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Leishmania Infection Impairs β_1-Integrin Function and Chemokine Receptor Expression in Mononuclear Phagocytes

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***Leishmania* spp. are intracellular parasites that cause lesions in the skin, mucosa, and viscera. We have previously shown that *Leishmania* infection reduces mononuclear phagocyte adhesion to inflamed connective tissue. In this study, we examined the role of adhesion molecules and chemokines in this process. Infection rate ($r = -0.826$, $P = 0.003$) and parasite burden ($r = -0.917$, $P = 0.028$) negatively correlated to mouse phagocyte adhesion. The decrease (58.7 to 75.0% inhibition, $P = 0.005$) in phagocyte adhesion to connective tissue, induced by *Leishmania*, occurred as early as 2 h after infection and was maintained for at least 24 h. Interestingly, impairment of cell adhesion was sustained by phagocyte infection, since it was not observed following phagocytosis of killed parasites (cell adhesion varied from 15.2% below to 24.0% above control levels, $P > 0.05$). In addition, *Leishmania* infection diminished cell adhesion to fibronectin (54.1 to 96.2%, $P < 0.01$), collagen (15.7 to 83.7%, $P < 0.05$), and laminin (59.1 to 82.2%, $P < 0.05$). The $CD11b^{hi}$ subpopulation was highly infected (49.6 to 97.3%). Calcium and Mg^{2+} replacement by Mn^{2+}, a treatment that is known to induce integrins to a high state of affinity for their receptors, reverted the inhibition in adhesion caused by *Leishmania*. This reversion was completely blocked by anti-VLA4 antibodies. Furthermore, expression of CCR4 and CCR5, two chemokine receptors implicated in cell adhesion, was found to be downregulated 16 h after infection (2.8 to 4.1 times and 1.9 to 2.8 times, respectively). Together, these results suggest that mechanisms regulating integrin function are implicated in the change of macrophage adhesion in leishmaniasis.**

The protozoan parasites of the genus *Leishmania* are the causative agents of tegumentary and visceral leishmaniasis. Distinct species of *Leishmania* cause different forms of the disease, although some overlap has been reported (1, 8, 34). For example, *Leishmania amazonensis*, a pathogen mostly detected in cutaneous lesions, has been described to cause visceral disease (1). The mechanisms by which the parasite induces such different diseases are largely unknown and may involve both pathogen and host factors (8, 13, 21, 34). In all cases, *Leishmania* infection initiates when an infected phlebotominae sand fly inoculates the parasites into the dermis during feeding. Thereafter, the infective promastigotes enter and multiply inside the host phagocytic cells, eventually reaching the draining lymph node. The identity and fate of the cells that carry the organism away from the injection site are controversial (31, 32, 36, 37). However, the genesis of the lesions is closely linked to the presence of macrophages harboring *Leishmania* amastigotes (3). In different forms of the disease, one may find parasitized macrophages in the lymph nodes that drain the inoculation site (32) or in the blood (27). Since the amastigote forms are obligated intracellular parasites, their migration has to rely on the addressing information displayed on the surface of their phagocytic hosts, such as the type of adhesion molecules or chemokine receptors.

In fact, it has been reported that upon phagocytosis of *Leishmania major* (31, 32, 37), mononuclear phagocytes harboring live parasites migrate from the skin to the draining lymph node. These data suggest that *Leishmania* infection induces cellular changes leading to de-adhesion (a first step in cellular traffic) and migration of mononuclear phagocytes from the skin to draining lymph node. The capacity of these cells to home to the skin, to mucosae, or to internal organs may also be modified by the parasite (2). The mechanisms by which *Leishmania* parasites inhibit macrophage adhesion are unclear.

We have recently reported the development of an in vitro adhesion assay to study the interactions of mononuclear phagocytes with the connective tissue (11). Using this tool, we have shown that different *Leishmania* species reduce the adherence of phagocytes to the inflamed connective tissue and suggested that mechanisms regulating integrin affinity would be involved in such modulation of cell adhesion by *Leishmania* (11). Thus, the observed decrease of cell adherence early upon *Leishmania* infection may account for the first stage of this migratory process. Upon activation or infection with intracellular pathogens, phagocytes remain in the tissues, contributing to chronic inflammation, or carry pathogens around the body. Hence, the understanding of the mechanisms involved in the interactions between phagocytes and the connective matrix may be helpful in the design of therapeutics aimed at controlling disorders associated with inadequate homing of monocytes, as proposed for leishmaniasis (46).

In the present work, we examine the role of integrins as

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well as chemokine receptors in the *Leishmania*-mediated inhibition of macrophage adhesion to the connective tissue and the variables influencing this process, such as infection versus parasite products, parasite burden, and the time course of infection.

MATERIALS AND METHODS

Animals. Eight- to 12-week-old BALB/c or Swiss albino mice were obtained from the colony of the Gonçalo Moniz Research Center-FIOCRUZ (Salvador, Brazil). The animals were maintained under controlled environmental conditions of humidity, temperature, and light-dark cycle, with commercial, balanced mouse chow and water ad libitum. The experiments involving animals were conducted in accordance with the Oswaldo Cruz Foundation guidelines for research with animals (http://www.fiocruz.br/presidencia/vppdt/comceua.htm).

Mouse peritoneal exudate cells. Peritoneal exudate cells (PEC) were obtained by the intraperitoneal injection of 3 ml of a sterile thioglycolate (Sigma) solution at 3% (wt/vol). Four days after the injection, peritoneal cells were collected by washing the peritoneal cavity twice with cold Ca^{2+}/Mg^{2+}-free Hanks balanced salt solution (HBSS; Sigma) containing 20 IU/ml heparin. The cells were washed twice in HBSS, suspended in RPMI (Sigma) with 10% fetal bovine serum (FBS; Cultilab, Brazil), 60 μg/ml gentamicin, and 2 mM glutamine (complete RPMI), and cultured in nonadherent polypropylene tubes at 37°C and 5% CO_2. More than 80% of the cells obtained using this process had macrophage features, as evaluated by morphological analysis of Giemsa-stained slides and by flow cytometry using forward and side scatters or staining with anti-CD11b (clone M1/70; BD Pharmingen) and F4/80 (clone CI:A3-1; Caltag) antibodies. Viability was assessed by trypan blue exclusion. At least 85% of the cells used for the experiments were viable.

Sections of inflamed tissue. Dorsal subcutaneous inflammatory air pouches were produced in BALB/c mice by the injection of 2 ml of air and 200 μl of soy oil containing 0.1% croton oil. Animals were sacrificed 3 days after the injection. Transversal full-thickness slices of tissue from the inflammatory air pouch were collected, immersed in Histoprep (Fisher Scientific), frozen in liquid nitrogen, and preserved at −70°C until use. Serial 5-μm cryostat sections (perpendicular to the skin and to the wall of the inflammatory air pouch) were collected on glass slides previously coated with a gelatin film (0.5% solution in HBSS; Sigma). Sections were air dried for 10 min, fixed with cold (−20°C) acetone for 3 min, and then washed with phosphate-buffered saline (PBS). Ten-millimeter-diameter circles were drawn around each section using a nontoxic marker pen (Pap Pen; Daido Sangyo, Japan) to prevent the free flow of the cell suspension over the slides. The purpose of using gelatin-coated glass slides was to minimize the adherence of macrophages to the glass (11).

