C–C Chemokines Released by Lipopolysaccharide (LPS)-stimulated Human Macrophages Suppress HIV-1 Infection in Both Macrophages and T Cells

By Alessia Verani, Gabriella Scarlatti, Manola Comar, Eleonora Tresoldi, Simona Polo, Mauro Giacca, Paolo Lusso, Antonio G. Siccardi, and Donata Vercelli

From the Molecular Immunoregulation, Lymphocyte Differentiation, and Human Virology Unit, Department of Biological and Technological Research, San Raffaele Scientific Institute, 20132 Milan, and International Center for Genetic Engineering and Biotechnology, 34012 Trieste, and Department of Biology and Genetics, University of Milan, 20100 Milan, Italy

Summary

Human immunodeficiency virus-1 (HIV-1) expression in monocyte-derived macrophages (MDM) infected in vitro is known to be inhibited by lipopolysaccharide (LPS). However, the mechanisms are incompletely understood. We show here that HIV-1 suppression is mediated by soluble factors released by MDM stimulated with physiologically significant concentrations of LPS. LPS-conditioned supernatants from MDM inhibited HIV-1 replication in both MDM and T cells. Depletion of C–C chemokines (RANTES, MIP-1α, and MIP-1β) neutralized the ability of LPS-conditioned supernatants to inhibit HIV-1 replication in MDM. A combination of recombinant C–C chemokines blocked HIV-1 infection as effectively as LPS. Here, we report an inhibitory effect of C–C chemokines on HIV replication in primary macrophages. Our results raise the possibility that monocytes may play a dual role in HIV infection: while representing a reservoir for the virus, they may contribute to the containment of the infection by releasing factors that suppress HIV replication not only in monocytes but also in T lymphocytes.
Materials and Methods

Reagents. PE-conjugated anti-CD14 mAb P9 (anti-Leu-M3, IgG2b) and an isotype control were purchased from Becton Dickinson (Mountain View, CA). A neutralizing rat anti-human IL-10 mAb (J53-19F1, IgG2a) was a gift from Dr. J. Abrams (DNAX Research Institute, Palo Alto, CA). rTNF-α, recombinant C-C chemokines (RANTES, MIP-1α, and MIP-1β), and neutralizing goat polyclonal antibodies against IL-1 receptor antagonist (IL-1Ra: neutralizing dose, ND₅₀ = 5–10 μg/ml), MIP-1α (ND₅₀ = 10 μg/ml), MIP-1β (ND₅₀ = 40 μg/ml), and RANTES (ND₅₀ = 100–200 μg/ml) were obtained from R&D Systems (Minneapolis, MN). The mAbs used in the ELISA assay for soluble TNF receptor 1, and in the immunofluorescence analysis of membrane TNF-α expression were provided by Dr. A. Corti (Department of Biological and Technological Research, San Raffaele Scientific Institute). Concentrations of TNF-α, IL-6, MIP-1α, MIP-1β, and RANTES in culture supernatants were determined by ELISA (Quantikine, R&D Systems).

LPS from Salmonella minnesota and purified goat IgG were purchased from Sigma Chemical Co. (St. Louis, MO). The endotoxin content of all cell culture reagents was assayed by the Limulus amebocyte lysate assay (Whittaker Bioproducts, Walkersville, MD), and was always <0.125 EU/ml. Polymixin B sulfate was purchased from Calbiochem-Novabiochem (La Jolla, CA).

Isolation of MDM and HIV-1 Infected. PBMC were isolated by Ficoll-Hyphaque (Pharmacia Uppsala, Sweden) density gradient centrifugation from Buffy-coat preparations obtained from healthy donors. The cells were then resuspended in RPMI-1640 medium supplemented with 10% AB serum (Sigma), 20% FCS (Biological Industries, Israel), 2 mM glutamine, 50 μg/ml streptomycin, and 100 U/ml penicillin, and cultured at a concentration of 1 × 10⁶ cells/cm² for 5 d at 37°C in 6-well tissue culture plates (Nunc, Roskilde, Denmark), in a 3 ml volume. Non-adherent cells were then removed by extensive washing with medium. The MDM preparations contained >90% CD14⁺ cells, as assessed by immunofluorescence.

Cells were infected with the monocytotropic HIV-1²⁹⁶(lo viral strain (tissue culture infectious dose₅₀, TCID₅₀, 110/10⁶ MDM) grown in primary MDM and never previously passaged in continuous cell lines, or with HIV-1²⁹⁶(lo grown in PHA-activated PBMC (TCID₅₀, 45/10⁶ MDM). Furthermore, cells were infected with primary viral isolates (HIV-1²⁹⁶(lo and HIV-1₁₈₁₉) with the biological characteristics of non-syncytium-inducing (NSI) strains.

For infection, MDM were incubated with the viral strains at a concentration of 500 pg/ml of p24 Ag in RPMI-1₆₄₀, 20% FCS, in a total volume of 2 ml of cell-free viral supernatant. After overnight incubation, unbound virus was removed by extensive washing, fresh medium (3 ml) was added, and the cultures were further incubated at 37°C. Supernatants were harvested every day for p24 Ag detection and reverse transcriptase (RT) determination. Culture medium was fully replaced every 3–4 d, without washing.

Isolation of Lymphocytes and HIV-1 Infection. Normal peripheral blood lymphocytes depleted of monocytes by two cycles of adherence to plastic were activated by a 3-d incubation with PHA (1.5 μg/ml; Sigma). The resulting PHA blasts were collected, resuspended at 2.5 × 10⁶ cells/ml in medium containing 10% FCS, and supplemented with polybrene (2 mg/ml; Sigma) and IL-2 (10 U/ml; Amersham, Buckinghamshire, UK), and incubated overnight in the presence of HIV-1²⁹⁶(lo. Subsequently, free virus was removed by washing twice in RPMI-1₆₄₀, and the cells (1.5 × 10⁶/ml) were cultured in 6-well plates in the presence of IL-2. Culture supernatants were harvested every 3–4 d, and tested for the presence of HIV-1 p24 Ag by ELISA.

