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Lipopolysaccharide Inhibits HIV-1 Infection of Monocyte-Derived Macrophages Through Direct and Sustained Down-Regulation of CC Chemokine Receptor 5

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It is now well established that HIV-1 requires interactions with both CD4 and a chemokine receptor on the host cell surface for efficient infection. The expression of the CCR5 chemokine receptor in human macrophages facilitates HIV-1 entry into these cells, which are considered important in HIV pathogenesis not only as viral reservoirs but also as modulators of altered inflammatory function in HIV disease and AIDS. LPS, a principal constituent of Gram-negative bacterial cell walls, is a potent stimulator of macrophages and has been shown to inhibit HIV infection in this population. We now present evidence that one mechanism by which LPS mediates its inhibitory effect on HIV-1 infection is through a direct and unusually sustained down-regulation of cell-surface CCR5 expression. This LPS-mediated down-regulation of CCR5 expression was independent of de novo protein synthesis and differed from the rapid turnover of these chemokine receptors observed in response to two natural ligands, macrophage-inflammatory protein-1α and -1β. LPS did not act by down-regulating CCR5 mRNA (mRNA levels actually increased slightly after LPS treatment) or by enhancing the degradation of internalized receptor. Rather, the observed failure of LPS-treated macrophages to rapidly restore CCR5 expression at the cell-surface appeared to result from altered recycling of chemokine receptors. Taken together, our results suggest a novel pathway of CCR5 recycling in LPS-stimulated human macrophages that might be targeted to control HIV-1 infection.

Peripheral blood monocytes and tissue macrophages play critical roles in both the natural history and the pathogenesis of HIV-1 infection. Cells of the macrophage lineage are among the first cells colonized as viral infection is established in the body (1). The observation that macrophages infected in vitro show little evidence of HIV-1-induced cytopathicity suggests that, in vivo, infected mononuclear cells may persist as long-lived viral reservoirs and contribute to the longitudinal propagation of HIV infection (2). For this reason, the control of HIV-1 infection in macrophages is an attractive target for anti-HIV therapy.

The surface marker CD4, expressed in a wide variety of cell types, was the first cell-surface molecule found to bind HIV-1. Recently, several members of an unrelated family of surface proteins that specifically bind ligands of the chemokine superfamily have been shown to function as HIV-1 coreceptors; that is, HIV must engage CD4 in combination with one of several distinct chemokine receptors for efficient entry of the virus into cells. These HIV-1 coreceptors belong to a large family of seven transmembrane domain G-protein-coupled receptors. This family of receptors can be subdivided into two major groups designated CC and CXC chemokine receptors after the subclass of chemokine (CC or CXC) that binds the receptor (3, 4). Individual chemokines affect the proliferation, differentiation, trafficking, phenotype, and activation of particular populations of hematopoietic cells, and the differential expression of chemokine receptors by distinct cell types is an important determinant of the specificity of chemokine-induced biological effects (5). In parallel, the preferential tropism that some HIV-1 strains show for specific cell types can be explained, at least partially, by selective adaptation to specific subclasses of chemokine receptors. For example, lymphotropic strains of HIV-1 (such as NL4-3, NDK, and Bru) preferentially infect activated T cells through binding to CXCR4, whereas CCR5 has been demonstrated to be the main coreceptor for macrophage-tropic strains (such as ADA and JRFL) (6, 7). The requirement of CCR5 for productive infection of macrophages by HIV-1 correlates with the observation that an increase in CCR5 expression in monocyte-derived macrophages (MDMs) during the first week after isolation from the blood coincides with an increase in permissiveness for HIV-1 (8–10). It is of particular note that patients presenting a genetically mutated CCR5 have been shown to be resistant to HIV-1 infection despite multiple exposures to the virus (11–13). In addition to the key role played by chemokines and their receptors in HIV pathogenesis (14), they regulate hematopoiesis (15, 16), tumor cell growth (17), and angiogenesis (18, 19).

