MAJOR ARTICLE

# Intermediate Monocytes Contribute to Pathologic Immune Response in *Leishmania braziliensis* Infections

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**Ulcer development in patients with cutaneous leishmaniasis (CL) caused by *Leishmania braziliensis* is associated with high levels of tumor necrosis factor (TNF). We found that early after infection, before ulcer development, the frequency of $CD16^+$ (both intermediate [$CD14^+CD16^+$] and nonclassical [$CD14^{dim}CD16^+$]) monocytes was increased in the peripheral blood of patients with *L. braziliensis*, compared with uninfected controls. These results suggest that $CD16^+$ monocytes might promote disease. Also, we found that intermediate monocytes expressed CCR2 and that increased levels of CCL2 protein were present in lesions from patients, suggesting that intermediate monocytes are more likely than nonclassical monocytes to migrate to the lesion site. Finally, we found that the intermediate monocytes produced TNF. Our results show that intermediate monocytes are increased in frequency soon after infection; express CCR2, which would promote their migration into the lesions; and, owing to their production of TNF, can enhance the inflammatory response.**

***Keywords.*** monocytes; *L. braziliensis*; immune response.

Cutaneous leishmaniasis (CL) is a disease with a wide spectrum of clinical presentations, ranging from self-limiting lesions to severe mucosal disease. The magnitude of the severity of the disease is due in large part to the inflammatory response, which is characterized by the recruitment of both lymphoid and myeloid cells to the site of infection. Many studies have characterized the participation of $CD4^+$ and $CD8^+$ T cells in mediating both the protection from and pathology of human CL [1–6]. However, less attention has been given to the contribution of monocyte subsets, even though monocytes migrate from the peripheral blood to inflammatory sites, differentiate into dendritic cells and macrophages, and play an important role in antigen presentation and *Leishmania* killing [7, 8].

Monocytes are divided into 3 subsets based on the expression of CD14, a coreceptor for lipopolysaccharide (LPS), and CD16, a low-affinity Fc receptor (FcγRIII) [9–11]. Originally, monocytes were divided into 2 subsets, $CD14^+CD16^-$ (termed classical monocytes) and $CD14^{low}CD16^+$ (termed nonclassical monocytes), but now are often divided into the following 3 subsets: $CD14^+CD16^-$ (classical), $CD14^+CD16^+$ (intermediate), and $CD14^{low}CD16^{++}$ (nonclassical). The distinction between the 3 subsets is not merely phenotypic, as they have major differences in terms of their capacity to produce cytokines, produce chemokines, and present antigen [12, 13]. It has been reported that patients with CL exhibit increased frequencies of $CD16^+$ monocytes in the blood, compared with healthy individuals, a phenomenon seen in other inflammatory diseases, such as arthritis and sepsis [13–18]. However, whether both intermediate and nonclassical monocyte subsets are increased during CL, when the increase in frequency occurs during infection, and how they function in leishmaniasis remains unknown.

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*Leishmania braziliensis* is the most common cause of CL in South America. Early after *L. braziliensis* infection, there is a dramatic enlargement of lymph node draining the site of infection, followed by the development of a papule at the site of the sand fly bite, which will turn into an ulcer after 1–4 weeks [19, 20]. Patients in the preulcerative phase of the disease are considered as having early cutaneous leishmaniasis (ECL), whereas patients with CL have classical ulcerated cutaneous lesions. In both preulcerative and ulcerative lesions, the predominant mononuclear cell infiltrate is composed of T cells and mononuclear phagocytes [21]. A significant feature of *L. braziliensis* infections is that the disease is caused in large part by the inflammatory response associated with the infection, rather than by uncontrolled parasite growth. Consistent with these findings, several inflammatory cytokines, including interleukin 17, interleukin 1β, and tumor necrosis factor (TNF), have been associated with the severity of disease [22, 23]. TNF is particularly important since there is a positive correlation between the proportion of T cells producing TNF and lesion size [24] and a high frequency of $CD68^+$ cells (a marker for monocytes and macrophages) producing TNF at the lesion site [25]. Moreover, the blockade of TNF in combination with antimony treatment increases the cure rate among patients infected with *L. braziliensis* [26–28]. Exposure to soluble *Leishmania* antigen (SLA) or *L. braziliensis* infection of murine macrophages, human peripheral blood mononuclear cells (PBMCs), and monocyte-derived macrophages leads to production of high levels of TNF [29–32]. However, the role of monocytes and monocyte subsets in TNF production in patients with CL has not been determined.

