$CD16^+$ monocytes in human cutaneous leishmaniasis: increased ex vivo levels and correlation with clinical data

George Soares, Aldina Barral, Jackson M. Costa, Manoel Barral-Netto, and Johan Van Weyenbergh[1]

LIMI, LIP, Gonçalo Moniz Research Center, Oswaldo Cruz Foundation (FIOCRUZ), Salvador-Bahia, Brazil; and Institute for Immunological Investigation, São Paulo-SP, Brazil

Abstract: Peripheral blood CD16 (Fc receptor for immunoglobulin G III)-positive monocytes have been shown to expand in different pathological conditions, such as cancer, asthma, sepsis, human immunodeficiency virus infection, and AIDS progression, but data in leishmaniasis are lacking. We found that cutaneous leishmaniasis patients (n=15) displayed a significant increase in the percentage (3.5 vs. 10.1) as well as mean fluorescent intensity (13.5 vs. 29.2) of ex vivo CD16 expression in monocytes as compared with healthy controls. We observed a significant positive correlation between the percentage of ex vivo $CD16^+$ monocytes and lesion size ($P=0.0052$, r=0.75) or active transforming growth factor-β plasma levels ($P=0.0017$, r=0.78). In addition, two patients with nonhealing lesions during a 3-year follow-up had high (9.1–19.4%) CD16 levels at diagnosis. Our data suggest a deleterious role for CD16 in human leishmaniasis, as well as its possible use as a marker for disease severity and/or adverse disease outcome. *J. Leukoc. Biol.* 79: 36–39; 2006.

Key Words: Leishmania · *TGF-β* · *Fc receptor* · *whole blood* · *lesion size*

Peripheral blood CD16-expressing monocytes have gained considerable attention as a subclass of phenotypically distinct, inflammatory monocytes (reviewed in ref. [1]). Their unique migratory capacity has been defined recently by the fractalkine/CX3C chemokine receptor 1 chemokine ligand-receptor pair [2, 3]. They have been shown to expand in different pathological conditions, such as cancer, asthma, sepsis, human immunodeficiency virus (HIV) infection, and AIDS progression [1, 4], but data about their possible role in leishmaniasis are lacking. Leishmaniasis is endemic in several parts of the world, with a global prevalence of over 12 million cases and 1.5 million new cases emerging every year [5]. The infection is caused by protozoan parasites of the genus *Leishmania,* transmitted trough the bite of the sand fly vector. Several *Leishmania* species are able to cause a wide spectrum of clinical manifestations, ranging from the mild cutaneous form, the disfiguring mucosal form, and the life-threatening visceral form, also known as kala-azar. The course of *Leishmania* infection presents several clinical features, depending on parasite virulence and tissue tropism, besides host immune response (reviewed in ref. [6]). Fc receptors (FcRs) compose an interface between humoral and cellular immune response [7], which are, respectively, detrimental and protective in human as well as murine leishmaniasis. Kima et al. [8] have shown that *Leishmania mexicana* infection in the murine host does not establish in the absence of circulating antibody and FcRs. In addition, human $CD16^+$ monocytes have been demonstrated to differentiate preferentially into dendritic cells in vitro [9], thus classifying them as potential targets for immunotherapy. Therefore, we quantified the FcR for immunoglobulin G III (CD16) surface expression in peripheral blood monocytes from patients with localized cutaneous leishmaniasis as compared with healthy controls and correlated CD16 expression to clinical data.

The Ethics Committee of the University Hospital Professor Edgard Santos (Salvador, Bahia, Brazil) approved this study, and informed consent was obtained from the patients and healthy controls. Peripheral blood (10 mL) was obtained from 15 patients (11 male, age 32.3±6.2 years) and 15 healthy controls (5 male, age 31.2±2.3 years) by venipuncture using heparin as an anticoagulant. Patients were attended and treated in two outpatient clinics (Jequié and Jiquiriçá, Bahia state, Northeast of Brazil), a rural area with a low socioeconomic status and a high incidence of infection with *Leishmania braziliensis* [10]. One or more of the following confirmed diagnosis of cutaneous leishmaniasis: characteristic lesion morphology, positive skin test [11], seropositivity toward *Leishmania* antigen [12], and the presence of parasites in the lesion. Clinical data of the patients are listed in **Table 1**. Whole blood (50 μl) was diluted with an equal volume of phosphate-buffered saline–1% bovine serum albumin–0.1% sodium azide, followed by staining for 30 min on ice with fluoresceinated anti-CD16 and lineage markers [CD14, CD64 (monocytes), CD3 (T cells), CD19 (B cells), CD16b (neutrophils), CD49d (eosinophils), and CD56 (natural killer cells)] or isotype-matched control antibodies (all from Immunotech-Coulter, Marseille, France), followed by fixation and erythrocyte lysis (whole blood lysing solution, Becton Dickinson, San Jose, CA). For each sample, 10,000 events were acquired in a cytoflu-