Preparation of LPS-conditioned and Monokine-depleted Supernatants. LPS-conditioned supernatants were prepared by incubating cultures of normal uninfected MDM in the presence or absence of LPS (1 μg/ml). 2 d later, supernatants were harvested, centrifuged, and stored at −20°C until used. To deplete LPS-conditioned supernatants of chemokines (MIP-1α, MIP-1β, RANTES), petri dishes were coated for 2 h at room temperature with neutralizing antibodies in PBS, at concentrations (10–30 μg/ml) expected to neutralize the amounts of cytokines found in culture supernatants. Control plates were coated with normal goat IgG (55 μg/ml). LPS-conditioned supernatants were incubated in the sensitized dishes overnight at 37°C, then collected and used immediately.

HIV-1 Detection. HIV-1 p24 Ag concentrations in the culture supernatants were determined by ELISA (17). In brief, p24 Ag from a detergent lysate of virions was captured by an immobilized anti-p24 Ag polyclonal antibody (D7320; Aalto Bio Research, Dublin, Ireland). Bound p24 Ag was then detected using an alkaline phosphatase-conjugated anti-p24 Ag monoclonal antibody (BC 1071; Allo Bio Research) and the AM-Pak ELISA amplification system (Dako A/S, Glostrup, Denmark).

RT activity in the supernatants of HIV-infected MDM was assayed as described in reference 18. In brief, 10 μl of cell-free culture supernatants were added to 50 μl of a mixture containing poly(A), oligo (dT) (Pharmacia), MgCl₂, and 3P-labeled deoxynucleoside triphosphate (dNTP) (Amersham) in a 96-well V-bottomed microtiter plate, and incubated 1.5 h at 37°C. 5 μl of the RT reaction mixture were then dotted onto DE81 paper (Whatman, Maidstone, England), dried, washed, and subsequently counted on a microplate scintillation counter (Packard Instrument Co., Meriden, CT).

Immunofluorescence. Expression of CD14 was detected by direct immunofluorescence, as previously described (19). Cultured monocytes and/or MDM in staining buffer (RPMI-1₆₄₀, 10% AB serum, containing 0.01% sodium azide) were incubated with fluorochrome-conjugated p9 mAb or isotype control for 40 min at 4°C. The cells were then extensively washed and fixed in 2% paraformaldehyde. Percentages of positive cells and mean fluorescence intensity (MFI) were analyzed by a FACScan (Becton Dickinson) gating on the monocyte population, as defined by forward and side light scatter.

Competitive PCR Amplification. This procedure was described in detail elsewhere (20). In brief, total RNA was extracted according to the guanidine thiocyanate procedure (21), and treated with RNAase-free DNase I (Boehringer, Mannheim, Germany) to remove traces of contaminating DNA. First-strand cDNA synthesis was obtained by priming with random hexamers and reverse transcription in 20 μl of RT mix containing 75 mM KCl, 50 mM Tris-HCl (pH 8.3), 3 mM MgCl₂, 0.4 mM each dNTP (Pharmacia), 400 U Moloney murine leukemia virus (MMLV)-RT (Promega, Madison, WI), 20 U RNasin (Promega). RNA was preheated at 65°C for 5 min and incubated with the reaction mix at 37°C. After 1 h, the reaction was stopped by incubation at 95°C for 5 min and samples were cooled on ice. Amplification of CC–CKR-5 cDNA was performed using primers CCR-9 (5'-CATCAT-CCTCTCTGACAATCG) and CCR-10 (5'-ATGGTGAAGATAGCCCTCACAG). Quantification of CC–CKR-5 mRNA levels in MDM was carried out by a competitive PCR procedure using a competitor DNA fragment carrying the primer recognition sites for β-actin (BA-1 and BA-4 [22]) and for CCR-5 (primers CCR-9 and CCR-10). β-actin is used as a standard to monitor the efficiency of total DNA extraction. A schematic representation of this competitor is shown in Fig. 6 A and its construction is described in the legend to Fig. 6.
Competitive PCR amplifications were carried out by adding to the sample increasing concentrations of the competitive templates, in 100 μl of PCR buffer (50 mM KCl, 10 mM Tris-HCl, 2 mM MgCl₂) containing the two primers (100 pmol each), the four dNTPs (200 μM each), and 2.5 U of Taq DNA polymerase (Perkin Elmer, Emeryville, CA). Samples were submitted to 50 cycles of amplification with the following cycle profiles: denaturation at 95°C for 30 s, annealing at 60°C (primer sets CC-CKR-5 and β-actin) for 30 s, extension at 72°C for 30 s. After amplification, 10 μl of each PCR reaction were resolved on a 8% non-denaturating polyacrylamide gel, visualized under UV light after ethidium bromide staining and photographed. Quantification of the amplification products was obtained by densitometric scanning.

Determination of Viral DNA Load by Semiquantitative PCR. High molecular weight DNA was extracted from MDM cultures exposed to HIV-HH1Ba-L for 14 h by overnight incubation at 37°C in lysis buffer (10 mM Tris-HCl, pH 8.3, 1 mM EDTA, 0.5% Nonidet-P-40, 0.5% Tween 20, 0.3 mg/ml protease K) followed by extraction with a phenol/chloroform/isamyl alcohol mixture (25:24:1) and ethanol precipitation. Samples were amplified using a sets of nested primers specific for the outer primer set, JA79/82; inner primer set, JA 80/81), as previously described (23). Viral DNA load was determined by progressively diluting the samples, and testing each dilution in five parallel reactions. As a control, HIV-1-infected cells were diluted in uninfected cells so as to contain 10 and 1 HIV-1 viral DNA copies per 10⁶ MDM donors were infected in vitro with the monocytotropic HIV-1Ba-L strain, in the presence or absence of LPS (1 μg/ml). MDM were washed 1 d later and further cultured, adding LPS every 3 d. Culture supernatants were harvested daily, and tested for p24 Ag secretion by ELISA. The data are representative of 10 (A), 3 (B), and 2 (D) separate experiments. In (C) MDM were infected with HIV-1Ba-L or HIV-15088 in the presence of decreasing concentrations of LPS. p24 Ag secretion was assessed 5 d after infection.

