

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

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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-1 α , and MIP-1 β) 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.

Monocyte/macrophages are key players in the pathogenesis of HIV-1 infection. Macrophages are major reservoirs for HIV-1 during all stages of infection (1, 2) and may be among the first cells to be infected by HIV-1 in patients (3, 4). Unlike T cells, HIV-infected monocytes show little or no virus-induced cytopathic effects *in vitro* (5, 6). HIV-infected macrophages therefore may persist in tissues for long periods of time and represent a vector for the spread of the infection to different tissues both within the patients and between individuals. In addition to this Trojan horse-like role, it has been recognized that monocytes may play a regulatory role during HIV infection by controlling the pace of disease progression through the release of soluble products (reviewed in 5, 7, 8).

Monocyte/macrophages are critically involved in the immune response to bacterial infections. LPS/endotoxin, the major constituent of the cell wall in gram-negative bacteria, has been shown to activate monocyte/macrophages by interacting with a specific receptor, CD14 (9), a glycosylphosphatidylinositol-linked glycoprotein expressed on the monocytic lineage at high density and less intensely on neutrophils (10, 11). CD14 plays a pivotal role in LPS-induced monokine release during infections and toxic shock (9, 12). More recently, LPS/CD14 interactions have been shown

to result in the induction of HIV expression in monocytoid tumor cell lines (13, 14), but to protect primary macrophages from productive infection by HIV-1 *in vitro* (15, 16). Notably, the concentrations of LPS that affect HIV-1 replication *in vitro* can be easily reached *in vivo* and may thus affect viral replication in patients with HIV superinfected with bacteria. The mechanisms underlying the complex effects of LPS on HIV-1 expression in monocytic cells have not been elucidated so far.

We have studied the effects of LPS on HIV-1 expression in cultures of monocyte-derived macrophages (MDM)¹ and T cells isolated from normal donors, and infected with different strains of HIV-1. Our results show that LPS-dependent inhibition of HIV infection affected T lymphocytes, as well as MDM, and involved the release of suppressive factors, most notably, the C–C chemokines RANTES (regulated upon activation, normal T expressed and secreted), macrophage inflammatory protein (MIP)-1 α , and MIP-1 β .

¹Abbreviations used in this paper: MDM, monocyte-derived macrophages; MIP, macrophage inflammatory protein; NSI, nonsyncytium-inducing; RANTES, regulated upon activation, normal T expressed and secreted; RT, reverse transcriptase.

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-1 α , and MIP-1 β), and neutralizing goat polyclonal antibodies against IL-1 receptor antagonist (IL-1Ra: neutralizing dose, ND₅₀ = 5–10 μ g/ml), MIP-1 α (ND₅₀ = 10 μ g/ml), MIP-1 β (ND₅₀ = 40 μ g/ml), and RANTES (ND₅₀ = 100–200 μ g/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-1 α , MIP-1 β , 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 assessed by the Limulus amoebocyte 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 Infection. PBMC were isolated by Ficoll-HyPaque (Pharmacia, Uppsala, Sweden) density gradient centrifugation from buffy-coat preparations obtained from healthy donors. The cells were then resuspended in RPMI-1640 (GIBCO BRL, Gaithersburg, MD) supplemented with 10% AB⁺ serum (Sigma), 20% FCS (Biological Industries, Israel), 2 mM glutamine, 50 μ g/ml streptomycin, and 100 U/ml penicillin, and cultured at a concentration of 1×10^6 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 $\geq 90\%$ CD14⁺ cells, as assessed by immunofluorescence.