Phagocyte infections and phagocytosis of killed *Leishmania*. *Leishmania braziliensis* (MHOM/BR/3456) and *L. amazonensis* (Leila strain, MHOM/BR88/BA-125) were grown in Schneider's insect medium (Sigma) containing 10% (*L. amazonensis*) or 20% (*L. braziliensis*) FBS at 24°C (47). Parasites were washed three times in HBSS, suspended in complete RPMI, and incubated with the phagocytes. Control phagocytes were either cultured in medium alone or in medium containing 3-μm-diameter latex beads (Sigma). For some experiments, the parasites were killed by incubation with absolute ethanol for 20 min on ice, washed three times in HBSS, and incubated with the phagocytes. For the flow cytometry analyses, *Leishmania* parasites were stained with a red fluorescent dye (PKH26; Sigma) according to the manufacturer's instructions before being incubating with the phagocytes. Briefly, 10^8 parasites were suspended in 2 ml of PBS, mixed to a 4 μM PKH26 solution in 2 ml of PBS, and incubated for 5 min at room temperature in the dark under periodic agitation. Staining was stopped by the addition of 4 ml of FBS, incubation at 37°C for 30 min, and washing three times in HBSS. Preliminary experiments showed that the dye does not escape from stained parasites or alter its viability and infectivity in vitro (data not shown). Promastigotes or amastigote forms of *Leishmania*, obtained according to a protocol described elsewhere (47) were used in these experiments with similar results. Ten parasites or latex particles per cell were used where not otherwise stated. Samples of the cells from each group were centrifuged onto glass slides, fixed with methanol, Giemsa stained, and examined by light microscopy to check the percentage of infected phagocytes and the number of parasites per infected cell. At least 1,000 mononuclear cells were counted.

Leukocyte-connective tissue adhesion assay. Leukocyte-connective tissue adhesion assays were performed according to a previously described method (11). Briefly, inflamed connective tissue sections were incubated with HBSS containing 1% (wt/vol) bovine serum albumin (BSA; Sigma) for 30 min at room temperature to block nonspecific reactions. Cells were washed twice in HBSS and suspended at 2×10^6 cells/ml in HBSS containing 1% BSA. Aliquots of 4×10^5 cells were placed over the tissue sections. They were incubated under agitation (80 cycles per minute on a circular shaker), washed three times in PBS with Ca^{2+} and Mg^{2+} (250 μM) to remove nonadherent cells, and fixed for 30 min in 1% glutaraldehyde. Slides were then stained with hematoxylin and eosin and examined under light microscopy. Measurements were performed using a CX41 microscope (Olympus) coupled to a digital imaging system. Nonoverlapping areas of inflamed connective tissue represented in all sections were selected for the measurements. Five microphotographs were taken from equivalent areas in each section, and the adhered cells were counted using the Image-Pro Plus 4.5 software (Media Cybernetics). Results are expressed as means ± standard errors of the means of the counts obtained in each treatment replicate. When different experiments are compared, the data are presented in relative value (percentage of cells adhered compared to untreated controls) to adjust for variations in cell counts from the control groups of different experiments. Four to six sections were used per group of cells subjected to different treatments.

Leukocyte-extracellular matrix component adhesion assay and cell adhesion blocking experiments. Sterile 96-well enzyme-linked immunosorbent assay (ELISA) plates were coated with collagen, fibronectin, and laminin. For coating the wells with collagen, plates were incubated overnight with 50 μl of a type I collagen solution at 300 μg/ml (Sigma), in ammonia atmosphere, at room temperature. To increase fibronectin binding to the wells, 100 μl of a 10 μg/ml fibronectin (Sigma) solution was added to wells previously coated with collagen. Laminin (Sigma) was used at a concentration of 20 μg/ml, 100 μl per well. The plates were incubated for 1 h at 37°C and washed three times in HBSS. Samples of the cells (200-μl volumes containing 8×10^4 cells) were seeded in each well and incubated for 1 h at 37°C and 5% CO_2. In the experiments of divalent cation replacement, cells were washed with Ca^{2+}/Mg^{2+}-free HBSS, incubated with 1 mM EDTA for 5 min on ice, washed three times in Ca^{2+}/Mg^{2+}-free HBSS, and suspended in Ca^{2+}/Mg^{2+}-free HBSS containing 1% BSA and either 250 μM Ca^{2+} and 250 μM Mg^{2+} or 500 μM Mn^{2+}. In some experiments, VLA4 interactions with fibronectin were blocked by incubation of the cells with anti-CD49d antibody (clone R1-2; BD Pharmingen) at 10 μg/ml for 20 min on ice before they were added to the plate. Control cells were incubated with an isotype-matched antibody (rat immunoglobulin G1, clone R3-34; BD Pharmingen). After the incubation, plates were carefully washed four times in warm HBSS, and the adhered cells were fixed with 1% glutaraldehyde for 30 min. Six replicates were used for each treatment. Three different nonoverlapping random areas per well were taken for quantification, similar to what was described above, in an inverted microscope. Results are expressed as the means of the counts obtained with each treatment.

Flow cytometry analysis. Swiss albino mice PEC were analyzed for surface expression of CD11a (clone 2D7), CD11b (M1/70), CD18 (C71/16), CD49d (9C10), CD62L (MEL-14), CD102 (3C4), and CD106 (429/MVCAM.A). All antibodies were fluorescein conjugates obtained from BD Pharmingen. Noninfected or *Leishmania*-infected cells were suspended in PBS containing 1% BSA and 0.05% sodium azide and blocked with 5% mouse serum plus 5% FBS for 30 min on ice. The cells were then incubated with labeled antibodies or isotype controls (A95-1 and R35-95 from BD Pharmingen) in a dilution of 1:200 for 30 min. The data shown are representative of four independent experiments. Cells were washed and analyzed on a FACScan flow cytometer using the CellQuest software (Becton-Dickinson). The histograms and bitmaps of distribution of cell populations were constructed using the FlowJo Software (Tree Star, Inc.). Fifty thousand events were analyzed per sample.