In the present work, we found that the frequency of $CD16^+$ monocytes is increased in *L. braziliensis*–infected patients in the preulcerative phase of the disease, suggesting that $CD16^+$ monocytes may be causal in the inflammatory response. We also found that both intermediate and nonclassical monocytes are increased in frequency and that the intermediate subset expressed CCR2. In addition, CCL2, the chemokine that drives the recruitment of CCR2-expressing cells, was expressed in the lesions, suggesting that intermediate monocytes may be recruited to lesions. Finally, we found that the intermediate monocytes were the only monocyte subset to respond to leishmania antigen through the production of TNF, identifying for the first time the intermediate monocytes as potential mediators of disease.

## MATERIAL AND METHODS

### Patients

Patients with ECL or CL were recruited at the health post in Corte de Pedra, Bahia, Brazil, which is a well-known area of *L. braziliensis* transmission. Patients with ECL had a papula or papula with small exo-ulcerative lesion, large lymphadenopathy and duration of disease less than 30 days (Table 1). Patients with late CL had 1–3 ulcers with a raised border and a duration of disease ranging from 30 to 70 days (Table 1). The frequency of monocyte subsets in uninfected controls, patients with ECL, and patients with CL and the lesion and lymph node sizes in patients with ECL and those with CL are shown in Table 2. The criteria for diagnosis were parasite isolation or an *L. braziliensis*–positive polymerase chain reaction result. Additionally, all patients had histological features of CL in skin biopsy specimens. In all cases, the immunological analysis was performed before therapy. This research was conducted with the approval of the Ethical Committee of Hospital Prof. Edgard Santos (Salvador, Bahia, Brazil) and CONEP (Brazil), and informed consent was obtained from each participant.

**Table 1. Epidemiological and Clinical Data From Uninfected Controls and Patients With Early Cutaneous Leishmaniasis (ECL) or Cutaneous Leishmaniasis (CL)**

| Characteristics | Uninfected Controls | Patients With ECL | Patients With CL | *P* value[a] |
|---|---|---|---|---|
| Age, y | 32 ± 12 | 36 ± 9 | 29 ± 12 | |
| Female:male ratio | 25:75 | 39:61 | 20:80 | |
| Illness duration, d | . . . | 50 ± 95 | 32 ± 11 | .0006 |
| Lymphadenopathy | . . . | 92 | NI | |
| Lesion size, mm | . . . | 6 ± 4 | 19 ± 6 | .0001 |
| Lesion type | | | | |
| Papule | . . . | 23 | 0 | |
| Nodule | . . . | 38 | 0 | |
| Exo-ulceration | . . . | 39 | 0 | |
| Ulcer | . . . | 0 | 100 | |
| Overall | . . . | 100 | 100 | |

Data are median value ± SD or percentage of patients.

Abbreviations: CL, cutaneous leishmaniasis; ECL, early cutaneous leishmaniasis; NI, not investigated; SD, standard deviation.

[a] By the nonparametric Mann–Whitney test.

### Sources of Antigen and Parasite

By use of an *L. braziliensis* isolate obtained from a patient with CL, SLA was prepared by sonication; tested for endotoxin, using the Limulus amebocyte lysate test; and used at a concentration of 5 μg/mL. An *L. braziliensis* isolated from another patient with CL was cultivated in Schneider's medium and maintained in the laboratory.

### Assays of Peripheral Blood and Biopsy Specimens

PBMCs were obtained from heparinized venous blood layered over a Ficoll-Hypaque gradient (GE Healthcare), washed, and resuspended in Roswell Park Memorial Institutes 1640 medium. Cells were cultured at 37°C in 5% $CO_2$ in the presence of SLA (5 μg/mL) and LPS (5 ng/mL). After 6 or 72 hours, cells were stained for flow cytometry, as described below, or supernatants were collected for cytokine measurement by enzyme-linked immunosorbent assay (ELISA; R&D Systems). In

