Leishmania infantum enhances human immunodeficiency virus type-1 replication in primary human macrophages through a complex cytokine network

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Abstract

Leishmaniasis has emerged as an important potential opportunistic disease among patients infected with human immunodeficiency virus type-1 (HIV-1). It has been reported that the visceral form of leishmaniasis accelerates the course of HIV-1 disease progression and shortens the life expectancy of persons in areas where both diseases are endemic. As both pathogens can in a productive manner the same target cell, that is, the macrophage, we examined the possible modulatory effect of the protozoan parasite Leishmania infantum on the biology of HIV-1 in primary human monocyte-derived macrophages (MDMs). We found that coinfection of MDMs with Leishmania and HIV-1 resulted in a significant enhancement of both virus transcription and release of progeny virus. The Leishmania-directed increase in HIV-1 production was associated with an increased secretion of the proinflammatory cytokines TNF-α and IL-1α. Altogether, these findings indicate that the presence of Leishmania and HIV-1 within the same cellular microenvironment leads to an enhancement of virus gene expression. The present work also underscores the importance of studying the possible complex interactions between two human pathogens in a physiological cellular reservoir.

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Introduction

Human immunodeficiency virus type 1 (HIV-1) infection leads to the functional impairment and progressive depletion of CD4+ T lymphocytes, followed by the emergence of opportunistic infections, which is clinically defined as the acquired immunodeficiency syndrome (AIDS) [1,2]. Among the various opportunistic pathogens that can be seen in AIDS patients, the leishmaniases are a diverse group of diseases caused by protozoan parasites of the genus Leishmania. Multiple species of Leishmania are known to cause human diseases involving the skin (cutaneous leishmaniasis) and mucosal surfaces (mucocutaneous leishmaniasis), or the visceral reticuloendothelial organs (visceral leishmaniasis, VL). VL has emerged as an important potential opportunistic disease among patients infected with HIV-1. According to an epidemiologic survey made by the World Health Organization, the areas where HIV-1/Leishmania coinfection is distributed are extensive [3]. To date, 33 countries worldwide have reported such coinfections. In southern Europe, 25% to 70% of adult VL cases are associated with HIV-1, and 1.5% to 9% of AIDS patients suffer from newly acquired or reactivated VL. Since more than one third of AIDS patients live in zones where leishmaniasis is endemic [2], the overlap of HIV-1 and VL represents a severe health problem. A considerable body of data from clinical studies has been gathered over the past two decades on the possible role that VL may play in the pathogenesis of HIV-1 infection. It has been found that VL promotes the clinical progression of AIDS, a process leading to a diminution of the life expectancy of such dually infected subjects [4]. For example, the median survival period of HIV-1-infected patients with VL is ranging from only 13 to 19 months [5].
Previous works have provided evidence indicating that regulation of HIV-1 gene expression is resulting from a complex network of extracellular signals, such as those provided by certain cytokines, together with transcriptional and posttranscriptional effects mediated endogenously by regulatory viral proteins such as Tat and Rev [6]. The regulatory network of cytokines has been shown to affect virtually every step of the virus life cycle, from entry into the target cell to budding of new progeny virions [7]. Research efforts aimed at monitoring the exact contribution of Leishmania parasite to the virus life cycle suggested that Leishmania can be seen as a potential cofactor in HIV-1 pathogenesis [8–10] and can modulate the cytokine profile in the coinfected patients [11–13]. In vitro studies demonstrated the ability of Leishmania to increase expression of proinflammatory cytokines such as TNF-α [14], IL-1α [15], and IL-6 [16]. Considering that those cytokines can modulate HIV-1 replication (reviewed in [17]), it is tempting to speculate that Leishmania affects HIV-1 production by altering cytokine production in the dually infected subjects.