**Results**

LPS Suppresses HIV-1 Replication in MDM Cultures Infected in Vitro. To characterize the effects of LPS on the replication of HIV-1 in monocytic cells, MDM from normal donors were infected in vitro with the monocytotropic HIV-1Ba-L strain, in the presence or absence of LPS (1 μg/ml). Fig. 1 A shows that p24 Ag secretion in untreated MDM cultures rapidly reached high levels, which were maintained for over 10 d. In contrast, p24 Ag secretion by LPS-treated MDM remained extremely low throughout the culture time. RT activity in the same cultures showed a similar pattern (data not shown). Fig. 1 B shows that LPS-dependent inhibition of p24 Ag secretion was also observed in MDM cultures infected in vitro with HIV-15088, a primary isolate from an asymptomatic HIV-1-infected patient with the biological characteristics of an NSI isolate. LPS had a potent inhibitory effect on the replication of both HIV-1Ba-L and HIV-15088. Fig. 1 C shows that p24 Ag secretion was inhibited by >70% using LPS at a concentration of 1 ng/ml. Notably, inhibition was still apparent when LPS was added at 10 μg/ml, a physiologically significant concentration (13). Interestingly, LPS addition did not inhibit HIV-1 expression in MDM cultures infected with the SI laboratory strain, HIV-111B (Fig. 1 D). The surprisingly high levels of replication of our HIV-111B in MDM are likely to result from multiple passages of the viral stock in human primary PBMC. Addition of LPS did not result in significant cell death, nor in apoptosis, as assessed by Trypan blue or propidium iodide staining (data not shown).

LPS-induced inhibition of HIV-1 replication was dependent on the time of addition of LPS to the culture. Fig. 2 shows that HIV-1 expression was completely blocked when LPS was added at the time of infection or 1 d later, but was progressively less affected when LPS was added 2 or 3 d after infection with HIV-1. Notably, viral replication was completely inhibited by pretreating MDM with LPS for 48 h before infection. However, the inhibitory effect of LPS pretreatment was abolished if the cells were subsequently washed before virus addition (data not shown). These data suggest that LPS interferes with early events in HIV-1 infection.

The Expression of CD14, the LPS Receptor, Is Upregulated in LPS-Treated, HIV-1-Infected MDM. LPS has been shown to upregulate the expression of its own receptor, CD14, in whole blood (25). Therefore, we asked whether a modulation of CD14 expression may contribute to the effects of LPS on HIV-1 replication in MDM. Immunofluorescence analysis of MDM cultures 2 d after HIV-1 infection showed that CD14 expression was upregulated not only in uninfected, LPS-treated MDM, but also in vitro HIV-1-infected, LPS-untreated cells (Fig. 3). Interestingly, LPS and HIV-1 synergized in upregulating CD14 expression. These data suggest that the combined effects that HIV-1 infection and LPS stimulation have on CD14 expression may amplify the LPS-induced, CD14-mediated suppression of HIV-1 replication.

LPS-induced HIV-1 Suppression Is Not Mediated by an Effect on the Secretion of IL-6 and TNF-α. A number of cy-
Chemokines have been described that regulate HIV-1 expression. In particular, TNF-α and IL-6 enhance HIV-1 replication in acutely infected MDM. The HIV-1–inducing effect of TNF-α is mainly, if not exclusively, mediated by the activation of NF-κB, which activates LTR-driven viral RNA transcription (26). IL-6 induces expression of viral proteins and RT activity to levels comparable to those induced by TNF-α, but unlike TNF-α, does not increase significantly the levels of steady-state viral mRNA (27). Therefore, we investigated whether a decrease in the production of these HIV-1 stimulatory cytokines may underlie LPS-dependent inhibition of HIV-1 replication in MDM. The HIV-1–inducing effect of TNF-α is mainly, if not exclusively, mediated by the activation of NF-κB, which activates LTR-driven viral RNA transcription (26). IL-6 induces expression of viral proteins and RT activity to levels comparable to those induced by TNF-α, but unlike TNF-α, does not increase significantly the levels of steady-state viral mRNA (27). Therefore, we investigated whether a decrease in the production of these HIV-1 stimulatory cytokines may underlie LPS-dependent inhibition of HIV-1 replication in MDM. The HIV-1–inducing effect of TNF-α is mainly, if not exclusively, mediated by the activation of NF-κB, which activates LTR-driven viral RNA transcription (26). IL-6 induces expression of viral proteins and RT activity to levels comparable to those induced by TNF-α, but unlike TNF-α, does not increase significantly the levels of steady-state viral mRNA (27). Therefore, we investigated whether a decrease in the production of these HIV-1 stimulatory cytokines may underlie LPS-dependent inhibition of HIV-1 replication in MDM. Fig. 4 shows that LPS-induced IL-6 secretion was vigorous and comparable in both uninfected and HIV-1–infected MDM cultures. In contrast, infected cultures treated with LPS showed an impairment in their ability to sustain TNF-α secretion over time. However, stimulation with LPS released high and comparable levels of TNF-α (>40 ng/ml) from uninfected and infected cells at the initiation of the culture, before removal of unbound virus. The decrease in TNF-α detected after ≥2 d of culture did not result from masking by shed soluble TNF receptors, nor from a selective upregulation of membrane TNF-α (data not shown). Addition of rTNF-α (10 and 100 U/ml) did not restore HIV-1 expression, as detected by p24 Ag (data not shown). Thus, the decrease in TNF-α was not responsible for the inhibitory effect of LPS on HIV-1 replication. Loss of sensitivity of HIV-1–infected MDM to TNF-α–mediated upregulation of HIV expression, rather than decreased levels of TNF-α, may be involved in LPS-induced inhibition of HIV infection. The mechanisms involved in TNF-α suppression are currently under investigation.

LPS-induced Inhibition of HIV Replication Is Mediated by Soluble Factors Active on Both MDM and T Lymphocytes. The finding that pretreatment with LPS inhibited HIV-1 infection only if the cells were not washed before adding the virus prompted us to investigate whether the effects of LPS are mediated by soluble factors. To this purpose, LPS-conditioned supernatants were obtained from MDM cultures stimulated with LPS for 24 h, and LPS was neutralized by the addition of polymixin B (15 μg/ml). Normal MDM were then infected with HIV-1 and cultured either with LPS, or with these supernatants (100% vol/vol) in the absence of LPS. Table 1 shows that the supernatants from LPS-treated MDM inhibited HIV-1 replication as actively as LPS itself, even in the presence of polymixin B. Interestingly, the effect of the soluble inhibitory factor(s) was not
MDM specific. Indeed, Table 2 shows that the same LPS-conditioned supernatants also suppressed viral expression in T lymphocytes infected with the NSI strains HIV-1Ba-L and HIV-1181, a primary isolate. The inhibitory effect of LPS-conditioned MDM supernatants on HIV replication in T cells was particularly remarkable, because LPS per se had no effect when added directly to purified infected T cells. However, LPS-conditioned supernatants failed to suppress the replication of an SI primary isolate, HIV-15233, in T cells. These results suggest that suppressive monokines released by MDM upon stimulation with LPS are responsible for the observed inhibition of HIV replication.