**Table 2. Frequency of Monocyte Subsets and, When Applicable, Lesion Size, and Lymph Node Size Among Uninfected Controls and Patients With Early Cutaneous Leishmaniasis (ECL) or Cutaneous Leishmaniasis (CL)**

| | Monocyte Subset, Patients, % | | | | |
|---|---|---|---|---|---|
| Study Group, Patient ID | Classical | Intermediate | Nonclassical | Lesion Size, mm | Lymph Node Size, mm |
| Uninfected controls | | | | | |
| UC1 | 79 | 17 | 4 | . . . | . . . |
| UC2 | 91 | 5 | 4 | . . . | . . . |
| UC3 | 64 | 24 | 12 | . . . | . . . |
| UC4 | 88 | 6 | 6 | . . . | . . . |
| UC5 | 76 | 14 | 10 | . . . | . . . |
| UC6 | 92 | 7 | 1 | . . . | . . . |
| UC7 | 92 | 7 | 1 | . . . | . . . |
| UC8 | 76 | 14 | 10 | . . . | . . . |
| UC9 | 76 | 10 | 14 | . . . | . . . |
| UC10 | 69 | 27 | 3 | . . . | . . . |
| UC11 | 93 | 5 | 2 | . . . | . . . |
| Patients with ECL | | | | | |
| ECL1 | 53 | 24 | 23 | 2 | 4 |
| ECL2 | 59 | 31 | 9 | 10 | 24 |
| ECL3 | 77 | 19 | 5 | 5 | 38 |
| ECL4 | 85 | 14 | 1 | 12 | 34 |
| ECL5 | 75 | 20 | 5 | 10 | 18 |
| ECL6 | 75 | 22 | 4 | 5 | 65 |
| ECL7 | 87 | 9 | 4 | 6 | 40 |
| ECL8 | 79 | 15 | 6 | 4 | 30 |
| ECL9 | 73 | 23 | 4 | 9 | 42 |
| ECL10 | 63 | 28 | 8 | 4 | 30 |
| ECL11 | 79 | 19 | 2 | 1 | 40 |
| ECL12 | 41 | 30 | 29 | 5 | 72 |
| ECL13 | 56 | 36 | 8 | 6 | 20 |
| ECL14 | 74 | 23 | 3 | 12 | 10 |
| ECL15 | 59 | 38 | 4 | 13 | 4 |
| Patients with CL | | | | | |
| CL1 | 75 | 11 | 13 | 8 | NI |
| CL2 | 65 | 26 | 9 | 10 | NI |
| CL3 | 66 | 28 | 6 | 7 | NI |
| CL4 | 42 | 40 | 18 | 10 | NI |
| CL5 | 69 | 29 | 2 | 12 | NI |
| CL6 | 65 | 32 | 3 | 9 | NI |
| CL7 | 87 | 11 | 2 | 11 | NI |
| CL8 | 53 | 35 | 12 | 12 | NI |
| CL9 | 57 | 39 | 4 | 25 | NI |
| CL10 | 62 | 28 | 10 | 14 | NI |
| CL11 | 78 | 16 | 6 | 14 | NI |
| CL12 | 62 | 31 | 7 | 6 | NI |
| CL13 | 83 | 14 | 3 | 10 | NI |
| CL14 | 74 | 23 | 3 | 31 | NI |
| CL15 | 77 | 20 | 2 | 10 | NI |

Abbreviations: CL, cutaneous leishmaniasis; ECL, early cutaneous leishmaniasis; NI, not investigated.

some experiments, infection with *L. braziliensis* was done at a ratio of 5 parasites to 1 monocyte for 2 hours. Extracellular parasites were washed and cells were incubated for 4 hours for intracellular TNF detection by flow cytometry. Biopsies were performed using a 4-mm punch, specimens were treated with Liberase TL (Roche) for 1 hour at 37°C, 5% $CO_2$, and tissue

dissociation was done using cell strainers. Dissociated cells were stained for flow cytometry, or biopsy specimens were cultured at 37°C in 5% $CO_2$ for 72 hours, with CCL2 levels in supernatants determined by ELISA (R&D Systems).

### Flow Cytometry

For flow cytometry, $10^6$ PBMCs were stained with fluorochrome-conjugated antibodies for surface markers (CD14, CD16, HLA-DR, and CCR2 [BD-Bioscience]) and fixed by using 2% formaldehyde. For intracellular staining, fixed cells were permeabilized with the cytofix/cytoperm kit (BD-Bioscience) and stained intracellularly with anti-TNF antibody (BD-Bioscience). Samples were evaluated on a FACSCanto II flow cytometer (BD Pharmingen), and analysis was performed using FlowJo software (Tree Star). The cells were gated on live cells, and the monocyte population was defined on the basis of size and complexity.

### Statistical Analysis

The Mann–Whitney, nonparametric unpaired test was used to assess differences between the groups studied. The paired *t* test was used to assess differences between different conditions from the same patient.