Cells of the mononuclear phagocyte lineage represent a major target for HIV-1 and serve also as an important cellular reservoir for viral persistence and as a vehicle for viral dissemination [18,19]. Since the mid-1980s, the susceptibility of monocytes/macrophages to HIV-1 has been very well documented both in vivo and in vitro [20–25]. A more recent review article illustrated the idea that infection of macrophages represents a strategy used by HIV-1 to evade immune pressure [26]. Interestingly, Leishmania resides primarily in host mononuclear phagocytes and has developed mechanisms to subvert the microbicidal activity of macrophages (reviewed in [8,27]). Thus, HIV-1 and Leishmania are able to infect and multiply within the same target cell, that is, monocytes/macrophages. Moreover, both pathogens can establish a latent infection following the primary infection. The presence of the two different intracellular pathogens within the same target cell might have functional consequences for the overall immune response and might also modulate the replicative cycle of HIV-1 and/or Leishmania.

The central objective of this study was to examine the modulatory effect of Leishmania on HIV-1 production in a physiologically relevant cell target, that is, human monocyte-derived macrophages. We report here that Leishmania behaves as a potent inducer of HIV-1 production in macrophages. The Leishmania-mediated augmentation of HIV-1 replication was linked with secretion of the proinflammatory cytokines TNF-α and IL-1α, which are known to exert stimulatory effects with regard to virus gene expression.

Materials and methods

Production of HIV-1 virus stocks

Viruses were produced by transient calcium phosphate transfection of human 293T cells with pJR-CSF, an R5 infectious molecular clone of HIV-1. Reporter viruses were pseudotyped by cotransfection of 293T cells with pNL4-3.Luc.R.E and an expression vector coding for the vesicular stomatitis virus envelope glycoprotein G (VSV-G). All virus preparations underwent a single freeze–thaw cycle before virus infection. Virus stocks were normalized for virion content using a p24 antibody capture assay developed in our laboratory (see below).

Leishmania parasites

Leishmania infantum promastigotes were maintained at 25°C with a weekly passage in semidefined culture medium containing fetal bovine serum (FBS) and antibiotics. The promastigotes used for infection were prepared from Day 7 or Day 8 cultures (stationary phase). Before infection, promastigotes were washed once in RPMI medium supplemented with FBS. Promastigotes were then resuspended in the same medium.

Isolation and culture of monocyte-derived macrophages

Human peripheral mononuclear cells (PBMCs) were isolated from healthy blood donors by density-gradient centrifugation on Ficoll-Hypaque. Monocytes were purified by adherence to plastic in RPMI medium supplemented with 10% FBS and antibiotics. PBMCs (3 × 10⁶/ml) were first seeded into 48-well plates, and nonadherent cells were removed after 2 h of plating by several washes with warm phosphate-buffered saline (PBS). Freshly isolated monocytes were allowed to differentiate into monocyte-derived macrophages (MDMs) in RPMI supplemented with human recombinant M-CSF (100 ng/ml) for 6 days.

HIV-1 infection assay

The possible effect of Leishmania infantum parasites on virus transcriptional activity was measured by using recombinant HIV-1-based reporter viruses. In brief, MDMs were exposed to Leishmania infantum parasites for up to 3 h. Free parasites were washed out with warm PBS. Next, VSV-G pseudotypes were added and the cells were incubated for another 2 days. At the end of the experiment, cells were lysed and luciferase units representing HIV-1 transcriptional activity were measured with a microplate luminometer (MLX: Dynex Technologies, Chantilly, VA).

Experiments were also conducted to define the modulatory effect of Leishmania infantum on replication of fully infectious progeny virus. The kinetics of HIV-1 replication were analyzed by serial measurements of extracellular p24 using an in-house double antibody enzymatic test as described previously [28]. Briefly, MDMs were exposed to Leishmania infantum (at different parasites/cell ratio) for up to 3 h. Free parasites were washed out with warm PBS. Fully competent JR-CSF viruses were then added (2 ng/ml of p24), left to incubate for 2 h, before washing out the free
virus. On the indicated numbers of days postinfection, cell culture supernatants were collected and viral lysates were added to an anti-p24 antibody precoated plate. After exposing the samples to a second anti-p24 antibody, which recognizes a p24 epitope different from the one recognized by the first antibody, the unknown p24 values were calculated on the basis of regression analysis of p24 standards prepared from known p24 concentration samples.