Bacterial LPS, a major constituent of the cell wall of Gram-negative bacteria (20), is a potent stimulus for macrophages. Exposure to low levels of LPS activates multiple macrophage effector functions that serve to coordinate host-protective immune and inflammatory responses. However, increased exposure to LPS drives

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macrophage activation, which leads to tissue injury and, in extreme cases of endotoxemia, to circulatory collapse, multiorgan failure, shock, and death (21–23). Several recent studies have demonstrated that LPS treatment protects primary macrophages from productive HIV-1 infection in vitro (24–27). In agreement with these previous reports, we describe in this paper that preincubation of MDM with LPS markedly decreased HIV-1 infection via inhibition of viral entry. We further demonstrate that the inhibitory effect of LPS was secondary to a direct and rapid down-regulation of CCR5 receptors from the cell surface, which was independent of de novo protein synthesis. Low levels of cell-surface chemokine receptor expression were maintained for an extended period compared with down-regulation mediated by binding of natural ligands. These results suggest that receptor down-regulation is the main mechanism of LPS-mediated resistance of macrophages to HIV-1 infection.

Materials and Methods

Reagents

HIV-1AAda was obtained from Dr. H. E. Gendelman (Department of Pathology and Microbiology, University of Nebraska Medical Center, Omaha, NE) (28). HIV-1 primary isolates 92US660, 92US657, and 92U021 were obtained from the AIDS Research and Reference Reagent Program (Rockville, MD). LPS from Escherichia coli 0127:BB was purchased from Difco (Detroit, MI). The cell culture medium DMEM was obtained from Life Technologies (Gaithersburg, MD). Normal human serum was obtained from Difco (Detroit, MI). The cell culture medium DMEM was obtained from Life Technologies (Gaithersburg, MD). Normal human serum was obtained from Difco (Detroit, MI). FBS (10% heat-inactivated normal human serum, 2 mM glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin) was obtained from the AIDS Research and Reference Reagent Program. Cell culture medium was DMEM (Life Technologies). Dinitrophenyl chloroformate was from DuPont NEN (Boston, MA). Fura 2-acetoxymethyl ester was from Molecular Probes (Eugene, OR). 

Isolation and differentiation of human MDMs

Human PBMCs were isolated from buffy coats of healthy seronegative donors (Long Island Blood Services, Melville, NY) by Ficoll density gradient centrifugation (Ficoll-Paque PLUS, Pharmacia Biotech, Piscataway, NJ) (29). PBMCs were cultured in Primaria flasks (Becton Dickinson, DuPont NEN, Boston, MA) and FBS was obtained from Life Technologies. Human recombinant M-CSF, the bacterial cytocheximide, and the human recombinant calcineurin calcium salt from Streptomyces conglobatus were obtained from Sigma (St. Louis, MO). BSA, fraction V, was purchased from USB-Amersham (Cleveland, OH). Human recombinant chemokines macrophage-inflammatory protein (MIP)-1α, MIP-1β, and RANTES were from PeproTech (Rocky Hill, NJ) and radiolabeled chemokines (125I)MIP-1α and (125I)MIP-1β were from DuPont NEN (Boston, MA). Fura 2-acetoxymethyl ester was from Molecular Probes (Eugene, OR).

Calcium signaling studies

Macrophages cultured in suspension for 7 days in Teflon flasks were suspended in a prewarmed (37°C) HBSS containing 2 mM Ca2+ and 10% FBS at a density of 1 x 10^6 cells/ml. Five microliters of fura 2 (from a 1-nM stock) was added and cells were incubated at 37°C for 20 min. The cell suspension was then diluted 3-fold in prewarmed HBSS containing 10% FBS for 5 min at 37°C. The cells were centrifuged for 5 min at 700 rpm and the pellet was resuspended in 2 ml of HBSS containing 2 mM Ca2+. Three aliquots of 600 μl each were dispensed in Eppendorf centrifuge tubes and spun for 5 s. Cell pellets were resuspended in fresh HBSS and transferred to luminescence spectrometer cuvets for readings. The data acquisition and analysis were performed using the software FL-winlab (Perkin-Elmer, Norwalk, CT).

