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

Sara Passos,[1,3,a] Lucas P. Carvalho,[1,2,3,a] Rúbia S. Costa,[1,a] Taís M. Campos,[1] Fernanda O. Novais,[4] Andréa Magalhães,[1] Paulo R. L. Machado,[1,3] Daniel Beiting,[4] David Mosser,[5] Edgar M. Carvalho,[1,3] and Phillip Scott[4]

[1]Serviço de Imunologia, Complexo Hospitalar Prof. Edgard Santos, [2]Instituto de Ciências da Saúde, Universidade Federal da Bahia, and [3]Instituto Nacional de Ciências e Tecnologia-Doenças Tropicais, Salvador, Brazil; [4]Department of Pathobiology, School of Veterinary Medicine, University of Pennsylvania, Philadelphia; and [5]Department of Cell Biology and Molecular Genetics, University of Maryland, College Park

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.

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

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.

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.

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)

Study Group, Patient ID	Monocyte Subset, Patients, %			Lesion Size, mm	Lymph Node Size, mm
	Classical	Intermediate	Nonclassical		
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 fluoro-chrome-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 1A). As previously reported, we found that the frequency of CD16+ monocytes was higher in patients with CL (Figure 1B). 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,
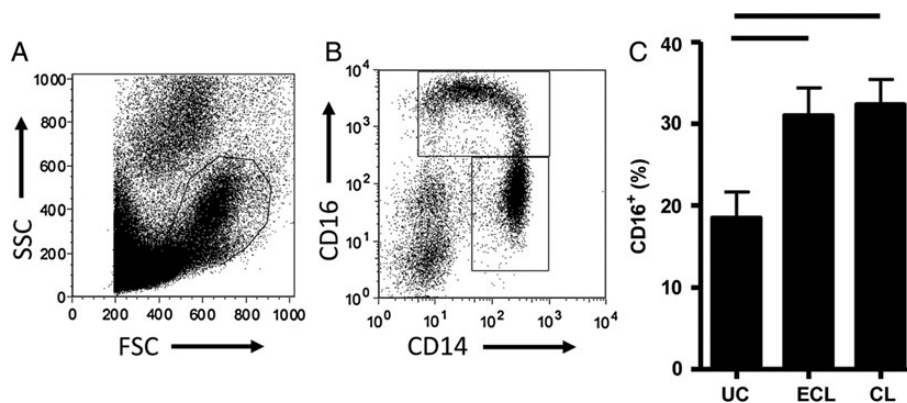

