Dendritic cells (DCs) play a critical role in the initiation of the immunological response against Leishmania parasites. However, the receptors involved in amastigote-dendritic cell interaction are unknown, especially in the absence of opsonizing antibodies. We have studied the interaction of Leishmania pifanoi axenic amastigotes with the C-type lectin DC-specific intercellular adhesion molecule (ICAM)-3-grabbing nonintegrin (DC-SIGN, CD209), a receptor for ICAM-2. ICAM-3, human immunodeficiency virus gp120, and Ebola virus. L. pifanoi amastigotes interact with immature human dendritic cells and CD209-transfected K562 cells in a time- and dose-dependent manner. Leishmania amastigote binding to human dendritic cells and DC-SIGN-transfected cells is inhibited by a function-blocking DC-SIGN-specific monoclonal antibody. More importantly, this monoclonal antibody dramatically reduces internalization of Leishmania amastigotes by immature human DCs. These results constitute the first description of a non-viral pathogen ligand for DC-SIGN and provide evidence for a relevant role of DC-SIGN in Leishmania amastigote uptake by dendritic cells. Our finding has important implications for Leishmania host-cell interaction and the immunoregulation of cutaneous leishmaniasis.

Leishmaniasis is a vector-borne parasitic disease with a broad range of clinical manifestations, from local cutaneous lesions to life-threatening visceral disease, mainly caused by differences among Leishmania species and the immunological status of the mammalian host. The parasite exists in two developmental stages: the flagellated promastigote is transmitted with the bite of the sand fly (insect vector) to the mammalian host, where it transforms into the amastigote stage. Leishmania amastigote infects mononuclear phagocytes, a key factor of the immune response against the parasite. This intracellular location potentially allows the parasite to subvert the effector and regulatory functions of these cells.

Cutaneous antigen-presenting cells, more specifically, epidermal Langerhans cells and dermal dendritic cells (DCs), are actively involved in the surveillance of their environment (1, 2). Given their proximity to the site of parasite delivery, the role of dendritic cells must be critical in initiating Leishmania-specific immune responses (3). For Leishmania amazonensis (Leishmania mexicana complex), both amastigotes and metacyclic promastigotes infect DCs (4), although the ability of Langerhans cells and DCs to take up other Leishmania species is still a matter of debate (5, 6). Langerhans cells and DCs within cutaneous lesions are parasitized by Leishmania in vivo in both human (7–9) and experimental murine (10, 11) cutaneous leishmaniasis.

To our knowledge, specific receptors for Leishmania on DCs have not yet been characterized. Dendritic cells express a wide variety of pathogen-associated molecular pattern receptors, including numerous C-type lectin and lectin-like receptors (12). Given the fact that Leishmania spp. surface displays an abundance of mannose-rich glycoconjugates (13), a reasonable hypothesis is that lectin-oligosaccharide interactions are involved in parasite recognition.

Dendritic cell-specific ICAM-3-grabbing nonintegrin (DC-SIGN, CD209) is a cell surface C-type lectin expressed on dendritic cells and involved in cell-cell interactions through its capacity to bind ICAM-3 and ICAM-2 (14, 15). DC-SIGN is also capable of binding HIV-1 gp120 (16) and Ebola virus (17). In the present report we have assessed the participation of DC-SIGN on Leishmania pifanoi axenic amastigote binding and uptake by human monocyte-derived dendritic cells (MDDCs). Our results demonstrate that amastigotes bind specifically and with high avidity to DC-SIGN, suggesting a role for this novel receptor in the outcome of the immune responses against Leishmania.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—The DC-SIGN-specific monoclonal antibody (MR-1) has been described previously (18). It was characterized (IgG1) and quantified by sandwich enzyme-linked immunosorbent assay (SBA Clonotyping System; SouthernBiotech, Birmingham, AL). The monoclonal antibody TSI1/18 (IgG1, anti-CD18) was used as isotype-matched control. Soluble mannan from Saccharomyces cerevisiae (M-7504; Sigma) was prepared as a 2 mg/ml stock solution in PBS, sterile-filtered, and used at the indicated concentrations. L. pifanoi amastigote glycosylinositolphospholipids were purified by successive extraction of whole parasite with organic solvents as described previously (19), ana-
lyzed by TLC, and stored as a 10 mg/ml sterile-filtered stock solution in PBS (1% Me₂SO). Recombinant human ICAM-3/Fc chimera was purchased from R&D Systems (Abingdon, United Kingdom).