Cells were infected with the monocytotropic HIV-1_{Ba-L} 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-1_{IIIIB} grown in PHA-activated PBMC (TCID₅₀: 45/10⁶ MDM). Furthermore, cells were infected with primary viral isolates (HIV-1₅₀₈₈ and HIV-1₁₈₁) 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 μ g/ml; Sigma). The resulting PHA blasts were collected, resuspended at 2.5×10^6 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-1_{Ba-L}. Subsequently, free virus was removed by washing twice in RPMI-1640, and the cells (1.5×10^6 /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 μ g/ml). 2 d later, supernatants were harvested, centrifuged, and stored at –20°C until used. To deplete LPS-conditioned supernatants of chemokines (MIP-1 α , MIP-1 β , RANTES), petri dishes were coated for 2 h at room temperature with neutralizing antibodies in PBS, at concentrations (10–30 μ g/ml) expected to neutralize the amounts of cytokines found in culture supernatants. Control plates were coated with normal goat IgG (55 μ g/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 Reagents, Dublin, Ireland). Bound p24 Ag was then detected using an alkaline phosphatase-conjugated anti-p24 Ag monoclonal antibody (BC 1071-AP; Aalto Bio Reagents) and the AMPAK 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 μ l of cell-free culture supernatants were added to 50 μ l of a mixture containing poly(A), oligo (dT) (Pharmacia), MgCl₂, and ³²P-labeled deoxythymidine 5'-triphosphate (dTTP) (Amersham) in a 96-well V-bottomed microtiter plate, and incubated 1.5 h at 37°C. 5 μ l 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 RNase-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 μ l 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 CKR-9 (5'-CATCAT-CCTCCTGACAATCG) and CKR-10 (5'-ATGGTGAAGATA-AGCCTCACAG). 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 (BA1 and BA4 [22]) and for CKR-5 (primers CKR-9 and CKR-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 μ l of PCR buffer (50 mM KCl, 10 mM Tris-HCl, 2 mM MgCl₂) containing the two primers (100 pmol each), the four dNTPs (200 μ M 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 μ l of each PCR reaction were resolved on a 8% non-denaturing 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-HH1_{Ba-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 proteinase K) followed by extraction with a phenol/chloroform/isoamyl alcohol mixture (25:24:1) and ethanol precipitation. Samples were amplified using a sets of nested primers specific for the *pol* gene (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 positive control, HIV-1-infected cells were diluted in uninfected cells so as to contain 10 and 1 HIV-1 viral DNA copies. The number of viral DNA copies per 10⁶ MDM was calculated according to the Poisson distribution formula. The sensitivity of the PCR assay was shown to be one copy of HIV-1 per 10⁵ cells (24).

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-1_{Ba-L} strain, in the presence or absence of LPS (1 μ g/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-1_{Ba-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 pg/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-1_{IIIB} (Fig. 1 D). The surprisingly high levels of replication of our HIV-1_{IIIB} in MDM are likely to result from multiple passages of the viral stock in human primary PBMC. Addition of LPS did not result

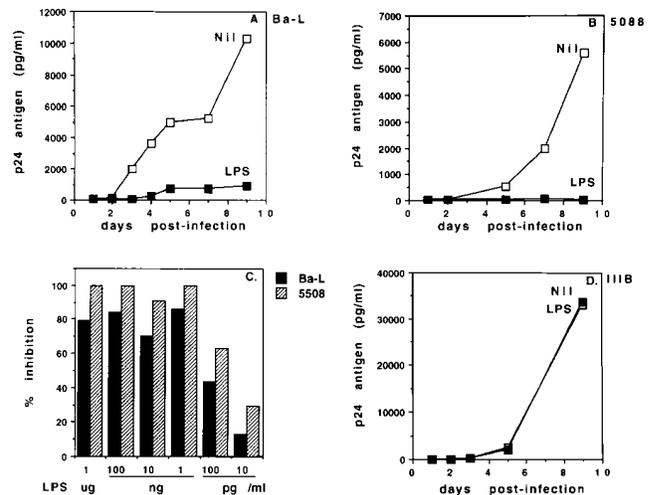


Figure 1. LPS suppresses HIV-1 replication in MDM cultures infected in vitro. MDM from healthy donors were infected with HIV-1_{Ba-L} (A), the primary NSI isolate HIV-1₅₀₈₈ (B), or HIV-1_{IIIB} (D), all at 500 pg/ml, in the presence or absence of LPS (1 μ g/ml). MDM were washed 1 d later and further 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-1_{Ba-L} or HIV-1₅₀₈₈ in the presence of decreasing concentrations of LPS. p24 Ag secretion was assessed 5 d after infection.