Chemokine receptor expression analysis by real-time PCR. We analyzed six different chemokine receptors known to be present on monocytic cells (CCR1, 3, 4, 5, and 7 and CXCR4) (35, 42, 43, 54, 55). Total RNA was extracted using the RNeasy RNA extraction kit (QIAGEN) according to the manufacturer's protocol. Real-time reverse transcription-PCR was performed on an ABI Prism 7000 sequence detection system (Applied Biosystems) using SYBR green PCR master mix (Applied Biosystems) after reverse transcription of 1 μg RNA using Superscript II reverse transcriptase (Invitrogen). The relative level of gene expression was determined by the comparative cycle threshold (C_T) method, as described by the manufacturer, whereby each sample was normalized to 18S and expressed as a relative change compared with untreated controls. Primer3 software (Whitehead Institute for Biomedical Research) (40) was used to design the specific primers (Table 1).

Analysis of results. The statistical significance of the difference between groups was assessed using the two-tailed Student *t* test or one-way analysis of variance (ANOVA), followed by Newman-Keuls post test, with a critical level of significance at a P value of <0.05. Trends were measured using Pearson's cor-

TABLE 1. Sequences of primers used in this study

Gene	Primer sequence (5′–3′)		Accession no.
	Sense	Antisense	
18S rRNA	CACGGCCGGTACAGTGAAAC	CCCGTCGGCATGTATTAGCT	M27358
CCR1	CAAAGGCCCAGAAACAAAGT	TGGTCAGGAATAATAGCTTCTGAAT	BC011092
CCR3	TGTTATCTCTGTTTCATTAGCAGTG	CAGTCTTGATTTCATCTGTGTTGA	U29677
CCR4	GACTGTCCTCAGGATCACTTTC	GGCATTCATCTTTGGAATCG	U15208
CCR5	CTCCTAGCCAGAGGAGGTGA	TGTCATAGCTATAGGTCGGAACTG	U83327
CCR7	CTACAGCCCCCAGAGCAC	TGACCTCATCTTGGCAGAAG	NM_007719
CXCR4	GGTAACCACCACGGCTGTA	AGTCTCCAGACCCCACTTCTT	NM_009911

relation coefficient (*r*). Statistical analyses were performed using the GraphPad Prism, version 3.00, program.

RESULTS

Intensity of infection determines adhesion rate to connective tissue of mouse monocytic phagocytes. We first evaluated the effect of infection intensity on phagocyte adhesion using different doses of parasites per cell. Twenty-four hours after the infection, the percentage of infected cells inversely correlated with the number of adhered cells ($r = -0.734$; $P < 0.0001$) (Fig. 1A). The number of parasites per infected phagocyte also negatively correlated with the connective tissue adhesion ($r = -0.780$; $P < 0.0001$) (Fig. 1B). These correlations followed a polynomial distribution, reaching a plateau at about 50% of infected phagocytes (Fig. 1A) or at three parasites per infected cell (Fig. 1B).

***Leishmania* infection modulates phagocyte adhesion as early as 2 h after infection.** We next tested whether the observed effect on *Leishmania*-infected macrophage adhesion was dependent on the time of exposure to the parasite. Observing different time points after in vitro infection (Fig. 2), we noticed a significant reduction in adhesion (25.1 to 41.8% of the control levels; ANOVA, $P < 0.01$) 2 h after infection. The inhibitory effect of *Leishmania* on cell adhesion was partially reverted between 6 and 8 h after infection. The difference between the levels of phagocyte adhesion at this early stage of more intense inhibition (2 to 4 h) and after the partial recovery (6 to 8 h) was statistically significant in 2 of 3 experiments (paired *t* test, $P = 0.01$, $P = 0.03$, and $P = 0.08$ in each of the three experiments).

A critical rate of infection has to be achieved to change the adherence of mononuclear phagocytes to the connective tissue 2 h after infection. As shown in Fig. 3A, 2 h after PEC infection with 0.6 parasites per cell, cell adhesion was not significantly decreased. It remained at 92.8 to 93.4% of the control levels (ANOVA $P > 0.05$), while infection with 2.5 parasites per cell significantly reduced the adhesion of the cells to inflamed connective tissue (53.0 to 69.5% of the control levels, ANOVA $P < 0.05$). After 24 h of infection with 0.6 live parasite per cell, however, we observed a significant reduction in the adhesion of

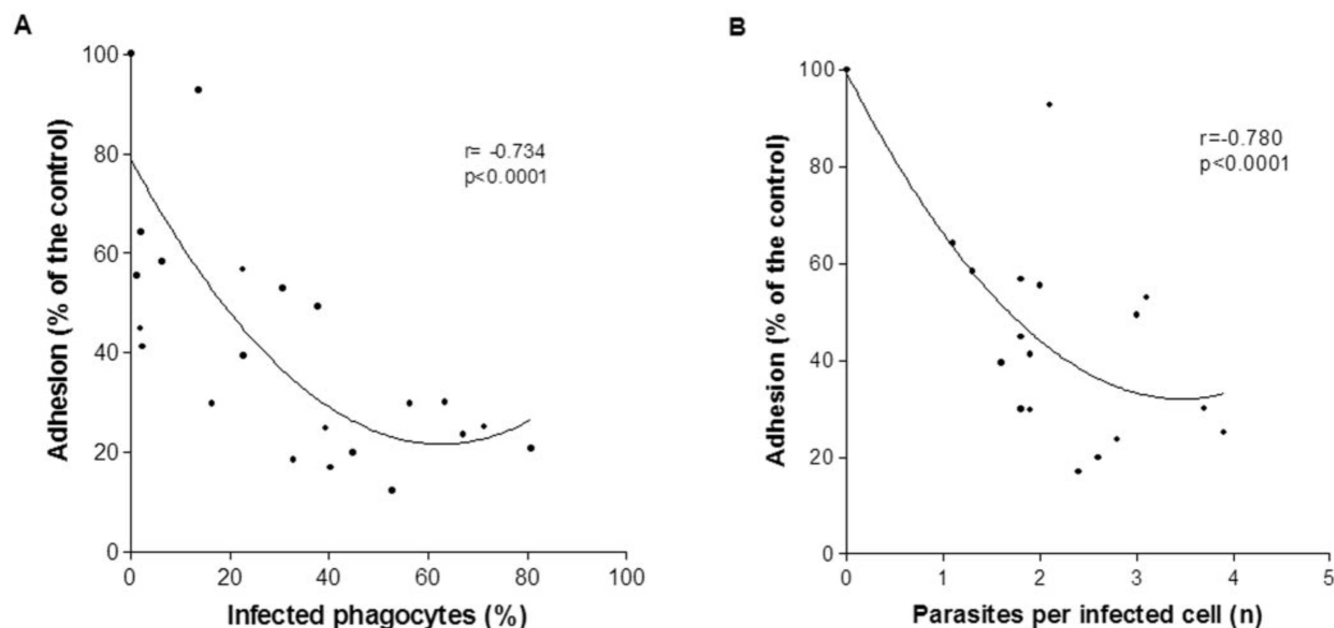

FIG. 1. Correlation between adhesion of peritoneal exudate cells to inflamed connective tissue and the intensity of infection, as measured by the percentage of infected phagocytes (A) and the number of parasites per infected cell (B). Twenty-four hours after infection, adherence to the connective tissue inversely correlated to both the percentage of infected phagocytes ($r = -0.734$, $P < 0.0001$) and the number of parasites per infected cell ($r = -0.780$, $P < 0.0001$). The dots represent data from five independent experiments.