C–C Chemokines Released by LPS-stimulated MDM Mediate the Suppression of HIV Replication. Several monokines have been reported to suppress HIV-1 replication. Among them, IL-10 blocks HIV replication by inhibiting the secretion of endogenous TNF-α and IL-6 (28), cytokines that upregulate HIV expression. IL-1Ra, on the other hand, has been described to be produced by HIV-infected MDM in excess relative to IL-1α and IL-1β, and thus effectively counteracts IL-1-mediated induction of HIV expression (29). We tested whether the release of these monokines was responsible for the LPS-induced inhibition of HIV-1 expression in MDM. To this purpose, neutralizing anti-IL-10 or anti-IL-1Ra antibodies were added to MDM cultures infected with HIV-1 and stimulated with LPS. Fig. 5 shows that addition of neither antibody reversed the suppression of HIV-1 replication caused by LPS, thus ruling out a role of IL-10 and IL-1Ra in HIV-1 suppression.

CD8+ T lymphocytes release soluble factors that inhibit HIV-1 replication in CD4+ T cells in a manner not restricted by the major histocompatibility complex (30). HIV-1 inhibition was recently shown to depend on the presence of the C–C chemokines RANTES, MIP-1α, and MIP-1β (31), the natural ligands of C–C CKR-5, the second recep-

Table 1. LPS-induced Inhibition of HIV Replication in MDM Is Mediated by the Release of Soluble Factors

| Culture | Supernatant added | Polymixin | HIV-1 p24 Ag release |
|---------|-------------------|-----------|---------------------|
| MDM + HIV-1 | Nil | – | 3,209 | 12,616 |
| MDM + HIV-1 | Nil | + | 3,620 | 13,917 |
| MDM + HIV-1+1LPS | Nil | – | 108 | 992 |
| MDM + HIV-1+1LPS | Nil | + | 2,953 | 12,408 |
| MDM + HIV-1 | Untreated MØ | + | 3,048 | 13,726 |
| MDM + HIV-1 | LPS-treated MØ | + | 100 | 300 |

MDM from healthy donors were infected in vitro with HIV-1, in the presence of LPS (1 μg/ml), LPS-conditioned supernatants (100% vol/vol), or polymixin B sulfate (15 μg/ml). Supernatants from infected cultures were harvested at different timepoints, and assayed by ELISA for p24 Ag secretion.
tor for primary NSI strains (32, 33, 34). Therefore, in preliminary experiments, we assessed whether CC–CKR-5 is expressed in MDM, and whether stimulation with LPS induces the release of these chemokines. Competitive PCR experiments were carried out to quantitatively determine the levels of CC–CKR-5 mRNA in total cDNA isolated from MDM. Quantification was achieved by using a DNA fragment that acts as a dual competitor for PCR amplification of both β-actin (as an internal standard) and CC–CKR-5 cDNA (Fig. 6A). Fig. 6B shows that high levels of CC–CKR-5 mRNA were expressed by MDM at the time of infection. Stimulation with LPS did not upregulate the expression of CC–CKR-5 in infected MDM (data not shown).

Next, we investigated whether stimulation with LPS induces MDM to release C-C chemokines. Table 3 shows that addition of LPS resulted in vigorous production of these C-C chemokines by MDM, both uninfected and infected in vitro with HIV. Then, we investigated whether the C-C chemokines released in LPS-conditioned supernatants played a role in the inhibition of HIV-1 replication. The simultaneous neutralization of RANTES, MIP-1α, and MIP-1β has been shown to be required to abrogate the HIV suppressive effects of CD8+ T cell supernatants. Thus, high concentrations of antibodies are necessary to achieve neutralization (31). Because monocytes and MDM express all types of Fcγ receptors (CD64, CD32, and CD16), the engagement of which is known to modulate HIV expression (35), supernatants from LPS-stimulated MDM cultures were simultaneously depleted of RANTES, MIP-1α, and MIP-1β by adsorption on specific antibodies immobilized on plastic. After polymixin B was added to neutralize LPS, the chemokine-depleted supernatants were added to HIV-1-infected MDM from different donors. In the representative experiment shown in Table 4, LPS-conditioned supernatants completely inhibited p24 Ag secretion. Depletion of C-C chemokines neutralized the inhibitory activity of the supernatants. In contrast, supernatants adsorbed on control goat IgG were almost as inhibitory as the undepleted ones. Our data suggest that the LPS-dependent release of HIV-1 suppressive chemokines plays a major role in the inhibition of HIV-1 replication in MDM.

Table 2. Soluble Factors Released by LPS-treated MDM Inhibit the Replication of NSI HIV-1 Strains in T Lymphocytes

| Culture | Supernatant added | Polymixin | Ba-L | 181 | 5233 |
|---------|------------------|-----------|------|-----|-----|
| T cells+HIV-1 | − | − | 2,155 | 8,755 | 7,057 |
| T cells+HIV-1 | − | + | 2,355 | 6,390 | 7,592 |
| T cells+HIV-1+LPS | − | − | 2,344 | 7,795 | 6,793 |
| T cells+HIV-1+LPS | − | + | 2,086 | 7,885 | 7,738 |
| T cells+HIV-1 | Untreated MØ | + | 2,225 | 8,927 | 7,462 |
| T cells+HIV-1 | LPS-treated MØ | + | 47 | 281 | 7,198 |

Lymphocytes from healthy donors were infected in vitro with two NSI HIV-1 strains, HIV-1_321 and HIV-1_332, or with an SI strain, HIV-1_5233, in the presence or absence of LPS (1 μg/ml), LPS-conditioned supernatants (100% vol/vol) or polymixin B, sulfate (15 μg/ml). Supernatants from infected cultures were harvested 5 d after infection, and assayed by ELISA for p24 Ag secretion.