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-

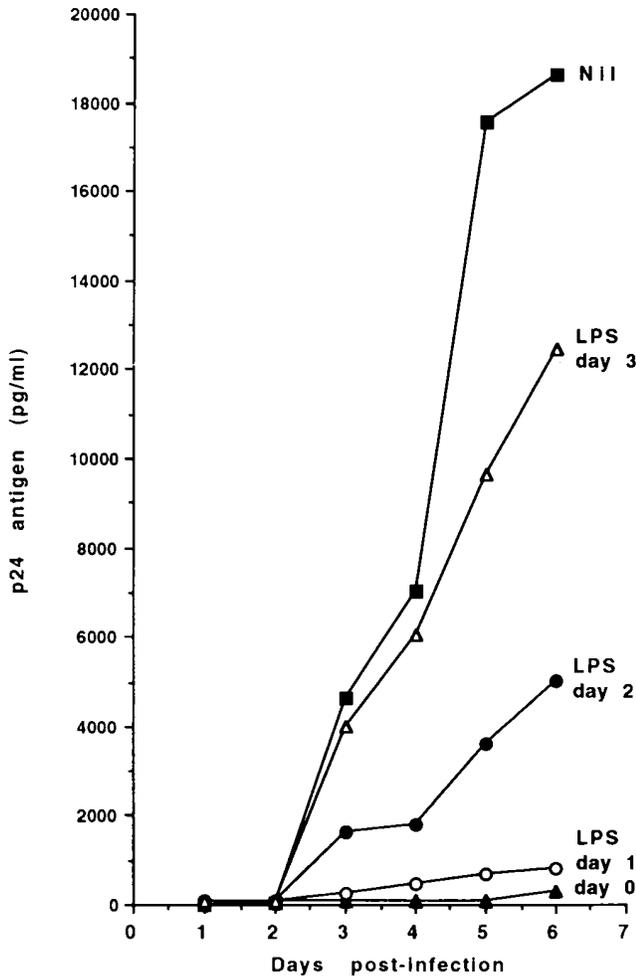


Figure 2. LPS-induced inhibition of HIV-1 expression in MDM cultures is dependent on the time of addition of LPS. MDM were infected with HIV-1_{Ba-L} (500 pg/ml), and stimulated with LPS (1 μ g/ml) at different times from the initiation of the culture. Culture supernatants were harvested daily, and tested for p24 Ag secretion by ELISA. The data represent the mean of two separate experiments.

tokines 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- κ B, 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

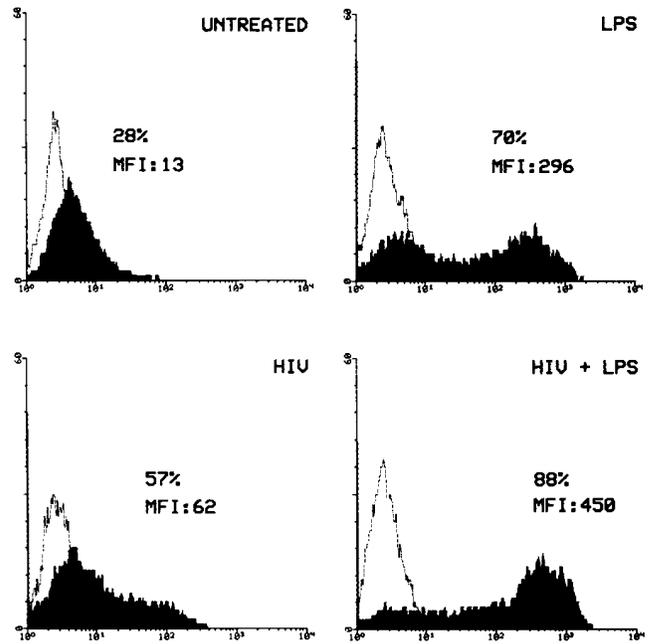


Figure 3. LPS and HIV-1 synergize in upregulating CD14 expression in MDM. MDM were infected with HIV-1_{Ba-L} (500 pg/ml) in the presence or absence of LPS (1 μ g/ml). After 2 d of culture, CD14 expression was assessed by direct immunofluorescence, using PE-conjugated mAb P9 and an unrelated isotype control. The data are representative of three separate experiments.

and infected cells at the initiation of the culture, before removal of unbound virus. The decrease in TNF- α detected after \geq 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 μ g/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