Figure 1. CD16+ monocyte frequency is increased early after *Leishmania braziliensis* infection. Peripheral blood mononuclear cells from uninfected controls (UCs), patients with early cutaneous leishmaniasis (ECL), and patients with cutaneous leishmaniasis (CL) were obtained, and the CD16+ monocyte frequency 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.
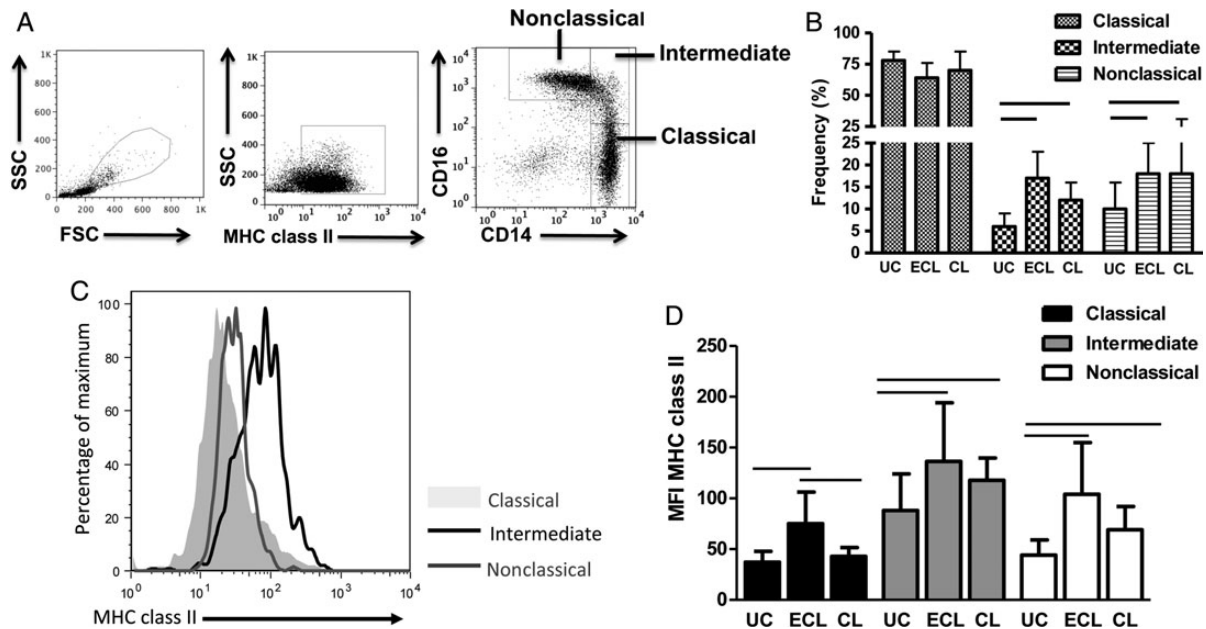
Figure 2. Patients with cutaneous leishmaniasis (CL) have an increased frequency of intermediate and nonclassical monocytes early after *Leishmania braziliensis* infection. Peripheral blood mononuclear cells from uninfected controls (UCs), patients with early CL (ECL), and patients with CL were obtained, 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 2A). 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 2B). 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 2C and 2D). 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 3A). 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 3B). We then assessed CCR2 expression on monocytes obtained from patients. Consistent with studies of monocytes from
