Interference of Interleukin-10 with Human Immunodeficiency Virus Type 1 Replication in Primary Monocyte-Derived Macrophages

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Previously we demonstrated an inhibitory effect of interleukin-4 (IL-4) on establishment of human immunodeficiency virus type 1 (HIV-1) infection in primary macrophages. The reported similarities between the biological effects of IL-4 and IL-10 prompted us to study the effect of IL-10 on HIV-1 replication. Treatment of primary macrophages with IL-10 resulted in inhibition of HIV-1 infection. This inhibitory effect was specific for macrophages, since IL-10 did not interfere with HIV-1 replication in primary T cells. Semiquantitative PCR analysis excluded an inhibitory effect of IL-10 on virus entry and reverse transcription. Effects of IL-10 on HIV-1 long terminal repeat-driven chloramphenical acetyltransferase activity also could not be demonstrated in a transient expression system in primary derived macrophages. In agreement with this, Northern (RNA) blot analysis demonstrated equal amounts of viral RNA species irrespective of IL-10 treatment, also excluding an inhibitory effect on elongation of virus transcription. Monocyte-derived macrophages (MDM) treated with IL-10 after HIV-1 inoculation showed accumulation of apparently mature p24 protein suggestive of an inhibitory effect at the level of virus assembly. IL-10 treatment of MDM prior to HIV-1 inoculation did not result in accumulation of p24 protein. Immunoblot analysis indeed showed the absence of mature p24 and gp120 but accumulation of the Pr53 gag-encoded protein in HIV-1-inoculated, IL-10-pretreated MDM, suggesting an inhibitory effect at the level of protein processing. A combination of IL-4 and IL-10 resulted in a cumulative inhibitory effect on HIV-1 replication in MDM. The recent observation that in the course of HIV-1 infection a shift occurs in the production of IL-2/gamma interferon toward enhanced IL-4 and IL-10 production and the reported shift from preferential macrophage-tropic towards preferential T-cell-tropic HIV-1 variants with progression of disease suggest that cytokines have an important role in the in vivo regulation of HIV-1 tropism.

Macrophages play an important role in the course of human immunodeficiency virus type 1 (HIV-1) infection. Macrophage-tropic HIV-1 variants are present during all stages of infection (20, 30, 31), which indicates their importance in viral persistence. In the asymptomatic stage of infection, macrophage-tropic HIV-1 variants are predominant (30). By virtue of their macrophage tropism, they may escape from host immune surveillance. In peripheral blood, where CD4+ T cells are the major target cell population (19, 29), macrophage-tropic HIV-1 variants can be detected, suggesting that T cells may become infected by progeny of HIV-1-infected tissue macrophages. Indeed, in tissues, macrophages are the predominantly infected cells (9, 12). Later on in infection, when the host immune system is compromised, an increase in the frequency of infected cells due to selective expansion of more T-cell-tropic HIV-1 variants is observed, while the amount of macrophage-tropic HIV-1 variants remains constant (30).

HIV-1 infection of macrophages can be regulated by several cytokines, such as interleukin-3 (IL-3), granulocyte-macrophage colony-stimulating factor, IL-1, IL-6, tumor necrosis factor alpha, gamma interferon, and IL-13 (15, 16, 22, 26, 33, 34). We previously demonstrated that IL-4, probably by inducing terminal differentiation of monocytes, inhibited the proliferative capacity of monocyte-derived macrophages (MDM) and concomitantly prevented productive HIV-1 infection (33). It appeared that for establishment of productive infection of MDM, besides a certain degree of differentiation, cell proliferation is required. In nonproliferating MDM, the process of reverse transcription is disturbed, resulting in only incomplete proviral DNA species (32, 33). In many biological processes, IL-4 and IL-10 have the same effects. For example, both inhibit the production of IL-1α, IL-1β, IL-6, IL-8, tumor necrosis factor alpha, granulocyte-macrophage colony-stimulating factor, and granulocyte colony-stimulating factor by monocytes at the transcriptional level (5, 25, 37). These observations prompted us to study whether IL-10, in analogy to IL-4, has an effect on HIV-1 replication in MDM.