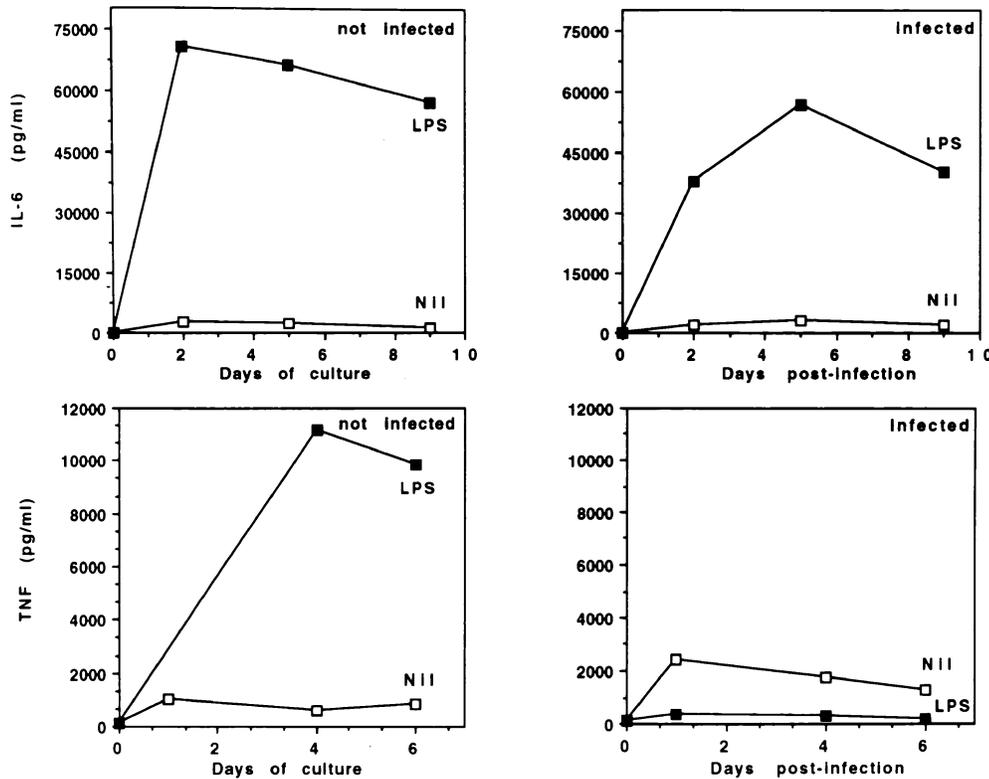


Figure 4. Effects of LPS stimulation and/or HIV-1 infection on IL-6 and TNF- α secretion by MDM. Uninfected or HIV-1_{Ba-L}-infected MDM were cultured in the presence or absence of LPS (1 μ g/ml). LPS was added to the cultures every 3 d. IL-6 and TNF- α concentrations in the supernatants were measured by ELISA. The data are representative of four separate experiments.

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-1_{Ba-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-1 α and IL-1 β , 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 re-

stricted by the major histocompatibility complex (30). HIV-1 inhibition was recently shown to depend on the presence of the C-C chemokines RANTES, MIP-1 α , and MIP-1 β (31), the natural ligands of CC-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	
			Day 4	Day 7
			<i>pg/ml</i>	
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 μ g/m), LPS-conditioned supernatants (100% vol/vol), or polymixin B sulfate (15 μ g/ml). Supernatants from infected cultures were harvested at different timepoints, and assayed by ELISA for p24 Ag secretion.

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

Culture	Supernatant added	Polymixin	HIV-1 p24 Ag release		
			Ba-L	181	5233
				<i>pg/ml</i>	
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_{Ba-L} and HIV-1₁₈₁, or with an SI strain, HIV-1₅₂₃₃, 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.

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. 6 A). Fig. 6 B 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.

