Leishmania infantum Promastigotes Reduce Entry of HIV-1 into Macrophages through a Lipophosphoglycan-Mediated Disruption of Lipid Rafts

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Visceral leishmaniasis is now recognized as an opportunistic disease in patients infected with human immunodeficiency virus type 1 (HIV-1). We report here that Leishmania infantum promastigotes enhance HIV-1 replication in monocyte-derived macrophages (MDMs) at late time points in the virus growth curve but also that, surprisingly, a reduction in HIV-1 production is seen during the initial days after infection. This early effect is caused by a Leishmania-mediated inhibition of virus entry into MDMs through the action of lipophosphoglycan (LPG), the major promastigote surface glycolipid. The impact of LPG in the observed phenomenon was confirmed using LPG-defective lpg1/H11546/H11546 knockout mutant promastigotes. Our results suggest that the LPG-mediated effect results from the disruption of lipid rafts. Altogether, these findings suggest that the presence of Leishmania within the same cellular microenvironment leads to 2 opposite, time-dependent effects on HIV-1 replication. Leishmania and HIV-1 can thus establish complex interactions in their common natural host cells.

The protozoan parasite Leishmania causes a variety of chronic diseases in humans, affecting the skin, mucous membranes, and viscera [1]. Motile flagellated promastigotes are transmitted through the blood meal of sand flies and are quickly internalized within macrophages. At this point, they differentiate into amastigotes inside an intracellular vacuolar compartment. Besides being a major tropical disease, visceral leishmaniasis, which is caused mainly by the closely related species Leishmania infantum, Leishmania chagasi, and Leishmania donovani, has emerged as an important opportunistic disease among HIV-1–positive individuals and patients with AIDS [2, 3]. Several factors have contributed to this situation. First, both pathogens are endemic in several regions of the world, such as sub-Saharan Africa, southern Asia, South America, and countries of the Mediterranean basin. Second, both Leishmania and HIV-1 share some of the same target cells, namely, macrophages and dendritic cells. Finally, coinfection has been reported in intravenous drug users who use contaminated needles [4].

Because these 2 human pathogens infect the same cells, their combined impact on the immune response—and on each other’s biology—may profoundly affect the development of their respective diseases. Interestingly, it has been reported that Leishmania replication is enhanced in HIV-1–infected macrophages [5]. Conversely, it has also been shown that Leishmania can modulate the life cycle of HIV-1, enhancing both HIV-1 gene transcription and virus production through the production of proinflammatory cytokines [6].

Lipophosphoglycan (LPG) is the major surface molecule found on Leishmania promastigotes and consists of repeating phosphoglycan units linked to a glycan core that is inserted into the membrane by a phosphatidyl-
inositol anchor [7]. The amastigote stage of most *Leishmania* species, such as *L. infantum* and *L. donovani*, considerably down-regulate LPG expression [8]. Interestingly, LPG has been shown to exert a dual effect with respect to HIV-1. Indeed, it can promote HIV-1 production in both CD4$^+$ T cells and macrophages [6, 9], and it can also inhibit syncytium formation and virus entry at a postbinding step in CD4$^+$ T cells [10, 11].

Given the complex interactions between HIV-1 and *Leishmania*, we investigated the impact of the parasite on the life cycle of HIV-1 in physiologically relevant human monocyte-derived macrophages (MDMs). Although we confirm our previously reported observations that *L. infantum* enhances HIV-1 production in this particular cell type [6], we also show that HIV-1 entry is reduced in promastigote-infected MDMs. We also determined that this modulatory effect on virus replication is mediated by the action of LPG.

**MATERIALS AND METHODS**

**Isolation and culture of MDMs.** Monocytes were purified by adherence to plastic in RPMI 1640 medium (Wisent) supplemented with 5% human serum and antibiotics. Freshly isolated monocytes were allowed to differentiate into macrophages in culture medium supplemented with recombinant human macrophage colony stimulating factor, at a final concentration of 100 ng/mL for 6 days.