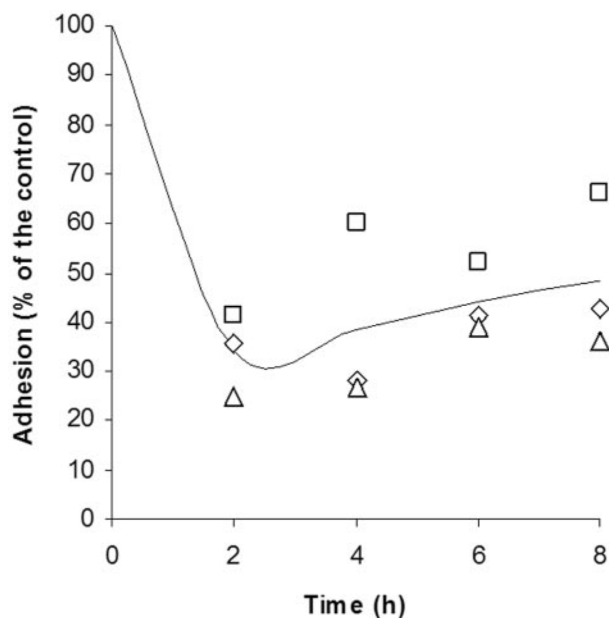

FIG. 2. Effect of infection time on the adhesion of mouse mononuclear phagocytes to the connective tissue. PEC were cultivated with medium alone or medium containing live *Leishmania* for 2, 4, 6, or 8 h and left to adhere for 30 min to a section of inflamed skin as described in Materials and Methods. The loss of adhesion of infected phagocytes was evident by 2 h after infection. This effect is partially reverted between 2 and 6 h after infection. Geometric forms represent three independent experiments, and the line represents the mean. Note that the tendency is maintained in all experiments in spite of the variation among different assays.

PEC to connective tissue (55.4 to 74.2% of the control levels; ANOVA $P < 0.001$) (Fig. 3B).

Modulation of mononuclear phagocyte adherence to inflamed connective tissue is sustained by infection and not by phagocytosis of killed parasites. To examine if the effect on leukocyte adhesion could be reproduced by products of dead parasites, phagocytes were incubated with killed *Leishmania*. Incubation with 10 parasites per cell for the short period of 2 h reduced the adhesion of PEC to the connective tissue (26.2 to 50.5%; ANOVA $P < 0.001$) (Fig. 3A). The same process was not observed when phagocytes were incubated for 2 h with 3-μm latex beads (the adhesion was 97.1 $\pm$ 23.6% of the control levels; data not shown). Additionally, when the incubation period of phagocytes with killed parasites was extended to 24 h, the adhesion of phagocytes to the connective tissue did not change (varied from 78.8 to 124.0% of the control levels, ANOVA $P > 0.05$) (Fig. 3B). At this later time point, the number of parasite-containing phagocytes in the group incubated with 10 killed parasites per cell was equivalent to that observed in the group infected with 2 live parasites per cell (22.7 versus 28.5%; t test $P = 0.7$), where significant reduction in adhesion was observed. Identical results were obtained using killed promastigotes or amastigotes (not depicted).

Since we observed some differences between the results from experiments performed at early (2 to 4 h) or after longer periods of *Leishmania* infection, we considered the possibility that distinct mechanisms influencing phagocyte adhesion would be taking place at these two stages of infection. Hence, we performed some of the subsequent experiments, taking into account those two times of infection.

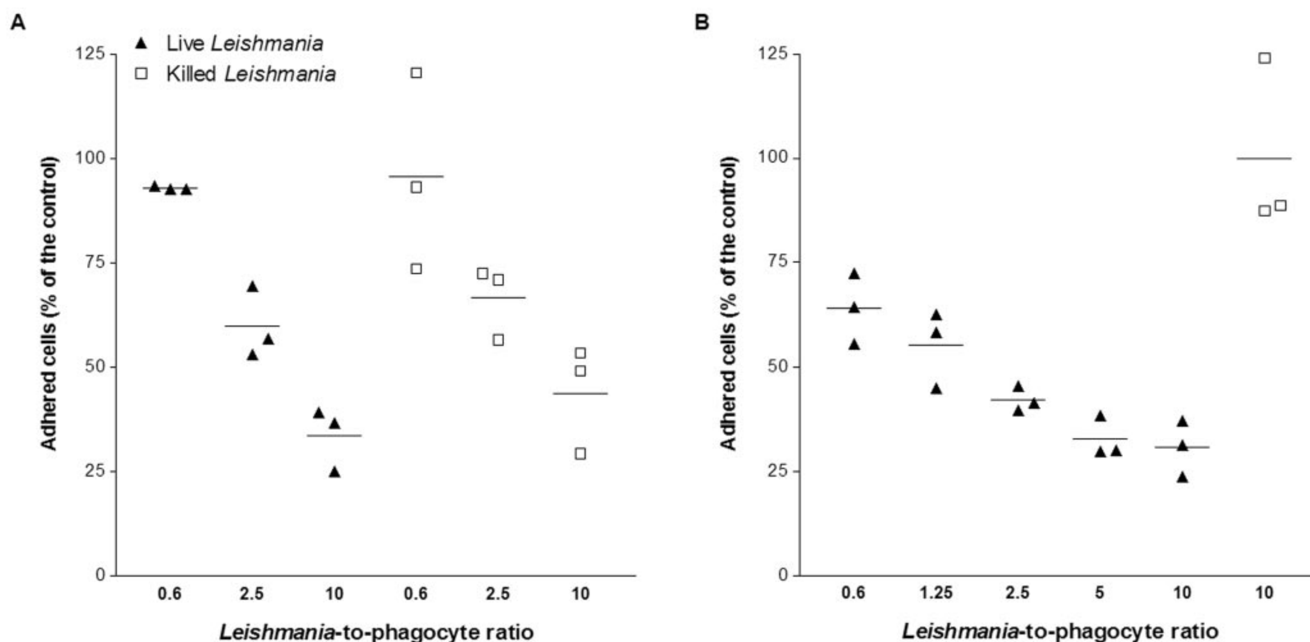

FIG. 3. Effect of infection with different *Leishmania*-to-phagocyte ratios in cell adhesion to connective tissue 2 h (A) or 24 h (B) after infection. Monocytes were cultivated with medium alone (controls) or with medium containing either live (filled triangles) or ethanol-killed (empty squares) *Leishmania* and were left to adhere for 30 min to sections of inflamed skin as described in Materials and Methods. Incubation of the phagocytes with small amounts of *Leishmania* (0.6 per phagocyte) led to a decrease in the adhesion 24 h after infection ($P < 0.001$) but not 2 h after infection ($P > 0.05$). Data were extracted from three independent assays.