Parasites—L. pifanoi MHOM/VE/80Ltrd axenic amastigotes were grown at 31 °C in simplified F29 medium containing 20% heat-inactivated fetal bovine serum (20). For binding experiments, parasites were labeled with the fluorescent dye CFSE (Molecular Probes, Leiden, The Netherlands) before cell adhesion, as described previously (4). This process neither alters nor impairs parasite multiplication (21), as determined in control experiments.

Cells—Immature MDDCs were prepared from peripheral blood monocytes using interleukin 4 (1000 units/ml) and granulocyte macrophage colony-stimulating factor (1000 units/ml) (18, 22). K562 cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM glutamine, and 10 μg/ml gentamicin (complete medium). K562 cells transfected with DC-SIGN (K562-CD209 cells) (18) were grown in complete medium supplemented with 300 μg/ml G418.

Parasite Binding Assays—Immature MDDCs, K562 cells, or K562-CD209 cells were resuspended in complete medium and aliquoted in 24-well plates (2 × 10⁵ cells/well). CFSE-labeled parasites were added onto the cells at the indicated ratio, and cells were incubated at 37 °C for the period of time indicated in each experiment. Afterward, cells were fixed with 1% paraformaldehyde (PBS) for 1 h at room temperature, mounted in Mowiol (Calbiochem), and analyzed on a Nikon Eclipse E800 microscope (Nikon, Melville, NY) equipped for epifluorescence. Alternatively, cells with and without attached parasites were resolved by flow cytometry in two peaks of low and high fluorescence intensity, using an EPICS-CS (Coulter Cientifica, Madrid, Spain). For inhibition assays, cells were washed with PBS-1 mM EDTA and preincubated for 10 min at room temperature with either MR-1 antibody (1.2 μg/ml, unless otherwise indicated), irrelevant antibodies (100 μg/ml human immunoglobulins or 16 μg/ml anti-CD18 TS1/18 isotype control), EGTA (5 mM), soluble mannan (300 μg/ml), or purified L. pifanoi amastigote glycosylinositolphospholipids (5 μg/ml) in complete medium before parasite addition.

DC-SIGN Adhesion Assays—DC-SIGN-dependent adhesion of MDDCs was evaluated using ICAM-3/Fc. 96-well microtiter EIA II-Linbro plates were coated overnight with ICAM-3/Fc at 3 μg/ml in 100 mM NaHCO₃, pH 8.8, at 4 °C, and the remaining sites were blocked with 0.4% bovine serum albumin for 2 h at 37 °C. MDDCs were labeled in complete medium with the fluorescent dye 2',7'-bis-(2-carboxyethyl)-5-((and-6)-carboxyfluorescein acetoxymethyl ester (Molecular Probes) and then preincubated for 20 min at 37 °C in RPMI 1640 medium containing 0.4% bovine serum albumin and function-blocking antibodies against CD209 (MR-1), CD18 (TS1/18), or soluble mannan in complete medium against CD209 (MR-1), CD18 (TS1/18), or soluble mannan in complete medium before parasite addition. After removal of unbound parasites by washing, DC-SIGN samples were incubated at 34 °C for 15 min at 37 °C. Unbound cells were removed by three washes with warm RPMI 1640 medium, and adherent cells were quantified using a fluorescence analyzer.

Infection of DCs with Leishmania Parasites—DCs were adjusted to 10⁵ cells/well in 24-well plates and incubated at 37 °C with parasites at different ratios for 1 h. For inhibition assays, cells were washed with PBS-1 mM EDTA and preincubated for 10 min at room temperature with either MR-1, TS1/18, or soluble mannan in complete medium before parasite addition. After removal of unbound parasites by washing, DC-SIGN samples were incubated at 34 °C for 48 h. Afterward, cells were washed with PBS, fixed with methanol, and stained with Giemsa to visualize amastigote-infected DCs. The percentage of infected DCs and the parasite:DC ratio were determined by light microscopy examination of at least 200 cells.