**Production of virus stocks.** Viruses were produced by transient calcium phosphate transfection of human 293T cells with pNL4-3balenv, a R5-tropic infectious molecular clone of HIV-1 [12]. Virus preparations underwent a single freeze-thaw cycle before infection. Virus stocks were normalized for virion content using a homemade p24 test [13]. Envelope-defective HIV-1–based particles pseudotyped with the vesicular stomatitis virus glycoprotein (VSV-G) and harboring a luciferase reporter gene were generated, and luciferase activity on infection was measured by incubating MDMs with 10 mmol/L methyl-$\beta$-cyclodextrin (M$\beta$CD) (Sigma) in serum-free medium at 37°C for 30 min before virus infection.

To estimate virus entry, MDMs ($2 \times 10^5$) were first incubated with parasites at a parasite to cell ratio of 10:1 for 3 h, and free parasites were washed out with warm PBS. Infected macrophages were then incubated for different time intervals. Cells were then pulsed with HIV-1 (10 ng of p24 per $1 \times 10^5$ cells) for 2 h at different time intervals. After virus entry, cells were washed, trypsinized to remove noninternalized virus, and lysed in ice-cold lysis buffer (20 mmol/L HEPES [pH 7.4], 150 mmol/L sodium chloride, and 0.5% Triton X-100). The amounts of internalized virus were estimated by the p24 assay.

**Modulation of cholesterol content.** Cholesterol depletion was done by incubating MDMs with 10 mmol/L methyl-$\beta$-cyclodextrin (M$\beta$CD) (Sigma) in serum-free medium at 37°C for 30 min. Cholesterol (Sigma) replenishment was achieved by treating cells for 30 min with exogenous purified cholesterol at a final concentration of 1 mmol/L.

**Flow cytometry analyses.** MDMs infected with *L. infantum* promastigotes or treated with 10 µg/mL purified LPG were stained with a saturating amount of either anti–CD4–fluorescein isothiocyanate (FITC), anti–CCR5–phycoerythrin (PE) (BD Pharmingen), anti–DC-SIGN–FITC (eBiosciences), or anti–mannose receptor (Abcam) antibody or an appropriate isotype-matched irrelevant control antibody for 30 min at 4°C. Cells stained with the anti–mannose receptor antibody were further incubated with a secondary mouse PE-conjugated antibody. Cells were then washed with PBS, fixed with 2% paraformaldehyde, and analyzed on a cytofluorometer (EPICS Elite ESP; Coulter Electronics). Further analysis was obtained using WinMDI software (version 2.9).

**Confocal microscopy of lipid rafts.** MDMs were seeded on coverslips and exposed to LPG (10 µg/mL) for 3 h. The cells were then fixed with 4% paraformaldehyde and permeabilized using 0.1% Triton X-100, and nonspecific binding was blocked with...
1% bovine serum albumin and 10% human serum in PBS. Cells were stained for DNA with DRAQ5 (Biostatus) and for the ganglioside M1 component of lipid rafts with the Alexa 594–labeled B subunit of cholera toxin (Molecular Probes). Cells were then mounted with Fluoromount (Southern Biotech, InterScience) and visualized with an Olympus FluoView FV300 confocal microscope (Olympus). Digital images were produced by use of Adobe Photoshop software (version 6.0; Adobe Systems).

Statistical analysis. The statistical significance of the results was defined by performing a 1-way analysis of variance with Dunnett’s posttests to compare treated and control samples. All analyses were performed on crude data (i.e., p24 concentrations) using InStat software (version 3.05; GraphPad Software). Differences were considered significant at $P < .05$.

**RESULTS**

**Decrease in early replication of HIV-1 and virus entry into MDMs caused by *L. infantum* promastigotes.** In agreement with previous observations [6], virus production is increased by the prior infection of MDMs with *L. infantum* promastigotes beginning on day 6 ($P < .01$) and up to 15 days after infection ($P < .01$) (figure 1A). Surprisingly, the data also suggested that virus production is reduced at the earliest time point tested after infection (i.e., 3 days) ($P = .0017$). Next, we estimated HIV-1 production during earlier periods. The results showed a significant inhibition of HIV-1 replication at 1 ($P = .0011$) and 2 ($P = .0095$) days after infection in MDMs previously infected with promastigotes (figure 1B). This suggests that *Leishmania* possibly affects an early event in the viral cycle in such coinfected macrophages. To demonstrate that p24 recovered during the initial days after infection results from de novo virus production and not from input virus, similar experiments were performed in the presence of antiviral drugs. The data shown in figure 1C indicate that the p24 detected from days 1 to 3 after infection of MDMs results from new viral protein synthesis.