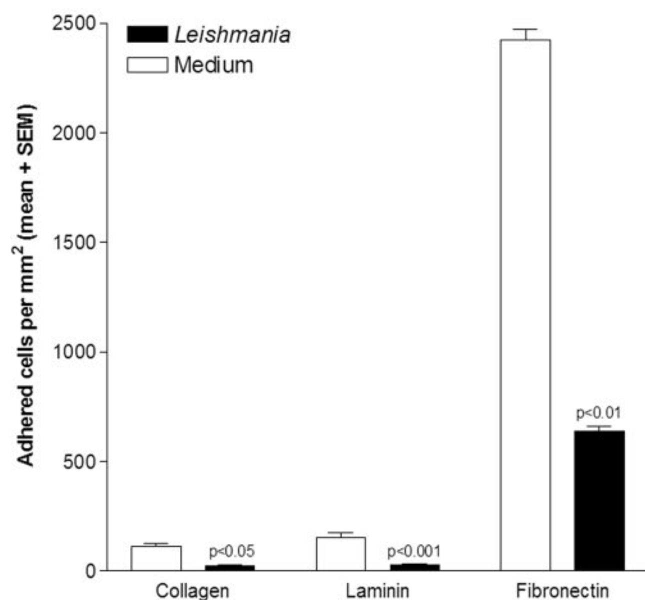

FIG. 4. Effect of *Leishmania* infection on the adhesion of mouse mononuclear phagocytes to purified extracellular matrix components. Cells cultivated with medium alone (empty bars) or medium containing live *Leishmania* (filled bars) were washed and adhered for 30 min to precoated ELISA plate wells, as described in Materials and Methods. *Leishmania* infection reduced adhesion to collagen (112.5 versus 25.0 cells/mm^2; ANOVA, $P < 0.05$), laminin (153.1 versus 28.1 cells/mm^2; ANOVA, $P < 0.001$), and fibronectin (2,421.9 versus 637.5 cells/mm^2; ANOVA, $P < 0.01$). The data shown are representative of results from five different experiments.

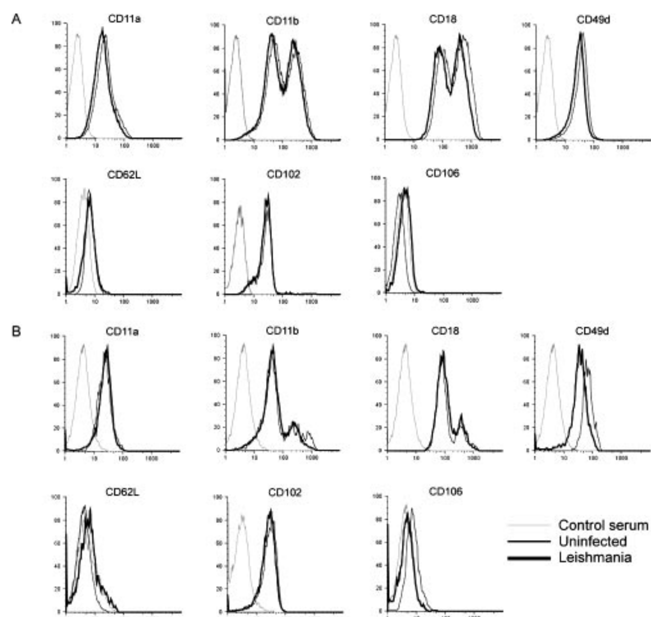

FIG. 5. Cell surface expression of adhesion molecules on mouse peritoneal exudate cells 2 h (A) or 24 h (B) after infection with *Leishmania*. Cells cultivated with medium alone (thin lines) or with live *Leishmania* (bold lines) were washed and stained with the appropriate fluorochrome-linked antibody, as described in Materials and Methods. Gray lines represent the isotype controls. The data depicted are representative of results from four independent analyses.

***Leishmania* infection decreases mononuclear phagocyte adhesion to purified extracellular matrix components.** To identify the cell-connective tissue adhesion pathways altered in the *Leishmania* infection, we have performed a series of experiments using fibronectin, collagen, and laminin as substrates for cell adhesion (39). The adhesion of PEC to all of the tested extracellular matrix components was reduced upon infection of the cells with *Leishmania* (Fig. 4). Adhesion to fibronectin was reduced to 26.3% (45.9 to 3.8; ANOVA $P < 0.01$) of the control levels. Moreover, the number of cells adhered to collagen or laminin was small and further reduced upon *Leishmania* infection, 22.3% (84.3 to 16.3%; ANOVA $P < 0.05$) and 17.8% (40.9 to 17.8%; ANOVA $P < 0.05$) of the control levels, respectively.

Expression of adhesion molecules is not modulated by *Leishmania* either 2 or 24 hours after infection. Since the experiments described above indicated impairment of a wide range of integrins involved in phagocyte-connective matrix interactions, we decided to compare the surface expression of adhesion molecules between infected and noninfected phagocytes. The PEC infected with *Leishmania* displayed only a slight shift toward a decrease of CD49d (VLA4 α_4 chain, a fibronectin receptor) in two of four experiments (Fig. 5). No significant changes in the intensity of expression of other adhesion molecules involved in cell-cell or cell-matrix interactions such as CD11a (LFA-1 α_L chain), CD11b (Mac-1 α_M chain), CD18 (β_2 integrin chain), CD62L (L-Selectin), CD102 (LFA-1 ligand ICAM-2), or CD106 (VLA4 ligand VCAM-1) were observed in four independent experiments (Fig. 5). Even when a highly infected phagocyte population was compared with noninfected cells, only small and nonconsistent variations on the expression of adhesion molecules were observed for CD49d, CD102, and CD116 (data not shown).

Differences in the profile of adhesion molecule expression were, however, associated with the intensity of phagocyte infection (Fig. 6): the CD11b^{hi} population showed a higher percentage of infection than the CD11b^{neg} and CD11b^{lo} populations (49.6 to 97.3% versus 13.5 to 20.7% and 15.8 to 26.1%, respectively). Almost all highly infected cells were in the CD11b^{hi} population. The CD11a^{neg} population had a higher percentage of infected cells than the CD11a^{pos} population (59.0 to 91.1% versus 24.1 to 30.9%). Almost all of the CD11a^{neg} population was highly infected. CD49d showed a pattern of infection similar to the one observed with the CD11a staining (66.6 to 83.5% infected cells in the CD49d^{neg} population and 23.5 to 37.0% in the CD49d^{pos} population). Also, most CD49d^{neg} cells were highly infected.