**MATERIALS AND METHODS**

**Cell isolation and culture.** Monocytes were isolated from peripheral blood of HIV-1-seronegative plasmapheresis donors by Ficoll density gradient centrifugation followed by centrifugal elutriation, as described previously (11). The monocyte fraction was >95% pure. The virtual absence of T cells in this population was demonstrated by the lack of [3H]thymidine incorporation in these cells above background levels upon stimulation with phytohemagglutinin (PHA). To obtain MDM, the monocytes were cultured in endotoxin-free
Iscove's modified Dulbecco's medium supplemented with 10% human pooled serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). Cells were cultured at a concentration of 10^6/ml in 96-well plastic tissue culture plates (100 μl per well) or tissue culture flasks (Nunc, Roskilde, Denmark) and maintained at 37°C in a humidified atmosphere supplemented with 5% CO₂.

The medium was changed every week.

Peripheral blood mononuclear cells (PBMC) were isolated from peripheral blood of HIV-1-seronegative donors by Ficoll density gradient centrifugation. PBMC were cultured for 2 days before inoculation in Iscove's modified Dulbecco's medium supplemented with 10% fetal calf serum (Hyclone Laboratories Inc., Logan, Utah), penicillin (100 U/ml), streptomycin (100 μg/ml), and PHA (0.1 or 0.05 μg/ml) (Murex Diagnostics Ltd., Dartford, England) at a concentration of 2.10^6/ml in 24-well tissue culture plates (Nunc) and maintained at 37°C in a humidified atmosphere supplemented with 5% CO₂. After inoculation, PBMC were cultured at a concentration of 1.10^6/ml in Iscove's modified Dulbecco's medium supplemented with 10% fetal calf serum, IL-2 (10 U/ml), penicillin (100 U/ml), and streptomycin (100 μg/ml). The medium was changed every 4 days.

**Virus.** For cell-free infection, the macrophage-tropic HIV-1 Ba-L isolate (12) was used. After 5 days of culture, MDM were exposed to an inoculum of 10^4.5 50% tissue culture-infective doses per ml; in our experiments, this corresponded to a multiplicity of infection of approximately 0.005. After 24 h, unabsorbed virus was removed and MDM were further cultured in fresh medium as described above.

For cell-free infection of PBMC, the T-cell line-tropic HIV IIB isolate (27) was used. After 2 days of stimulation with PHA, PBMC were exposed to an inoculum corresponding to a multiplicity of infection of approximately 0.001. After 2 hours, unabsorbed virus was removed and PBMC were cultured in fresh medium containing IL-2 as described above.

**Virus production.** Viral production was measured in a p24 antigen capture enzyme-linked immunosorbent assay (ELISA) (36). A culture was considered positive when the extinction was over two times that of the negative control. For detection of intracellular p24, MDM were cultured as described above. At day 14 after inoculation, cells were washed and lysed in 0.2% Triton X-100 and lysates were tested by ELISA.

**Cytokines.** Recombinant IL-3 (rIL-3; 10 ng/ml) was from Gist-Brocades (Delft, The Netherlands). rIL-10 (100 U/ml) and polyclonal anti-rIL-10 serum were kindly provided by R. de Waal-Malefijt and J. de Vries, DNAX Research Institute (Palo Alto, Calif.). rIL-2 (10 U/ml) was from Cetus Corporation (Emeryville, Calif.). rIL-4 was from Sandoz (Basel, Switzerland).

