunosorbent assay technique. Correlation analysis between levels of CXCL-9 and IL-10 (A), CXCL-10 and IL-10 (B), TNF and IL-10 (C), and IFN-γ and IL-10 (D). Data were analyzed by the Spearman correlation test. Statistical significance, $P < .05$. Abbreviations: a, alpha; r, recombinant.

DISCUSSION

Understanding the factors involved in the progression of the papular lesion found in ECL to the ulcer characteristic of CL, and why patients with ECL have a high rate of failure to Sb^5, may identify target molecules that are associated with ulcer development. In the present study, we observed that early after *L braziliensis* infection, the inflammatory response is already upregulated because PBMCs produce high levels of CXCL-9, CXCL-10, TNF, IFN-γ, IL-1β, and MMP-9 upon stimulation with SLA. Similar to what was observed in CL patients, exogenous addition of IL-10 decreased CXCL-9, TNF, and IFN-γ in ECL. However, whereas IL-10 decreased CXCL-10 production in CL, it enhanced CXCL-10 production in ECL patients. Moreover, although antimony therapy decreased TNF and IFN-γ levels in CL, the production of these cytokines was enhanced during Sb^5 in ECL patients. We recognized that the low numbers of patients with ECL who were cured may have prevented us from documenting a correlation between a particular cytokine with failure to therapy. However, our data indicate that a failure to down-modulate the inflammatory response is associated with progression from ECL to CL.

Similar to our previous observations, the majority of ECL failed to Sb^5 [14, 30]. However, we did not find an association between clinical findings or 1 particular cytokine with failure to therapy. Thus, a hierarchical cluster was performed, considering all the cytokines evaluated. There was a positive, although weak, correlation between increased levels of cytokines and failure to therapy. This led us to extend our immunologic study, and we aimed to better characterize the inflammatory response in ECL and identify inflammatory targets that would explain the progression from a papular lesion to an ulcer despite therapy.

The immune response in CL is characterized by an overproduction of proinflammatory chemokines and cytokines [8, 31]. In previous studies, we showed that TNF and IFN-γ levels were lower in ECL than in CL [14]. In this study, we extended these observations and showed that PBMCs from ECL produce high levels of CXCL-9, CXCL-10, TNF, and IL-1β after stimulation with SLA. The role of CXCL-9 and CXCL-10 in