## RESULTS

### The Frequency of Circulating CD16$^+$ Monocytes Is Increased Early During *L. braziliensis* Infection

During *L. braziliensis* infection, the frequency of circulating monocyte subsets change, such that there are higher levels of CD16$^+$ monocytes in patients, compared with uninfected controls from areas of endemicity [18]. The significance of this observation remains unclear, but since an increased frequency of the CD16$^+$ monocyte population has been associated with several chronic diseases, we considered the possibility that these cells might contribute to the disease. Alternatively, the increased frequency of the CD16$^+$ monocyte subset could simply be a consequence of the inflammatory response associated with the development of ulcerated cutaneous lesions. To address this issue, we assessed the frequency of CD16$^+$ monocytes in patients in the early phase of the disease, to determine whether changes were evident prior to the development of cutaneous ulcers. Patients with ECL exhibit a papula that has been present for <30 days. PBMCs were collected and stained for CD16 and CD14 (Figure 1*A*). As previously reported, we found that the frequency of CD16$^+$ monocytes was higher in patients with CL (Figure 1*B*). More importantly, we also found an increase in CD16$^+$ monocytes in patients with ECL, indicating that the alterations in the frequency of monocyte subsets is not likely due to the severe inflammation associated with ulcer development.

### The Frequency of Circulating Intermediate and Nonclassical Monocytes Is Increased in Patients With CL

Many of the early studies examining the role of monocytes in disease only compared two subsets: CD14$^{++}$CD16$^-$ and CD14$^+$CD16$^+$. However, monocytes can be divided into at least 3 subsets, termed classical (CD14$^{++}$CD16$^-$), intermediate (CD14$^{++}$CD16$^+$), and nonclassical (CD14$^+$CD16$^{++}$). These subsets appear to differ not only in the expression of CD14 and CD16, but also in function [9, 11, 33, 34]. Therefore, we next asked whether the increased frequency of CD16$^+$ monocytes that we observed in *L. braziliensis*–infected patients occurred preferentially in either the intermediate or the nonclassical population. PBMCs were collected from patients; stained for major histocompatibility complex (MHC) class II,

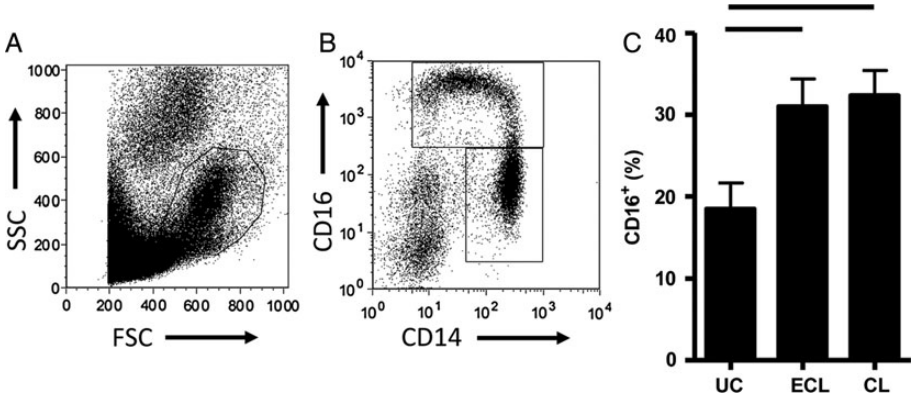


**Figure 1.** CD16$^+$ monocyte frequency is increased early after *Leishmania braziliensis* infection. Peripheral blood mononuclear cells from uninfected controls (UCs), patients with early cutaneous leishmaniasis (ECL), and patients with cutaneous leishmaniasis (CL) were obtained, and the CD16$^+$ monocyte frequency was assessed by flow cytometry. *A*, Gating strategy to assess CD16$^+$ monocytes. *B*, Frequency of CD16$^+$ monocyte in UCs and patients with ECL or CL. The results expressed are the mean (±SD) from 15 individuals from each group. Lines above the bars indicate statistically significant differences between corresponding groups ($P < .05$). Abbreviations: FSC, forward scatter; SD, standard deviation; SSC, side scatter.