[1] Correspondence: FIOCRUZ, LIMI-CPqGM, Rua Waldemar Falcão 121, 40295-001 Salvador-BA, Brazil. E-mail: johan@cpqgm.fiocruz.br

Received January 22, 2005; revised September 8, 2005; accepted September 27, 2005; doi: 10.1189/jlb.0105040.

TABLE 1. Clinical Data of Cutaneous Leishmaniasis Patients

Patient number	Lymphadenopathy	Disease duration (days)	Lesion size (cm)	Anti-*Leishmania* DTH	Anti-*Leishmania* serology	Healing time (days)	Parasites in lesion	% $CD16^+$ monocytes
1	+	45	4	+	–	60	ND	15.8
2	–	30	ND	–	–	60	ND	1.0
3	+	20	ND	+	+	ND	ND	2.6
4	+	40	ND	–	–	45	ND	13.2
5	+	60	2.0	+	+	ND	+	13.2
6	–	90	6.0	+	–	60	ND	32.1
7	–	150	1.5	+	–	150	ND	12.6
8	+	30	1.0	+	+	60	–	5.9
9	+	60	1.0	+	+	>1200	–	9.1
10	–	60	1.5	+	+	120	ND	13.2
11	–	45	5.0	+	–	>1200	–	19.4
12	+	21	2.5	+	+	180	+	4.8
13	–	90	2.0	+	–	150	+	0.0
14	+	90	1.0	+	–	30	ND	6.6
15	–	75	2.5	ND	–	120	+	1.2

DTH, Delayed-type hypersensitivity; ND, not determined.

orometer (FACSort, Becton Dickinson) and analyzed using CellQuest software. Monocytes were gated according to their characteristic forward- and side-scatter (**Fig. 1A**) and were confirmed to be $CD14^+$, $CD64^+$ and $CD3^-$, $CD19^-$, $CD16b^-$, $CD56^-$ (Fig. 1B and not shown). Transforming growth factor-β1 (TGF-β1; active form) in plasma was quantified using a commercial enzyme-linked immunosorbent assay (ELISA) kit (DuoSet, R&D Systems, Minneapolis, MN); total TGF-β1 levels (active+latent form) were measured following acidification in siliconized tubes. All results are expressed as mean ± SEM. Statistical analysis of data was performed using GraphPad Prism 3.0. As all data had a normal distribution, as assessed by the Kolmogorov-Smirnov test, parametric analysis (Student's *t*-test with Welch correction and Pearson's correlation) was used; a P value <0.05 was considered significant.

As shown in **Figure 2, A** and **B**, we found a significant increase in the percentage (3.2±1.2 vs. 10.1±2.2, $P=0.016$) as well as mean fluorescent intensity (MFI; 13.5±4.6 vs. 29.2±4.1, $P=0.017$) of ex vivo CD16 expression in monocytes from patients with localized cutaneous leishmaniasis, as compared with healthy controls. To test the hypothesis that these elevated levels of $CD16^+$ monocytes might be clinically relevant, we analyzed possible correlation with disease duration, lesion size, or healing time or possible association with lymphadenopathy, anti-*Leishmania* DTH, or anti-*Leishmania* serology. As shown in **Figure 3A**, we observed a highly significant, positive correlation between the percentage of ex vivo monocytes expressing CD16 and lesion size ($P=0.0052$, r=0.75), but not other clinical data (not shown), in patients with localized cutaneous leishmaniasis. As CD16 expression can be induced by TGF-β in monocytes and macrophages [13], we quantified active as well as total (active+latent) TGF-β levels in plasma from controls and patients. Active but not total TGF-β plasma levels strongly correlated to the percentage of $CD16^+$ monocytes (r=0.78, $P=0.0017$), as shown in Figure 3B. Active TGF-β levels also correlated significantly, but less strongly, to lesion size (r=0.70, $P=0,011$, not shown), suggesting an indirect effect of the cytokine on lesion formation, which might be CD16-mediated. In human leishmaniasis, in situ demonstration of TGF-β in lymphoblastoid cell lines or mucosal leishmaniasis patients is restricted to early lesions [14]. In addition, the percentage of $CD16^+$ monocytes (3.6±1.1% vs. 13.5±2.7%, $P=0.005$) but not TGF-β (53±31 pg/ml vs. 137±47 pg/ml, $P=0.29$) was significantly lower in