**PCR analysis.** To monitor reverse transcription, a semiquantitative PCR assay amplifying the R/U5 fragment or a conserved pol fragment was used. After 5 days of culture in the presence of cytokines where indicated, MDM were infected with HIV-1 Ba-L and cultured in the presence or absence of IL-10. The cell-free inoculum was DNase (RQI; 200 ng/ml; Promega Corp., Madison, Wis.) treated for 45 min in medium supplemented with 6 mM MgCl₂ and filtered through a 0.22-μm-pore-size filter. As a control, MDM were treated in parallel with 10 μM zidovudine for 30 min prior to inoculation to detect newly synthesized proviral DNA. MDM were harvested 2, 8, and 24 h after inoculation and washed five times. DNA was extracted from 10^6 cells and finally dissolved in 100 μl of water. For all PCR primer sets, the MgCl₂ concentration and thermal cycling were optimized. The HIV-1 R/U5 region was amplified in the presence of 2 mM MgCl₂ with the primer set M667-AA55 (39). To amplify a conserved sequence of the HIV-1 pol region (2) in the presence of 3 mM MgCl₂, we used the primers pol-D (5'-TTA GTC AGT GCT GGA ATC AGG-3' [positions are relative to nucleotides 4447 to 4467 of the HxB2D proviral genome]) and pol-F (5'-5'GTA ACT GCT ACC AGG-3' nucleotide [positions 4199 to 4219]). As a control for the general efficiency of PCR amplification of the DNAs, all of the DNAs were subjected to PCR analysis in the presence of 3 mM MgCl₂ with the primer set PC03-PC04 (5'-ACA CAA CTG TGT TCA CTA GC-3' and 5'-CAA CTG TCA CC-3' respectively), amplifying part of the human β-globin gene (28). The PCR amplification cycles were as follows: 5 min at 95°C once; 1 min at 95°C, 2 min at 37°C, and 2 min at 72°C repeated 25 times; and then an extra 5-min extension at 72°C and subsequent cooling to 4°C. For quantification, PCR products were separated on 1% agarose gels, blotted onto GeneScreen Plus membranes, and hybridized with [α-32P]dATP end-labelled oligonucleotide pol-C (5'-GCC ATG CAT GGA CTA GAC TGT AGT CCA GG-3' [nucleotide positions 4373 to 4404]) for pol-D-polF-amplified fragments, LTR-B (5'-GCA CTC AAG GAC GTA TTT ATT GAG GAC GC-3') for M667-AA55-amplified fragments, and R506-4 (5'-CGC ACT CTT GAG GAA TCT GCC GTT ACT GCC TGG G-3') for PC03-PC04-amplified fragments. Dependent on the specific activity of the probes, exposure to X-ray films was performed for 10 to 48 h at −70°C with intensifying screens.

**Transfection and chloramphenicol acetyltransferase (CAT) assay.** MDM were transected by electroporation. MDM were cultured for 5 days in the presence or absence of IL-3, IL-10, or a combination thereof. Cells were then harvested after 10 min of incubation in 5 mM EDTA by scraping with a rubber policeman. Cells were subsequently washed and resuspended in RPMI supplemented with 20% fetal calf serum to a final concentration of 20 × 10^6/ml. For each transfection, 5 × 10^6 cells and 10 μg of plasmid DNA were placed on ice for 10 min. Cells were electroporated at 250 V and 960 μF in disposable cuvettes (Bio-Rad Laboratories, Richmond, Calif.). Cells were transfected with either a long terminal repeat (LTR)-CAT construct in which the complete HIV-IIB LTR is linked to CAT (1) or a construct containing two nuclear factor κB (NF-κB) binding sites linked to the thymidine kinase promoter in front of the CAT gene (24). The LTR-CAT construct was cotransfected with a simian virus 40-tat construct (14) in which the tat gene is under control of the simian virus 40 promoter. After electroporation, cells were placed on ice for 10 min resuspended in culture medium to a final concentration of 2 × 10^6/ml, and cultured in six-well tissue culture plates. After 24 h, cell extracts were prepared for CAT assay.

**RNA isolation and Northern blot analysis.** MDM were inoculated with HIV-1 Ba-L after 5 days of culture in the presence or absence of IL-10. Virus was removed after 24 h, and the MDM culture was continued in the presence or absence of IL-10. Four days after infection, MDM were harvested for RNA isolation. Total RNA of 50 × 10^6 cells was isolated with RNA-zol (Cinna/Biotecx Laboratories Inc., Houston, Tex.) and then precipitated with isopropanol. Total RNA was separated on a 1% agarose-formaldehyde gel and transferred to GeneScreen Plus membranes (Du Pont Co. Biotechnology Systems, Boston, Mass.). Hybridization was performed with a 3′ LTR probe which recognizes all HIV-1 mRNA transcripts and a β-actin probe to measure the total RNA. The probes were radiolabelled with [α-32P]dATP by the random-primer method. Dependent on the specific activity of the probes, exposure to X-ray films was performed for 10 to 48 h at −70°C with intensifying screens.