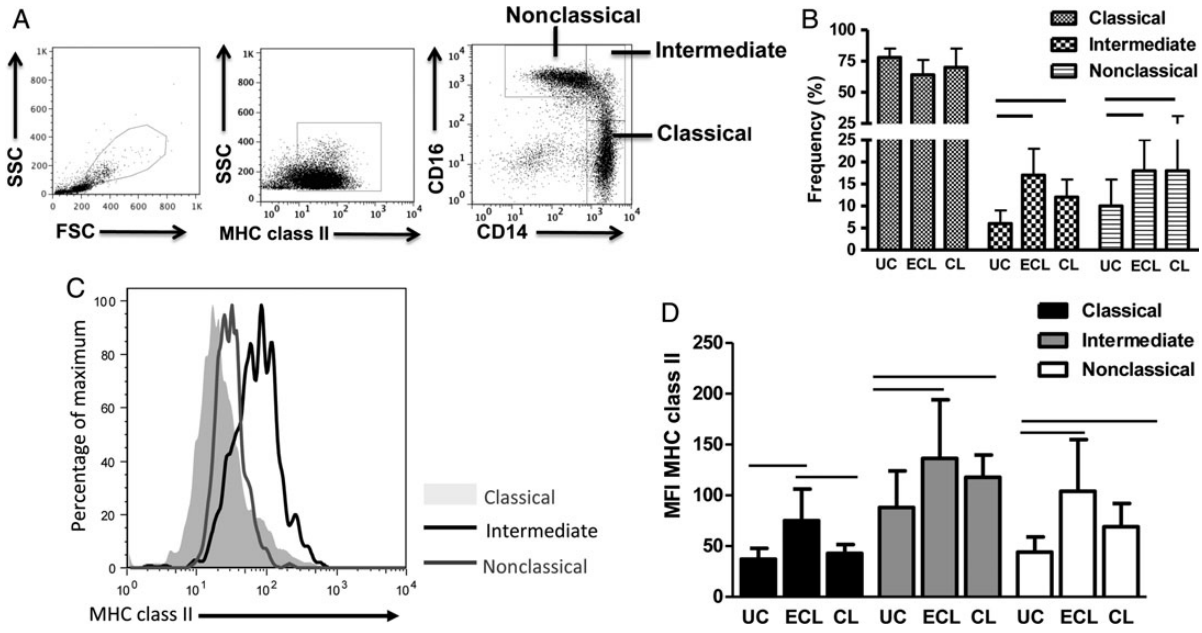


**Figure 2.** Patients with cutaneous leishmaniasis (CL) have an increased frequency of intermediate and nonclassical monocytes early after *Leishmania braziliensis* infection. Peripheral blood mononuclear cells from uninfected controls (UCs), patients with early CL (ECL), and patients with CL were obtained, and the monocyte subset frequency was assessed by flow cytometry. *A*, The gating strategy to assess monocyte subsets was based on size and granulosity, followed by major histocompatibility complex (MHC) class II expression. *B*, Frequency of monocyte subsets in UCs (n = 12), patients with ECL (n = 18), and patients with CL (n = 15). *C*, MHC class II expression in different monocytes populations. *D*, Mean fluorescence intensity (MFI) of MHC class II expression in monocyte subsets (n = 15). Lines above the bars indicate statistically significant differences between corresponding groups ($P < .05$). Abbreviations: FSC, forward scatter; SSC, side scatter.

CD14, and CD16; and analyzed by flow cytometry. The gate strategy was done primarily on the basis of size and granulosity and then on the basis of MHC class II, CD14, and CD16 expression to define the classical, intermediate, and nonclassical monocytes (Figure 2*A*). We then compared the frequency of the subsets in patients and found that the frequency of intermediate and nonclassical monocytes was increased both in patients with ECL and those with CL (Figure 2*B*). The intermediate subset is known to express higher levels of MHC class II, compared with either the classical or nonclassical subset. Therefore, to confirm the distinct identity of this population, we examined MHC class II expression on the monocyte subsets, and as predicted we found that the intermediate subset expressed the highest MHC class II levels (Figure 2*C* and 2*D*). These results demonstrate that alterations in the frequencies of monocyte subsets precedes the development of an ulcerated lesions, indicating that such changes are not a consequence of inflammation associated with severe disease.

### CCL2 Is Expressed in *L. braziliensis* Lesions

The chemokine CCL2 (MCP-1), which binds to its receptor CCR2, is critical in recruiting monocytes into leishmanial lesions [36] and is expressed preferentially on classical and intermediate monocytes. In contrast, nonclassical monocytes fail to express CCR2 but instead express high levels of CX3CR1, which binds to fractalkine expressed on the endothelium of blood vessels [37]. The interactions between CX3CR1 and fractalkine contribute to the patrolling behavior of the nonclassical subset [37]. We tested whether PBMCs from patients produced CCL2 and fractalkine following stimulation with SLA. PBMCs were collected from uninfected controls (UCs) and from patients with ECL or CL and were cultured with or without SLA. None of the participants produced detectable levels of fractalkine in the absence or presence of SLA (data not shown). CCL2 levels were relatively low in cells from UCs and did not increase upon stimulation with antigen. In contrast, there was a significant increase in CCL2 levels in cells from patients with ECL and those with CL (Figure 3*A*). The production of CCL2 was evident with or without leishmanial antigen, suggesting that the cells were activated to produce CCL2 in vivo. To detect CCL2 gene expression, we then obtained lesion biopsy specimens from another set of patients with CL and skin biopsy specimens from healthy subjects, extracted RNA, and assessed CCL2 gene expression by unbiased microarray ex vivo. CCL2 gene expression was significantly higher in CL lesions, compared with skin specimens from healthy subjects (Figure 3*B*). We then assessed CCR2 expression on monocytes obtained from patients. Consistent with studies of monocytes from