**Cytokine production**

Levels of TNF-α, IL-1α, and IL-6 in cell-free culture supernatants were quantified with commercially available enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer’s instructions (Cedarlane Laboratories Limited). Pentoxifylline (1-5’-oxohexyl)-3,7-dimethyl xanthine) (Sigma; 100 μM) or a neutralizing antibody specific for each cytokine tested (R and D systems, 500 ng/ml for anti-TNF-α, 100 ng/ml for anti-IL-1α, and 500 ng/ml for anti-IL-6) was added to the culture medium at the time of infection and then every 3 days with each medium change.

**Statistical analysis**

Results presented are expressed as the mean value ± SD of quadruplicate samples. Statistical significance was determined by nonparametric equivalent Wilcoxon (Mann–Whitney) test. Calculations were made with prism. P values less than 0.1 (instead of 0.05, given that primary cells were used in this study) were considered statistically significant.

**Results**

**Transcriptional activity of HIV-1 in MDMs is increased upon concurrent infection with Leishmania infantum**

It was shown several years ago that *Leishmania* can mediate the release of progeny virus from two cell lines of monocytoid origin latently infected with HIV-1 (i.e., U1 and OM10.1) [8]. To closely approximate the complex molecular events taking place in a more natural cellular reservoir for both microorganisms, we performed coinfection studies in primary human MDMs. We initially tested the ability of *Leishmania* parasites to augment HIV-1 transcription in such cells. This goal was achieved by first inoculating MDMs with increasing concentrations of *Leishmania infantum* ranging from 5 to 20 parasites per target cell. The parasitic infection was allowed to establish itself for 3 h before infection with VSV-G pseudotyped HIV-1-based reporter viruses. Forty-eight hours later, the virus-encoded luciferase activity was monitored in cell lysates. As depicted in Fig. 1, HIV-1 LTR-driven reporter gene activity is augmented upon addition of *Leishmania infantum* (i.e., 40.1 ± 2.8 RLU for samples infected with HIV-1 only and 110.2 ± 5.4 RLU for samples infected with HIV-1 and *Leishmania* at a 10:1 parasites/cell ratio; P value of 0.06), therefore suggesting that virus transcription in MDMs is enhanced by this protozoan parasite. It should be noted that *Leishmania infantum* was used in the present work because it is the most common *Leishmania* species found in individuals coinfected with both pathogens.

**Growth of infectious R5-tropic viruses in MDMs is increased by Leishmania infantum**

Replication of HIV-1 is recognized as a complex process regulated by proteins of viral and cellular origin that has been shown to work in cis and in trans. Therefore, to provide physiological significance to previous data on virus transcription, experiments were also carried out with a macrophage-tropic, CCR5-dependent infectious isolates of HIV-1 (i.e., strain JR-CSF). Results from Fig. 2 indicate that replication of fully competent R5 progeny virus is increased by coinfecting primary human MDMs with *Leishmania infantum* (i.e., 2850 ± 71 pg/ml of p24 for samples inoculated with HIV-1 only and 7300 ± 213 pg/ml of p24 for samples infected with HIV-1 and *Leishmania* at a 10:1 parasites/cell ratio at 6 days postinfection; P value of 0.03). It should be noted that comparable findings were made when virus particles were applied before addition of *Leishmania* parasites (data not shown).