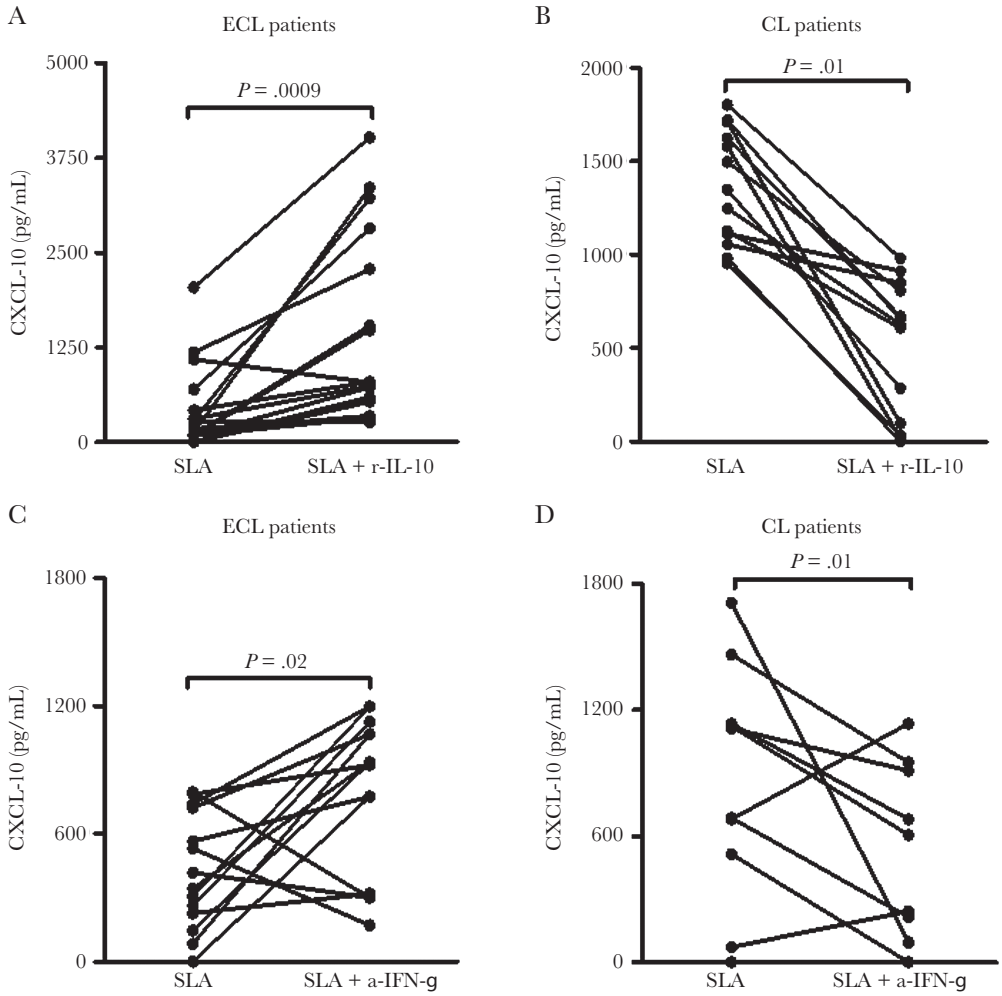

Figure 5. Effect of exogenous addition of interleukin (IL)-10 and interferon (IFN)-γ neutralization on the production of CXCL-10. Peripheral blood mononuclear cells from patients with early cutaneous leishmaniasis (N = 17) and cutaneous leishmaniasis (N = 14) before treatment with glucantime were stimulated with soluble *Leishmania* antigen (5 μg/mL) in the presence or absence of recombinant IL-10 (100 ng/mL) (A and B) or anti-IFN-γ (C and D) antibody (5 μg/mL). The production of CXCL-9 and CXCL-10 was evaluated by enzyme-linked immunosorbent assay. Statistical analysis was performed by Wilcoxon matched pairs test ($P < .05$). Abbreviation: TNF, tumor necrosis factor.

CL is not specifically defined, but the high production of these chemokines may influence the clinical outcome of *L braziliensis* infection, because both cytokines recruit cells to the lesion site [32]. There is also evidence that IL-1β and TNF are associated with pathology in *L braziliensis* infection [8–10, 24]. Moreover, MMP-9 produced by monocytes is upregulated by TNF [18]. Metalloproteinases degrade extracellular matrix and participate in tissue injury. Metalloproteinase genes are over expressed in CL [18] and are associated with severity of ATL and failure to therapy [33]. The ability of unstimulated PBMCs from ECL to produce MMP-9 and the enhancement of this molecule after stimulation with SLA indicate that MMP-9 production occurs early in *L braziliensis* infection, and its role in ulcer development deserves further investigation.

A direct correlation between IFN-γ and TNF with lesion size is documented in CL, and IFN-γ is the main cytokine inducer of CXCL-9 and CXCL-10 [9, 23, 34]. However, the correlation between CXCL-9 and CXCL-10 and TNF with IFN-γ production in ECL was weak, suggesting that others factors than IFN-γ are inducing these cytokines production. In fact, CXCL-10 synthesis may occur even in absence of IFN-γ [35] and TNF, and IFN-γ may induce CXCL-10 by different signaling pathways [36]. To test this hypothesis, we neutralized IFN-γ with monoclonal antibodies against this cytokine in cell cultures stimulated with SLA and measured CXCL-9 and CXCL-10. Although in CL neutralization of IFN-γ decreased CXCL-10 production, an enhancement in the production of this chemokine was observed in ECL. This phenomenon was observed with CXCL-10 but not with CXCL-9. CXCL-10 is produced by a variety of cells but mainly by monocytes [37], and the intermediate monocytes are the main source of proinflammatory cytokines [17, 38]. Because this monocyte subpopulation is increased in ECL, it is possible that monocytes play a major role in the inflammatory response and in the pathology observed in ECL. It is clear that macrophages and T cells prevent *Leishmania* growth and dissemination [4, 39]. However, in