Figure 5. Effects of neutralizing antibodies against HIV-1-inhibitory cytokines. MDM were infected with HIV-1_321 and stimulated with LPS (1 μg/ml), in presence or absence of neutralizing anti-IL-1Ra or anti-IL-10 antibodies (10 μg/ml). Culture supernatants were harvested daily, and tested for p24 Ag secretion by ELISA. The data represent the mean of two separate experiments. Control antibodies had no effect on p24 Ag secretion.
HIV-1Ba-L for 14 h, in the presence or absence of LPS and LPS-conditioned supernatants. Viral DNA load was determined by a semiquantitative nested PCR procedure, using two primer sets specific for the pol gene (23, 24). In a representative experiment, 5,860 viral DNA copies were detected in 10³ infected MDM 14 h after infection. Addition of LPS or LPS-conditioned supernatants reduced the number of viral DNA copies to 407 and 585 per 10³ MDM, respectively, thus decreasing viral load by 93 and 90%. The finding that LPS treatment suppressed the rate of proviral DNA formation at an early time after MDM infection is consistent with the reported ability of C–C chemokines to interfere with HIV-1 entry.

Recombinant C–C chemokines inhibit HIV-1 replication in human MDM. To assess whether C–C chemokines are sufficient to inhibit HIV-1 replication in MDM, recombinant RANTES, MIP-1α, and MIP-1β were added to HIV-infected MDM, alone or in combination. Fig. 7 (left) shows that a combination of the three chemokines, each at a concentration of 50 ng/ml, inhibited the replication of HIV-1Ba-L in infected MDM by 76%. In the same experiments, addition of LPS reduced p24 Ag release by 75%. Among the three chemokines, RANTES was the most effective one, because it inhibited HIV-1Ba-L infection as efficiently as LPS when used at a concentration of 250 ng/ml. Notably, the inhibitory effect of C–C chemokines on HIV-1 replication was even more pronounced in MDM cultures infected with NS1 primary viral isolates. Indeed, Fig. 7 (right) shows that RANTES, MIP-1α, and MIP-1β blocked the replication of HIV-1Ba-L by >75% even when used individually at a concentration as low as 10 ng/ml. The combination of the three chemokines suppressed HIV-1Ba-L by over 90%. The concentrations of recombinant chemokines used in our experiments were physiologically significant. Indeed, the assessment of the concentrations of endogenous chemokines released by MDM during the overnight incubation with virus and LPS before washing (data not shown) demonstrated that at the time of in vitro infection, HIV is exposed to similar amounts of chemokines. These results show that recombinant chemokines are sufficient to inhibit HIV infection in human MDM.

Discussion

For several years, it has been known that stimulation with bacterial LPS protects macrophages from productive infection by HIV-1 in vitro (15, 16). Despite the potential implications of this finding for the pathogenesis and treatment of HIV infection, the mechanisms responsible for the HIV suppressive effect of LPS have remained unknown. Our present results indicate that LPS stimulates human MDM to release soluble factors, the C–C chemokines RANTES, MIP-1α, and MIP-1β, that strongly inhibit HIV replication, not only in macrophages but also in T lymphocytes. These data may help redefine our current understanding of the role played by monocyte/macrophages in the pathogenesis of HIV infection. Macrophages have been viewed mostly negatively, as major targets for infection (3, 4), reservoirs for the virus (1, 2), triggers for T cell apoptosis (36, 37), and last but not least, as a source of soluble factors...
Suppression of HIV-1 Infection by LPS-induced Chemokines

TNF-α, IL-1, IL-6) that sustain viral replication (27, 38, 39). The potential for a defensive role of macrophages only became clear after C–C chemokines have been shown to exert a potent inhibitory effect on HIV replication (31). These chemoattractants are vigorously secreted not only by CD8+ T lymphocytes, the cells traditionally implicated in HIV-1 suppression, but also by activated monocyte/macrophages (40). Important indications about the possible mechanism of action of these HIV-suppressive chemokines have emerged from a series of recent reports, which demonstrated that selected chemokine receptors act as critical surface membrane cofactors for HIV infection (32–34, 41–43). These include CC–CKR-5, a RANTES, MIP-1α, and MIP-1β receptor that is used by most primary NSI strains, and LESTR/fusin, the receptor for the lymphocyte chemoattractant SDF-1 (44, 45), used by cell line–adapted SI strains, as well as CC–CKR-2b and CC–CKR-3, which may also be used by a limited number of isolates. C–C chemokines block membrane fusion and HIV-1 entry, either by competing for the HIV-1 binding site on CC–CKR-5, and/or through the downregulation of surface receptor expression (32, 33, 34).

Within this context, our data suggest that a receptor for RANTES, MIP-1α, and MIP-1β is a major cofactor for HIV-1 entry in macrophages, as well as T cells. This hy-

**Table 3. C–C Chemokine Secretion in MDM Cultures**

|          | MIP-1α | MIP-1β | RANTES |
|----------|--------|--------|--------|
| Day 2    | pg/ml  | pg/ml  | pg/ml  |
| Experiment 1 |       |        |        |
| Nil      | 1,710  | 591    | 198    |
| LPS      | 39,297 | 17,164 | 12,740 |
| HIV      | 2,040  | 627    | 281    |
| HIV + LPS| 34,452 | 20,253 | 8,380  |
| Experiment 2 |       |        |        |
| Nil      | 380    | 50     | 38     |
| LPS      | 22,680 | 37,590 | 12,080 |
| HIV      | 1,750  | 2,330  | 47     |
| HIV + LPS| 21,710 | 20,110 | 8,890  |

Uninfected or HIV-1 Ba-L–infected MDM were cultured in the presence or absence of LPS (1 μg/ml). LPS was added to the cultures every 3 d. Supernatants were harvested after 2 and 5 d of culture. The concentrations of C–C chemokines in the supernatants were measured by ELISA.