Immunofluorescence microscopy

Human MDMs were obtained as described above and treated with LPS (100 ng/ml) or medium alone for 3 h. For visualization of cell-surface CCR5 expression, MDMs were subjected to the following immunostaining protocol at the end of either the 3-h incubation with LPS or the 24-h period after the removal of LPS. Cells were washed and then incubated with an unlabeled mouse monoclonal anti-CCR5 Ab (2D7) for 30 min, after which the cells were washed and resuspended in PBS. All the incubations were done with cells in suspension and carried at 4°C. Small aliquots of cells were submitted to cyto centrifugation and then analyzed using an immunofluorescence microscope (Olympus, Melville, NY) or a confocal system in an inverted microscope (PCM 2000; Nikon, Melville, NY). Acquired images were later assembled using the Microsoft PowerPoint program. In experiments analyzing intracellular as well as extracellular CCR5 expression, MDMs were first stained for cell-surface CCR5 as described above and then were incubated with a fixing and permeabilizing solution (Cytofix/Cytopern; Pharmingen, San Diego) for 20 min, washed twice with Perm/Wash (Pharmingen) solution, and then incubated again with unlabeled 2D7 Ab for 30 min, always in the presence of the permeabilizing solution. The cells were washed and incubated with a rhodamine-labeled secondary Ab for 30 min. The cells were extensively washed and resuspended in PBS. Cells were processed and analyzed for fluorescence as described above.

RNase protection assay

Total RNA was isolated (Ultraprep RNA isolation system, Biotex Laboratories, Houston, TX) from macrophages treated with LPS or medium alone at different time points after the treatment, and 2 μg of RNA was used in an RNase protection assay (Riboquant, Pharmingen). Samples were separated by SDS-PAGE and quantified by electronic radiography in a Direct Imaging system (Packard).
Results

Pre-incubation with LPS renders MDMs resistant to infection with HIV-1

A previous study demonstrated that LPS, when present both at the time of infection of macrophages with HIV-1 and during subsequent cultivation of infected cells, potently inhibited HIV-1 infection (27). To determine whether continuous exposure to LPS was required for this effect or whether brief treatment of macrophages with LPS might be sufficient to induce an antiviral state, MDMs were exposed to LPS for 2 h, then washed extensively, and cultured for various periods in fresh medium without LPS until the time of infection (time 0) with HIV-1_\text{ADA}_. A 2-h preincubation with LPS was sufficient to produce a profound and lasting inhibition of HIV-1 infection (Fig. 1). HIV infection was assessed by PCR with primers specific for the early products of reverse transcription (primers LTR/gag), and PCR product was quantified autoradiographically after Southern hybridization with ^32^P-labeled probe. PCR standards were prepared by amplification of serial dilutions of a lysate from 8E5/LAI cells, which contain one HIV-1 proviral copy per cell (44). The results are shown for one experiment of two performed with cells from different donors, each yielding comparable results.

Incubation with LPS results in a decrease of MIP-1α and MIP-1β binding in a time- and dose-dependent manner

Because CCR5 expression is required for HIV-1 infection of MDMs, ligand binding studies were undertaken to investigate whether cell-surface CCR5 expression was regulated by LPS treatment. MDMs were first treated with increasing concentrations of LPS and then analyzed for ^[125]I\text{MIP-1α} binding. A dose-dependent decrease of ^[125]I\text{MIP-1α} binding was observed with increasing LPS concentration (Fig. 2a). There is some donor variability in LPS sensitivity, therefore LPS at 100 ng/ml (a dose that abrogated binding and calcium signaling in nearly every donor) was used for all additional experiments. As shown in Fig. 2b, LPS-induced down-regulation of MIP-1α binding also correlated with time of exposure to LPS, and substantial inhibition of MIP-1α binding occurred as early as 1.5 and 2 h after the addition of LPS.

In addition to expressing CCR5, differentiated macrophages express a second MIP-1α binding receptor, CCR1, and the total ^[125]I\text{MIP-1α} binding activity observed in Fig. 2, a and b, might be
attributable to binding to one or both of those receptors (34). To specifically determine the fate of CCR5 after LPS stimulation, parallel binding studies with \([^{125}I]\)MIP-1\(\alpha\), which has been reported to bind exclusively to CCR5 receptors, were performed (4). As can be observed in Fig. 2c, preincubation of macrophages with LPS resulted in a 90% reduction of MIP-1\(\beta\) binding, confirming that CCR5-mediated chemokine binding was down-modulated as a consequence of exposure to LPS.