Analyses of cells gated at the *Leishmania*^{pos} and *Leishmania*^{neg} subpopulations (Fig. 6) revealed that CD11b expression was bimodal in the infected population showing discrete CD11b^{lo} and CD11b^{hi} peaks. Although the CD11b^{hi} peak was blunt in the uninfected subpopulation, the proportions of the cell populations in gates CD11b-negative, -low, and -high (Fig. 6) were unchanged, comparing the *Leishmania* to the control group (negative, 3.7 to 16.7% versus 4.1 to 17.6%; low, 67.0 to 88.9% versus 56.9 to 71.0%; high, 8.3 to 21.7% versus 10.8 to 39.8).

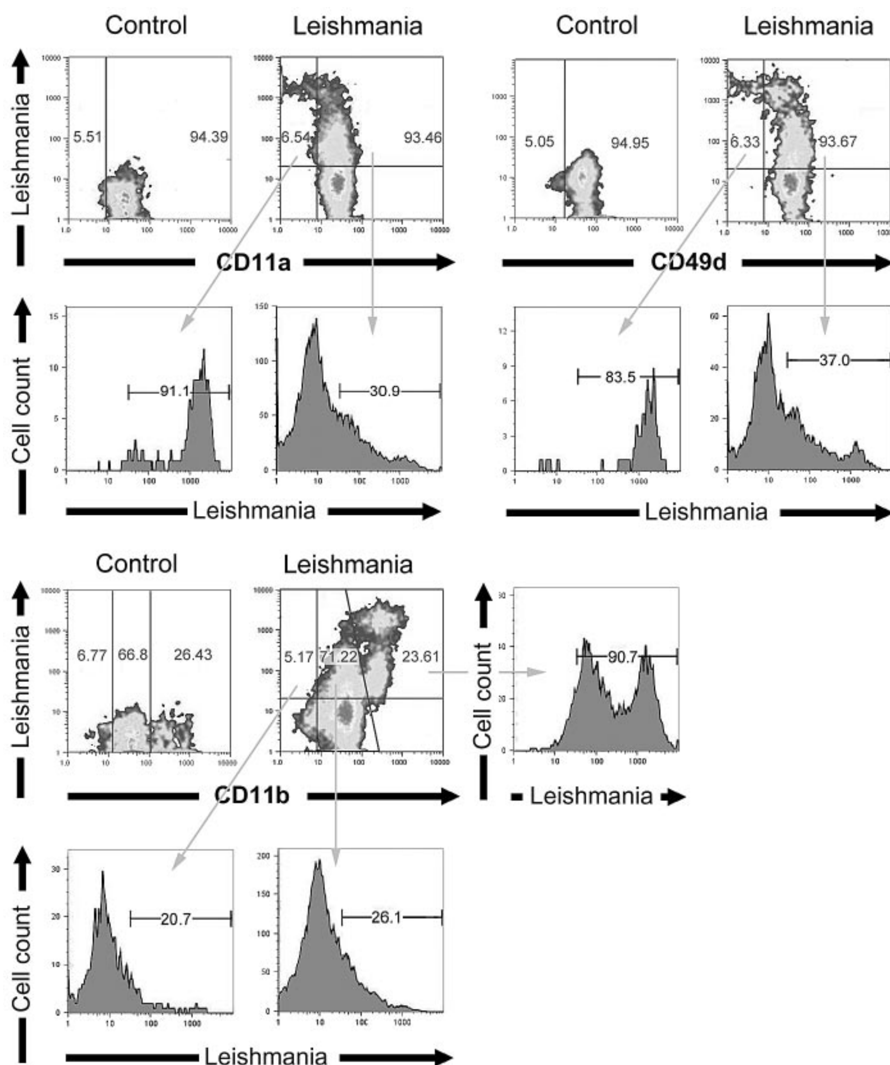

FIG. 6. Cell surface expression of adhesion molecules and infection rate in subpopulations of mouse peritoneal exudate cells 24 h after culture. Cells cultivated with medium alone (control) or medium containing live fluorescently stained *Leishmania* (*Leishmania*) were washed and stained with fluorescein isothiocyanate-conjugated antibodies as described in Materials and Methods. Numbers in the contour plots represent the proportions of the populations defined by the fluorescence intensity in the *x* axis. The filled histograms express the percentages of the infected population in the gates negative, positive/lo, and positive/high defined in the plots. The data presented are representative of results from four independent experiments.

VLA4 function is modulated by *Leishmania* infection. Manganese regulates integrin function and activates most integrins to its highest affinity form without modifying membrane clustering formation or surface expression levels (9, 45). To confirm that the impairment in the infected-cell adhesion to fibronectin resulted from changes in very late activation antigen 4 (VLA4) function instead of from the expression of this integrin, we conducted experiments replacing Ca^{2+} and Mg^{2+} with Mn^{2+} on infected cells during the adhesion assays (Fig. 7). Calcium and Mg^{2+} replacement by Mn^{2+} increased the adhesion of both infected cells (447.9 to 3,961.0 cells/mm^2) and uninfected cells (2,193.0 to 3,945.0 cells/mm^2) to fibronectin (ANOVA $P < 0.001$), reverting the inhibitory effect of *Leishmania* infection on the adhesive capabilities of these cells (ANOVA $P > 0.05$). Anti-VLA4 treatment abrogated the effect produced by Mn^{2+} on the adhesion of these cells (ANOVA $P < 0.001$).

***Leishmania* down-regulates CCR4 and CCR5 gene expression by mononuclear phagocytes.** Since our data suggested that integrin function, instead of adhesion molecule expression, was altered in *Leishmania*-infected cells, we investigated possible changes in the expression of chemokine receptors by infected cells. We found that 2 h after infection (Fig. 8A), CCR1, CCR5, and CCR7 were up-regulated about two times compared to the control group (1.59 to 2.89, 1.68 to 3.74, and 1.92 to 2.36 times, respectively). By 16 h after infection (Fig. 8B), however, live *Leishmania* parasites caused a down-regulation in the expression of CCR4 (−2.82 to −4.05 times) and CCR5 (−1.88 to −2.84 times). Killed *Leishmania* parasites increased the expression of CCR4 at this same time point (1.66 to 5.43