*Leishmania*-dependent augmentation of HIV-1 replication in MDMs is linked with enhanced secretion of TNF-α and IL-1α

*Leishmania* and its major surface molecule, the lipophosphoglycan, have been shown to mediate secretion of several
cytokines, including TNF-α, IL-1α, and IL-6 [8,11,29,30]. Therefore, in an attempt to define whether secretion of such cytokines could be modulated in the studied experimental cell system, MDMs were exposed to Leishmania and HIV-1 either used alone or in combination, and cytokine production was measured in culture supernatants. Results from Figs. 3A and B demonstrate that Leishmania infantum by itself is a weak inducer of TNF-α and IL-1α (56.5 ± 4.1 and 160.9 ± 44.2 pg/ml for TNF-α and IL-1α, respectively). In contrast, HIV-1 acts as a much more potent activator of TNF-α and IL-1α secretion (149.4 ± 18.8 and 459.6 ± 49.9 pg/ml for TNF-α and IL-1α, respectively). More importantly, higher levels of both cytokines were produced upon coinfection of primary human MDMs with Leishmania and HIV-1 (297.1 ± 34.7 and 803.7 ± 103.7 for TNF-α and IL-1α, respectively; P values of 0.03 and 0.06 for TNF-α and IL-1α, respectively). Virus infection of MDMs resulted in significant production of IL-6 (Fig. 3C). However, a nonstatistically significant increase in IL-6 secretion was detected following infection of MDMs with both HIV-1 and Leishmania infantum (932.5 ± 52.7 pg/ml for samples infected with HIV-1 only and 1182.5 ± 102.7 pg/ml for samples infected with HIV-1 and Leishmania; P value of 0.2).

The contribution of TNF-α, IL-1α, and IL-6 in the observed increase in HIV-1 production that is due to Leishmania was next evaluated by performing infection studies with appropriate blocking compounds. A series of investigations was performed with pentoxifylline (PTX), a phosphodiesterase inhibitor that abolishes TNF-α production [31,32], and neutralizing antibodies specific for TNF-α, IL-1α, or IL-6. Treatment of primary human MDMs with each of the tested neutralizing agents led to a decrease of virus release in the culture supernatant for samples inoculated with HIV-1 only (a diminution of 29%, 40%, 26%, and 43% upon treatment with PTX, anti-TNF-α, anti-IL-1α, and anti-IL-6, respectively; P value of 0.07) (Fig. 4A). However, a slightly more important diminution in production of progeny virus was observed upon a similar treatment of MDMs coinfected

Fig. 2. Replication of a competent macrophage-tropic HIV-1 variant in MDMs is enhanced by Leishmania. MDMs were either left uninfected or inoculated with the listed doses of Leishmania infantum promastigotes before the addition 3 h later of fully infectious macrophage-tropic HIV-1 (i.e., JR-CSF at 2 ng of p24 per 5 x 10^5 target cells). Half of the supernatant was harvested from each well at the indicated time points. Virus production was assessed by measuring p24 released into the cell-free culture supernatant. Data shown are the mean ± SD of quadruplicate samples and are representative of independent experiments performed with three different donors.

Fig. 3. Dual infection of MDMs with Leishmania and HIV-1 results in an augmented secretion of TNF-α, IL-1α, and IL-6. MDMs were left uninfected or inoculated either with Leishmania infantum (10 parasites per target cell), HIV-1 (JR-CSF at 2 ng of p24 per 5 x 10^5 cells), or both pathogens. At 24 h postinfection, cell supernatants were collected, and levels of TNF-α, IL-1α, and IL-6 were measured using commercial enzymatic tests. Data shown are the mean ± SD of quadruplicate samples and are representative of independent experiments performed with three different donors.
with HIV-1 and *Leishmania* (a reduction of 34%, 57%, and 51% following treatment with PTX, anti-TNF-α, and anti-IL-1α; *P* value of 0.04) (Fig. 4A). A comparable decrease in HIV-1 release was seen upon treatment of dually infected MDMs with anti-IL-6 (a reduction of 43%), therefore indicating that IL-6 is not playing an active role in the parasite-mediated enhancement of HIV-1 production.