**Table 4. Antibody-mediated Depletion of C–C Chemokines Neutralizes the HIV Suppressive Activity of LPS-conditioned Supernatants**

| Culture       | Supernatant added | Polymixin | Depletion | HIV-1 p24 Ag pg/ml |
|---------------|-------------------|-----------|-----------|-------------------|
| MDM + HIV-1   |                   |           |           | 4,516             |
| MDM + HIV-1   |                   | +         |           | 4,426             |
| MDM + HIV-1 + LPS |              |           |           | 597               |
| MDM + HIV-1 + LPS |              | +         |           | 4,500             |
| MDM + HIV-1   | Untreated MØ      | +         |           | 5,739             |
| MDM + HIV-1   | LPS-treated MØ    | +         |           | 176               |
| MDM + HIV-1   | LPS-treated MØ    | +         | Anti-chemokines | 3,597             |
| MDM + HIV-1   | LPS-treated MØ    | +         | Normal goat IgG | 806               |

MDM from healthy donors were infected in vitro with HIV-1 Ba-L, in the presence of LPS-conditioned supernatants (100% vol/vol), undepleted or depleted of monokines by adsorption on specific neutralizing antibodies or control IgG immobilized on plastic. Polymixin B sulfate was added at a concentration of 15 μg/ml. Supernatants from infected cultures were harvested after 4 d of culture, and assayed by ELISA for p24 Ag secretion. The table shows the results of a representative experiment.
has been recently shown that both macrophages and CD4
and HIV-1Ba-L Env-expressing cells (34). Furthermore, it
detected and critically regulated by the interactions between
potently inhibited fusion between primary macrophages
replication of both macrophages and T cells is genetically deter-
tion of HIV-1IIIB (31), because this SI strain, unlike HIV-1Ba-L
observation that C–C chemokines do not block the replica-
and the NSI strains, requires LESTR/fusin, rather than
CC–CKR-5, as a coreceptor for entry and fusion (41).

The suppressive effect of C–C chemokines on MDM in-
fection by NSI HIV-1 strains is supported by the observa-
tion that recombinant RANTES, MIP-1α, and MIP-1β
potently inhibited fusion between primary macrophages
and HIV-1GaL Env-expressing cells (34). Furthermore, it
has been recently shown that both macrophages and CD4+ T
cells from multiply exposed uninfected individuals (46)
resistant to infection by primary NSI isolates of HIV-1, while
remaining susceptible to infection by SI strains (47). It is not
yet clear whether resistance results from a defect in second
receptor usage secondary to C–C chemokine hyperpro-
duction (46) and/or from mutations in the CC–CRK-5
gene that generate a nonfunctional receptor unable to sup-
port cell fusion and infection by NSI HIV-1 strains (48).
However, it seems likely that susceptibility to HIV-1 infec-
tion of both macrophages and T cells is genetically deter-
moved, and critically regulated by the interactions between
C–C chemokines and their receptors.

The issue of C–C chemokine-induced inhibition of
HIV-1 replication in macrophages is still somewhat contro-
versial. Dragic et al. (33) recently reported that entry of
NSI HIV-1 strains into primary macrophages was relatively
insensitive to C–C chemokines. This discrepancy with our
results is likely to be caused by differences in the experi-
mental conditions. The readout in our experiments was p24
Ag secretion in MDM cultures infected with several HIV-1
strains, including primary isolates from HIV-1-infected pa-
tients. In contrast, Dragic et al. assessed virus entry by a sin-
gle-cycle infection with an env-deficient virus, which also
carrying the luciferase reporter gene, complemented by en-
velope glycoprotein expressed in trans (33). This assay, al-
though elegant, is necessarily artificial, and may not capture
the full complexity of virus-host cell interactions in mac-
rophages infected with naturally occurring HIV-1 strains.
On the other hand, a number of differences in culture con-
ditions (i.e., MDM propagation and stimulation and/or vi-
rus source and/or activity of recombinant chemokines) may
have determined the lack of HIV suppression observed by
Schmidt Mayerova et al. (49). In our hands, different batches
of recombinant C–C chemokines have reproducibly sup-
pressed infection by different HIV-1 NSI strains over sev-
eral months.

While the results obtained with recombinant chemok-
ines clearly show that these chemokines are sufficient
to suppress HIV replication in MDM, it is possible that
LPS-conditioned supernatants contain additional factor(s) with
HIV suppressive effects. Preliminary experiments in our
laboratory indicate that the replication of some HIV-1
strains is insensitive to C–C chemokine-mediated inhibi-
tion, but is blocked by LPS-conditioned supernatants (Ve-
rami, A., G. Scarlatti, and D. Vercelli, manuscript in prepa-
ration). The nature of other potential HIV-suppressive
factor(s) contained in the LPS-conditioned MDM superna-
tants from infected cultures (Ve-
rami, A., G. Scarlatti, and D. Ver-
celli, manuscript in preparation).

Figure 7. Recombinant C–C chemokines inhibit HIV-1 repli-
cation in human MDM. MDM from healthy donors were in-
fected in vitro with HIV-13GaL (left) or with the NSI primary vi-
rual isolate HIV-13GaL (right), in the presence or absence of LPS
(1 μg/ml) and recombinant chemokines. Chemokines were
added to HIV-13GaL-infected cultures at a concentration of 250
ng/ml when used individually, and 50 ng/ml each when used
in combination. For HIV-13GaL-
infected cultures, chemokines were
used at 10 ng/ml, individually
and in combination. Superna-
tants from infected cultures
were harvested at different time
points, and assayed by ELISA for
p24 Ag secretion.
tion in both macrophages and T cells may prompt a reinter-
pretation of the role played by bacterial superinfections
in the pathogenesis and progression of HIV infection. It has
been recently shown that CD14 is not just the receptor for
LPS of gram-negative bacteria (9) but is a multipurpose
receptor for foreign lipopolysaccharidies of gram-positive bacteria
and mycobacteria (52, 53). Thus, a vast array of exogenous
stimuli derived from microbial pathogens may conceivably
trigger intense chemokine release. In this perspective, the
effect of bacterial superinfections in patients with HIV-1
immunodeficiency may be complex and somewhat coun-
terintuitive. The chemokine response triggered by the in-
fecious agent upon interaction with the macrophages of
the host in fact may contribute to the containment of HIV-1
infection in the main targets of the virus, T cells, and
mononuclear phagocytes.