To understand how the presence of promastigotes in MDMs affects the early time points in the virus growth curve, we first investigated the impact of *Leishmania* infection on HIV-1 entry. A significant ($P < .01$) 2-fold inhibition of viral entry was observed up to 3 days after the initial infection with *L. infantum* promastigotes, compared with HIV-1 entry in uninfected MDMs (figure 2). These results were observed for several donors.

**Figure 1.** Kinetics of virus replication after infection of monocyte-derived macrophages (MDMs) with either HIV-1 alone or *Leishmania* and HIV-1. MDMs were first either left unexposed or exposed to *Leishmania infantum* (Li) promastigotes for 3 h, washed, and incubated with HIV-1 for 2 h, with excess virus removed (A). Virus production was assessed by measuring the p24 content in cell-free supernatants at 3, 6, 9, 12, and 15 days after infection. In another set of experiments, p24 content was measured at 1, 2, and 3 days after infection (B). Additionally, MDMs were either left untreated (control) or treated with efavirenz or zidovudine (AZT) for 30 min before exposure to virus (C). Data are mean ± SE values for triplicate samples and are representative of 3 independent experiments.

**Figure 2.** Kinetics of virus entry after infection of monocyte-derived macrophages (MDMs) with either HIV-1 alone or *Leishmania* and HIV-1. MDMs were first either left unexposed or exposed to *Leishmania infantum* (Li) promastigotes for 3 h, washed, maintained in medium, and pulsed with HIV-1 for 2 h at the indicated time points. Excess virus was removed, cells were lysed in lysis buffer, and the amounts of internalized virus were estimated by the p24 test. Data are mean ± SE values for triplicate samples and are representative of 6 independent experiments.

**HIV-1 Entry Is Affected by *Leishmania***
promastigote-infected macrophages were transferred to a new MDM culture before HIV-1 exposure, no negative impact on viral entry was observed in these cells (data not shown). These observations suggest that neither the expression of the tested viral receptors nor the secretion of soluble factors affect the process of HIV-1 entry into *Leishmania*-infected MDMs.

**Association between inhibition of HIV-1 entry and LPG-mediated disruption of membrane lipid rafts.** It has been reported that cholesterol depletion and dispersion of cholesterol-enriched microdomains known as “lipid rafts” by MβCD decreases HIV-1 entry, rendering cells highly resistant to HIV-1 infection [14]. We therefore examined whether subjecting MDMs to promastigotes affects the integrity of cell membrane lipid rafts. As expected, we observed that addition of MβCD significantly reduces HIV-1 entry into MDMs (figure 3A). Cholesterol replenishment in such MβCD-treated MDMs reestablishes virus entry levels very close to those found in untreated cells. We then determined whether cholesterol would also restore HIV-1 entry into promastigote-infected MDMs. In agreement with our previous findings, we observed a diminution in virus entry into *Leishmania*-infected MDMs compared with uninfected cells (figure 3B). This inhibition was found up to 3 days after the initial uptake of the parasite by MDMs. It is interesting that a treatment with exogenous purified cholesterol restored virus entry to some extent in the promastigote-infected MDMs. This observation suggests that *L. infantum* promastigotes affect HIV-1 entry into MDMs by disrupting membrane lipid rafts. Similar observations were made when MDMs were preincubated with heat-killed promastigotes (data not shown).