**LPS-mediated inhibition of MIP-1\(\alpha\) binding is maintained for an extended period of time compared with chemokine-induced down-regulation**

To study the kinetics of receptor down-regulation and reappearance at the cell surface, MDM cultures were incubated with either LPS or a mixture of chemokines, and receptor binding was analyzed as a function of time (Fig. 3, a–c). A 3-h incubation with LPS resulted in complete inhibition of \([^{125}I]\)MIP-1\(\alpha\) binding for up to 24 h after LPS was removed from the cell culture. About 40% of \([^{125}I]\)MIP-1\(\alpha\) binding activity was recovered by 48 h, and by 96 h MIP-1\(\alpha\) binding activity was fully restored. When cells were rechallenged with LPS 5 days after primary challenge, cell-surface CCR5 was again down-regulated (120 h point; Fig. 3a). To rule out the possibility that these LPS-induced changes in cell-surface CCR5 expression were a reflection of LPS-induced cytotoxicity, the viability of untreated and LPS-treated cultures was calculated. No significant effect on cell viability was observed up to 5 days after LPS stimulation (at 100 ng/ml) as measured by MTT enzymatic assay (Thiazolyl blue) or neutral red viability assay (data not shown).

In sharp contrast to the sustained down-regulation of MIP-1\(\alpha\) binding induced by LPS, ligand-mediated down-regulation of MIP-1\(\alpha\) binding, though nearly 90% at time 0 (Fig. 3b), was rapidly reversed after the removal of chemokines (Fig. 3c). The inhibition of MIP-1\(\alpha\) binding observed upon restimulation with a fresh mixture of chemokines 5 days (120 h) after removal of the initial chemokine-conditioning stimulus shows that the cultures had fully regained their chemokine sensitivity (see Fig. 1).

**LPS abrogates calcium signaling responses to chemokines and prolongs cell desensitization to chemokines**

To assess whether LPS-mediated down-regulation of the ligand-binding activity of CCR5 chemokine receptor affected intracellular calcium signaling induced by chemokine binding, calcium concentrations were estimated in LPS-treated macrophages in response to MIP-1\(\alpha\) or MIP-1\(\beta\) by a fluorometric method. Preincubation of cells with LPS for 2 h completely abrogated the calcium response elicited by MIP-1\(\alpha\) (Fig. 4a) or MIP-1\(\beta\) (Fig. 4b). To evaluate the kinetics of this effect, calcium signaling studies were performed at different times after an LPS preincubation period. As illustrated in Fig. 4c, the cells exposed to LPS remained unresponsive to exogenous chemokines 24 h after LPS exposure (at which time chemokine binding is still undetectable (Fig. 3a)), and an attenuated response was seen after 48 h. By 72 h after LPS exposure, calcium signaling responses to exogenous chemokine (MIP-1\(\beta\)) were approaching normal. Therefore, LPS reduces the number or the functional activity of CCR5 receptors on macrophages.

**LPS-mediated internalization of CCR5 analyzed by immunofluorescence microscopy**

To directly analyze the effect of LPS treatment on CCR5 expression at the single-cell level, macrophages were stained with FITC-labeled mAb (2D7) specific for CCR5 protein to identify cell-surface receptors. The same cells were then permeabilized and stained with unlabeled 2D7 and then with secondary rhodamine-labeled anti-mouse Ig to detect newly exposed intracellular CCR5. Samples were evaluated by fluorescence microscopy and quantitatively analyzed using an imaging system (Metamorph; Universal Imaging, West Chester, PA). Fig. 5 compares dual staining in untreated (top panel) and LPS-treated cells (bottom panel) and shows a substantial decrease in membrane staining (green) in conjunction with an increase in internal staining (red).

**FIGURE 3.** Analysis of receptor recycling. MDM cultures were pre-incubated with 100 ng/ml LPS (a) or with a mix of chemokines containing 50 nM each of MIP-1\(\alpha\), MIP-1\(\beta\), and RANTES (b) for 3 h. Control samples were incubated with complete medium alone. At the end of the preincubation, the cells were extensively washed, and incubation with \([^{125}I]\)MIP-1\(\alpha\) was initiated immediately after washing (time 0) or at indicated time points. At the 120-h-time point, the cells were restimulated with a fresh solution of LPS or chemokines. In the experiment represented in c, MDM cultures were incubated with 0.5 nM of MIP-1\(\alpha\) for 1 h, after which it was removed by extensive washing before being returned to 37°C. Cell samples were submitted to binding with \([^{125}I]\)MIP-1\(\alpha\) at 2, 5, 10, and 30 min after the removal of MIP-1\(\alpha\). The bars show the mean ± SD percentage of MIP-1\(\alpha\) binding with the control representing 100%. The graphs present the average of specific binding obtained with cells from two different donors, each assessed in duplicate.
FIGURE 4. LPS abrogates calcium signaling responses of monocyte-derived macrophages to MIP-1α and MIP-1β. 

