LPS reduces HIV-1 replication in primary human macrophages partly through an endogenous production of type I interferons

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Abstract It has been proposed that the systemic immune activation state seen in HIV-1-infected patients is caused by circulating microbial products such as lipopolysaccharide (LPS). Given that macrophages play a key role in HIV-1 pathogenesis, we investigated the LPS-mediated effect on HIV-1 replication in cells of the myeloid lineage. We demonstrate that LPS promotes virus gene expression in a monocytic cell line while it diminishes virus production in primary human monocyte-derived macrophages (MDM). The incapacity of LPS to drive HIV-1 production in MDM was not due to its inability to activate the ubiquitous transcription factor NF-κB even in virus-infected cells. Neutralization of type I interferons (IFN) with B18R, a soluble vaccinia virus-coded type I IFN receptor, significantly but not totally diminished the antiviral activity of LPS. Therefore, inhibition of HIV-1 replication in MDM treated with microbial-derived LPS resulted from the induction of type I interferons and a yet to be defined soluble factor.

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Introduction

During their lifespan, macrophages are exposed to a large variety of microorganisms, such as bacteria, fungi, parasites and viruses. These cells express pathogen recognition receptors (PRRs) that are responsible for the detection of pathogen-associated molecular patterns (PAMPs). PAMPs are molecules found on the surface of invading microbes, and can take the form of various pathogenic constituents such as lipopolysaccharide (LPS), peptidoglycans, or flagellin. Notably, LPS is a major endotoxin derived from Gram-negative bacteria, which is known to trigger the signaling cascade that induces septic shock and organ failure. It has been recently demonstrated that at all stages of an HIV-1 infection, the immune system conferring protection to various infections in the intestinal and genital tracts is compromised by an
exhaustive depletion of CD4+ T lymphocytes [1,2]. This breach in the mucosal immune system favors infections from the resident flora and recurrent and invasive co-infections. Interestingly enough, the level of circulating LPS, which is used as an indicator of microbial translocation, is significantly increased in HIV-1-infected individuals [3]. It is thus possible that HIV-1-infected cells can be exposed to circulating bacterial products including LPS.

Toll-like receptors (TLRs) along with C-type lectins and glycosylphosphatidylinositol (GPI)-anchored receptors are important PRRs found on innate immune cells (reviewed in [4]). It has been reported that macrophages express all known TLRs [5], as well as the receptor responsible for binding LPS, the GPI-anchored CD14 [6,7]. CD14 is a surface protein that lacks an intracellular domain and a coreceptor is thus required for signaling to occur. TLR4 has been shown to play that role in the responsiveness to LPS [8]. The CD14/TLR4 complex triggers signaling through both MyD88-dependent and -independent signal transduction pathways (reviewed in [9]). The MyD88-dependent signaling events lead notably to the activation and nuclear translocation of the transcription factor NF-κB [10,11]. On the other hand, the MyD88-independent pathway results in a diminished NF-κB induction and is initiated after the MyD88-dependent signal following CD14/TLR4 engagement (i.e. about 30 min later) [12]. This pathway leads to the activation of the transcription factor IRF3 [13], which is responsible for the sequential production of the antiviral cytokines interferon (IFN)-β and IFN-α. The antiviral activities of these two type I IFNs are related to their capacity to regulate viral mRNA translation and induce degradation of viral RNA (reviewed in [14]).

The regulatory elements of HIV-1 (i.e. long terminal repeat/LTR) harbor two NF-κB binding sites [15], and NF-κB is thus actively participating in the transcriptional activity of this retrovirus. Several studies have illustrated that LPS induces HIV-1 gene expression in established cell lines of the monocytic lineage through a NF-κB-dependent pathway [16–20]. Surprisingly, when similar studies were performed in primary human macrophages, LPS was found to exert an opposite effect since it reduces virus replication [21,22]. Thus, the central objective of the present study was to perform a comparative analysis of the LPS-mediated effect on HIV-1 gene expression in monocytic cells and primary human macrophages in an attempt to shed light on the previous contradictory observations with respect to the modulatory effect of LPS on HIV-1 replication. We confirm that LPS promotes virus production in a monocytic cell line, whereas it strongly inhibits virus replication in primary human monocyte-derived macrophages (MDM). We provide evidence that the LPS-directed inhibition of HIV-1 expression in MDM is at least partly associated with the secretion of type I IFNs. We conclude that circulating microbial products such as LPS might affect HIV-1 pathogenesis by exerting an effect on virus replication in macrophages which are thought to constitute a crucial reservoir of actively replicating virus.