**Immunoblot analysis.** MDM were cultured in the presence of HIV-1 pol region (2) in the presence of 3 mM MgCl₂, we used the primers pol-D (5'-TTA GTC AGT GCT GGA ATC AGG-3' [positions are relative to nucleotides 4447 to 4467 of the HxB2D proviral genome]) and pol-F (5'-5'GTA ACT GCT ACC AGG-3' nucleotide [positions 4199 to 4219]). As a control for the general efficiency of PCR amplification of the DNAs, all of the DNAs were subjected to PCR analysis in the presence of 3 mM MgCl₂ with the primer set PC03-PC04 (5'-ACA CAA CTG TGT TCA CTA GC-3' and 5'-CAA CTG TCA CC-3' respectively), amplifying part of the human β-globin gene (28). The PCR amplification cycles were as follows: 5 min at 95°C once; 1 min at 95°C, 2 min at 37°C, and 2 min at 72°C repeated 25 times; and then an extra 5-min extension at 72°C and subsequent cooling to 4°C. For quantification, PCR products were separated on 1% agarose gels, blotted onto GeneScreen Plus membranes, and hybridized with [α-32P]dATP end-labelled oligonucleotide pol-C (5'-GCC ATG CAT GGA CTA GAC TGT AGT CCA GG-3' [nucleotide positions 4373 to 4404]) for pol-D-polF-amplified fragments, LTR-B (5'-GCA CTC AAG GAC GTA TTT ATT GAG GAC GC-3') for M667-AA55-amplified fragments, and R506-4 (5'-CGC ACT CTT GAG GAA TCT GCC GTT ACT GCC TGG G-3') for PC03-PC04-amplified fragments. Dependent on the specific activity of the probes, exposure to X-ray films was performed for 10 to 48 h at −70°C with intensifying screens.
or absence of IL-10 (100 U/ml) for 5 days prior to inoculation with HIV-1. At day 6 or 10 after inoculation, cell lysates were prepared for immunoblot analysis. MDM (5 × 10⁶) were harvested and solubilized in 4% sodium dodecyl sulfate (SDS)-10% 2-mercaptoethanol. In parallel, 20 × 10⁶ MDM were solubilized in a lysis buffer containing 0.1% SDS and viral glycoproteins were enriched by using lentil lectin Sepharose 4B (Pharmacia), precipitated from the eluate with cold acetone, and washed with acetone. The pellets were dried and dissolved in 4% SDS-10% 2-mercaptoethanol. The samples were boiled for 10 min and centrifuged, and the supernatant was applied to a 1.5-mm-thick 8 or 10% acrylamide gel or a 3-mm-thick 11% acrylamide gel in the presence of SDS. After gel electrophoresis, proteins were transferred to nitrocellulose membranes that were subsequently blocked with gelatin as previously described (13). A polyclonal antibody raised against recombinant full-length p24 was used to identify gag-encoded proteins, and a polyclonal antibody raised against recombinant vaccinia virus-expressed gp120 was used to identify gp120 and gp160 in the different preparations. To visualize the antigen-antibody complex, horseradish peroxidase-conjugated horse antibodies against rabbit immunoglobulin G (CLB PK17E) were used in combination with 3',3'-diaminobenzidine as the substrate. Total protein on nitrocellulose membranes was visualized by amido black staining.

RESULTS

Dose-dependent inhibition of HIV-1 replication in MDM by IL-10. The effect of IL-10 on HIV-1 replication in MDM was studied. MDM were cultured for 5 days prior to inoculation with HIV-1 Ba-L in the presence of increasing concentrations of IL-10 (0, 10, 50, 100, 150, and 250 U/ml). In parallel experiments, increasing concentrations of IL-10 were only added 24 h after inoculation and kept in the cultures for 5 days. Cumulative p24 production was measured 7 and 14 days after inoculation.

Treatment with IL-10 before inoculation with HIV-1 resulted in dose-dependent inhibition of virus infection, which was complete at a concentration of 100 U of IL-10 per ml (Fig. 1a). Addition of IL-10 after inoculation also resulted in dose-dependent inhibition of HIV-1 replication (Fig. 1b), but even in the presence of the highest dose of IL-10 used, complete inhibition of virus production could not be achieved.