(a) and (b), Monocyte-derived macrophage cultures were incubated in the presence or absence of 100 ng/ml of LPS for 3 h. After loading with fura 2 (see Materials and Methods), cells were analyzed in a luminescence spectrometer. After a baseline was obtained, MIP-1α (a) or MIP-1β (b) at 200 nM was added to the cell suspension. Increases in cytoplasmic calcium lead to fura 2 luminescence, which was recorded with the aid of a Perkin-Elmer program (FL Winlab). The luminescence ratio (340/380 nm) is proportional to intracellular free calcium levels. The increase in cytoplasmic calcium elicited by the addition of the ionophore, ionomycin, demonstrates that the cells were efficiently loaded with fura 2 and do not exhibit “leakage” effects. 

(c), Cells were prepared as in a except that loading with fura 2 was performed at 0, 24, or 72 h after the end of the 3-h LPS treatment period. Calcium signaling responses were analyzed after the addition of MIP-1β.
LPS-dependent inhibition of HIV infection is sustained after removal of LPS

To determine whether LPS-induced down-regulation of CCR5 correlated with susceptibility of macrophages to HIV-1 infection, we inoculated LPS-stimulated MDM cultures with HIV-1 at various intervals after pretreatment with LPS. Viral entry was evaluated by PCR using primers specific for the early products of reverse transcription (R/U5). As shown in Fig. 7, susceptibility of cells to HIV-1 infection was inhibited during the first 24 h after LPS stimulation but returned to control levels by 72 h, which is in good correlation with the kinetics of CCR5 recovery. In additional experiments in which the 48-h time point was analyzed, viral entry was still 50% inhibited compared with control (data not shown).

LPS-mediated down-regulation of CCR5 does not require de novo protein synthesis

LPS induces general stimulation of macrophages and promotes, among many other activation-specific responses, secretion of the proinflammatory cytokines TNF and IL-1 as well as of the chemokines MIP-1α and MIP-1β. These LPS-induced mediators could modulate macrophage expression of CCR5 in an autocrine manner, as has been suggested previously (35). Our observation that after a 1-h exposure to LPS (at a time when MIP-1 peptide levels were undetectable by ELISA; data not shown) MIP-1α binding was inhibited by 60% (Fig. 2b) suggested that the down-regulation occurred too rapidly to be mediated by chemokine secretion, which occurs over a 3- to 6-h time course. To further investigate possible mechanisms by which LPS might affect CCR5 receptor expression and activity, cells were incubated with either media alone or LPS in the presence or absence of the protein synthesis inhibitor cycloheximide. Binding activity for [125I]MIP-1β was then assessed as a measure of cell-surface CCR5 expression. Cells treated with LPS showed significantly decreased binding of MIP-1β, whether stimulated with LPS in the presence or absence of cycloheximide (Fig. 8a). Control cells not exposed to LPS exhibited the same MIP-1β binding activity in the presence or absence of cycloheximide. Further addition of a chemokine mixture containing MIP-1α, MIP-1β, and RANTES at 10 nM each to cultures exposed to LPS in the presence of cycloheximide did not further reduce MIP-1β binding (Fig. 8a). Therefore, the LPS-mediated CCR5 down-regulation in macrophages as a consequence of LPS exposure does not require synthesis of new protein.

To further investigate whether LPS-induced mediators could be responsible for the prolonged LPS-initiated down-regulation of chemokine receptors, [125I]MIP-1α binding studies were performed on MDMs that were incubated with LPS for 3 h, washed free of LPS, and incubated for 24 h, at which time they were again washed and replenished with medium alone or medium containing cycloheximide for an additional 3 h. In case secreted chemokines were involved in CCR5 down-regulation, it would be expected that the last 3-h incubation without cycloheximide would result in receptor down-regulation, whereas in the presence of cycloheximide no CCR5 down-regulation would be observed. However, comparable levels of inhibition of MIP-1α binding between the MDMs replenished with medium in the presence or absence of cycloheximide was observed (Fig. 8b). This result demonstrates that new protein synthesis is not required for the LPS-induced prolonged down-regulation of CCR5.

