

# Nitric Oxide Regulates MIP-1 $\alpha$ Expression in Primary Macrophages and T Lymphocytes: Implications for Anti-HIV-1 Response

Barbara Sherry,<sup>1</sup> Helena Schmidtmayerova,<sup>1,2</sup> Gabriele Zybarth,<sup>1</sup> Larisa Dubrovsky,<sup>1</sup> Tobias Raabe,<sup>1,3</sup> and Michael Bukrinsky<sup>1</sup>

<sup>1</sup>The Picower Institute for Medical Research, Manhasset, New York, U.S.A.

<sup>2</sup>Institute of Virology, Slovak Academy of Sciences, Bratislava, Slovakia

<sup>3</sup>Present address: Department of Internal Medicine, Ohio State University, Columbus, Ohio, U.S.A.

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## Abstract

**Background:** Chemokines and chemokine receptors have been shown to play a critical role in HIV infection. Chemokine receptors have been identified as coreceptors for viral entry into susceptible target cells, and several members of the  $\beta$  chemokine subfamily of cytokines, MIP-1 $\alpha$ , MIP-1 $\beta$ , and RANTES, have been identified as the major human immunodeficiency virus (HIV)-suppressive factors produced by activated CD8<sup>+</sup> T lymphocytes. In macrophages, HIV-1 infection itself was shown to upregulate the production of MIP-1 $\alpha$  and MIP-1 $\beta$ . In the present study, we address the mechanisms by which HIV-1 infection regulates  $\beta$  chemokine responses in macrophages and lymphocytes.

**Material and Methods:** To address whether nitric oxide (NO), generated as a consequence of HIV-1 infection, regulates  $\beta$  chemokine responses in monocyte/macrophages and/or macrophage-depleted peripheral blood mononuclear cells (PBMCs) these two cell populations were isolated from HIV seronegative donors, placed in culture, and infected with HIV-1 in either the presence or absence of exogenous activators (e.g. lipopolysaccharide, phytohemagglutinin), inhibitors

of nitric oxide synthase (NOS), or chemical donors of NO. Cultures were analyzed for  $\beta$  chemokine responses by ELISA and RNase protection.

**Results:** LPS-induced MIP-1 $\alpha$  release is enhanced in HIV-1-infected, as compared to uninfected, monocyte/macrophage cultures, and this enhancing effect is partially blocked by the addition of inhibitors of NOS, and can be reproduced by chemical generators of NO even in the absence of HIV-1 infection. A similar strategy was used to demonstrate a role for NO in HIV-1-mediated induction of MIP-1 $\alpha$  in unstimulated macrophage cultures. NOS inhibitors also decreased MIP-1 $\alpha$  and MIP-1 $\beta$  production by phytohemagglutinin-stimulated monocyte-depleted PBMC cultures.

**Conclusions:** These results indicate that NO amplifies MIP-1 $\alpha$  responses in activated macrophages and lymphocytes, and suggests that this pleiotropic molecule might function as an enhancing signal that regulates secretion of  $\beta$  chemokines during HIV-1 infection. These findings reveal a novel mechanism by which NO might regulate the anti-HIV activity of immune cells.

## Introduction

Within the cytokine network there is a discrete family of highly homologous low molecular weight peptides with potent chemotactic and activating properties, called chemokines (chemo-

attractant cytokines) (1). Chemokines are secreted primarily by activated leukocytes (lymphocytes and monocytes) and have been implicated in a wide range of acute and chronic inflammatory processes (2). The superfamily's two main branches are classified as "C-X-C" or  $\alpha$  and "C-C" or  $\beta$  groups, as defined by the spacing of the first two cysteines in the conserved motif.

Relationships between human immunodeficiency virus (HIV)-1 infection and chemokine

Address correspondence and reprint requests to: Dr. Michael Bukrinsky, The Picower Institute for Medical Research, 350 Community Drive, Manhasset, NY 11030, U.S.A. Phone: (516) 365-4200; Fax: (516) 365-5090; E-mail: mbukrinsky@picower.edu

production are complex and not fully understood.  $\beta$  Chemokines, RANTES, MIP-1 $\alpha$ , and MIP-1 $\beta$ , have been identified as the major HIV-suppressing factors produced by activated CD8<sup>+</sup>T lymphocytes (3). As we demonstrated some time ago, HIV-1 infection of monocytes results in the specific and selective induction of chemokines MIP-1 $\alpha$  and MIP-1 $\beta$ , but not pro-inflammatory cytokines tumor necrosis factor (TNF) and interleukin-6 (IL)-6 (4). This suggests that chemokine production could be a defensive response of monocytes to viral infection. On the other hand, virus-induced chemokines can produce pathological effects on surrounding tissue, as was shown in mice with Coxsackievirus-caused myocarditis (5). As potent mediators of cell migration and activation, chemokines are believed to play a central role in the early events of inflammation by localizing and enhancing the inflammatory reaction at the site of tissue damage, including the one caused by HIV-1 infection.