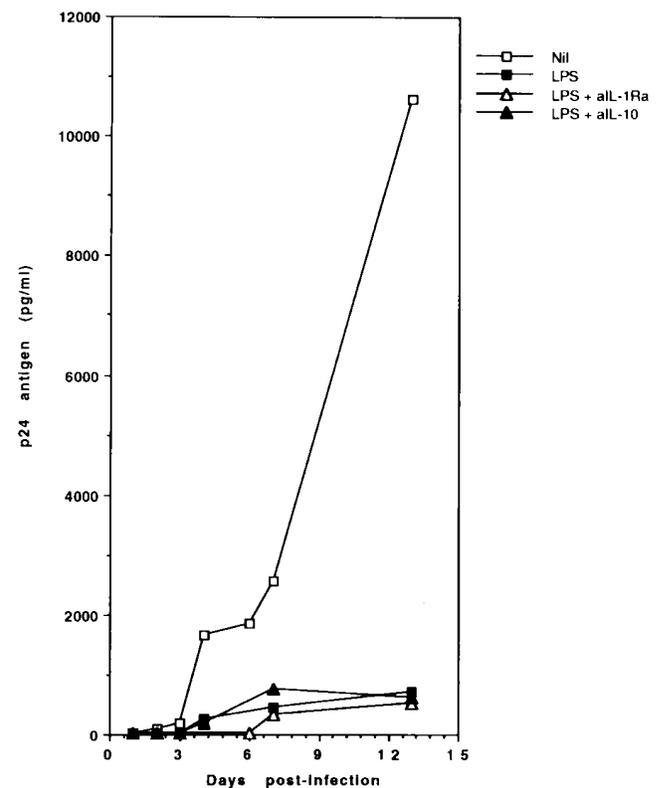


Figure 5. Effects of neutralizing antibodies against HIV-1-inhibitory cytokines. MDM were infected with HIV-1_{Ba-L} and stimulated with LPS (1 µg/ml), in presence or absence of neutralizing anti-IL-1Ra or anti-IL-10 antibodies (10 µg/ml). Culture supernatants were harvested daily, and tested for p24 Ag secretion by ELISA. The data represent the mean of two separate experiments. Control antibodies had no effect on p24 Ag secretion.

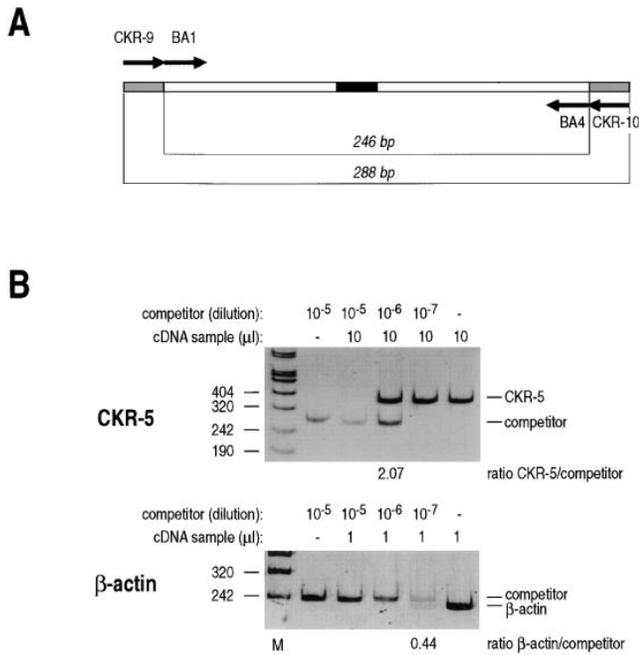


Figure 6. MDM express CC-CKR-5 mRNA. Total RNA was extracted from untreated MDM. RNA samples were treated with DNase I to remove traces of contaminating DNA and reverse transcribed using random hexameric primers. The cDNA products were mixed to scalar amounts of a synthetic competitor DNA fragment containing primer recognition sites for both β -actin and CC-CKR-5 amplification, and amplified with the respective primer pairs. (A) Schematic representation of the competitor DNA fragment used for the quantification of CC-CKR-5 and β -actin cDNA. The fragment contains a core sequence derived from the human β -actin cDNA, carrying a 20-bp insertion in the middle (closed box). Amplification with the β -actin-specific primer set BA1-BA4 detects a 226-bp product on human cDNA, and a 246-bp product from the competitor DNA. To this core sequence, the primer recognition sites for human CC-CKR-5 amplification were added at the two ends (indicated by gray boxes) by reamplification with composite primers corresponding to the CKR-9+BA1 sequence at one end and CKR-10+BA4 at the other end. Amplification with CKR-9 and CKR-10 generates a 288-bp fragment from the competitor template and a 368-bp fragment from the CC-CKR-5 cDNA. (B) Competitive PCR for the quantification of CC-CKR-5 and β -actin mRNAs. cDNA samples from untreated MDM were mixed with tenfold dilution of the competitor DNA fragment as indicated, and amplified with primer sets CKR-9/CKR-10 and BA1/BA4 for CC-CKR-5 and β -actin mRNA quantification. Amplification products were resolved by polyacrylamide gel electrophoresis, stained with ethidium bromide, and quantified by densitometric scanning. According to the principles of competitive PCR, quantification of the target molecules in the samples was obtained by estimation of the ratio between the amplification products, as reported at the bottom of each gel. Furthermore, since the same competitor DNA fragment acts as a competitor for quantification of both CC-CKR-5 and β -actin, standardization for mRNA input is obtained by estimating the ratio between the two measurements, as indicated at the bottom of the figure. M, molecular weight markers.