A similar observation was made in MDMs from different healthy donors when the antibodies (i.e., anti-TNF-α and anti-IL-1α) were used in combination. In the samples treated with HIV-1 only, virus production was diminished by the blocking antibodies by 33%, 46%, and 32% for donors 1, 2, and 3, respectively (*P* values of 0.7, 0.3, and 0.5), whereas in the samples treated with both HIV-1 and *Leishmania*, the blocking antibodies decreased virus production by 39%, 56%, and 64% for donors 1, 2, and 3, respectively (*P* values of 0.06, 0.03, and 0.03) (Fig. 4B).

**Discussion**

Patients infected with HIV-1 might develop fatal secondary infections with several opportunistic microorganisms, including protozoan parasites. This is the case in the Mediterranean basin where leishmaniasis has emerged as an extremely serious opportunistic disease in AIDS patients. For example, the visceral form of leishmaniasis, which is mainly caused by the *Leishmania infantum* species, is responsible for significant morbidity and mortality in AIDS subjects [33]. However, despite the fact that epidemiological studies have revealed that the protozoan parasite *Leishmania* plays an important role in the course and outcome of HIV-1 disease progression, there is a paucity of data on the underlying immunological mechanisms involved in *Leishmania*/HIV-1 coinfection. We previously reported that *Leishmania* can upregulate virus expression in two cell lines of monocytoid origin latently infected with HIV-1 (i.e., U1 and OM-10.1) [8–10]. More recently, we provided evidence that *Leishmania* can promote HIV-1 replication in human tonsillar tissue cultured ex vivo through an effect on proinflammatory cytokine production [34]. Although these studies are informative, they present some limitations. For example, it is more appropriate to conduct studies in a physiological cellular reservoir based on the idea that tumor cell lines may behave sometimes quite differently than primary human cells. Moreover, the analysis of the involvement of a specific cell type once tonsillar tissue is inoculated with both pathogens (i.e., HIV-1 and *Leishmania*) is complicated by the complex nature of histocultures of human lymphoid tissue. In an attempt to solve these issues and since there is no information on the possible influence of *Leishmania* on the life cycle of HIV-1 when both organisms are found concurrently within primary human macrophages, we performed a series of investigations to define whether *Leishmania* can modulate HIV-1 biology in MDMs.

Since the mid-1980s, macrophages have been recognized for their important role in initial infection with HIV-1 and contribution to the pathogenesis of the disease throughout the course of infection [21,24,25]. Both blood monocytes and tissue macrophages [35–37] express CD4 receptor and chemokine receptors CXCR4 and CCR5, which serve as HIV-1 coreceptors, and hence are considered as primary target cells for HIV-1. These cells produce HIV-1 for weeks to months without significant cytopathic effects [35,37,38] and persist as long-term stable reservoirs for HIV-1 capable of disseminating the virus to tissues. Following HIV-1 infection, effector functions carried out by monocytes/macrophages, such as phagocytosis, intracellular killing, chemotaxis and cytokine production, are impaired. Such defects eventually contribute to the overall pathogenesis of the disease allowing development of opportunistic infections. With respect to *Leishmania*, this protozoan can successfully invade macrophages and subvert multiple functions of the host mononuclear phagocyte (reviewed in [39]). *Leishmania* can survive and reproduce within the macrophage by
manipulating the host immune response. For example, *Leishmania* can inhibit multiple cellular defense mechanisms such as phagosome–endosome fusion process, hydrolytic enzymes, cell signaling pathways, and nitric oxide production (reviewed in [40]).

Using human MDMs as an in vitro experimental cell system, we first investigated the potential modulatory effect of *Leishmania infantum* on HIV-1 transcriptional activity. Reporter HIV-1-based viruses pseudotyped with VSV-G were used because the pseudotyping strategy allows bypassing the natural entry mode of HIV-1. This integrated virus not only widely broadens the natural virus tropism but also significantly enhances virus infectivity [41,42]. Our results revealed that *Leishmania* enhances HIV-1 LTR-directed gene expression (Fig. 1). We tested also the ability of *Leishmania* to influence replication of complete HIV-1 particles bearing a tropism for macrophages (i.e., JR-CSF). *Leishmania* was found to influence positively the replicative cycle of JR-CSF virions in MDMs (Fig. 2).