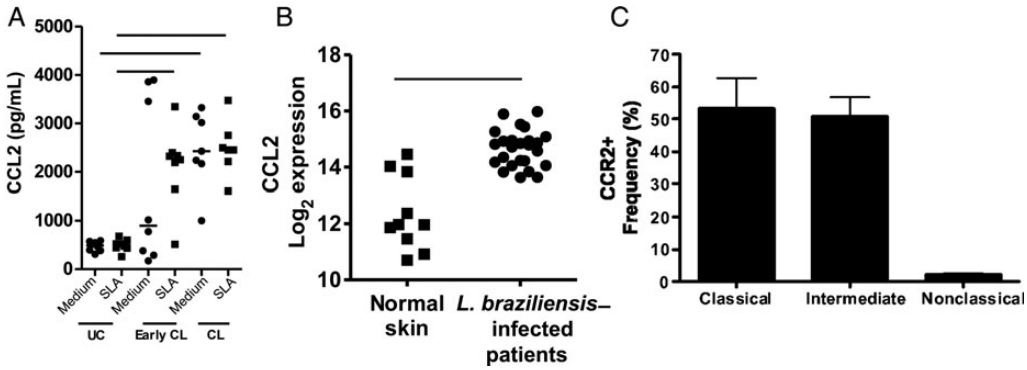


**Figure 3.** Nonclassical monocytes do not express CCR2. *A*, Peripheral blood mononuclear cells (PBMCs) from uninfected controls (UCs), patients with early cutaneous leishmaniasis (ECL), and patients with cutaneous leishmaniasis (CL) were cultured in the presence of soluble *Leishmania* antigen (5 μg/mL) for 72 hours, and CCL2 levels were determined by enzyme-linked immunosorbent assay on culture supernatants. *B*, Biopsy specimens from patients with CL and those with normal skin were obtained, and gene expression was assessed by unbiased microarray ex vivo. *C*, PBMCs from patients with CL were obtained, and expression of CCR2 on circulating monocyte subsets were determined by flow cytometry ex vivo. Lines above the bars indicate statistically significant differences between corresponding groups ($P < .05$). Abbreviations: *L. braziliensis, Leishmania braziliensis*; SLA, soluble *Leishmania antigen*.

healthy individuals, we found that classical and intermediate monocytes from patients with CL expressed CCR2, while nonclassical monocytes were negative for CCR2 (Figure 3*C*). Taken together, these results suggested that both classical monocytes and intermediate monocytes would be recruited to leishmanial lesions.

### The Intermediate Monocyte Subset Is a Source of TNF After *Leishmania* Infection

TNF can promote increased macrophage killing of leishmanial parasites but also plays a role in promoting increased pathology in leishmaniasis [38]. Thus, there is a correlation with increased TNF levels and increased disease, and blockade of TNF combined with chemotherapy improves the clinical outcome in *L. braziliensis*–infected patients [1, 31, 39, 40]. We found that TNF is produced by PBMCs early after infection (Figure 4*A*). These results are consistent with a role for TNF in promoting ulcer development. This led us to ask what cells were producing TNF. We stimulated PBMCs with SLA and found that monocytes from patients with CL produced significantly more TNF than $CD4^+$ and $CD8^+$ T cells (Figure 4*B* and 4*C*). Therefore, we next determined what monocyte subsets were contributing to TNF production in patients with CL. To do so, we cultured PBMCs from patients with CL with SLA and again assessed TNF production by intracellular staining. Our results indicated that the intermediate monocyte subset had the highest percentage of TNF-producing cells (Figure 4*D* and 4*E*).