FIGURE 5. Preincubation of MDMs with LPS down-regulates surface expression of CCR5. Treatment with LPS induces internalization of surface-exposed CCR5. The top panel shows control (untreated) MDMs, whereas the bottom panel shows MDMs pretreated with LPS for 3 h (see Materials and Methods). Untreated and LPS-treated cells were first stained with unlabeled monoclonal 2D7 and then with FITC-labeled anti-mouse IgG Ab (membrane, green) and then were permeabilized and stained for intracellular CCR5 with anti-CCR5 and rhodamine-labeled anti-mouse IgG Ab (cytoplasmic, red).

with stronger intracellular staining (red) in LPS-treated cells. Therefore, we conclude that LPS induces internalization of CCR5.

To assess whether LPS-mediated down-regulation of cell-surface CCR5 is comparable to ligand-induced down-regulation, MDM cultures were incubated either with a mixture of chemokines or with LPS for 3 h and then analyzed for cell-surface CCR5 expression by immunofluorescence. As seen in Fig. 6a (compare left panel and middle panel), a 3-h incubation with chemokines results in down-regulation of cell-surface CCR5. Similar results are observed after a 3-h incubation with LPS (compare left panel and middle panel of Fig. 6b). But surprisingly, cell-surface expression of CCR5 was still completely absent in the LPS-treated cells 24 h after removal of LPS, whereas surface expression in ligand-treated cells was recovered 24 h after chemokine was removed from the media. To further characterize the prolonged LPS-induced down-modulation of CCR5 expression, MDM cultures were stimulated with LPS for 3 h, washed, and then replenished with fresh media. Twenty-four hours after removal of LPS, cells were permeabilized and stained for CCR5 by first incubating with unlabeled primary Ab (2D7) and then incubating with a rhodamine-labeled secondary anti-IgG Ab. The intensity of intracellular staining for CCR5 observed in the cell population that had LPS removed 24 h earlier was comparable to the group that was stained immediately after the 3-h incubation with LPS (Fig. 6c). The control represents untreated cells that show a stronger CCR5 staining at the membrane. These results demonstrate that CCR5 surface receptors remain in the cytoplasmic compartment for a prolonged period of time after LPS-induced internalization and suggest that the receptors are not being targeted for degradation for at least 24 h after LPS stimuli is removed.
LPS mediates a slight early down-regulation and a more pronounced late up-regulation of the mRNA for CCR5

To determine the effect of LPS treatment on CCR5 mRNA expression, the levels of CCR5 mRNA were analyzed in LPS-stimulated macrophage cultures and compared with CCR5 mRNA levels in cells not exposed to LPS. Levels of CCR5 mRNA were found to be slightly decreased after LPS stimulation, showing a return to normal levels by 7 h after the addition of the endotoxin stimulus (Fig. 9). From this time until 75 h after LPS stimulation, message levels were higher than those in controls. In all experiments, a decrease in CCR5 mRNA was also observed for the control at the 3-h time point, returning to normal levels at later time points. All results were normalized according to GAPDH mRNA signals. Thus, we conclude that LPS-induced down-regulation of CCR5 is not mediated by reduction of CCR5 mRNA expression.
with IMIP-1 allowed to recover for 3 h at which time the cells were submitted to binding with fresh media supplemented with cycloheximide 24 h later and were replenished with fresh media. Cells which had been washed and replenished with fresh media were again washed and replenished with fresh media or with media supplemented with cycloheximide. A separate set of cells received, in addition to cycloheximide, LPS (100 ng/ml). The bars represent the average percent binding of IMIP-1 to LPS induced a sustained down-regulation of CCR5 receptors, presented in this paper indicate that stimulation of macrophages with LPS (100 ng/ml) was added to cell cultures for 3 h. Cell samples were collected at indicated time points after the stimulation for RNA purification. Two micrograms of total RNA was used in an RNase protection assay, the product was separated on an SDS-PAGE sequencing gel, and the corresponding bands for CCR5 and GAPDH were quantified on a Packard Direct Imager. Bar graph represents the quantification of the specific bands for CCR5. The data are presented for one representative experiment of five performed with cells from different donors.