To identify the *Leishmania* factor or factors responsible for diminishing HIV-1 entry, we took advantage of the biphasic life cycle of the parasite. We therefore examined the effect of *Leish-

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**Figure 3.** Necessity of intact lipid rafts for HIV-1 entry into monocyte-derived macrophages (MDMs). In panel A, MDMs were either left untreated, treated with a concentration of the diluent used to prepare the methyl-β-cyclodextrin (MβCD) solution (i.e., ethanol), or treated with MβCD (10 mmol/L) for 30 min. In some cases, purified cholesterol (CH; 1 mmol/L) was added exogenously onto MβCD-treated MDMs. All cells were then exposed to HIV-1 for 2 h, after which noninternalized virus was removed and the amounts of internalized HIV-1 were estimated by p24 ELISA of the cell lysates. In panel B, MDMs were exposed to *Leishmania infantum* (Li) promastigotes for 3 h, uninfected parasites were washed off, and cells were cultured for the indicated time periods. Uninfected MDMs were kept as controls. At this point, some cells were treated with CH (1 mmol/L) for 30 min. All MDMs were then pulsed with HIV-1 for 2 h, after which excess virus was removed and the amounts of internalized virus were estimated by p24 ELISA in cell lysates. Data are mean ± SE values for triplicate samples and are representative of 3 independent experiments. *P < .05; **P < .01.

**Figure 4.** No effect of amastigotes on virus entry into monocyte-derived macrophages (MDMs). Target cells were infected with *Leishmania infantum* axenic amastigotes (AMA) for 3 h, after which excess parasites were washed off and cells were cultured for the indicated time periods. Uninfected MDMs were kept as controls. All MDMs were then pulsed with HIV-1 for 2 h, after which noninternalized virus was removed and cells were lysed. The amounts of internalized virus were estimated by p24 ELISA of cell lysates. Data are mean ± SE values for triplicate samples and are representative of 3 independent experiments.
of promastigotes (figure 2). We did not find a significant decrease in HIV-1 entry into MDMs during the 3 days after infection with axenic amastigotes (figure 4). This suggested that some promastigote-specific molecules were responsible for limiting virus entry into MDMs. One such molecule, LPG, is the most abundant promastigote surface glycoconjugate [8]. Therefore, we examined the impact of LPG on virus entry into MDMs by use of purified LPG from \textit{L. donovani}, which is structurally similar to that from \textit{L. infantum} (S. Turco, personal communication).

MDMs were first incubated with increasing concentrations of LPG (1, 10, and 100 \(\mu\)g/mL) before HIV-1 exposure. The results, depicted in figure 5A, revealed a significant dose-dependent inhibition of HIV-1 entry into LPG-treated MDMs. More importantly, the process of HIV-1 entry was partially restored by replenishing the LPG-treated MDMs with exogenous purified cholesterol (figure 5B), suggesting that the impact of LPG on viral entry is linked to a loss in the integrity of cell membrane lipid rafts. To investigate this hypothesis, we stained both untreated and LPG-treated MDMs with fluorescently labeled B subunit of cholera toxin, which binds gangliosides (such as ganglioside M1) that are found concentrated in lipid rafts. Confocal microscopy analysis indicated that intact lipid microdomains were either less defined or absent in LPG-treated macrophages (figure 5C). Altogether, these observations strongly suggest that LPG inhibits HIV-1 entry into MDMs by disrupting the integrity of lipid raft microdomains.

Given that our previous observations were obtained with purified LPG, we further examined the putative contribution of this major surface constituent of promastigotes to the parasite-dependent effect on HIV-1 entry by use of a more physiological tool. First, to test further our hypothesis that LPG affects HIV-1 entry by targeting lipid rafts, we determined whether promastigotes inhibit the entry of HIV-1–based viruses pseudotyped with VSV-G. These chimeric viruses are internalized into endosomes without the need for intact lipid rafts [14, 17]. As shown in figure 6, there was no significant difference in the entry of VSV-G pseudotypes between uninfected and promastigote-infected MDMs (\(P > .3\)). Viral entry experiments were also performed in MDMs infected with either wild-type \textit{L. donovani} promastigotes or an isogenic LPG-defective \textit{Lpg1} knockout mutant [15]. As expected, wild-type \textit{L. donovani} promastigotes inhibited HIV-1 entry similarly to \textit{L. infantum} promastigotes (compare figures 2 and 7A), with half as much virus entering MDMs infected with either species of promastigotes. However,