Besides the effect of IL-10 on HIV-1 production in otherwise untreated MDM, we analyzed the effect of IL-10 in combination with IL-3, a cytokine known to enhance HIV-1 expression in primary macrophages (16, 33, 34). MDM pretreated with IL-3 (10 ng/ml) showed enhanced virus production compared with untreated controls. However, when IL-3 was used in combination with IL-10 before inoculation, complete inhibition of virus production was found at a concentration of 100 U of IL-10 per ml (Fig. 1a). IL-10 treatment of MDM 24 h after HIV-1 inoculation in combination with IL-3 pretreatment resulted in dose-dependent inhibition of virus production, but even at the highest concentration of IL-10 used, complete inhibition of virus production could not be established (Fig. 1b). To confirm that the observed inhibition was specifically due to IL-10, the IL-10 preparation was preincubated with neutralizing anti-IL-10 serum (5) for 45 min at 37°C. Inhibition of HIV-1 replication by IL-10 (50 to 100 U/ml), added either before or after inoculation, was completely abrogated after preincubation of the IL-10 preparation with 5 μg of anti-IL-10 serum per ml (Fig. 2).

Previously, we have shown that IL-4 induces terminal differentiation of MDM and concomitantly inhibits MDM prolifer-

![FIG. 1. Dose-dependent complete inhibition of HIV-1 replication in MDM cultured in the presence of IL-10 prior to inoculation but partial inhibition of HIV-1 replication in MDM by IL-10 treatment after inoculation. Cells were cultured for 5 days before inoculation in the absence or presence of IL-3 (10 ng/ml) in combination with increasing concentrations of IL-10 either before (a) or 24 h after (b) inoculation. p24 production at day 14 is shown. The results are means of four replicates and representative of three independent experiments.]
IL-10 does not interfere with HIV-1 entry or reverse transcription in MDM. We have previously shown that in IL-4-treated, nonproliferating MDM, only incomplete proviral DNA species are synthesized. This indicated normal virus entry but a block in the virus replication cycle at the level of reverse transcription (32, 33, 39). To monitor the effect of IL-10 treatment on the kinetics of virus entry and reverse transcription, MDM were cultured in the presence or absence of IL-10. After 5 days, the cells were inoculated with DNase-treated HIV-1 Ba-L and cultures were continued. MDM that were cultured in medium alone prior to inoculation were cultured in the presence of IL-10 starting 2 h after inoculation. DNA was isolated 2, 8, and 24 h after inoculation, and semiquantitative PCR analysis of the presence of proviral DNA was subsequently performed. Amplification of the R/U5 region, which corresponds to a relatively early product of reverse transcription, was successful within 2 h after inoculation, irrespective of IL-10 treatment (Fig. 4). The presence of proviral pol DNA, a relatively late product of reverse transcription, could be demonstrated within 2 h after inoculation in untreated MDM and within 8 h after inoculation in MDM treated with IL-10 before or after inoculation (Fig. 4). As a control for the general efficiency of PCR amplification of the DNAs, all DNAs were subjected to PCR amplification of part of the human β-globin gene (Fig. 4). Although reached with different kinetics, 24 h after inoculation, the amounts of both the R/U5 and pol proviral DNA species were comparable in all of the MDM cultures, irrespective of IL-10 treatment. This indicated that in contrast to the results we obtained with IL-4-treated MDM, normal virus entry and reverse transcription could proceed in IL-10-treated MDM.

IL-10 does not inhibit HIV-1 replication at the transcriptional level. Inhibitory effects of IL-10 on the production of several cytokines by monocytes have been reported (5). Expression of these cytokines and HIV-1 are both predominantly regulated by NF-κB (17, 23, 35). Here we analyzed whether the inhibition of HIV-1 replication by IL-10 was at the transcriptional level and associated with inhibition of NF-κB activation.