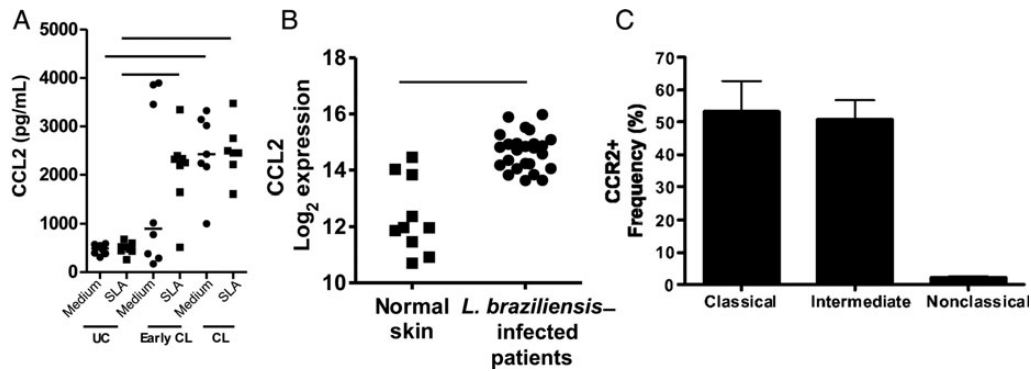
Figure 3. Nonclassical monocytes do not express CCR2. *A,* Peripheral blood mononuclear cells (PBMCs) from uninfected controls (UCs), patients with early cutaneous leishmaniasis (ECL), and patients with cutaneous leishmaniasis (CL) were cultured in the presence of soluble *Leishmania* antigen (5 μg/mL) 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 non-classical 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
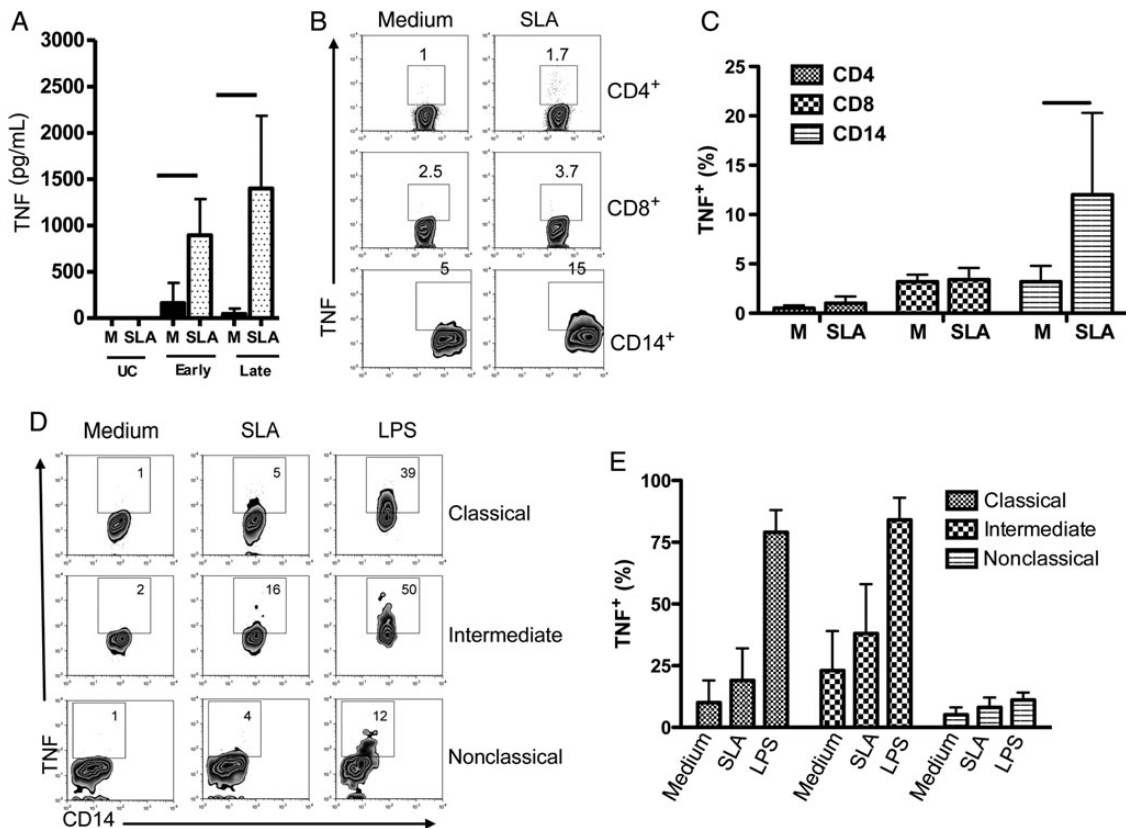
Figure 4. Intermediate monocytes retain inflammatory phenotype. *A*, Peripheral blood mononuclear cells (PBMCs) from uninfected controls (UC), patients with early cutaneous leishmaniasis (ECL), and patients with cutaneous leishmaniasis (CL) were stimulated with media (M) alone or 5 μg/mL of soluble *Leishmania* 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