**Materials and methods**

**Antibodies and reagents**

Accutase and lipopolysaccharide (LPS) were purchased from Sigma (St-Louis, MO). B18R was obtained from eBioscience.
San Diego, CA) and IFN-β from Cedarlane Laboratories (Burlington, ON). TNF-α was measured with the commercial Human TNF ELISA Set (BD Biosciences, San Diego, CA).

The mouse monoclonal antibodies anti-IκBα and anti-phosphorylated-IκBα were purchased from Cell Signaling Technology Inc. (Danvers, MA), whereas the HRP-coupled goat anti-mouse antibody was obtained from GE Healthcare (Chalfont St. Giles, UK). The mouse monoclonal antibody anti-CD14 (clone UCHM1) was purchased from Ancell (Bayport, MN), whereas the anti-TLR4 antibody (clone HTA125) was obtained from Imgenex (San Diego, CA).

Cells

Human embryonic kidney 293T cells were obtained from the ATCC while THP89GFP cells, a derivative of THP-1 cells that are latently infected with a fully competent dual-tropic viral strain (i.e. 89.6/R5X4) engineered to also express the enhanced green fluorescence protein (eGFP), were kindly provided by D. N. Levy (New York University College of Dentistry, New York, NY). Monocytes were purified by adherence to plastic in complete RPMI 1640 supplemented with 5% heat-inactivated autologous serum. Briefly, peripheral blood mononuclear cells (1.5 x 10^7 cells/mL) were plated in 75 cm² flasks. After 2 h, non-adherent cells were removed by several washes with phosphate-buffered saline (PBS) and freshly isolated monocytes were further cultured in the same medium for 7 days in the presence of M-CSF (100 ng/mL). MDM were recovered by scraping with a soft cell scraper following incubation with Accutase and plated in 48-well plates at a final concentration of 5 x 10^5 cells per well in complete RPMI medium supplemented with 5% autologous serum.

Flow cytometry analyses revealed that the studied MDM preparations were constituted mostly of CD14+ cells (i.e. ≥ 99%) and very few contaminating CD3+ cells (i.e. about 0.1%).

Plasmids and virus production

The full-length R5-tropic infectious molecular clone of HIV-1 pNL4.3Balenv was kindly provided by R. Pomerantz (Thomas Jefferson University, Philadelphia, PA). The luciferase-encoding pNL4.3 Luc'E' R' and the R5-tropic envelope-encoding pJR-FLenv vectors were kindly provided by N. R. Landau (The Salk Institute for Biological Studies, San Diego, CA). Fully infectious viruses were generated by transient transfection of 293T cells with pNL4.3Balenv, whereas single cycle pseudotyped reporter viruses were produced by cotransfection of 293T cells with pNL4.3 Luc'E' R' and pJR-FLenv. Virus stocks were normalized for virion content using a homemade p24 assay [23].

Measurements of eGFP, luciferase activity and p24 content

The percentage of eGFP-expressing cells was measured by flow cytometry. Briefly, THP89GFP cells were initially exposed to the tested stimuli and were recovered from the wells, washed with ice-cold PBS and then fixed with 2% paraformaldehyde for 30 min at 4 °C. Thereafter, cells were resuspended in a solution made of PBS 2% BSA and measurements were made with an Epics® XL FACS (Beckman Coulter; Fullerton, CA) (excitation, 490 nm; emission, 525 nm). In the case of MDM infected with luciferase-encoding viruses, reporter gene activity was monitored in cell lysates as described previously [24]. For p24 measurements, the cell-free supernatant from stimulated cells was loaded on an ELISA plate and we performed our in-house sensitive double-antibody sandwich ELISA test [23].