This work was supported by AIDS Project, Istituto Superiore di Sanità, Italy (grant 9306-39 to D. Vercelli,
9306-20 to G. Scarlatti, 9402-11 to M. Giacca, 9304-78 to P. Lusso, and 9405-04 to A.G. Siccardi). A. Ve-
rcani was the recipient of a fellowship from Istituto Superiore di Sanità.

Address correspondence to Donata Vercelli, M.D., Molecular Immunoregulation Unit, DIBIT, San Raffaele
Scientific Institute, Via Olgettina 58, 20132 Milan, Italy.

Received for publication 29 July 1996 and in revised form 2 December 1996.

References

1. Stoler, M.H., T.A. Eskin, S. Benn, R.C. Angerer, and L.M.
Angerer. 1986. Human T-cell lymphotropic virus type III
infection of the central nervous system. Preliminary in situ
analysis. JAMA. 256:2360–2364.

2. Koenig, S., H.E. Gendelman, J.M. Orenstein, M.C.D.
Canto, G.M. Pezeshkpour, M.Y. unglbluth, F. Jannotta, A. Ak-
samit, M.A. Martin, and A.S. Fauci. 1986. Detection of AIDS
virus in macrophages in brain tissue from AIDS patients with
encephalopathy. Science (Wash. DC). 233:1089–1093.

3. Gendelman, H.E., J.M. Orenstein, M.A. Martin, C. Ferrua,
R.Mitra, T. Phipps, L.A. Wahl, H.C. Lane, A.S. Fauci, D.S.
Burke et al. 1988. Efficient isolation and propagation of hu-
man immunodeficiency virus on recombinant colony-stimu-
lating factor 1–treated monocytes. J. Exp. Med. 167:1428–
1441.

4. Weiss, S.H., J.J. Goedert, S. Gartner, M. Popovic, D. Watters,
P.Markham, F. Di Mauro, Veronese, M.H. Gall, W.E. Bark-
ley, J. Gibbons et al. 1988. Risk of human immunodeficiency
virus infection among laboratory workers. Science (Wash. DC).
239:68–71.

5. Ho, D.D., T.R. Rota, and M.S. Hirsch. 1986. Infection of mono-
cytes/macrophages by human T lymphotropic virus type III.
J. Clin. Invest. 77:1712–1715.

6. Salahuddin, S.Z., R.M. Rose, J.E. Groopman, P.D. Mark-
ham, and R.C. Gallo. 1986. Human T lymphotropic virus
type III infection in human alveolar macrophages. Blood. 68:
281–284.

7. Ho, D.D., M.D. Roger, R.J. Pomerantz, and J.C. Kaplan.
1987. Pathogenesis of infection with human immuno-
deficiency virus. N. Engl. J. Med. 317:278–286.

8. Meltzner, M.S., D.R. Skillman, P.J. Gomatos, D.C. Kalter,
and H.E. Gendelman. 1990. Role of mononuclear phagocytes
in the pathogenesis of human immunodeficiency virus infec-
tion. Annu. Rev. Immunol. 8:169–194.

9. Right, S.D., R.A. Ramos, P.S. Tobias, R.J. Ulevitch, and
J.C. Mathison. 1990. CD14, a receptor for complexes of li-
poplysaccharide (LPS) and LPS binding protein. Science (Wash.
DC). 249:1431–1433.

10. Haziot, A., S. Chen, E. Ferrero, M.G. Low, R. Silber, and
S.M. Goyert. 1988. The monocyte differentiation antigen,
CD14, is anchored to the cell membrane by a phosphatidyli-
ositol linkage. J. Immunol. 141:547–552.

11. Simmons, D.L., S. Tan, D.G. Tenen, A. Nicholson-Weller,
and B. Seed. 1989. Monocyte antigen CD14 is a phospho-
lipid anchored membrane protein. Blood. 73:284–289.

12. Dentener, M.A., V. Bazil, E.J.U. Von Amsuth, M. Ceska,
and W.A. Buurman. 1993. Involvement of CD14 in li-
poplysaccharide-induced tumor necrosis factor-α, IL-6 and
IL-8 release by human monocytes and alveolar macrophages.
J. Immunol. 150:2885–2891.

13. Pomerantz, R.J., M.B. Feinberg, D. Trono, and D. Baltimore.
1990. Lipopolysaccharide is a potent monocyte/macrophage-
specific stimulator of human immunodeficiency virus type 1
expression. J. Exp. Med. 172:253–261.

14. Bagasra, O., S.D. Wright, T. Seshamma, J.W. Oakes, and
R.J. Pomerantz. 1992. CD14 is involved in control of human
immunodeficiency virus type 1 expression in latently infected
cells by lipopolysaccharide. Proc. Natl. Acad. Sci. USA. 89:
6285–6289.

15. Kornbluth, R.S., P.S. Oh, J.R. M unis, P.H. Cleveland,
and D.D. Richman. 1989. Interferons and bacterial lipopolysac-
charide protect macrophages from productive infection by
human immunodeficiency virus in vitro. J. Exp. Med. 169:
1137–1151.

16. Bernstein, M.S., S.E. Tong-Starken, and R.M. Locksley.
1991. Activation of human monocyte-derived macrophages
with lipopolysaccharide decreases human immunodeficiency
virus replication in vitro at the level of gene expression. J.
Clin. Invest. 88:540–545.

17. M oore, J.P., J.A. McKeating, Y.X. Huan, A. Ashkenazi,
and D.D. Ho. 1992. Virions of primary human immunodefi-
ciency virus type 1 isolates resistant to soluble CD4 (sCD4)
neutralization differ in sCD4 binding and glycoprotein gp120
retention from sCD4-sensitive isolates. J. Virol. 66:235–243.

18. Willey, R.L., D.H. Smith, L.A. Lasky, T.S. Theodore, P.L.
Earl, B. Moss, D.J. Copon, and M.A. Martin. 1988. In vitro
mutagenesis identifies a region within the envelope gene of
the human immunodeficiency virus that is critical for infec-
Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162:156–159.

Grassi, G., L. Zentilin, S. Tafuro, S. Diviacco, A. Falauchi, and M. Giaccia. 1994. A rapid procedure for the quantification of low abundance RNAs by competitive reverse transcription-polymerase chain reaction. Nucleic Acids Res. 22:4547–4549.