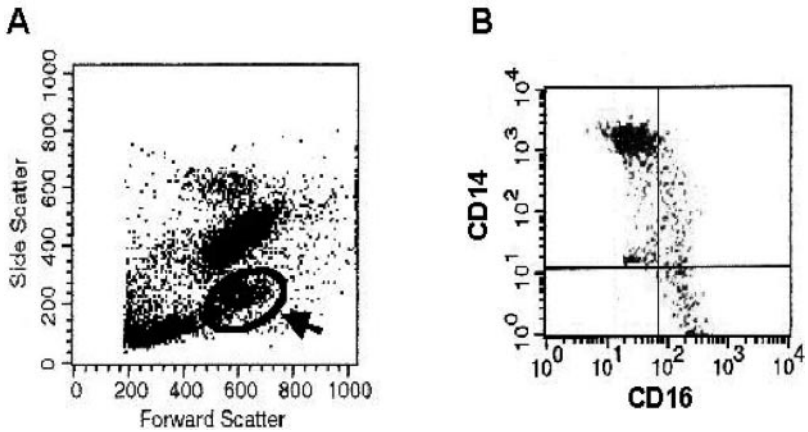

Fig. 1. Representative flow cytometry dot plots of (A) monocytes (arrow), gated by characteristic forward- and side-scatter in whole blood following erythrocyte lysis, and (B) CD16 expression in gated monocytes from a patient with cutaneous leishmaniasis. Total $CD16^+$ levels were measured, including CD14high and CD14low (upper- and lower-right quadrants, respectively).

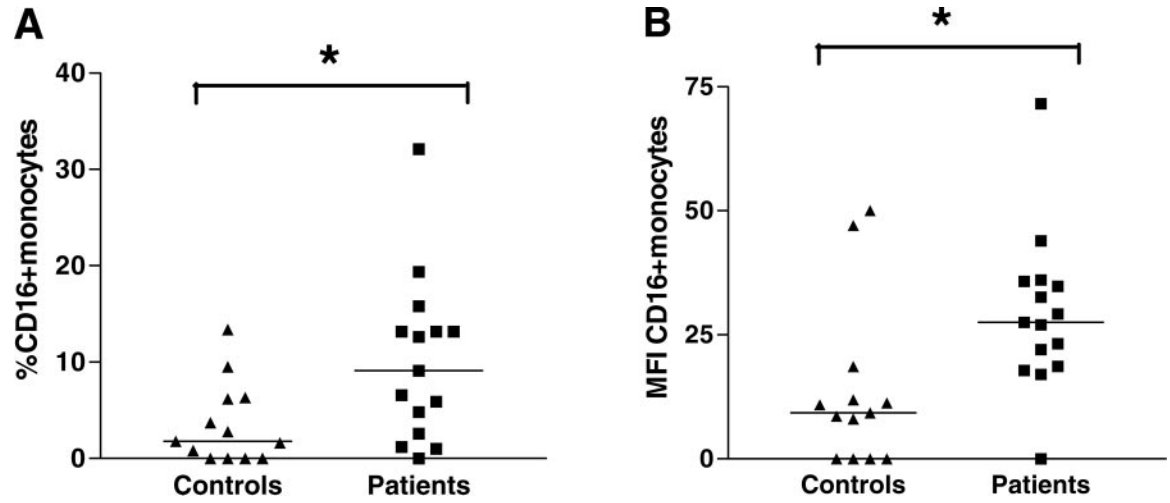

Fig. 2. CD16 expression in peripheral blood monocytes of healthy controls and patients with cutaneous leishmaniasis. (A) Percentage of positive cells; (B) MFI, quantified by flow cytometry (*, $P<0.05$).

patients with initial disease (<30 days of disease duration). Direct activation of latent TGF-β by cysteine proteases from live *Leishmania chagasi* promastigotes has been demonstrated [15] and might supposedly take place during the first few hours of infection. Taken together, these data suggest TGF-β production, systemic or in situ, might precede temporally and actually induce monocyte CD16 expression in the course of *Leishmania* infection.