HIV-1 infection and treatment of studied target cells

MDM were infected with a fixed amount of virus (i.e. 10 ng of p24 per 5 x 10^5 cells). In the case of pseudotyped virus infection,
viruses were left in the medium throughout the experiment. In the case of fully infectious viruses, they were left therein for 4 h; cells were then washed with PBS and fresh medium was added. Every 2–3 days, half the medium was collected and replaced.

THP89GFP cells and MDM were washed with PBS before stimulation. LPS was added in fresh medium using final concentrations ranging from 100 ng/mL up to 1 μg/mL. TNF-α was used at a final concentration of 10 ng/mL and B18R at 200 ng/mL. In some experiments, IFN-β was used at a final dose of 675 U/mL in the presence or absence of 100 ng/mL of B18R.

Electrophoresis and western blot

The non-phosphorylated and phosphorylated forms of IκBα were detected by western blot analysis using a standard procedure and visualized through the use of anti-IκBα (diluted 1:2000) and anti-phosphorylated IκBα antibodies (diluted 1:1000). The membrane was revealed using enhanced luminol substrate according to the manufacturer’s instructions (PerkinElmer Life Sciences, Wellesly, MA).

Flow cytometry analysis

To monitor CD14 expression on MDM, cells were recovered from wells by gently scraping following Accutase incubation. Fc receptors were blocked by treating MDM with 10% human serum for 30 min at 4 °C. Then, cells were incubated with an anti-CD14 or an isotype-matched control antibody for 30 min at 4 °C followed by a second incubation with an R-phycoerythrin-conjugated goat anti-mouse antibody. Cells were resuspended in 2% paraformaldehyde and analysed by flow cytometry. Cells were washed twice with ice-cold PBS between each step.

Results

LPS induces virus gene expression in monocytic cells latently infected with HIV-1

Our initial series of investigations was aimed at confirming the ability of LPS to induce HIV-1 gene expression in monocytic cells. To this end, the THP89GFP cell line was treated with increasing concentrations of LPS. These cells were also stimulated with TNF-α as a positive control since this pro-inflammatory cytokine is known to act as a potent inducer of HIV-1 gene expression. We first estimated virus gene expression following a 24 h period treatment. As shown in Figure 1A, there is a linear LPS-mediated dose-dependent induction of virus gene expression in THP89GFP cells. In parallel, we evaluated production of progeny virus by assessing the p24 content in cell-free supernatants from such similarly treated THP89GFP cells. Virus production was increased following treatment with LPS (Fig. 1B), thus confirming that LPS can increase both transcription of latent HIV-1 and virus production in a human monocytic cell line.

HIV-1 replication is reduced upon treatment of MDM with LPS

In order to evaluate the effect of LPS on HIV-1 transcriptional activity in a more relevant physiological cell system, primary human MDM were infected with luciferase-encoding viruses pseudotyped with the R5-tropic JR-FL envelope.

Figure 5 LPS-mediated NF-κB is functional in uninfected and virus-infected MDM. MDM (5 × 10^5) either uninfected or infected with NL4.3Balenv for 7 days were stimulated with LPS (100 ng/mL). Cell-free supernatants were collected at the indicated time periods following treatment and analyzed for the presence of TNF-α. The data correspond to the means ± SEM of triplicate samples and are representative of two independent experiments.