Discussion

Monocytes/macrophages are major targets for HIV-1 at early stages of infection. These cells play an important role in disease progression, functioning as stable reservoirs of virus production and facilitating the transmission of virus to CD4 lymphocytes. Chemokine receptor CCR5 appears to be the main coreceptor on macrophages for HIV-1 entry (36, 37). Several lines of evidence presented in this paper indicate that stimulation of macrophages with LPS induced a sustained down-regulation of CCR5 receptors, resulting in marked resistance to HIV-1 infection. Loss of cell-surface CCR5 was demonstrated by reduced binding of its natural ligands, by inhibition of chemokine-induced Ca$^{2+}$ flux, by decreased cell-surface expression of CCR5 and a concomitant increase in intracellular CCR5-specific staining, and by inhibition of HIV-1 infection at the step of virus entry.

Consistent with the above results, LPS added together with HIV-1 in macrophage cultures has been shown to increase viral entry into those cells, as assessed by accumulation of early products of reverse transcriptase (27). The observed increase in viral entry under these conditions might be explained by the overt LPS-induced internalization of CCR5, which would facilitate the transport of surface-bound HIV-1 to the intracellular compartment.

One possible explanation for the loss of cell-surface CCR5 seen after LPS treatment is that chemokines, which are secreted by the macrophages as a consequence of LPS stimulation, act in an autocrine fashion to down-modulate their own receptors. And, in fact, a recent report suggests that LPS-induced down-regulation of cell-surface CCR5 on macrophages is mediated by CC chemokines released as a consequence of activation (35). Our results argue against this hypothesis. Indeed, a 50% reduction of MIP-1$\alpha$ binding was observed as early as 1 h after the addition of LPS (Fig. 2b), when no chemokines were detected in the culture medium (data not shown). This suggests that de novo protein synthesis is not required for promoting the inhibitory effect. Further proof that down-regulation of CCR5 is chemokine release-independent was obtained in an experiment in which macrophages were stimulated with LPS in the presence of cycloheximide, a specific inhibitor of protein synthesis. Cycloheximide completely blocked synthesis and secretion of chemokines, as measured by ELISA (data not shown). Still, LPS-induced down-regulation of CCR5 (as judged by MIP-1$\beta$ binding) in cycloheximide-treated cells was comparable to that in control cultures and was not increased by exogenously added CC chemokine mixture (Fig. 8a), indicating that LPS is directly responsible for the down-regulation of CCR5. Interestingly, exogenously added chemokines reduced binding of MIP-1$\alpha$ to LPS-stimulated, cycloheximide-treated macrophages by ~50% (data not shown). Because MIP-1$\alpha$ binds to CCR1 as well as to CCR5 on macrophages, this result suggests that although for some receptors (e.g., CCR5) direct LPS-induced internalization constitutes the main mechanism of receptor deactivation, other receptors (e.g., CCR1) might be deactivated through secondary mechanisms such as LPS-stimulated chemokine secretion.

Surprisingly, recovery of CCR5 on the cell surface after LPS-mediated down-regulation was markedly delayed compared with
receptor recovery after exposure to a natural ligand, MIP-1α. Indeed, 100% inhibition of MIP-1α binding was observed 24 h after LPS treatment (Fig. 3a), whereas ~80% of the original binding was restored within 30 min after removal of MIP-1α (Fig. 3c). β Chemokine-induced CCR5 down-regulation and recovery was similar to that observed for CXCR4 in response to stromal cell-derived factor-1α (38). The delay in CCR5 surface re-expression after LPS treatment did not appear to be due to continuous down-regulation of chemokine receptors by LPS-induced release of chemokines in long term culture. This was demonstrated by the sustained down-regulation of MIP-1α binding in MDM cultures that had been stimulated with LPS 24 h in advance of receiving fresh media containing cycloheximide for 3 h before carrying out the binding experiments. If chemokine expression were responsible for down-regulation of CCR5 surface expression, cycloheximide would be expected to block further chemokine expression and 3 h should have sufficed to allow re-appearance of CCR5 at the cell surface. The failure of MIP-1α binding to be restored after LPS-induced mediators were removed from the culture and synthesis of new inflammatory mediators was blocked by cycloheximide demonstrated that the observed prolonged down-regulation of CCR5 is not chemokine-dependent (Fig. 3b). Although we have observed donor variability in both cell-surface expression of CCR5 and magnitude of calcium signaling response after chemokine stimulation, sustained LPS-dependent down-regulation of surface CCR5 was observed in all donors tested.