Competitive inhibition of HIV-1 coreceptor utilization by released chemokines is expected to result in the inhibition of HIV entry into MDM (32-34). Therefore, we tested the effects of LPS and LPS-conditioned supernatants on the early stages of the HIV-1 replication cycle by assessing the levels of proviral DNA in MDM incubated with HIV-1_{Ba-L} for 14 h, in the presence or absence of LPS and LPS-conditioned supernatants. Viral DNA load was deter-

mined 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 10^6 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 10^6 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-1 α , and MIP-1 β 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-1_{Ba-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-1_{Ba-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 NSI primary viral isolates. Indeed, Fig. 7 (right) shows that RANTES, MIP-1 α , and MIP-1 β blocked the replication of HIV-1₅₀₈₈ by >75% even when used individually at a concentration as low as 10 ng/ml. The combination of the three chemokines suppressed HIV-1₅₀₈₈ 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-1 α , and MIP-1 β , 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

Table 3. C-C Chemokine Secretion in MDM Cultures

	Day 2			Day 5		
	MIP-1 α	MIP-1 β	RANTES	MIP-1 α	MIP-1 β	RANTES
	<i>pg/ml</i>			<i>pg/ml</i>		
Experiment 1						
Nil	1,710	591	198	900	610	190
LPS	39,297	17,164	12,740	2,014	2,302	2,494
HIV	2,040	627	281	1,227	1,612	542
HIP+LPS	34,452	20,253	8,380	2,266	3,719	818
Experiment 2						
Nil	380	50	38	1,200	1,300	67
LPS	22,680	37,590	12,080	25,480	47,220	2,279
HIV	1,750	2,330	47	2,960	8,200	75
HIV+LPS	21,710	20,110	8,890	7,440	13,960	332

Uninfected or HIV-1_{Ba-L}-infected MDM were cultured in the presence or absence of LPS (1 μ g/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.

(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-1 α , and MIP-1 β 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-1 α , and MIP-1 β is a major cofactor for HIV-1 entry in macrophages, as well as T cells. This hy-

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
				<i>pg/ml</i>
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 M \emptyset	+	–	5,739
MDM+HIV-1	LPS-treated M \emptyset	+	–	176
MDM+HIV-1	LPS-treated M \emptyset	+	Anti-chemokines	3,597
MDM+HIV-1	LPS-treated M \emptyset	+	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 μ g/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.