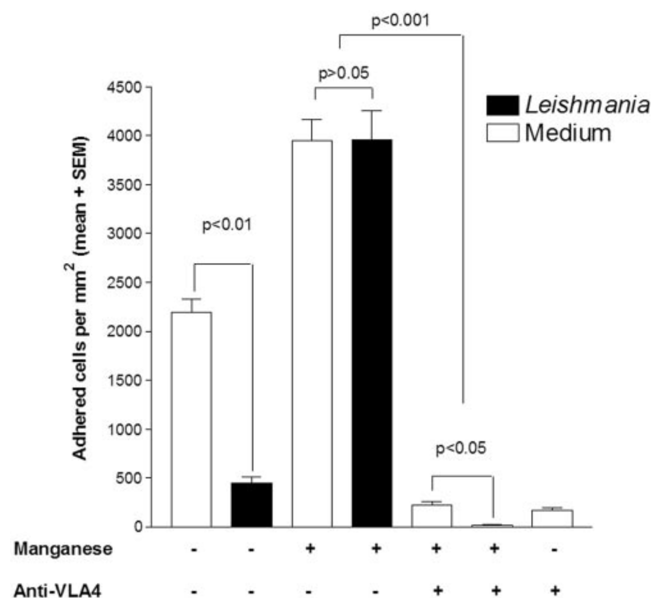

FIG. 7. Effect of manganese ions on *Leishmania*-infected phagocyte adhesion to fibronectin. Cells were cultivated for 24 h with medium alone (empty columns) or medium containing *Leishmania* (solid columns), washed, treated with 250 μM Ca^{2+} and 250 μM Mg^{2+} or with 500 μM Mn^{2+} in the presence of anti-α_4-integrin antibody (anti-VLA4) or isotype controls. Cells were allowed to adhere for 30 min to fibronectin-coated ELISA plate wells, as described in Materials and Methods. The addition of Mn^{2+} reverted the loss of phagocyte adhesion induced by *Leishmania* infection (ANOVA, $P > 0.05$). The increase in adhesion induced by Mn^{2+} addition was abrogated by the addition of an anti-α_4-integrin antibody (ANOVA, $P < 0.001$). +, present; −, absent.

times). The lipopolysaccharide (LPS)-treated controls showed a marked reduction in the expression of CXCR4 both at 2 h (−6.88 to −13.48 times) and 16 h (−1.81 to −3.74 times), in accordance with previous work (48, 51).

DISCUSSION

In the present study, we investigated the mechanisms underlying the modulation of the adhesion of mononuclear phagocytes to connective tissue by *Leishmania* parasites. This modulation, which has been previously described by de Almeida and collaborators (19) and by our group (11), may be important in the process of parasite dissemination to different tissues (30). Here, we demonstrate that phagocyte adherence to the inflamed connective tissue is modulated by the parasite burden in the cell and that the changes are sustained by infection, and not by phagocytosis of killed *Leishmania*. Our data suggest that the mechanisms which regulate cell surface β1-integrin activity, rather than the differential expression of these adhesion molecules, are implicated in the change of macrophage adhesion in leishmaniasis and that changes in the expression of chemokine receptors may be involved in this process.

In our previous report, we noticed that the reduction of adhesion observed after phagocyte infection by *Leishmania* was variable (11). Here, we show that differences in the intensity of infection cause such variation. A negative correlation was observed between the percentage of infected cells, the number of parasites per infected cell, and their adherence to the connective tissue. It has been estimated that, in the early stages of *Leishmania* infection in vivo, the rate of parasites per phagocytic cell is low, varying in the range of 0.03 to 0.1 (17). As the disease progresses, however, the number of parasites

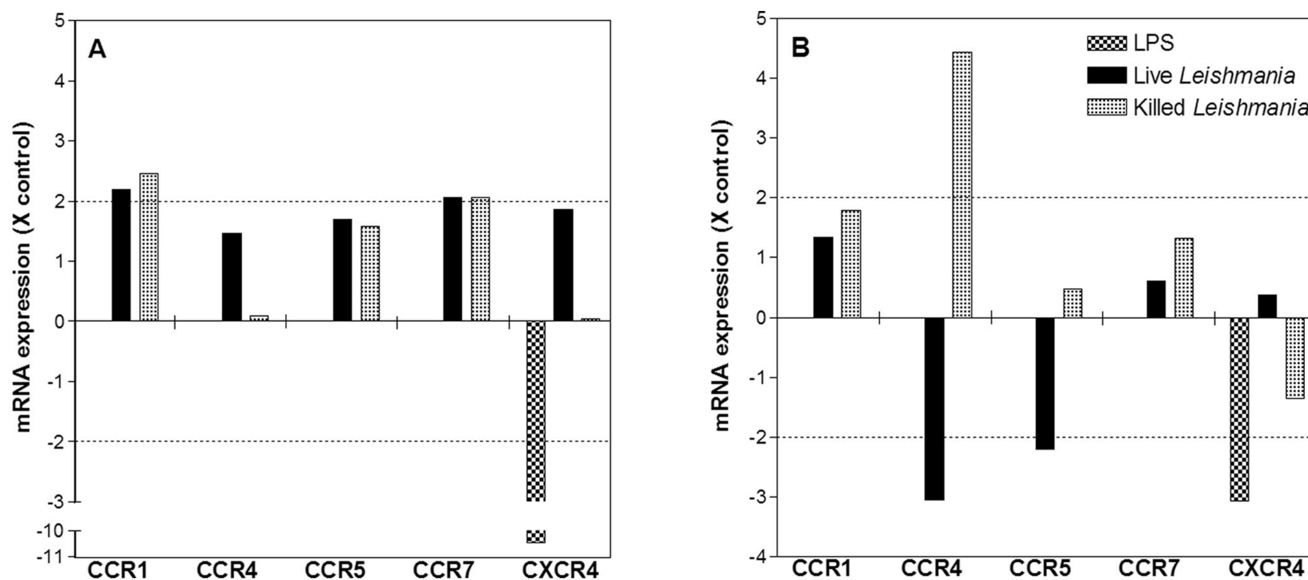

FIG. 8. Chemokine receptor mRNA expression by PEC 2 h (A) or 16 h (B) after incubation with *L. amazonensis*. Cells were cultivated in medium alone (baseline), in the presence of 1 μg/ml LPS (checkered columns), or in medium containing either live (solid bars) or killed (dotted bars) *Leishmania*. After incubation, total RNA was extracted and subjected to real-time reverse transcription-PCR, as described in Materials and Methods. Two hours after incubation (A), either live or ethanol-killed *Leishmania* caused an increase in the expression of CCR1, CCR5, and CCR7, while LPS decreased CXCR4 expression 10.4 times. Sixteen hours after incubation (B), killed parasites induced a fourfold increase in the expression of CCR4, while live parasites inhibited CCR4 (three times) and CCR5 (two times) expression. LPS decreased CXCR4 expression three times. These data are representative of the results from three independent experiments.

steadily increases and may reach a rate of 30 to 100 parasites per phagocytic cell (17, 28). The partial clearance of the parasite from the inoculation site after a period of intense proliferation correlates with the appearance of infected cells at distant sites (50). Our data show that infection with small numbers of *Leishmania* was not able to reduce phagocyte adhesion at 2 h postinfection. Nevertheless, significant reduction of phagocyte adhesion occurred by 24 h, when intracellular proliferation had taken place (33, 53). Indeed, increasing the amount of *Leishmania* parasites per cell significantly reduced phagocyte adhesion until reaching a plateau at 10 parasites per cell, even after 2 h of coincubation. Under conditions of such high parasite burden, connective tissue adhesion by infected phagocytic cells reached levels of about 20 to 30% of that observed with noninfected cells. We are now studying the migratory capabilities and pathways used by these cells in vivo.