Figure 6 CD14 expression is not altered by HIV-1 infection. MDM were either left uninfected (A) or infected (B) with fully infectious NL4.3Balenv for 7 days. The level of CD14 was assessed by flow cytometric analyses after staining with an isotype-matched control antibody (filled region) or anti-CD14 antibody (clone UCHM1) (empty region).
These replication-defective reporter viruses undergo a single cycle of replication. Following infection with such reporter viruses, MDM were stimulated with LPS and then lysed before monitoring virus-encoded luciferase activity. In sharp contrast to what is seen in established THP-89GFP cells, virus gene expression was markedly diminished upon treatment of such HIV-1-infected MDM with LPS (i.e., about 18-fold decrease) (Fig. 2). Interestingly, HIV-1-mediated gene expression was again increased with TNF-α but to a lesser extent than in THP-89GFP cells. To better reflect HIV-1 infection of macrophages in vivo, MDM were infected with fully infectious viruses (i.e., NL4.3Balenv) for 7 days to allow establishment of infection before treatment with LPS. Data illustrated in Figure 3 demonstrate that treatment of MDM acutely infected with HIV-1 with LPS at a final concentration of 100 ng/mL leads to a strong diminution in virus production. Similar observations were made when LPS was used at final doses of 250 and 500 ng/mL (data not shown). We also confirmed that TNF-α stimulation augments HIV-1 production as seen with the luciferase reporter viruses (data not shown). Altogether, these experiments indicate that a striking difference exists between the THP-89GFP cell line and primary human macrophages with respect to the LPS-mediated effect on HIV-1 replication.

NF-κB is activated and functional in uninfected and virus-infected MDM following LPS stimulation

As we observed a decrease in viral replication following LPS-mediated engagement of the CD14/TLR4 complex on the surface of MDM and given that this signal transduction pathway involves NF-κB activation, we next wanted to determine whether this transcription factor is induced in both uninfected and HIV-1-infected MDM. Previous studies have demonstrated that NF-κB is controlled by its inhibitory regulator IκBα, which, upon phosphorylation, is polyubiquitinated and degraded, therefore allowing NF-κB to migrate and translocate to the nucleus [25]. Western immunoblots using an antibody specific for phosphorylated IκBα were thus performed in MDM stimulated with LPS. As shown in Figure 4A, treatment of uninfected MDM with LPS results in a rapid phosphorylation of IκBα – within 5 min – and a second wave of phosphorylation took place after approximately 60 min of stimulation. The absence of IκBα phosphorylation between 10 and 30 min corresponds to protein degradation, which depletes the IκBα intracellular pool as depicted in Figure 4B where total IκBα is shown (i.e., non-phosphorylated and phosphorylated). Once the pool is regenerated, new IκBα is phosphorylated, which corresponds to the second wave of phosphorylation. Importantly, similar findings were made in LPS-treated MDM that were also infected with HIV-1 (Fig. 4B), thus indicating that the LPS-mediated signal transduction pathway involving IκBα phosphorylation is still effective in MDM despite virus infection. Although these results suggest that NF-κB was free to translocate to the nucleus, there is no indication on the functionality of this transcription factor. Given that TNF-α secretion requires induction of an active form of NF-κB, we next performed an ELISA test to measure TNF-α production in the supernatant of LPS-treated MDM. Treatment of MDM with LPS results in a time-dependent production of TNF-α (Fig. 5). Importantly, similar observations were made in HIV-1-infected MDM that were treated also with LPS. Altogether these results suggest that the LPS-mediated decrease in virus production is not due to defective signaling events specific for NF-κB.

LPS-induced inhibition of HIV-1 production in MDM is partially mediated by type I IFNs

To define the mechanism responsible for the LPS-mediated inhibition of virus production in MDM, we first assessed whether HIV-1 infection can reduce surface expression of receptors that can be engaged by LPS. Given that LPS binds to CD14 and TLR4 is required for signaling to occur upon ligation of CD14 and considering that the tested anti-TLR4 antibody (i.e., clone HTA125) was not working effectively in flow cytometry (data not shown), experiments were performed using an anti-CD14 antibody. Data depicted in Figure 6...
indicate that HIV-1 is not inducing a down-regulation of CD14 expression (i.e. 43.9% in uninfected versus 43.7% in virus-infected MDM).