Albert, J., and E.M. Fenyo. 1990. Simple, sensitive, and specific detection of human immunodeficiency virus type 1 in clinical specimens by polymerase chain reaction with nested primers. J. Clin. Microbiol. 28:1560–1564.

Birnbaum, J.R., J. Albert, and F. Vartdal. 1991. Few infected CD4+ T cells but a high proportion of replication-competent provirus copies in asymptomatic human immunodeficiency virus type 1 infection. J. Virol. 65:2019–2023.

Marchant, A., J. Duchow, J.-P. Delville, and M. Goldman. 1992. Lipopolysaccharide induces up-regulation of CD14 molecule on monocytes in human whole blood. Eur. J. Immunol. 22:1663–1665.

Cullen, B.R., and W.C. Green. 1989. Regulatory pathways governing HIV-1 replication. Cell 58:423–426.

Poli, G., P. Bresler, A. Kinter, E. Duh, W.C. Timmer, A. Rabson, J.S. Justement, S. Stanley, and A.S. Fauci. 1990. Interleukin 6 induces human immunodeficiency virus expression in infected monocyctic cells alone and in synergy with tumor necrosis factor by transcriptional and post-transcriptional mechanisms. J. Exp. Med. 172:151–158.

Weissman, D., G. Poli, and A.S. Fauci. 1994. Interleukin 10 blocks HIV replication in macrophages by inhibiting the autocrine loop of tumor necrosis factor and interleukin 6 induction of virus. AIDS Res. Hum. Retroviruses 10:1199–1206.

Zavaleta, C., A.-C. Riman, G. Bousin, D. Bermont, J.-F. Bach, and B. Descamps-Latscha. 1995. HIV predominantly induces IL-1 receptor antagonist over IL-1 synthesis in human monocytes. J. Immunol. 155:2784–2793.

Walker, C.M., D.J. Mody, D.P. Stites, and J.A. Levy. 1986. CD8+ lymphocytes can control HIV infection in vitro by suppressing virus replication. Science (Wash. DC) 234:1563–1566.

Cosentino, G., E. Soprana, C.P. Thienes, A.G. Siccardi, G. Viale, and D. Verrecchi. 1995. IL-13 downregulates CD14 expression and TNF-α secretion in human monocytes. J. Immunol. 155:3145–3151.

Comar, M., G. Marzio, P. Dagaro, and M. Giaccia. 1996. Quantitative dynamics of HIV type 1 expression. AIDS Res. Hum. Retroviruses 12:117–126.

Moor et al. 1996. HIV-1 entry into CD4+ cells is mediated by the chemokine receptor CC–CKR-5. Science (Wash. DC) 272:1955–1958.

Titsikov, E.N., R. Furein, K. Mclnosh, P.R. Scholl, and R.S. Geha. 1995. Cross-linking of Fcy receptors activates HIV-1 long terminal repeat-driven transcription in human monocytes. Nature Med. 1:129–134.

Finkel, T.H., G. Tudor-Williams, N.K. Banda, M.F. Cotton, T. Curiel, C. Monks, T.W. Baba, R.M. Ruprecht, and A. Kupfer. 1995. A poxvirus occurs predominantly in bystander cells and not in productively infected cells of HIV- and SIV-infected lymph nodes. Nature Med. 1:129–134.

Walker, C.M., D.J. Mody, D.P. Stites, and J.A. Levy. 1986. CD8+ lymphocytes can control HIV infection in vitro by suppressing virus replication. Science (Wash. DC) 234:1563–1566.

Bleul, C.C., M. Farzana, H. Choe, C. Parolin, I. Clark-Lewis, J. Sodroski, and T.A. Springer. 1996. The lymphocyte chemoattractant SDF-1 is a ligand for LESTR/fusin and blocks HIV-1 entry. Nature Med. 2:412–417.

Bohmer, E., A. Arma, F. Bacher, C. Bessa, J.-L. Virelizard, F. Arenzana-Seisdedos, O. Schwartz, J.-M. Heard, I. Clark-Lewis, D.F. Legler et al. 1996. The CXC chemokine SDF-1 is the ligand for LESTR/fusin and blocks HIV-1 entry. Nature Med. 2:412–417.

Paxton, W.A., S.R. Martin, D. Tse, T.R. O'Brien, J. Skurk, N.L. Van Devanter, N. Padian, J.B. Braun, D.P. Kotler, S.M. Wollinsky et al. 1996. Relative resistance to HIV-1 infection of CD4 lymphocytes from persons who remain uninfected despite multiple high-risk sexual exposures. Nature Med. 2:412–417.
Suppression of HIV-1 Infection by LPS-induced Chemokines

caucasian individuals bearing mutant alleles of the CCR-5 chemokine receptor gene. Nature (Lond.). 382:722–725.

49. Schmidtmayerova, H., B. Sherry, and M. Bukrinsky. 1996. Chemokines and HIV replication. Nature (Lond.). 382:767.

50. Gessani, S., U. Testa, B. Varano, P.D. Marzio, P. Borghi, L. Conti, T. Barberi, E. Tritarelli, R. Martucci, and D. Seripa. 1993. Enhanced production of LPS-induced cytokines during differentiation of human monocytes to macrophages. Role of LPS receptors. J. Immunol. 151:3758–3766.

51. Gendelman, H.E., L.M. Baca, J. Turpin, D.C. Kalter, B. Hansen, J.M. Orenstein, C.W. Dieffenbach, R.M. Friedman, and M.S. Meltzer. 1990. Regulation of HIV replication in infected monocytes by IFN-α. Mechanisms for viral restriction. J. Immunol. 145:2669–2676.

52. Pugin, J., D. Heumann, A. Tomasz, V.V. Krawchenko, Y. Akamatsu, M. Nishijima, M.P. Glauser, P.S. Tobias, and R.J. Ulevitch. 1994. CD14 is a pattern recognition receptor. Immunity. 1:509–516.

53. Kusunoki, T., E. Hallman, T.S.-C. Juan, H.S. Lichenstein, and S.D. Wright. 1995. Molecules from Staphylococcus aureus that bind CD14 and stimulate innate immune responses. J. Exp. Med. 182:1673–1682.