The effect of IL-10 on HIV-1 transcription was studied first in a transient expression system. MDM were cultured for 5 days in the presence or absence of IL-3 (10 ng/ml) with or without IL-10 (100 U/ml). Cells were then transfected with CAT constructs in which the transcriptional activity is under control of NF-κB linked to a heterologous promoter or the complete HIV-1 LTR. A modest stimulatory effect on both HIV-1 LTR- and NF-κB-driven CAT activities was observed when MDM were treated with IL-10 alone or in combination with IL-3 (Fig. 5a). Comparable plasmid recovery from the transfected cells by Hirt extractions excluded the possibility that differences in CAT activity were due to differences in transfection efficiency. Normal LTR-driven gene expression in the presence of IL-10 was confirmed by Northern blot analysis.
of HIV-1-specific mRNA expression. MDM were cultured for 5 days in the presence or absence of IL-10 (100 U/ml). Twenty-four hours after inoculation, unab sorbed virus was removed and cultures were continued. MDM that were cultured in medium alone prior to inoculation were cultured in the continuous presence of IL-10 starting 24 h after inoculation. Four days after inoculation, total RNA was isolated. Irrespective of IL-10 treatment, equal amounts of all HIV-1-specific mRNAs species could be demonstrated (Fig. 5b). Apparent differences in HIV-1-specific mRNA transcripts could be attributed to differences in the total amounts of RNA, as demonstrated by hybridization with a β-actin probe. These results also excluded the possibility of an inhibitory effect of IL-10 on elongation of RNA transcription.

Accumulation of p24 in IL-10-treated, HIV-1-infected MDM. The normal expression of viral RNA products but the absence of virus production in IL-10-treated MDM pointed to posttranscriptional interference by IL-10 in the HIV-1 replication cycle. To analyze this, MDM were cultured in the presence of IL-10 (100 U/ml) for 5 days either prior to or after inoculation with HIV-1 Ba-L, the postinoculation treatment starting 24 h after virus exposure. Fourteen days after inoculation, the presence of p24 was measured in culture supernatant and cell lysates. When MDM were treated with IL-10 before inoculation, p24 antigen could not be detected in culture supernatant or cell lysates. In contrast, p24 antigen was detected in cell lysates of MDM that had been treated with IL-10 starting 24 h after inoculation, the amount of p24 antigen in the culture supernatant being very low compared with that in the untreated control (Fig. 6). The accumulation of p24 in these cells point to an inhibitory event at the level of virus assembly.

Absence of processed viral proteins in MDM cultured in the presence of IL-10 prior to inoculation. The absence, as established by p24 ELISA, of accumulated p24 in MDM that had been treated with IL-10 prior to inoculation prompted us to study the expression of viral proteins by immunoblot analysis. MDM were cultured in the presence or absence of IL-10 (100 U/ml) for 5 days prior to inoculation with HIV-1 Ba-L, after which the cultures were continued. MDM that were cultured in medium alone prior to inoculation were cultured in the presence of IL-10 for 5 days starting 24 h after inoculation. At day 10 after inoculation, when the presence of p24 in the supernatants of both the untreated culture and the culture treated with IL-10 after inoculation indicated HIV-1 production, cell lysates were prepared for immunoblot analysis.

The presence of Gag proteins and Gag precursor proteins (7, 36) could be demonstrated in lysates of untreated MDM and MDM treated for 5 days with IL-10 after inoculation. However, in lysates of MDM that had been cultured in the presence of IL-10 prior to inoculation, no evidence of Gag proteins or Gag precursor proteins was observed (Fig. 7A). Viral gp120 and gp160 were also demonstrated in lysates of untreated MDM and MDM treated with IL-10 after inoculation but not in lysates of MDM cultured in the presence of IL-10 prior to inoculation (Fig. 7A). Even when viral glycoproteins were isolated from $2 \times 10^6$ MDM, no gp120 could be detected in MDM treated with IL-10 prior to inoculation (Fig. 7B). Differences in the amounts of viral proteins could not be
attributed to differences in the amounts of total protein as visualized on nitrocellulose by amido black staining (Fig. 7C).

When cell lysates were already prepared for immunoblot analysis at 6 days after inoculation, the presence of the Pr53 gag-encoded protein and the lower precursor form (Pr37) (7) could be demonstrated in lysates of untreated MDM. However, in lysates of MDM that had been cultured in the presence of IL-10 prior to inoculation, accumulation of the Pr53 gag-encoded protein, but no evidence of processed gag-encoded proteins, was observed (Fig. 7D). Since productive HIV-1 infection in MDM could not be measured at day 6 after inoculation, the amount of the p24 gag-encoded protein in these cell lysates may have been extremely low. The absence later on in infection of gag-encoded Pr53 in MDM treated with IL-10 prior to inoculation (Fig. 7A) may have been due to degradation of the Pr53 in these cells (cf. reference 38).