References

1. Carvalho LP, Passos S, Schriefer A, Carvalho EM. Protective and pathologic immune responses in human tegumentary leishmaniasis. Front Immunol **2012**; 3:301.
2. Santos Cda S, Boaventura V, Ribeiro Cardoso C, et al. CD8(+) granzyme B(+)-mediated tissue injury vs. CD4(+)IFNgamma(+)-mediated parasite killing in human cutaneous leishmaniasis. J Invest Dermatol **2013**; 133:1533–40.
3. Zaph C, Uzonna J, Beverley SM, Scott P. Central memory T cells mediate long-term immunity to Leishmania major in the absence of persistent parasites. Nat Med **2004**; 10:1104–10.
4. Keesen TS, Antonelli LR, Faria DR, et al. CD4(+) T cells defined by their Vbeta T cell receptor expression are associated with immunoregulatory profiles and lesion size in human leishmaniasis. Clin Exp Immunol **2011**; 165:338–51.
5. Anderson CF, Stumhofer JS, Hunter CA, Sacks D. IL-27 regulates IL-10 and IL-17 from CD4+ cells in nonhealing Leishmania major infection. J Immunol **2009**; 183:4619–27.
6. Novais FO, Carvalho LP, Graff JW, et al. Cytotoxic T cells mediate pathology and metastasis in cutaneous leishmaniasis. PLoS Pathog **2013**; 9:e1003504.
7. Leon B, Lopez-Bravo M, Ardavin C. Monocyte-derived dendritic cells formed at the infection site control the induction of protective T helper 1 responses against Leishmania. Immunity **2007**; 26:519–31.
8. Novais FO, Nguyen BT, Beiting DP, et al. Human classical monocytes control the intracellular stage of Leishmania braziliensis by reactive oxygen species. J Infect Dis **2014**; 209:1288–96.
9. Zawada AM, Rogacev KS, Rotter B, et al. SuperSAGE evidence for CD14++CD16+ monocytes as a third monocyte subset. Blood **2011**; 118:e50–61.
10. Ziegler-Heitbrock L, Ancuta P, Crowe S, et al. Nomenclature of monocytes and dendritic cells in blood. Blood **2010**; 116:e74–80.
11. Wong KL, Tai JJ, Wong WC, et al. Gene expression profiling reveals the defining features of the classical, intermediate, and nonclassical human monocyte subsets. Blood 118:e16–31.
12. Ansari AW, Meyer-Olson D, Schmidt RE. Selective expansion of proinflammatory chemokine CCL2-loaded CD14+CD16+ monocytes subset in HIV-infected therapy naive individuals. J Clin Immunol **2012**; 33: 302–6.
13. Belge KU, Dayyani F, Horelt A, et al. The proinflammatory CD14+ CD16+DR++ monocytes are a major source of TNF. J Immunol **2002**; 168:3536–42.
14. Ziegler-Heitbrock HW, Fingerle G, Strobel M, et al. The novel subset of CD14+/CD16+ blood monocytes exhibits features of tissue macrophages. Eur J Immunol **1993**; 23:2053–8.
15. Passlick B, Flieger D, Ziegler-Heitbrock HW. Identification and characterization of a novel monocyte subpopulation in human peripheral blood. Blood **1989**; 74:2527–34.
16. Fingerle G, Pforte A, Passlick B, Blumenstein M, Strobel M, Ziegler-Heitbrock HW. The novel subset of CD14+/CD16+ blood monocytes is expanded in sepsis patients. Blood **1993**; 82:3170–6.
17. Kawanaka N, Yamamura M, Aita T, et al. CD14+,CD16+ blood monocytes and joint inflammation in rheumatoid arthritis. Arthritis Rheum **2002**; 46:2578–86.
18. Soares G, Barral A, Costa JM, Barral-Netto M, Van Weyenbergh J. CD16+ monocytes in human cutaneous leishmaniasis: increased ex vivo levels and correlation with clinical data. J Leukoc Biol **2006**; 79:36–9.
19. Barral A, Barral-Netto M, Almeida R, et al. Lymphadenopathy associated with Leishmania braziliensis cutaneous infection. Am J Trop Med Hyg **1992**; 47:587–92.
20. Barral A, Guerreiro J, Bomfim G, Correia D, Barral-Netto M, Carvalho EM. Lymphadenopathy as the first sign of human cutaneous infection by Leishmania braziliensis. Am J Trop Med Hyg **1995**; 53:256–9.
21. Bittencourt AL, Barral A. Evaluation of the histopathological classifications of American cutaneous and mucocutaneous leishmaniasis. Mem Inst Oswaldo Cruz **1991**; 86:51–6.
22. Voronov E, Dotan S, Gayvoronsky L, et al. IL-1-induced inflammation promotes development of leishmaniasis in susceptible BALB/c mice. Int Immunol **2010**; 22:245–57.
23. Gonzalez-Lombana C, Gimblet C, Bacellar O, et al. IL-17 mediates immunopathology in the absence of IL-10 following Leishmania major infection. PLoS Pathog **2011**; 9:e1003243.
24. Antonelli LR, Dutra WO, Almeida RP, Bacellar O, Carvalho EM, Gollob KJ. Activated inflammatory T cells correlate with lesion size in human cutaneous leishmaniasis. Immunol Lett **2005**; 101:226–30.
25. Faria DR, Gollob KJ, Barbosa J Jr, et al. Decreased in situ expression of interleukin-10 receptor is correlated with the exacerbated inflammatory and cytotoxic responses observed in mucosal leishmaniasis. Infect Immun **2005**; 73:7853–9.
26. Bafica A, Oliveira F, Freitas LA, Nascimento EG, Barral A. American cutaneous leishmaniasis unresponsive to antimonial drugs: successful

treatment using combination of N-methilglucamine antimoniate plus pentoxifylline. Int J Dermatol 2003; 42:203–7.

27. Sadeghian G, Nilforoushzadeh MA. Effect of combination therapy with systemic glucantime and pentoxifylline in the treatment of cutaneous leishmaniasis. Int J Dermatol 2006; 45:819–21.

28. Ribeiro de Jesus A, Luna T, Pacheco de Almeida R, Machado PR, Carvalho EM. Pentoxifylline down modulate in vitro T cell responses and attenuate pathology in Leishmania and HTLV-I infections. Int Immunopharmacol 2008; 8:1344–53.