Besides its strong correlation to lesion size, CD16 expression might have a predictive value for clinical evolution. During a 3-year follow-up, two out of 15 patients (Numbers 9 and 11 in Table 1) did not heal and had elevated (9.1–19.4%) $CD16^+$ monocytes at the time of diagnosis. Patient 9 actually progressed to mucosal leishmaniasis and further increased CD16 levels to 30.2% at 6 months of treatment. One additional patient (for which no blood sample was available at diagnosis and thus, was not included in Table 1) relapsed 1 year after treatment and also displayed increased (11.2%) $CD16^+$ monocytes at the time of relapse. Whether CD16 plays a direct role in lesion formation, e.g., by provoking immune complex-mediated vasculitis [16], remains to be determined. Data from a murine model of cutaneous leishmaniasis [8] demonstrate the necessity of FcRs, including CD16 for lesion development. $CD14^+16^+$ monocytes have been shown to be high tumor necrosis factor (TNF) producers [17] and thus might be involved in tissue destruction in cutaneous and mucosal leishmaniasis [16], suggesting that increased CD16 expression at diagnosis or during follow-up might be a cause, rather than consequence, of increased disease severity. TNF inhibitors, such as pentoxyphillin, have been used successfully in cutaneous and mucosal leishmaniasis patients refractory to standard antimonial therapy [18, 19]. This apparently contradictory

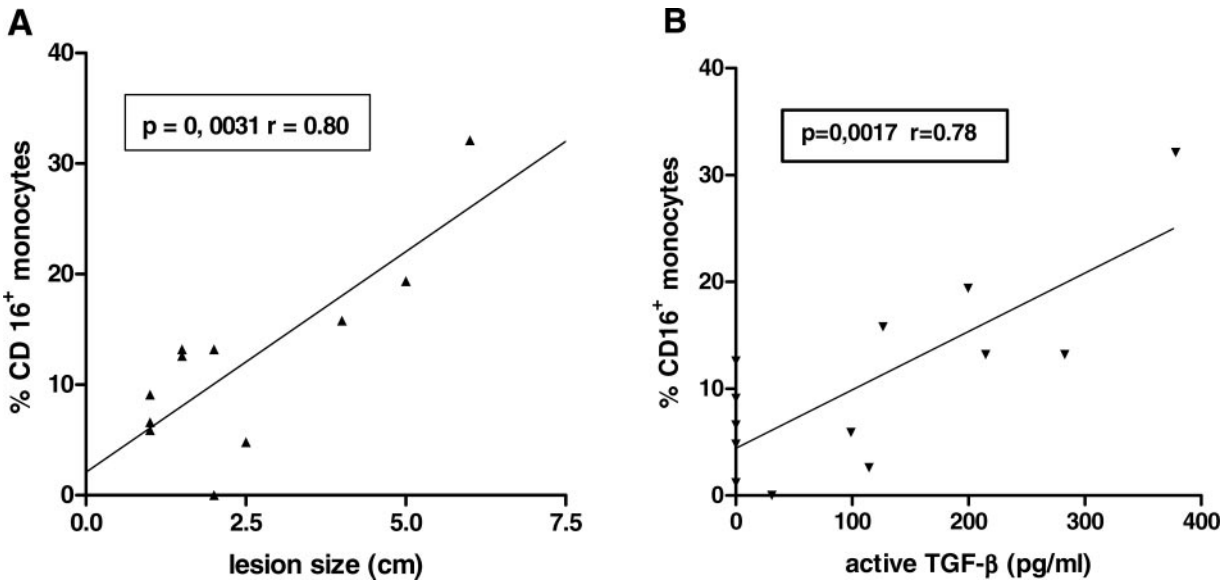

Fig. 3. Correlation between CD16-positive peripheral blood monocytes and (A) lesion size and (B) active TGF-β plasma levels in cutaneous leishmaniasis patients.