Cumulative inhibitory effect of IL-10 in combination with IL-4 on HIV-1 replication in MDM. Previously, we showed that IL-4 interferes with HIV-1 replication in MDM at the level of reverse transcription (33). Here we show that IL-10 interferes with HIV-1 replication most likely at a posttranslational level. We analyzed whether the IL-4 and IL-10 inhibitory effects on HIV-1 replication are synergistic. MDM were cultured for 5 days prior to inoculation with HIV-1 Ba-L in medium alone or in medium containing IL-4 (0.1 or 1 ng/ml) in combination with increasing concentrations of IL-10 (0, 1, 5, 10, 50, 100, 150, and 250 U/ml). Cumulative p24 production was measured 7 and 14 days after inoculation. Treatment with IL-10 alone resulted in a dose-dependent inhibition of HIV-1 replication in MDM, which was complete at a concentration of 100 U/ml (Fig. 8). The inhibitory effect of IL-10 when used together with IL-4 (0.1 ng/ml) was stronger than the inhibition observed in MDM treated with IL-10 alone, although it was still complete only at an IL-10 concentration of 100 U/ml. In combination with 1 ng of IL-4 per ml, complete inhibition of virus replication in MDM was already achieved at 1 U of IL-10 per ml. Analysis of the dose-response curves for IL-4 and IL-10 separately showed that the sum of the percentages of inhibition achieved by each of the cytokines alone was never exceeded by the percentage of inhibition observed when those concentrations of IL-4 and IL-10 were used in combination. This indicated that IL-10 and IL-4 in combination have a cumulative, but not a synergistic, inhibitory effect on HIV-1 replication in MDM.

DISCUSSION

In a previous study, we demonstrated that a 5-day culture of primary macrophages in the presence of IL-4 resulted in MDM that were completely refractory to HIV-1 infection. The induction of terminal differentiation probably abrogated their proliferative capacity and, concomitantly, their susceptibility to infection (33). Effects of IL-10 on macrophages, similar to those of IL-4 (5, 37), prompted us to study the effect of IL-10 on the HIV-1 replication cycle.

IL-10 interfered with virus production both in already infected MDM, although only partial inhibition was established in these cells, and in MDM treated with IL-10 prior to inoculation. Inhibition of virus production was also observed when IL-10 was used in combination with IL-3, a cytokine previously reported to enhance HIV-1 infection in MDM (16, 34). The inhibitory effect was specific to IL-10, since it was completely neutralized by anti-IL-10 serum. Virus entry and reverse transcription were not disturbed in IL-10-treated MDM. Moreover, IL-10 did not affect LTR-driven expression since Northern blot analysis showed equal amounts of HIV-1-specific mRNA species, also excluding an effect on elongation of virus transcription. Thus, both in MDM cultured in the presence of IL-10 prior to inoculation and in MDM treated with IL-10 after HIV-1 exposure, the virus replication cycle appeared to be normal up to the level of RNA transcription. The presence of apparently mature p24 protein in lysates of MDM treated with IL-10 after HIV-1 inoculation indicated accumulation of viral proteins, pointing to an inhibitory effect on virus assembly. To analyze whether the absence of accumulated gag-encoded proteins in MDM treated with IL-10 prior to inoculation was due to a defect in protein synthesis, immunoblot analysis was performed 6 days after inoculation, revealing accumulation of Pr53 gag-encoded precursor protein and complete absence of processed gag-encoded proteins in MDM. Immunoblot analysis performed 10 days after inoculation showed a complete absence of viral proteins p24 and gp120 in MDM that had been cultured in the presence of IL-10 prior to inoculation, not only suggesting the degradation of precursor proteins (38) but also indicating that the effect of IL-10 was not restricted to gag-encoded proteins alone. These results suggest an IL-10-mediated inhibitory effect at the level of protein processing. Although the inhibitory effects of IL-10 differ, depending on the time of administration relative to the time of inoculation, both pre- and postinoculation treatments interfered at a posttranslational level. The possibility that both treatments lead to interference early and late, respectively, in the same process of protein processing cannot be excluded. The monocyte differentiation state at the time of IL-10 administration may explain the differential response to IL-10 treatment.