**Figure 5.** Inhibition of HIV-1 entry and disruption of lipid rafts by purified lipophosphoglycan (LPG) in monocyte-derived macrophages (MDMs). MDMs were exposed to different concentrations of purified LPG from \textit{Leishmania donovani} for 3 h, washed, and either pulsed with HIV-1 for 2 h \(A\) or incubated with cholesterol (CH; 1 mmol/L) for 30 min and then pulsed with virus \(B\). Noninternalized virus was removed, cells were lysed, and amounts of internalized virus were estimated by p24 ELISA of the cell lysates. Data are mean ± SE values for triplicate samples and are representative of 3 independent experiments. \(^*P < .05; \quad **P < .01\). In panel \(C\), MDMs were either left unexposed or treated with LPG (10 \(\mu\)g/mL) for 3 h, fixed, and stained for DNA (blue) and the ganglioside M1 component of lipid rafts, using the B subunit of cholera toxin (red, arrows). Cells were mounted and processed for confocal microscopy analysis. Micrographs are representative of 50 cells observed in each condition, obtained from 2 donors (bar shows 5 \(\mu\)m).
MDMs that had internalized *L. donovani* lipophosphoglycan (LPG)–defective mutants were as susceptible to HIV-1 as uninfected MDMs. Inhibition of viral entry was restored when purified LPG was added along with the *lpg1* knockout mutants before HIV-1 exposure (figure 7B), thus confirming that LPG indeed interferes with an early step in the HIV-1 replicative cycle.

DISCUSSION

One of several emerging opportunistic infections associated with the development of AIDS, leishmaniasis is in itself a major tropical disease affecting millions worldwide [18, 19]. The overlap between areas endemic for visceral leishmaniasis and those of the AIDS epidemic was first evident in countries of the Mediterranean basin. There, in the last decade, as many as 70% of visceral leishmaniasis cases were associated with HIV-1 infection [2, 4, 20]. With the advent of highly active antiretroviral therapy, patients with AIDS have been better able to control initial *Leishmania* infections, but many experience relapses after antiparasitic drug treatments [4, 20, 21]. These situations, combined with the growing number of AIDS cases in countries in which visceral leishmaniasis is endemic, have prompted the need for a better understanding of the complex interactions between these 2 pathogens. Both HIV-1 and *Leishmania* target cells of the monocytic lineage, resulting in new and complex combined effects on the host’s immune system and at the level of the individual coinfected cell [22].

It has been demonstrated that macrophage functions are greatly affected after HIV-1 infection. For example, cellular activation and cytokine production, intracellular killing, and phagocytosis are altered or generally inhibited by HIV-1 [23]. This is particularly relevant because infected macrophages can remain in such a state for weeks and months, producing virus and acting as long-term HIV-1 cellular reservoirs [24]. Opportunistic intracellular pathogens, such as *Mycobacterium tuberculosis*, take advantage of macrophage dysfunction to better establish infection [25]. Although phagocytosis (but not intracellular survival) of such pathogens is inhibited by HIV-1 [26], that of *Leishmania* amastigotes is not. Indeed, HIV-1 infection promotes the intake of amastigotes and their replication [5]. Thus, *Leishmania* acts opportunistically and behaves synergistically with the virus in such coinfections.

Initial reports focusing on the *Leishmania*-mediated effect on HIV-1 replication in macrophages also proposed that both pathogens have a synergistic effect on their replication. Indeed, LPG activates HIV-1 gene expression in monocyte-derived U1 and OM-10.1 cells, through the effect of tumor necrosis factor (TNF)–α [27]. This corroborated further observations in promastigote-infected MDMs, in which HIV-1 production was at least 2-fold that in MDMs without parasites [6] and was dependent on the proinflammatory cytokines TNF-α and interleukin-12.
kin (IL–1α). This effect on HIV-1 was not limited to cells of monocytic lineage, because LPG itself can promote HIV-1 gene expression in CD4+ T cells through an NF-κB–dependent pathway [9]. In a more physiological context, it has been demonstrated that *Leishmania* promastigotes also enhance replication of HIV-1 in cultured lymphoid tissue, and this was linked to higher production of TNF-α and IL-1α [28]. In the present study, we initially set out to scrutinize further the possible *Leishmania*-induced modulatory effect on HIV-1 replication in MDMs at the early stages of viral infection. Therefore, we focused on the interactions between both pathogens within the infected host cell. Although our results paralleled those reported elsewhere [6], we detected a decrease in HIV-1 production in promastigote-infected MDMs during the early days after infection.