Increased production of nitric oxide (NO) has been linked to HIV-1 infection both in vivo and in vitro (6–10). Several studies described a suppressive effect of NO on HIV-1 replication in vitro through inhibition of viral enzymes, reverse transcriptase (11) and protease (12), or cellular transcription factor nuclear factor- $\kappa$ B (NF- $\kappa$ B) and long terminal repeat (LTR)-driven transcription (13–15). Our earlier work (16) demonstrated that NO contributed to hyper-responsiveness of HIV-1-infected macrophages, in particular by enhancing lipopolysaccharide (LPS) induced TNF $\alpha$  secretion. However, no link between NO and  $\beta$  chemokine production during HIV-1 infection has been established.

In this paper, we present evidence that NO generated in HIV-infected monocytes up-regulates expression of MIP-1 $\alpha$  induced in response to stimulation with LPS. We also demonstrate that HIV-1 infection activates expression of inducible nitric oxide synthase (NOS) in monocyte-depleted peripheral blood mononuclear cell (PBMC) cultures and NOS inhibitors decrease the amount of MIP-1 $\alpha$  produced by these cells in response to phytohemagglutinin (PHA) stimulation. These results suggest a novel role of NO in the control of anti-HIV responses and provide new insights into the possible mechanisms of MIP-1 $\alpha$  regulation in HIV-1-infected patients.

## Materials and Methods

### Reagents

Lipopolysaccharide (LPS), aminoguanidine (AG), N<sup>G</sup>-monomethyl-L-arginine (L-NMMA), D-NMMA, sodium nitroprusside (SNP), and s-nitroso-N-acetyl-penicillamine (SNAP) were purchased from Sigma, St. Louis, MO. Spermine bis(nitric oxide) adduct (NONOate) was obtained from Toronto Research Chemicals Inc., North York, Ontario, Canada.

### Cell Cultures and Infection with HIV-1

PBMC of HIV- and hepatitis B-seronegative donors were purified from whole blood by Ficoll-Hypaque gradient centrifugation. Macrophage cultures were prepared from PBMC by plastic adherence as described (7). Adherent cultures were >98% monocytes by the criteria of cell morphology and nonspecific esterase staining. Monocytes were allowed to differentiate in vitro for 6 days in the presence of 250 U/ml recombinant human macrophage-colony stimulating factor (rhM-CSF) (Sigma), then exposed to 100 ng p24 of a monocyto-tropic strain HIV-1<sub>ADA</sub> per 10<sup>6</sup> cells as described (4,7). After an overnight incubation, cells were washed and incubation was continued in Dulbecco's modified Eagle's medium (DMEM), supplemented with 20% human serum. Progress of infection was followed by reverse transcription (RT) activity in the culture supernatants. Non-adherent cultures (predominantly T and B lymphocytes) were treated with 5  $\mu$ g/ml PHA (Sigma) for 48 hr, washed, and inoculated with heat-inactivated (56°C, 2 hr) HIV-1<sub>LAI</sub> (RT activity of the inoculum prior to heat-inactivation was 8  $\times$  10<sup>5</sup> cpm per 10<sup>6</sup> cells). After a 1-hr absorption, excess of the virus was washed away, and cells were plated at a density 1  $\times$  10<sup>6</sup> cells/ml in (RPMI) 1640 supplemented with 20 U/ml of recombinant IL-2, 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (pen/strep) (Sigma).

### ELISA Assays

Human MIP-1 $\alpha$  and MIP-1 $\beta$  levels were each determined by a specific sandwich ELISA as described (4). Polyclonal goat anti-human MIP-1 $\alpha$  (or MIP-1 $\beta$ ) obtained from R&D Systems, Minneapolis, MN, USA was used as a coating antibody and polyclonal rabbit anti-human MIP-1 $\alpha$  (or MIP-1 $\beta$ ) was used as a

primary antibody. These two chemokine sandwich ELISAs are specific (no detectable MIP-1 $\alpha$ /MIP-1 $\beta$  cross-reactivity), and consistently detect concentrations of MIP-1 peptides above 50 pg/ml. RANTES ELISA was purchased from R&D Systems.