29. Bacellar O, Lessa H, Schriefer A, et al. Up-regulation of Th1-type responses in mucosal leishmaniasis patients. Infect Immun 2002; 70:6734–40.

30. Carvalho LP, Passos S, Bacellar O, et al. Differential immune regulation of activated T cells between cutaneous and mucosal leishmaniasis as a model for pathogenesis. Parasite Immunol 2007; 29:251–8.

31. Ribeiro-de-Jesus A, Almeida RP, Lessa H, Bacellar O, Carvalho EM. Cytokine profile and pathology in human leishmaniasis. Braz J Med Biol Res 1998; 31:143–8.

32. Giudice A, Vendrame C, Bezerra C, et al. Macrophages participate in host protection and the disease pathology associated with Leishmania braziliensis infection. BMC Infect Dis 2012; 12:75.

33. Grage-Griebenow E, Flad HD, Ernst M. Heterogeneity of human peripheral blood monocyte subsets. J Leukoc Biol 2001; 69:11–20.

34. Randolph GJ, Sanchez-Schmitz G, Liebman RM, Schakel K. The CD16 (+) (FcgammaRIII(+)) subset of human monocytes preferentially becomes migratory dendritic cells in a model tissue setting. J Exp Med 2002; 196:517–27.

35. Conrad SM, Strauss-Ayali D, Field AE, Mack M, Mosser DM. Leishmania-derived murine monocyte chemoattractant protein 1 enhances the recruitment of a restrictive population of CC chemokine receptor 2-positive macrophages. Infect Immun 2007; 75:653–65.

36. Quinones MP, Estrada CA, Jimenez F, et al. CCL2-independent role of CCR2 in immune responses against Leishmania major. Parasite Immunol 2007; 29:211–7.

37. Bjorkander S, Heidari-Hamedani G, Bremme K, Gunnarsson I, Holmlund U. Peripheral monocyte expression of the chemokine receptors CCR2, CCR5 and CXCR3 is altered at parturition in healthy women and in women with systemic lupus erythematosus. Scand J Immunol 2013; 77:200–12.

38. Melby PC, Andrade-Narvaez FJ, Darnell BJ, Valencia-Pacheco G, Tryon VV, Palomo-Cetina A. Increased expression of proinflammatory cytokines in chronic lesions of human cutaneous leishmaniasis. Infect Immun 1994; 62:837–42.

39. Oliveira F, Bafica A, Rosato AB, et al. Lesion size correlates with Leishmania antigen-stimulated TNF-levels in human cutaneous leishmaniasis. Am J Trop Med Hyg 2011; 85:70–3.

40. Carvalho LP, Petritus PM, Trochtenberg AL, et al. Lymph node hypertrophy following Leishmania major infection is dependent on TLR9. J Immunol 2011; 188:1394–401.

41. Goncalves R, Zhang X, Cohen H, Debrabant A, Mosser DM. Platelet activation attracts a subpopulation of effector monocytes to sites of Leishmania major infection. J Exp Med 2011; 208:1253–65.

42. Kavoosi G, Ardestani SK, Kariminia A. The involvement of TLR2 in cytokine and reactive oxygen species (ROS) production by PBMCs in response to Leishmania major phosphoglycans (PGs). Parasitology 2009; 136:1193–9.

43. Dimitrov S, Shaikh F, Pruitt C, et al. Differential TNF production by monocyte subsets under physical stress: blunted mobilization of proinflammatory monocytes in prehypertensive individuals. Brain Behav Immun 2012; 27:101–8.

44. Ziegler-Heitbrock L. The CD14+ CD16+ blood monocytes: their role in infection and inflammation. J Leukoc Biol 2007; 81:584–92.

45. Frankenberger M, Sternsdorf T, Pechumer H, Pforte A, Ziegler-Heitbrock HW. Differential cytokine expression in human blood monocyte subpopulations: a polymerase chain reaction analysis. Blood 1996; 87:373–7.

46. Chakrabarti S, Zee JM, Patel KD. Regulation of matrix metalloproteinase-9 (MMP-9) in TNF-stimulated neutrophils: novel pathways for tertiary granule release. J Leukoc Biol 2006; 79:214–22.