RESULTS

L. pifanoi Amastigotes Specifically Bind to DC-SIGN—Preliminary experiments revealed that L. pifanoi axenic amastigotes were efficiently internalized by human MDDCs (see below). In order to explore whether DC-SIGN was involved in amastigote binding, we first analyzed the interaction of axenic amastigotes with K562 cells transfected with DC-SIGN (K562-CD209 cells). L. pifanoi axenic amastigotes bound to transfected cells, but not to untransfected cells, in a dose-dependent manner, as evaluated by flow cytometry using CFSE-labeled parasites (Fig. 1). Maximal binding was observed within the 1:10 to 1:5 range by using distinct K562-CD209:amastigote ratios (1:1, 1:5, and 1:10). Accordingly, and to avoid an excess of parasites, a ratio of 1:5 was used for the rest of kinetic and competition assays. The binding of amastigotes to K562-CD209 was fast and reached saturation at 1 h (Fig. 2). Importantly, the anti-DC-SIGN MR-1 monoclonal antibody completely blocked amastigote binding to K562-CD209 cells (Fig. 2), confirming the involvement of DC-SIGN in this interaction. Soluble mannan (300 μg/ml) only caused a 30% reduction in amastigote binding (Fig. 2), whereas Leishmania glycosylinositolphospholipids, high mannose-containing molecules abundantly present on promastigote and amastigote plasma membranes (23, 24), did not inhibit amastigote binding at 5 μg/ml (roughly equivalent to 2 × 10⁷ amastigotes/ml) (25) (Fig. 2). Therefore, DC-SIGN functions as a receptor for L. pifanoi amastigotes, whose cell attachment can be partially competed by mannan.

MDDCs Bind L. pifanoi Axenic Amastigotes via DC-SIGN—
The role of DC-SIGN in amastigote uptake by dendritic cells was initially assessed by preincubation of human immature MDDCs with an excess of anti-DC-SIGN antibody or soluble mannan before the addition of CFSE-labeled axenic amastigotes (1:5 ratio, 2 h, 37 °C). Pretreatment of MDDCs with MR-1 antibody reduced parasite binding in a dose-dependent manner at more than 30 ng/ml (Fig. 3). The dose-dependent inhibitory effect of the MR-1 antibody demonstrates the specificity of the DC-SIGN-Leishmania amastigote interaction. As expected, binding was completely inhibited by EGTA, an inhibitor of C-type lectin-mediated interactions, but not by human immunoglobulins or the isotype control anti-CD18 TS1/18 antibody (Fig. 3). On the other hand, pretreatment with mannan weakly affected amastigote binding to MDDCs.

Essentially the same results were obtained after microscopic examination. As shown in Fig. 4A, the number of amastigotes bound per DC was reduced in the presence of 1.2 μg/ml anti-DC-SIGN MR-1 antibody (17.7 ± 4.9 versus 7.25 ± 2.0 amastigotes/DC), whereas no effect was observed with the isotype-matched TS1/18 antibody at 16 μg/ml. These results further confirmed that DC-SIGN contributes to amastigote binding onto the DC surface. In agreement with the flow cytometry data (Fig. 3), mannan had no significant effect on the number of amastigotes bound per DC (Fig. 4A), despite the fact that it effectively abrogated the DC-SIGN-dependent MDDC binding to immobilized ICAM-3 (Fig. 4B).

To test whether DC-SIGN-L. pifanoi amastigote interaction had a physiological significance in MDDC infection, immature cells were left untreated (control) or preincubated with MR-1 antibody or mannan (500 μg/ml) and then incubated with axenic amastigotes at a 1:5 DC:parasite ratio. After 48 h, infection was evaluated by examination of at least 200 Giemsastained DCs (Fig. 5). The percentage of infected DCs and the number of internalized amastigotes per infected cell were determined by light microscopy examination of Giemsastained preparations.