It has been reported that engagement of the CD14/TLR4 complex by LPS involves the MyD88-independent pathway that results in the production of type I IFNs such as IFN-β. Consequently, we hypothesized that the observed decrease in viral replication might be due to the presence of these antiviral cytokines in the medium following LPS stimulation. In order to explore this possibility, we made use of the soluble vaccinia virus-encoded protein B18R that has been demonstrated to inhibit the antiviral activity and cellular binding of the type I IFNs of different mammalian species (e.g. IFN-α, IFN-β and IFN-ω) [26,27]. We initially assessed the capacity of B18R to inhibit the antiviral efficacy of IFN-β in our experimental model system. As demonstrated in Figure 7A, this antiviral cytokine can potently inhibit HIV-1 production in MDM and the presence of B18R completely reverses the IFN-β-mediated inhibition of virus replication. To confirm the putative role played by type I IFNs in the observed LPS-mediated diminution of HIV-1 production in MDM, the virus-derived soluble type I IFN receptor B18R was added simultaneously with LPS in target cells infected with fully infectious NL4.3Balenv particles. We had previously observed a stronger difference in viral production after 6 days of LPS stimulation (Fig. 3) and therefore performed the measurements after that time length. When the inhibitor was added shortly after LPS treatment, we observed that B18R reduces the LPS-dependent antiviral activity by about 50% (Fig. 7B). A higher concentration of B18R did not further increase virus production (data not shown). Therefore, the secretion of type I IFNs that is seen upon treatment of MDM with LPS is partly responsible for the observed decrease in HIV-1 production.

Discussion

It is now well established that HIV-1 can productively infect two main groups of cells, namely CD4+ T cells and cells of myeloid lineage such as monocytes, macrophages and dendritic cells. The majority of the HIV-1 RNA found in plasma is thought to originate from productively infected CD4+ T lymphocytes [28,29] but tissue macrophages are now considered as an important stable reservoir for HIV-1 [30]. Although the general mechanism through which HIV-1 can achieve infection of CD4+ T cells and macrophages displays some similarities, some features are peculiar to the latter cell type. For example, compared with virus-infected CD4+ T lymphocytes, which have a half-life of 1 to 2 days, HIV-1-infected macrophages are relatively refractory to the cytopathic effects of HIV-1 infection both in vivo and in vitro and can release large amounts of virions for weeks and months following infection [31–34]. This is clearly illustrated by the demonstration that infected alveolar macrophages have an estimated half-life of about 2 months, whereas it has been estimated that HIV-1-infected microglia can survive for several years [35,36]. Interestingly, the establishment of viral reservoirs in both CD4+ T lymphocytes and macrophages is not prevented by initiating antiretroviral therapy early after infection (i.e. prior to seroconversion) [37–39]. Therefore, the long-term persistence of productive infection coupled with a resistance to virus-mediated cell death has conferred to macrophages the role of a stable cellular reservoir of HIV-1 that can perpetuate the chronic state of infection.

Myeloid cell lines and primary human MDM have been used as reference model systems to dissect out the possible modulatory effect of some specific soluble factors on HIV-1 gene expression in this cell type. A number of cytokines and chemokines have been shown to affect HIV-1 replication in cells of macrophage lineage (reviewed in [40]). LPS is a component of outer cell membrane of Gram-negative bacteria. It has been reported that LPS stimulates macrophages/dendritic cells and makes them secrete a vast array of cytokines, chemokines and type I IFNs [9,41–43]. It has been recently demonstrated that circulating LPS, which is used as an indicator of microbial translocation, is significantly increased in chronically HIV-1-infected individuals and rhesus macaques infected with simian immunodeficiency virus [3]. Therefore, exploring the LPS-mediated regulatory effect on HIV-1 production in cells of macrophage lineage is important in understanding the pleiotropic effects of exogenous stimuli such as bacterial LPS in the virus life cycle.