To evaluate whether the changes in phagocyte adhesion were dependent on the infection of the cells by the parasite or could be maintained by products of phagocytosed *Leishmania* parasites, mononuclear phagocytes were incubated with either live or ethanol-killed *Leishmania* parasites. We chose to use ethanol to kill *Leishmania* parasites, as this method would better preserve the carbohydrate structure as well as the morphology of the parasites (14). In experiments employing dead parasites, we only observed reduction in cell adhesion in the early stages of coincubation (2 h). Such inhibition in cell adhesion was reverted to control levels by 24 h of culture, even when the number of parasites inside the cells was maintained at rates that inhibited adhesion if live *Leishmania* had been used. In fact, when live parasites were used, an intense decrease in cell adhesion was observed 2 to 4 h after infection, with a slight reversion at later time points (6 to 8 h). A possible explanation for this process is a transient blocking of adhesion molecules on the surface of phagocytic cells by molecules present on the surface of the *Leishmania* (noninternalized parasites are still seen at 2 h after infection, some of them attached to the surface of phagocytes). *Leishmania* binds to Mac-1 (38) and other integrins (4), and these molecules are intimately linked to connective tissue-phagocyte and intercellular adhesion (9, 20, 52). Flow cytometry analyses of the cells used in this study show that the $CD11b^{hi}$ subpopulation is highly phagocytic or permissive to *Leishmania* infection. Such findings may be related to the use of this pathway in parasite binding and internalization by the phagocyte, producing a transient blocking of the interactions between this (and potentially of other integrins) and the connective tissue.

Integrins constitute the main molecules used by monocytic phagocytes to interact with connective tissue cells and extracellular matrix components like collagen, laminin, and fibronectin (9). Integrins α_4/β_1 (VLA4) and α_5/β_1 (VLA5) are important in the interaction of monocytes with fibronectin (5, 10, 49). We have previously shown that monocytic phagocytes rely mainly on β_2- and α_4-integrins to adhere to the inflamed connective tissue (11). Here, we observed that the infection by *Leishmania* reduces macrophage adhesion to fibronectin, laminin, and collagen. By using flow cytometry analyses, however, we did not observe a significant decrease in the cell surface expression for any of the examined adhesion molecules. This is true for the whole group of cells coincubated with *Leishmania* (11) and for the subset of amastigote-containing cells, also examined in this work. Calcium and Mg^{2+} replacement by Mn^{2+}, a treatment that is known to induce integrins to a high state of affinity for their receptors, reverted the inhibition in adhesion caused by *Leishmania*. This finding further indicates that *Leishmania* infection interferes primarily with mechanisms controlling the function of phagocyte integrins rather than with integrin expression. It is now long recognized, however, that signaling via heptahelical G-protein-coupled chemokine receptors may modify the binding activity of integrins without altering their cell surface expression levels (20, 26, 29). Integrin function may be modulated by altering their lateral mobility/clustering and three-dimensional molecular conformation (22, 45). Consistent with these findings, we have shown herein that the adhesion of infected cells was almost completely restored by manganese, a phenomenon that was blocked by antibodies to the α_4-integrin. As manganese is known to change the integrin from a low-affinity to a high-affinity state (45), these results indicate that the control of integrin affinity may be impaired in infected phagocytes. On the other hand, the possibility that a high parasite burden switches off some phagocyte integrins may not be excluded, since a large proportion of the highly infected cells were $CD11a^{neg}$ or $CD49d^{neg}$.

We also showed that CCR1, CCR5, and CCR7 expression were increased during the early stages of in vitro infection with *Leishmania* and that CCR4 and CCR5 were decreased at later time points (16 h). The beta chemokine receptor 4 (CCR4) has been described as an important homing molecule, directing lymphocytes specifically to the inflamed skin in response to its ligands CCL17 (TARC) and CCL22 (MDC) (7). Immunohistochemistry of the inflammatory air pouches that we used in our adhesion assays revealed a strong expression of CCL17 (data not shown). Knockout mice lacking CCR4 show normal macrophage recruitment to the peritoneal cavity early after LPS injection. However, by 24 h, the number of these cells is much lower than that found in wild-type mice. Reduction of peritoneal macrophage numbers was accompanied by resistance to LPS-induced shock and decreased macrophage-derived serum cytokine levels (12). These results indicate the importance of the impairment of the CCR4-dependent pathway in the kinetics of macrophage mobilization in an inflammatory setting. In our experiments, live, but not killed, *Leishmania* parasites induced down-regulation of CCR4 and loss of phagocyte adherence to the connective tissue 24 h after infection. This may indicate the involvement of this pathway in the migration of monocytic phagocytes from the *Leishmania* inoculation site and in the development of a systemic/adaptive response.

The main ligand for CCR5 (RANTES or CCL5) has been shown to modulate the adhesion of eosinophils to ICAM-1 without altering the expression of β_2-integrins (23). It has also been shown that the CCR5 ligands CCL3 (MIP-1α) and CCL5 stimulate firm adhesion of monocytes to VCAM-1 and fibronectin-coated plates in a β_1-integrin-dependent way as a result of affinity regulation (49). Our results show that live *Leishmania* up-regulated the expression of CCR5 at the early stages of infection, as observed by Dasgupta and collaborators (15). Such effect, however, is rapidly reversed to down-regulation as the infection progresses, in accordance with the findings of Steigerwald and Moll, who showed that the *L. major*-induced down-regulation of CCR2 and CCR5 decreased the

capacity of bone marrow-derived dendritic cells to migrate toward its respective ligands CCL2 and CCl3 (44). The possible participation of such down-regulation of CCR5 in lowering the capacity of infected cells to adhere to connective tissue is now being studied in our laboratory.

Previous work has shown that immunological hyporesponsiveness in leishmaniasis may be due to an active inhibition of the antigen-presenting functions of monocytic phagocytes and that these effects are related to parasite burden in these cells (18, 25, 41). In this work, we showed that integrin functions are impaired in these cells in a manner that is also dependent on parasite burden. Besides their functions in leukocyte trafficking, integrins have been shown to be necessary for the formation of immunological synapses and for antigen-presenting cell functions, stabilizing the antigen-presenting cell–T-cell interaction and allowing the relatively low-affinity T-cell receptor ligands to stimulate T cells (16, 24). It could be possible, therefore, that, in addition to changes in B7 expression (25, 41), the impairment in integrin function in mononuclear phagocytes with high *Leishmania* burden may also contribute to the differential stimulation of $CD4^+$-T-helper cells during antigen presentation that occurs in the distinct forms of the disease. Thus, the impairment of integrin function observed in the work reported herein is in accordance with evidence suggesting that *Leishmania* parasites produce a wide spectrum of suppressive changes in the infected phagocyte (6). The intracellular mechanisms altered by *Leishmania* infection that could mediate these effects deserve further study. The identification of these intracellular pathways may disclose potential targets to be aimed at in new therapeutic strategies for aggressive forms of leishmaniasis and other diseases associated with inadequate homing of phagocytes (46).

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