## DISCUSSION

The pathology associated with CL is caused by the recruitment of inflammatory cells from the blood, and in this study we focused on the role that monocytes might play in the immunopathology associated with human CL. Human monocytes are heterogeneous and can be subdivided into subsets, termed classical, intermediate, and nonclassical, on the basis of the differential expression of CD14 and CD16 [9]. We found that there was an increase in the frequency of intermediate and nonclassical monocytes in the blood of patients with CL and, importantly, that these changes also occurred in patients with ECL. This suggests that changes in the frequency of monocyte subsets are not the consequence of full-blown disease but rather might contribute to the development of disease. Further analysis indicated that the intermediate subset may contribute to disease, as these cells produced TNF in response to SLA.

These results indicate that intermediate subset of monocyte might have pathologic role during *Leishmania* infection and that preferential migration of these cells to lesion sites may influence outcome of disease. It was previously shown that migration of mononuclear phagocytes to inflamed sites, including during leishmaniasis, is dependent on CCR2 expression [41]. Thus, the next question we had was whether there were differences regarding CCR2 expression, a chemokine receptor that binds to CCL2, among monocyte subsets from patients with CL. While classical and intermediate populations express CCR2, nonclassical monocytes did not express this molecule. The fact that nonclassical monocyte frequency positively correlates with smaller lesions and that these cells do not express CCR2 indicates that patients with CL would probably benefit in case this population could migrate to lesion site. Although classical and intermediate monocytes expressed high levels of CCR2, the frequency of intermediate monocytes are quite low, compared with classical monocytes. Thus, future studies should address migration ability of monocyte subsets in CL and determine the frequency of these cells at lesion sites. We also assessed the expression of MHC class II as a marker of monocyte