Immunofluorescence analysis of CCR5 receptor expression (Figs. 5 and 6) immediately after and 24 h after chemokine or LPS treatment indicate that CCR5 is rapidly internalized in response to both stimuli but that in the case of ligand-induced internalization, cell-surface receptor expression is quickly restored, whereas in the case of LPS-induced internalization, the receptor remains degraded and sequestered within the cell for >24 h. These results suggest that LPS treatment induces an alternate route of intracellular trafficking of the receptors after internalization. It is important to note that the striking difference in the recovery rate of cell-surface CCR5 after LPS- vs chemokine-induced down-regulation might be even more significant if we consider that the recovery of surface receptors after chemokine treatment apparently occurs between 45 and 60 min, as suggested by earlier binding studies (Fig. 3c). This is not an unprecedented phenomenon because a similar inhibition of CCR5 recycling after internalization recently has been reported for a modified form of RANTES (aminooxypentane-RANTES) (39). These results suggest that chemokine receptors can undergo differential intracellular pathways after the internalization process, depending on the nature of the triggering stimuli.

Although the molecular mechanisms by which LPS down-regulates surface expression of chemokine receptors are not yet defined, immunofluorescence analysis (Fig. 5) suggests that they involve internalization of receptors. It has been documented that LPS, via activation of p38 mitogen-activated protein kinase, can induce rearrangement of actin filaments and thus promote endocytosis (40, 41). Experiments analyzing whether CCR5 colocalizes with markers of endosomes and/or lysosomes are in progress and are expected to reveal whether LPS-mediated down-regulation of this receptor occurs via enhanced endocytosis or whether another mechanism is involved. In addition, such experiments may elucidate the fate of the receptor after its internalization.

Interestingly, in contrast to previous work by others (42), there was no significant decrease in CCR5 mRNA levels in LPS-treated compared with control macrophages (Fig. 9). On the contrary, message levels for CCR5 progressively increased starting 7 h after LPS stimulation, returning to levels comparable with controls in the subsequent 30 h. This temporary increase in CCR5 mRNA may be important for eventual replenishing of the receptor on the cell membrane, which is observed around 96 h after the removal of LPS. The absence of CCR5 mRNA down-regulation supports the hypothesis that LPS-induced down-modulation of CCR5 is through inhibition of recycling and/or trafficking of the receptors to the membrane.

The results presented herein suggest a model whereby CCR5 receptors on macrophages are down-modulated in response to LPS exposure independently of autocrine chemokine stimulation, leading to resistance to HIV-1 infection. In addition, the data strongly suggest the use of a pathway of chemokine receptor recycling that differs from recycling initiated by ligand binding and delays the return of biologically active CCR5 to the cell surface. In a recent publication, LPS was shown to induce a prolonged down-modulation of CXCR1 and CXCR2 in macrophages through a tyrosine kinase-dependent pathway (43). Whether such a pathway is involved in LPS-dependent CCR5 down-modulation remains to be evaluated. Preliminary experiments in our lab indicate that LPS-mediated down-modulation of surface CCR5 is a specific effect because the cell-surface expression of the FMLP receptor, another G protein-coupled seven-transmembrane receptor, was not affected by LPS treatment (data not shown). In addition, our results suggest that CCR1 and CCR5 (receptors for MIP-1α) have different sensitivity to LPS. It will be important to determine whether this effect is restricted to LPS or whether other macrophage activators elicit the same response. Our results raise the important clinical question of whether certain states of tissue macrophage activation (e.g., concomitant Gram-negative bacterial infection) render such cells more resistant to HIV infection, thus slowing the spread of the virus. Further studies on the elucidation of the unique intracellular pathway that CCR5 undergoes as a consequence of LPS stimulation will provide a more complete understanding of the intricacies of CCR5 receptor regulation and may reveal new targets for anti-HIV therapies.

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