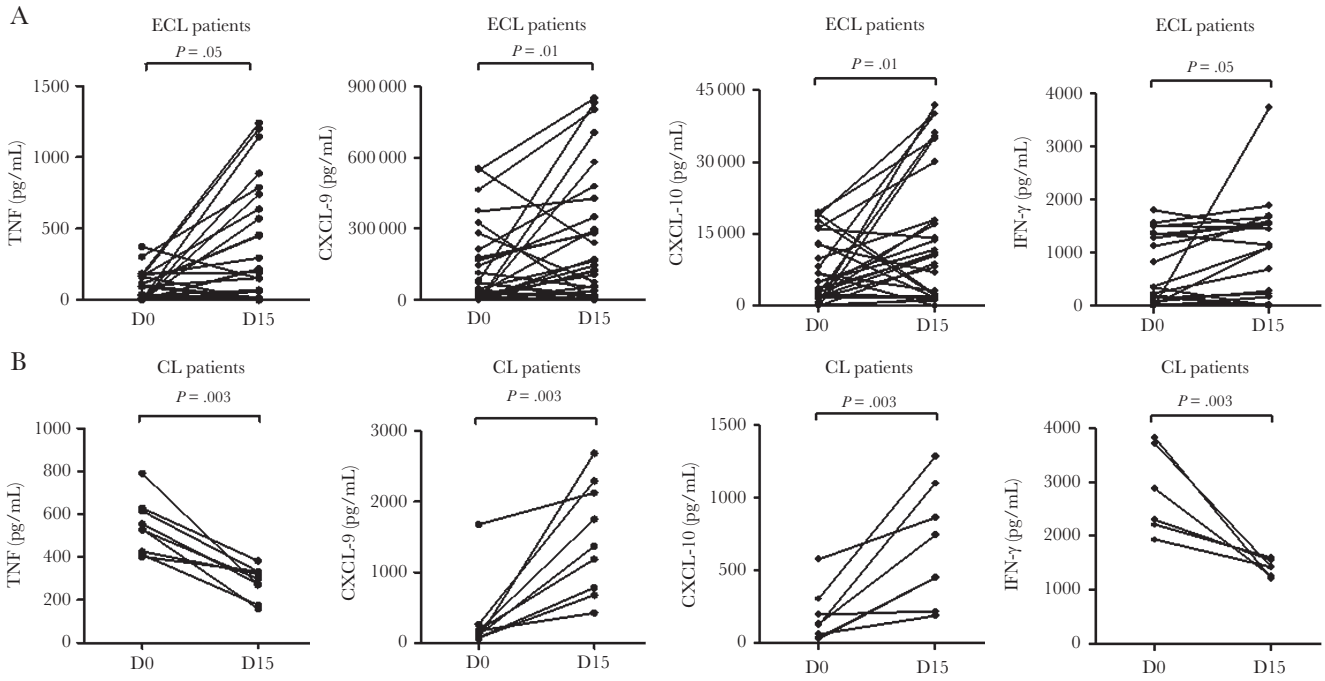

Figure 6. Production of cytokines and chemokines before and during treatment with glucantime. Peripheral blood mononuclear cells from patients with early cutaneous leishmaniasis ([ECL] N = 44) and cutaneous leishmaniasis ([CL] N = 13) before treatment and in day 15 of treatment with glucantime were stimulated with soluble *Leishmania* antigen (5 μg/mL). The chemokines and cytokines levels in ECL (A) and CL (B) patients were evaluated by enzyme-linked immunosorbent assay. Statistical analysis was performed by Wilcoxon matched pairs test ($P < .05$). Abbreviations: IFN, interferon; TNFs, tumor necrosis factor.

L braziliensis infection, monocytes can induce a strong inflammatory response and pathology [17, 40]. Therefore, it is likely that the enhanced inflammatory response induced by monocytes in ECL is directly involved in the progression of the papular lesion to the classic ulcer observed in CL. Consistent with this idea, there is a direct correlation between macrophages ($CD68^+$ cells) and the inflammatory reaction in ECL [13].

Monocytes are the main source of IL-10 [41], a cytokine known to share anti-inflammatory and regulatory functions [42]. A direct correlation between proinflammatory cytokines and IL-10 levels was observed in ECL, suggesting an attempt to down-modulate the inflammatory response. Exogenous addition of IL-10 decreased CXCL-9, TNF, and IFN-γ production in ECL as it does in CL patients [29]. However, although IL-10 down-modulated CXCL-10 production in CL, it upregulated CXCL-10 synthesis in ECL. The proinflammatory effects of IL-10 with enhancement in the production of CXCL-10 have been previously observed in human endotoxemia [43]. CXCL-10 binds to the CXCR3 receptor to exert its biological effects [44]. In addition to cell recruitment, CXCL-10 modulates innate and adaptive immune responses and regulates apoptosis [45, 46]. CXCL-10 has been associated with the pathogenesis of autoimmune and chronic inflammatory diseases such as multiple sclerosis and human T-lymphotropic virus-1-associated myelopathy [47, 48]. We do not know why IL-10 is unable to downregulate CXCL-10. However, because IL-10 was not able to down-modulate CXCL-10 in ECL (in contrast to other cytokines), the relationship between the downstream and upstream signaling pathways of CXCL-10 should be investigated, to evaluate CXCL-10 as a novel therapeutic target in leishmaniasis.

Changes in immunologic responses are documented during therapy and after cure of CL. As soon as 15 days after initiation of Sb^5, patients with CL exhibit a decrease in the production of TNF and IFN-γ [14, 18, 20, 49]. This contrasts with our findings that cytokine production increased in ECL patients after treatment. We do not know why this occurs, but it may be due to release of parasite antigens that enhance the response or simply an evolution of the immune response from the initial to a later phase of the infection that occurs independent of treatment. However, these data, in conjunction with the observation that after therapy CXCL-9 and CXCL-10 levels were higher in ECL than CL, support our hypothesis that during therapy of ECL patients, there is an increase in the inflammatory response that leads to the progression of the initial papular lesion to the classic ulcer characteristic of CL.

CONCLUSIONS

We have previously shown that combined therapy with a leishmanicidal drug and agents able to down-modulate the inflammatory response is more effective than antimony alone and can accelerate the healing time in CL patients [50]. In this study, we further our understanding of ECL with data suggesting a critical role for the inflammatory response in the evolution of ECL to CL despite therapy, and, therefore, we argue that treatment

with an immunomodulatory agent combined with a leishmanicidal drug in ECL may reduce the high rate of treatment failure in this phase of the disease.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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Potential conflicts of interest. All authors: No reported conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest.

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