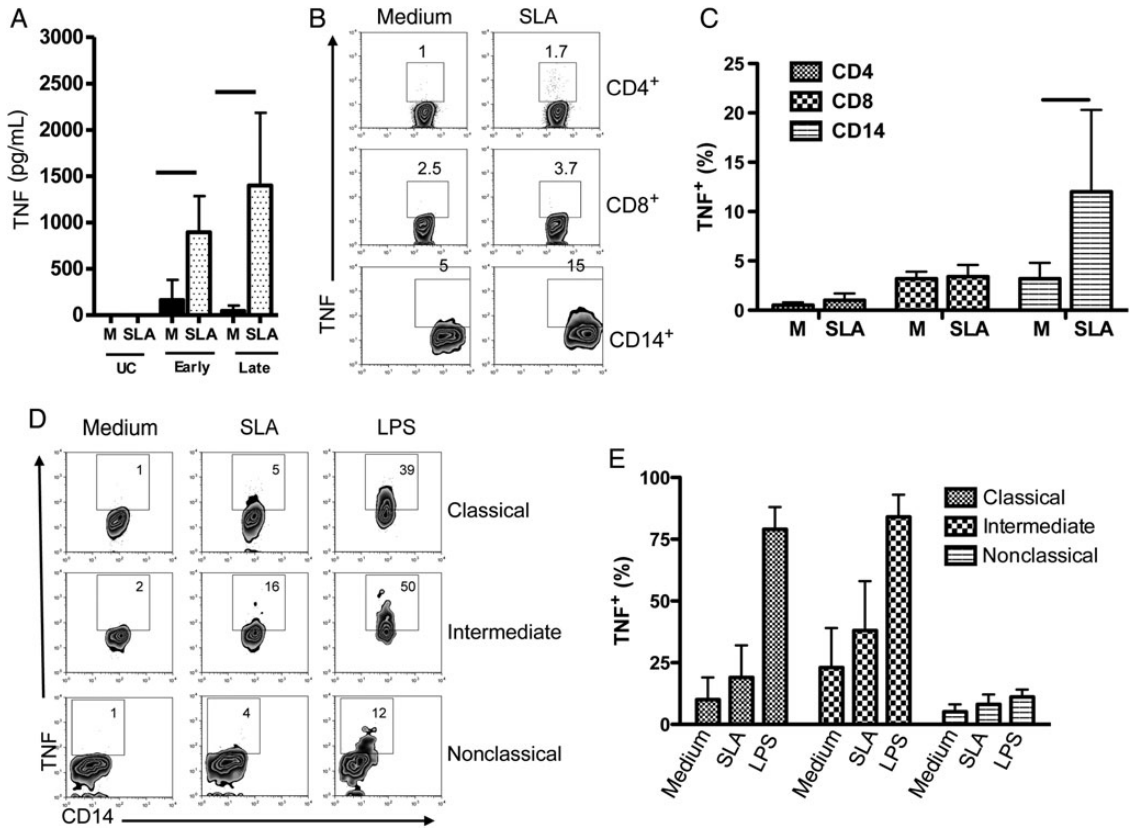


**Figure 4.** Intermediate monocytes retain inflammatory phenotype. *A*, Peripheral blood mononuclear cells (PBMCs) from uninfected controls (UC), patients with early cutaneous leishmaniasis (ECL), and patients with cutaneous leishmaniasis (CL) were stimulated with media (M) alone or 5 μg/mL of soluble *Leishmania* antigen (SLA) for 72 hours. Cytokines levels were determined on cell cultures supernatants by enzyme-linked immunosorbent assay. Results are mean (±SD) from 15 individuals from each group. *B*, PBMCs from patients with CL were pulsed with 5 μg/mL of SLA or lipopolysaccharide (10 ng/mL), and after 6 hours in the presence of Stop Golgi, intracellular staining was performed to determine the frequencies of tumor necrosis factor (TNF)–producing cells by flow cytometry. *B*, Representative plots showing the frequency of $CD4^+$, $CD8^+$, and $CD14^+$ cells producing TNF. *C*, Mean values (±SD) for 5 patients with CL. *D*, Representative plots showing frequencies of monocyte subsets producing TNF. *E*, Mean values (±SD) for 8 patients with CL. Lines above the bars indicate statistically significant differences between corresponding groups ($P < .05$). Abbreviations: LPS, lipopolysaccharide; SD, standard deviation.

activation and found that, early after infection, classical and nonclassical monocytes were activated. In contrast, nonclassical monocytes expressed very low levels of MHC class II in *L. braziliensis*–infected individuals, suggesting that these cells do not participate during leishmaniasis.

TNF is the main inflammatory cytokine secreted during culture by SLA-stimulated PBMCs obtained from patients with CL and is also highly expressed in lesions from these individuals. Recently, a positive correlation between TNF level and disease severity was documented [24, 29]. Here, we evaluated the immune response in patients with CL before the ulcer developed. We found that, early after infection, levels of TNF were increased in these individuals. These data support the hypothesis that cells other than T cells might be playing a major role in inducing ulcer development in CL. As monocytes can rapidly respond to stimuli and can be important sources of TNF, we wanted to know whether TNF could be produced by monocytes shortly after contact with *Leishmania* products. SLA and LPS induced a high frequency of TNF-producing monocytes after 6 hours. One question that will be addressed in the near future is which components present in *L. braziliensis* antigen trigger TNF production by monocytes. It has been reported that a few components of other *Leishmania* species, such as LPG and DNA, activate cells through Toll-like receptor 2 (TLR2) and TLR9, respectively, and we are currently investigating whether signaling through these TLRs can trigger immune responses in human monocytes infected by *L. braziliensis* [40, 42]. Our previous observation studying monocyte-derived macrophages from healthy subjects, individuals with *L. braziliensis* subclinical infection, and patients with CL showed that macrophages from these individuals secreted different amounts of inflammatory mediators after been infected with *Leishmania*, suggesting that the heterogeneity in population of monocytes among these individuals might influence the immune response during the disease [32]. One of the major functional differences between monocyte subsets has been reported to be about the

ability of these cells to produce cytokines, and controversial results have been published regarding the ability of $CD16^+$ subsets to produce more TNF. While most studies show that $CD16^+$ monocytes are the main source of TNF, a few documented that $CD16^-$ monocytes were the main source of this cytokine [9, 13, 43–45]. Because $CD16^+$ populations can be divided into intermediate and nonclassical monocytes, we wanted to know the contribution of the 3 monocyte subsets to the production of TNF in patients with CL. Our results showed that classical and intermediate monocytes secreted more TNF than nonclassical monocytes in response to SLA and LPS. These data are in concordance with what has been seen in healthy subjects, in whom the nonclassical population is less responsive to LPS [11]. One reason why nonclassical monocytes do not respond well to LPS may be the low expression of CD14 within this population. The fact that nonclassical monocytes also produced low levels of TNF in response to SLA has to be further investigated.

Our data show that intermediate monocytes are the main inflammatory cells in patients with CL. The increasing frequency of this population and TNF production occur early after infection, before the ulcer appearance, and remains with progression of the infection to ulcer development. Many pathological events preceding and during inflammatory responses can be attributed to the effect of TNF. For instance, TNF increases cytotoxicity, expression of metalloproteinases, and necrosis [46]. Therefore, our data not only contribute to the understanding of pathogenesis of CL, but also emphasize the role of monocyte subsets in the development of the pathology observed in *L. braziliensis* infection.

## Notes

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***Potential conflicts of interest.*** All authors: No reported conflicts.

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