In the present study, we investigated the modulation of HIV-1 infection and replication by LPS in both monocyte cells and primary human MDM. We confirm previous published data showing that extracellular LPS displays a dichotomic effect with respect to HIV-1 infection in cells of macrophage lineage. Indeed, LPS acts as a strong inducer of virus gene expression in an experimental model system for latently infected macrophages (i.e. monocyte THP89GFP cells). However, a completely different outcome was seen in MDM since virus production is reduced by a treatment with LPS. We provide evidence that the LPS-mediated inhibition of HIV-1 production in MDM is not due to an inability of this exogenous stimuli to mediate signal transduction leading to nuclear translocation of NF-κB even in virus-infected cells. We observed that the anti-HIV-1 activity induced by bacterial LPS in MDM in vitro is due in part to an endogenous production of type I IFNs. The involvement of such soluble factors in the LPS-directed suppression of HIV-1 replication in MDM was illustrated through the use of B18R, a vaccinia virus-derived soluble receptor that has a high affinity for type I IFNs and thus protects cells from their antiviral effects. It can be proposed that IFN-β is involved based on the previously reported role of this soluble cytokine in mediating the LPS-induced antiviral activity through CD14/TLR4 signal transduction events [44]. Indeed, the study by Doyle et al. revealed that this type I IFN is up-regulated in macrophages by the CD14/TLR4 signaling pathway [44]. Treatment of MDM with exogenously added IFN-β corroborated that this cytokine acts as a potent anti-HIV-1 agent and its antiviral potency is totally abrogated by B18R, thereby confirming the importance of IFN-β in the LPS-dependent inhibitory effect on HIV-1 gene expression seen in primary human MDM. However, the contribution of other type I IFNs such as IFN-α cannot be totally excluded because it is a TLR4-mediated secondary response gene [44]. Unfortunately, experiments conducted with neutralizing anti-IFN-α and anti-INF-β were not conclusive (data not shown) probably because MDM express significant levels of Fc receptors and it is possible that HIV-1 gene expression in MDM is affected upon engagement of Fc receptors by the tested anti-IFN-α and anti-INF-β antibodies. That is why we used the vaccinia virus-derived soluble receptor B18R to block type I IFNs in MDM to
circumvent any potential effect made by the Fc portion of antibodies. The LPS-mediated suppression of virus production was not totally abrogated in the presence of B18R thus suggesting that another soluble factor in addition to type I IFNs is involved in the inhibitory effect mediated by LPS.

HIV-1 transcription is controlled by viral and cellular factors that can bind the virus regulatory elements better known as LTR. It has been demonstrated that the HIV-1 LTR carries three CCAAT/enhancer binding protein (C/EBP) sites in its S’ negative regulatory element (NRE) region [45] that can bind the transcription factor C/EBPβ [46]. These three sites are essential for the control of viral replication in macrophages but not in CD4+ T cells [46]. A previous work has revealed that C/EBPβ can exist under two distinct isoforms. A 30–37 kD C/EBPβ isoform that can physically interact with histone acetyltransferase complexes and positively affect HIV-1 LTR-driven transcription [47]. A smaller isoform of C/EBPβ (i.e. 10–20 kD) has been shown to exert a completely opposite effect since it can inhibit HIV-1 LTR-dependent transcription and virus replication [48]. Additional studies confirmed that this transcriptional repressor abrogates HIV-1 LTR promoter activity and viral replication in both primary macrophages and monocyctic cell lines [46, 49]. Interestingly, a recent study has demonstrated that LPS induces the anti-inflammatory cytokine IL-10, which inhibits HIV-1 transcription in macrophages by inducing the C/EBPβ repressor [50]. It can thus be postulated that the LPS-mediated repression of HIV-1 production in MDM is due to the combined action of type I IFNs and IL-10. Studies are currently in progress to illuminate the precise contribution of IL-10 in the observed phenomenon.

Our data confirm that LPS has a dichotomous effect on HIV-1 replication in cells of the monocytic lineage. Both activation of latent HIV-1 infection and inhibition of active replication can be seen in vitro in monocytic cells and primary human MDM, respectively. Although immortal myeloid cell lines are considered as useful experimental tools to decipher the complexity of interactions between HIV-1 and cells of the myeloid lineage, the current work is an additional evidence that primary MDM represent a more appropriate system than immortal cell lines. The previously reported resistance of most monocyctic cell lines to infection with RS-tropic isolates of HIV-1 further confirms the benefit of using primary human MDM as a model system to define the characteristics of acute and chronic HIV-1 infection in vitro. More importantly, the present study leads us to propose that pathogen-derived products such as LPS can modulate HIV-1-associated pathology by modulating virus replication in macrophages, a major cellular reservoir thought to play a key role in virus persistence.

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