The immunopathogenic mechanism(s) whereby *Leishmania* may enhance HIV-1 production under in vivo conditions was recently proposed to involve either antigen-specific or non-antigen-specific pathways [43]. We provide evidence here that *Leishmania* modulates virus replication by virtue of its capacity to induce secretion of some cytokines known to up-regulate HIV-1 gene expression, therefore via a non-antigen-specific pathway. Indeed, coinfection of MDMs with *Leishmania* and HIV-1 resulted in an enhanced production of soluble factors such as TNF-α and IL-1α (Fig. 3), all of which are recognized as inducers of HIV-1 gene expression [44]. In addition, the direct implication of these two cytokines in the *Leishmania*-dependent enhancement of virus release came from experiments performed with blocking agents (Fig. 4).

The proinflammatory cytokine TNF-α is usually considered as essential to mount a protective immunity. However, when produced in excess, it may have deleterious effects on the host. For example, high secretion of proinflammatory cytokines such as TNF-α in disseminated visceral leishmaniasis is thought to be responsible for hepatosplenomegaly, weight loss, fever, and other constitutional symptoms [45]. The serum levels of TNF-α have been suggested to be used as markers of disease severity and to monitor efficacy of therapeutic interventions aimed at controlling growth of *Leishmania* [14]. In AIDS, a dysregulated secretion of proinflammatory cytokines has been proposed to contribute actively to the pathogenesis of the disease. More specifically, there is a direct correlation between levels of circulating TNF-α and plasma viral load [46]. Virus gene expression that is seen upon treatment with TNF-α is due to nuclear translocation of the ubiquitous mammalian transcription factor NF-κB [43]. Thus, it can be hypothesized that the *Leishmania*-dependent release of TNF-α in the present work may function in an autocrine/paracrine manner to induce virus gene expression in MDMs harboring integrated viral genome. This observation is in agreement with a previous study showing that TNF-α plays a pivotal role in *Leishmania*-mediated induction of virus replication in an established human T cell line latently infected with HIV-1 [47]. IL-1α is another proinflammatory cytokine that is produced by activated macrophages and other cell types during inflammatory reactions in combination with TNF-α and IL-6 [1]. IL-1α has also been demonstrated to activate HIV-1 production in cultured primary MDMs [48]. Similarly to TNF-α, IL-1α has been reported to promote virus gene expression following transfection of human T lymphoid cells (i.e., Molt-4 and Jurkat) [49,50] and mouse cell lines [51] with reporter gene constructs driven by the HIV-1 regulatory sequences. It should be emphasized that the peak levels of studied cytokines were detected at 24 h postinfection, which is well before maximal amounts of HIV-1 were released by infected MDMs (i.e., 6 days postinfection). This finding when coupled with the reported high concentrations of proinflammatory cytokines in disseminated leishmaniasis patients [11,52] prompts us to suggest that *Leishmania* is not directly affecting virus gene expression but is rather modulating the life cycle of HIV-1 through an indirect phenomenon that is linked with induction of elevated levels of TNF-α and IL-1α.

In summary, our data indicate that *Leishmania* has important modulatory effects on the biology of HIV-1 in human MDMs, a cell type known to act as an important cellular reservoir for both pathogens. Given that *Leishmania* is a protozoan parasite that commonly coinfests HIV-1-infected persons in developing countries and macrophage can act as a major cellular reservoir for both pathogenic agents, it can be proposed that the observed *Leishmania*-mediated increase in cytokine production will affect virus production not only in macrophages, but also in HIV-1-infected CD4+ T lymphocytes in persons dually infected with *Leishmania* and HIV-1. There is clearly a great need for additional data to more precisely address the clinical course of *Leishmania*/HIV-1 coinfection in areas where both infections are endemic. The knowledge gained from such studies might help the development of therapeutic approaches to combat the *Leishmania*/HIV-1 epidemics in such parts of the world.

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