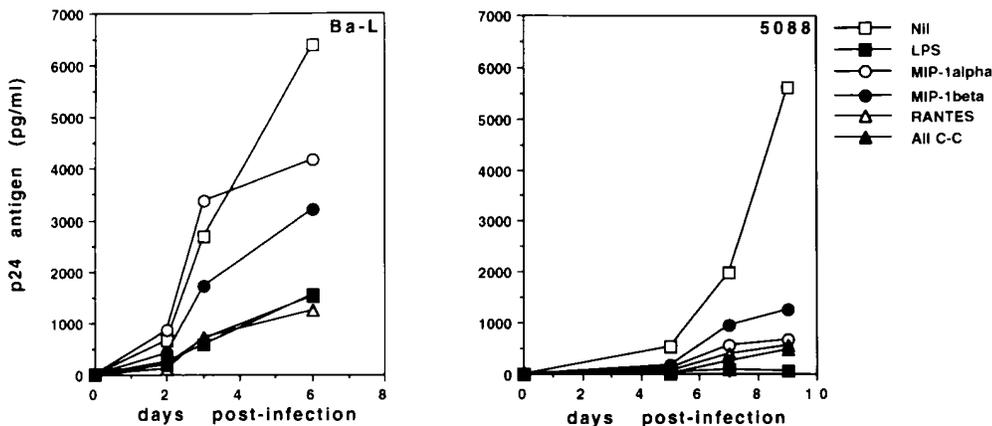


Figure 7. Recombinant C–C chemokines inhibit HIV-1 replication in human MDM. MDM from healthy donors were infected in vitro with HIV-1_{Ba-L} (left) or with the NSI primary viral isolate HIV-1₅₀₈₈ (right), in the presence or absence of LPS (1 μ g/ml) and recombinant chemokines. Chemokines were added to HIV-1_{Ba-L}-infected cultures at a concentration of 250 ng/ml when used individually, and 50 ng/ml each when used in combination. For HIV-1₅₀₈₈-infected cultures, chemokines were used at 10 ng/ml, individually and in combination. Supernatants from infected cultures were harvested at different timepoints, and assayed by ELISA for p24 Ag secretion.

pothesis is supported by a number of findings presented in this paper: (a) at the time of infection, MDM expressed mRNA for CC–CKR-5, the β -chemokine receptor; (b) stimulation with LPS induced the release of endogenous C–C chemokines, and reduced viral DNA load in infected MDM by >90% as early as 14 h after infection; (c) depletion of C–C chemokines strongly reduced the HIV-suppressive capacity of LPS-conditioned MDM supernatants; (d) recombinant chemokines at physiologically significant concentrations inhibited the replication of HIV-1 NSI strains in MDM, and (e) activation of MDM with LPS inhibited the replication of NSI primary isolates, but not of HIV-1_{IIB}, an SI laboratory strain. This pattern is consistent with the observation that C–C chemokines do not block the replication of HIV-1_{IIB} (31), because this SI strain, unlike HIV-1_{Ba-L} 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 infection by NSI HIV-1 strains is supported by the observation that recombinant RANTES, MIP-1 α , and MIP-1 β potently inhibited fusion between primary macrophages and HIV-1_{Ba-L} Env-expressing cells (34). Furthermore, it has been recently shown that both macrophages and CD4⁺ T cells from multiply exposed uninfected individuals (46) resist 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 hyperproduction (46) and/or from mutations in the CC–CKR-5 gene that generate a nonfunctional receptor unable to support cell fusion and infection by NSI HIV-1 strains (48). However, it seems likely that susceptibility to HIV-1 infection of both macrophages and T cells is genetically determined, 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 controversial. 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 experimental 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 patients. In contrast, Dragic et al. assessed virus entry by a single-cycle infection with an *env*-deficient virus, which also carries the luciferase reporter gene, complemented by envelope glycoprotein expressed in *trans* (33). This assay, although elegant, is necessarily artificial, and may not capture the full complexity of virus–host cell interactions in macrophages infected with naturally occurring HIV-1 strains. On the other hand, a number of differences in culture conditions (i.e., MDM propagation and stimulation and/or virus source and/or activity of recombinant chemokines) may have determined the lack of HIV suppression observed by Schmidtmayerova et al. (49). In our hands, different batches of recombinant C–C chemokines have reproducibly suppressed infection by different HIV-1 NSI strains over several months.

While the results obtained with recombinant chemokines clearly show that these chemoattractants 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 inhibition, but is blocked by LPS-conditioned supernatants (Verani, A., G. Scarlatti, and D. Vercelli, manuscript in preparation). The nature of other potential HIV-suppressive factor(s) contained in the LPS-conditioned MDM supernatants, and their function vis-a-vis chemokines, is currently under investigation. IFN (α and/or β) is a good candidate, because it is known to be released by LPS-stimulated MDM (50), and to block HIV replication (51).

Finally, the observation that LPS-stimulated macrophages release soluble factors that effectively inhibit HIV replica-

tion in both macrophages and T cells may prompt a reinterpretation 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 lipoglycans 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 counterintuitive. The chemokine response triggered by the infectious 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.

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