### Table I

| MR-1 antibody inhibits MDDC infection by L. pifanoi axenic amastigotes |
|-----------------|-----------------|-----------------|
| Immature human MDDCs were left untreated (control), preincubated with a monoclonal antibody specific for DC-SIGN (MR-1), an irrelevant antibody (TS1/18), or mannan, and then infected with *L. pifanoi* axenic amastigotes. The percentage of infected DCs and the number of internalized amastigotes per infected cell were determined by light microscopy examination of Giemsa-stained preparations. |

| DC:parasite ratio | % infected DC | No. of parasites/infected DC |
|-------------------|--------------|-----------------------------|
| Control           | 82.5         | 2.3                         |
| MR-1              | 7.4          | 2.4                         |
| TS1/18            | 91           | 1.1                         |
| Mannan            | 34           | 2.3                         |
stained cells. L. pifanoi axenic amastigotes were efficiently internalized by human immature MDDCs in the absence of opsonizing antibodies (82.5% of DCs infected), whereas pre-treatment of MDDCs with MR-1 antibody significantly reduced the number of infected cells (82.5% versus 7.4%) (Table I). As observed in binding experiments, incubation with anti-CD18 did not reduce the percentage of infected cells (91.0%). Again, mannan partially inhibited Leishmania internalization into MDDCs. Taken together, these results demonstrate that, in the absence ofFc receptor-mediated interactions, DC-SIGN is a functionally relevant receptor for binding and internalization of L. pifanoi amastigotes on dendritic cells.

**DISCUSSION**

Early events occurring at the site of delivery of Leishmania include infection of host cells by metacyclic promastigotes followed by their intracellular transformation into amastigote. Once released into the extracellular milieu, amastigotes invade new cells and thereby disseminate the infection. In the first rounds of this expansive cycle, specific antibodies are absent, precluding opsonizing mechanisms from contributing to parasite uptake. Infection of murine and human DCs by amastigotes is clearly documented in the literature (7–11), although the receptors involved remain unknown. According to the available literature, opsonization by specific antibodies may play a major role in targeting and entry of the parasite into the host cells (26). Because most of the Leishmania amastigote-dendritic cell studies were carried out with tissue-derived parasites, known to be opsonized by antibodies, the participation of opsonization-independent mechanisms might have been masked. In the present study we have used axenic amastigotes, devoid of opsonizing antibodies, in order to analyze the participation of the receptor DC-SIGN in binding and internalization of Leishmania amastigotes. Our results demonstrate that axenic amastigotes specifically bind to DC-SIGN both on K562-CD209 cells and MDDCs. Because MR-1 antibody dramatically reduced amastigote binding to and internalization into DCs, our results suggest an important role for this receptor in the early infection of DCs by Leishmania.

Unlike MDDC binding to ICAM-3, mannan did not completely block amastigote binding to dendritic cells, in agreement with previous data on the failure of mannan to inhibit Leishmania amastigote-macrophage interactions (27). Several alternative explanations might account for the incomplete inhibitory effect of mannan on the DC-SIGN-Leishmania interaction. First, Leishmania binding sites on DC-SIGN might differ from those involved in mannan binding. In this regard, site-directed mutagenesis has shown that ICAM-3- and HIV gp120-binding sites are not completely identical and that HIV gp120 recognition by DC-SIGN is not dependent on glycosylation (28). Second, the overall affinity of the Leishmania ligand(s) for DC-SIGN might exceed that of mannan. This alternative is supported by the fact that subtle changes in the oligosaccharide structure of related mannose-rich molecules lead to substantial differences in their affinity for DC-SIGN (29). Furthermore, because the parasite could behave as a multivalent ligand, the possibility of cooperative effects on the interaction between Leishmania and DC-SIGN-expressing cells cannot be ruled out.

To our knowledge, this is the first description of DC-SIGN as a receptor for nonviral pathogen. Moreover, because pathogens are known to alter dendritic cell effector functions (30), it is tempting to speculate that DC-SIGN-Leishmania interactions might condition the initiation of the pathological immune response caused by L. mexicana complex parasites. Further studies on the DC-SIGN-Leishmania interaction will undoubtedly increase our understanding of the Leishmania infection process and will pave the way for alternative strategies to fight the disease.

**Acknowledgments—**We thank Dr. P. Lastres and Dr. M. A. Ollacarizqueta for assistance with flow cytometry and epifluorescent microscopic analyses, respectively.

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