TGF-β–TNF-α loop points at a complex interplay and a delicate balance between both autocrine macrophage products with antagonistic activity on its own leishmanicidal capacity [14], which hampers the possible use of either cytokine as a biological marker. However, peripheral monocyte CD16 expression might be considered as a candidate biological marker in cutaneous leishmaniasis, which should be tested in larger studies in other endemic areas and possibly in other forms of the disease.

In light of HIV–*Leishmania* coinfection, which is of increasing epidemiological concern in Brazil as well as in Southern Europe, easy-to-use biological markers should be valuable to monitor high-risk populations, in addition to viral load and parasite burden. CD16 has already been proposed as a sensitive marker for AIDS progression [20], probably through its induction by TGF-β, which our group and others [21, 22] have shown to increase viral load in HIV-1-infected human macrophages in vitro. As shown in this study, CD16 can be useful as an easy-to-quantify possible biological marker of disease severity in human leishmaniasis in endemic areas. To this aim, one drop (100 μl) of whole blood can be stained under field conditions and processed for flow cytometry 24–48 h later with similar results (G. Soares and J. Van Weyenbergh, unpublished results).

In conclusion, this study is the first demonstration of a possible deleterious role for FcRs in human leishmaniasis, considering increased CD16 expression in peripheral blood monocytes, strong correlation to lesion size and active TGF-β plasma levels, and its possible use as a predictor of adverse clinical outcome.

ACKNOWLEDGMENTS

Financial support was provided by TMRC, PRONEX, CNPq, and the INSERM-FIOCRUZ Collaborative Program. A. B. is a CNPq senior investigator; G. S. is a recipient of a FAPESB fellowship. We thank the patients and the volunteers who participated in this study, Eliane Góes Nascimento and the staff from PIEJ, and Jiquiriça outpatient clinics; Jorge Tolentino for his collaborative effort; and Theolis Barbosa and André Bafica for helpful discussions and critical reading of the manuscript. We are grateful to Juana Wietzerbin and the INSERM-FIOCRUZ Collaborative Program for providing flow cytometry and ELISA reagents.