Inhibitory effects of IL-10 on cytokine expression in monocytes at the transcriptional level have already been described (5). Moreover, IL-10 treatment resulted in decreased expression of cell surface molecules such as major histocompatibility complex class II (6) and B7 (8), but in those studies the
FIG. 7. Absence of processed viral proteins in HIV-1-inoculated MDM cultured in the presence of IL-10 for 5 days prior to inoculation but normal expression of processed viral proteins in untreated MDM and MDM treated with IL-10 after inoculation. MDM were cultured with or without IL-10 (100 U/ml) for 5 days prior to inoculation with HIV-1 Ba-L and then cultured further. MDM not treated with IL-10 prior to inoculation were cultured with IL-10 starting at 24 h after inoculation. At day 10 after inoculation, when p24 could be detected in the culture supernatant of the untreated culture and the culture treated with IL-10 after inoculation, lysates of 5 x 10^6 MDM were prepared for immunoblot analysis. Electrophoresis was performed with a 1.5-mm-thick 10% acrylamide gel, and the proteins were transferred to nitrocellulose. The immunoblot was stained for gag-encoded proteins and gp120-gp160 (A). Viral glycoproteins were isolated from 20 x 10^6 MDM and prepared for immunoblot analysis. Electrophoresis was performed with a 1.5-mm-thick 8% acrylamide gel, and the proteins were transferred to nitrocellulose. The immunoblot was stained for gp120-gp160 (B). The total protein on nitrocellulose membranes was visualized by amido black staining (C). (D) MDM were cultured with or without IL-10 (100 U/ml) for 5 days prior to inoculation with HIV-1 Ba-L, and lysates of 5 x 10^6 MDM were prepared for immunoblot analysis 6 days after inoculation. Gel electrophoresis was performed on a 3-mm-thick 11% acrylamide gel, and the proteins were transferred to nitrocellulose. The immunoblot was stained for gag-encoded proteins.

FIG. 8. IL-4 and IL-10 have a cumulative effect on HIV-1 replication in MDM. MDM were cultured for 5 days prior to inoculation with HIV-1 Ba-L in medium alone or in the presence of IL-4 (0.1 or 1 ng/ml) in combination with increasing concentrations of IL-10 (0, 1, 5, 10, 50, 100, 150, and 250 U/ml). p24 production at day 7 is shown. The results are means of two replicates and are representative of four independent experiments.
towards increased IL-4 and IL-10 production in response to mitogen (3, 4).

IL-10 and IL-4 both interfered with HIV-1 replication in MDM, albeit at different levels of the virus replication cycle. When IL-10 treatment was performed in combination with IL-4, cumulative inhibition of HIV-1 production by MDM could be demonstrated. In the light of our current and previous (33) findings, elevated production of IL-4 and IL-10 may thus decrease macrophage susceptibility for HIV-1 infection. Moreover, preferential infection of Th2 cells, but not Th1 cells, by HIV-1 has been suggested (18). The shift from low frequencies of predominantly macrophage-tropic HIV-1 clones towards high frequencies of preferentially T-cell-tropic clones, as we previously observed with progression of disease (30), may be associated with this altered cytokine production profile. This observation may thus be suggestive of an important contribution of cytokines to the in vivo regulation of HIV-1 tropism.

The presence of macrophage-tropic HIV-1 variants during all stages of infection has pointed to an important role for macrophages in viral persistence. Therefore, the inhibitory effect of IL-10 on both susceptibility to infection and virus replication in already infected MDM suggests that this cytokine could be a candidate for use in therapeutic intervention. Moreover, IL-10 had no effect on virus replication in primary T cells. The possible role, however, of both IL-4 and IL-10 in the cross-regulation of Th phenotypes and impaired immunity, most likely associated with a skewing to Th2 cells (21), argues against the use of these cytokines to treat HIV-1-infected individuals.

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REFERENCES