It is known that, once internalized in macrophages, *Leishmania* promastigotes modulate the phagosomal compartment in order to survive and establish a productive infection [29, 30]. Furthermore, this provides time for promastigotes to eventually differentiate into amastigotes, which are better adapted to the harsh phagolysosomal milieu. LPG is an important factor for early promastigote survival within the phagosome, but is eventually down-regulated on *Leishmania* amastigotes [8]. In the case of coinfection with HIV-1, we now provide evidence that *Leishmania* can exert 2 different and opposing effects on the virus replication cycle in MDMs. Indeed, *Leishmania* can enhance HIV-1 production but can also reduce virus entry. The positive impact of the protozoan parasite *Leishmania* on virus replication would mostly occur when promastigotes enter cells already infected with HIV-1, which is highly relevant given the reported susceptibility of immunocompromised HIV-1–infected individuals to *Leishmania* infection. On the other hand, we now suggest that the negative effect of *Leishmania* on HIV-1 entry is a consequence of the LPG-dependent impact on lipid raft integrity in MDMs. Concerning this mechanism, the presence of LPG in the mammalian cell is limited to the early steps of the parasitic infection. It is thus less likely to act simultaneously on incoming new viruses. Nevertheless, LPG inhibits HIV-1 syncytium formation between infected CD4+ T cells [10, 11], possibly also as a consequence of its effect on the plasma membrane lipid microdomains. Finally, our results showing that axenic amastigotes do not inhibit virus entry indicate that *Leishmania* infection will most likely increase the overall viral load in HIV-1–infected patients. This is suggested by our observations for longer (>3-day) MDM coinfections, in which most promastigotes would probably have differentiated into amastigotes. It is also possible that different cytokines may be secreted in response to amastigote or promastigote infections.

Although several groups have reported that LPG affects lipid raft integrity [31–33], the precise mechanisms remain unknown. It has been proposed that the insertion of LPG into the cellular membrane, through its GPI-linked anchor, elicits a steric hindrance phenomenon in lipid microdomains [34]. Moreover, the negatively charged carbohydrate chains of LPG are thought to interfere with the clustering and interactions of proteins in rafts [33]. The shedding of LPG by internalized promastigotes and its subsequent insertion into the host cell membrane [35] would therefore eventually have an impact, in the case of newly bound HIV-1 particles, on the entry of the virus. Indeed, it has been demonstrated that the HIV-1 primary receptor CD4 and coreceptor CCR5 must both be colocalized in lipid rafts to enable membrane fusion and HIV-1 entry [14]. However, it is of particular interest that the effects of LPG are counterbalanced by the addition of cholesterol. Cholesterol is indeed essential for HIV-1 entry [36] and is an important component of lipid rafts [37]. The exact mechanism by which cholesterol reverses the LPG-mediated inhibition of HIV-1 entry remains to be elucidated. Insights might be gained by further investigating the impact of LPG on viral fusion, as has been done with other viruses (such as Sendai and influenza virus) [34, 38, 39].

In coinfections, the complex interactions between HIV-1, *Leishmania*, and the host macrophage are determined by a variety of factors. Our findings clearly show that the effect of *Leishmania* on HIV-1 replication changes in relation to the form of the parasite and which pathogen infects the macrophage first. It is also possible that different HIV-1 strains and other *Leishmania* species react differently in coinfections. Furthermore, as suggested elsewhere for HIV-1 and *Mycobacterium tuberculosis* coinfections [40], differences in MDM isolation techniques should also be considered. This will be of particular importance in future studies of HIV-1 and *Leishmania* coinfections in other monocyte-derived cells, such as immature and mature dendritic cells. Additional studies are needed to help us understand the impact of *Leishmania* on HIV-1 replication in persons afflicted with this increasingly common combination of severe infections.

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