REFERENCES

1. Ziegler-Heitbrock, H. W. (1996) Heterogeneity of human blood monocytes: the CD14+CD16+ subpopulation. *Immunol. Today* **17,** 424–428.
2. Geissmann, F., Jung, S., Littman, D. R. (2003) Blood monocytes consist of two principal subsets with distinct migratory properties. *Immunity* **19,** 71–82.
3. Ancuta, P., Rao, R., Moses, A., Mehle, A., Shaw, S. K., Luscinskas, F. W., Gabuzda, D. (2003) Fractalkine preferentially mediates arrest and migration of CD16+ monocytes. *J. Exp. Med.* **197,** 1701–1707.
4. Thieblemont, N., Weiss, L., Sadeghi, H. M., Estcourt, C., Haeffner-Cavaillon, N. (1995) CD14lowCD16high: a cytokine-producing monocyte subset which expands during human immunodeficiency virus infection. *Eur. J. Immunol.* **25,** 3418–3424.
5. Desjeux, P. (2004) Leishmaniasis: current situation and new perspectives. *Comp. Immunol. Microbiol. Infect. Dis.* **27,** 305–318.
6. Melby, P. C. (2002) Recent developments in leishmaniasis. *Curr. Opin. Infect. Dis.* **15,** 485–490.
7. Heijnen, I. A., van de Winkel, J. G. (1997) Human IgG Fc receptors. *Int. Rev. Immunol.* **16,** 29–55.
8. Kima, P. E., Constant, S. L., Hannum, L., Colmenares, M., Lee, K. S., Haberman, A. M., Shlomchik, M. J., McMahon-Pratt, D. (2000) Internalization of *Leishmania mexicana* complex amastigotes via the Fc receptor is required to sustain infection in murine cutaneous leishmaniasis. *J. Exp. Med.* **191,** 1063–1068.
9. Randolph, G. J., Sanchez-Schmitz, G., Liebman, R. M., Schakel, K. (2002) The CD16+ (FcγRIII+) subset of human monocytes preferentially becomes migratory dendritic cells in a model tissue setting. *J. Exp. Med.* **196,** 517–527.
10. Miranda, J. C., Reis, E., Schriefer, A., Goncalves, M., Reis, M. G., Carvalho, L., Fernandes, O., Barral-Netto, M., Barral, A. (2002) Frequency of infection of Lutzomyia phlebotomines with *Leishmania braziliensis* in a Brazilian endemic area as assessed by pinpoint capture and polymerase chain reaction. *Mem. Inst. Oswaldo Cruz* **97,** 185–188.
11. Reed, S. G., Badaro, R., Masur, H., Carvalho, E. M., Lorenco, R., Lisboa, A., Teixeira, R., Johnson Jr., W. D., Jones, T. C. (1986) Selection of a skin test antigen for American visceral leishmaniasis. *Am. J. Trop. Med. Hyg.* **35,** 79–85.
12. Badaro, R., Reed, S. G., Barral, A., Orge, G., Jones, T. C. (1986) Evaluation of the micro enzyme-linked immunosorbent assay (ELISA) for antibodies in American visceral leishmaniasis: antigen selection for detection of infection-specific responses. *Am. J. Trop. Med. Hyg.* **35,** 72–78.
13. Welch, G. R., Wong, H. L., Wahl, S. M. (1990) Selective induction of Fc γ RIII on human monocytes by transforming growth factor-β. *J. Immunol.* **144,** 3444–3448.
14. Barral, A., Teixeira, M., Reis, P., Vinhas, V., Costa, J., Lessa, H., Bittencourt, A. L., Reed, S., Carvalho, E. M., Barral-Netto, M. (1995) Transforming growth factor-β in human cutaneous leishmaniasis. *Am. J. Pathol.* **147,** 947–954.
15. Gantt, K. R., Schultz-Cherry, S., Rodriguez, N., Jeronimo, S. M., Nascimento, E. T., Goldman, T. L., Recker, T. J., Miller, M. A., Wilson, M. E. (2003) Activation of TGF-β by *Leishmania chagasi*: importance for parasite survival in macrophages. *J. Immunol.* **170,** 2613–2620.
16. Correia Silva, T. M., Barral, A., Pompeu, M. M. L., Costa, J., Lessa, H., Carvalho, E. M., de Freitas, L. A. R. (1998) Immune complex-mediated vasculitis in human cutaneous leishmaniasis. *Mem. Inst. Oswaldo Cruz* **93** (Suppl. II), 49–50.
17. Belge, K. U., Dayyani, F., Horelt, A., Siedlar, M., Frankenberger, M., Frankenberger, B., Espevik, T., Ziegler-Heitbrock, L. (2002) The proinflammatory CD14+CD16+DR-monocytes are a major source of TNF. *J. Immunol.* **168,** 3536–3542.
18. Bafica, A., Oliveira, F., Freitas, L. A., Nascimento, E. G., Barral, A. (2003) American cutaneous leishmaniasis unresponsive to antimonial drugs: successful treatment using combination of N-methilglucamine antimoniate plus pentoxifylline. *Int. J. Dermatol.* **42,** 203–207.
19. Lessa, H. A., Machado, P., Lima, F., Cruz, A. A., Bacellar, O., Guerreiro, J., Carvalho, E. M. (2001) Successful treatment of refractory mucosal leishmaniasis with pentoxifylline plus antimony. *Am. J. Trop. Med. Hyg.* **65,** 87–89.
20. Allen, J. B., Wong, H. L., Guyre, P. M., Simon, G. L., Wahl, S. M. (1991) Association of circulating receptor Fc γ RIII-positive monocytes in AIDS patients with elevated levels of transforming growth factor-β. *J. Clin. Invest.* **87,** 1773–1779.
21. Lima, R. G., Van Weyenbergh, J., Saraiva, E. M., Barral-Netto, M., Galvao-Castro, B., Bou-Habib, D. C. (2002) The replication of human immunodeficiency virus type 1 in macrophages is enhanced after phagocytosis of apoptotic cells. *J. Infect. Dis.* **185,** 1561–1566.
22. Lazdins, J. K., Klimkait, T., Woods-Cook, K., Walker, M., Alteri, E., Cox, D., Cerletti, N., Shipman, R., Bilbe, G., McMaster, G. (1991) In vitro effect of transforming growth factor-β on progression of HIV-1 infection in primary mononuclear phagocytes. *J. Immunol.* **147,** 1201–1207.