#### *RNase Protection Assay*

RNase protection was performed according to published procedures (17). MIP-1 $\alpha$  probe yielded a protected fragment of 168 nucleotides; MIP-1 $\beta$  probe, of 140 nucleotides; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe, of 95-nucleotide fragment on a sequencing-type gel.

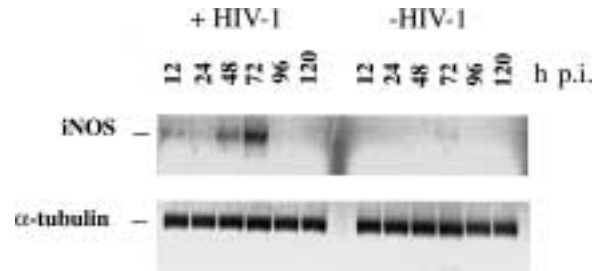
#### *Reverse Transcription Polymerase Chain Reaction (RT-PCR) Analysis*

Total cellular RNA was extracted from 10<sup>6</sup> PBMCs with RNazol (Biotecx Laboratories, Inc., Houston, TX). RNA was reverse transcribed using random hexamer primers and murine leukemia virus (MLV) reverse transcriptase (Gibco BRL, Rockville, MD). PCR was performed with cDNA using primers specific for iNOS [forward: 5'-CTGTCCTTG-GAAATTTCTGTT-3'; reverse: 5'-TGGCCAGAT-GTTCCTCTATT-3'; probe: 5'-CTCAGCAAGCA-GCAGAATGAGT-3' (18)] and  $\alpha$ -tubulin (19) as previously described (7). iNOS-specific PCR fragment of 458 bp was revealed either by ethidium bromide staining of the gel or by Southern hybridization as described (7).

## Results

#### *HIV-1 Induced iNOS mRNA in Monocyte-depleted PBMC Cultures*

Previously, we presented evidence that infection with HIV-1 leads to induction of iNOS and production of NO by HIV-1-infected macrophage cultures (7). This effect did not require productive infection of the target cells, since it could be reproduced by inoculation of macrophage cultures with heat-inactivated virus or T cell line-tropic strains of HIV-1 that did not replicate in monocytes (H. S. and M. B., unpublished results). To determine whether HIV-1 can similarly induce iNOS in T lymphocytes, another major target of the virus, we analyzed expression of iNOS gene by RT-PCR in monocyte-depleted PBMCs inoculated with heat-inactivated HIV-1<sub>LAT</sub>. Results presented in Figure 1 demonstrate

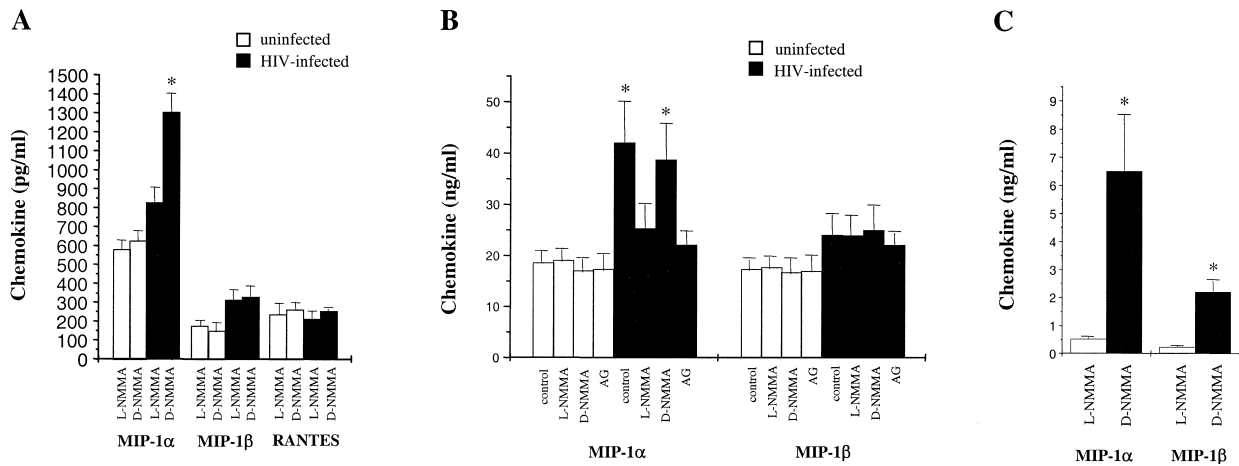


**Fig. 1. Demonstration of iNOS RNA induction in monocyte-depleted PBMC cultures.** At the indicated intervals after inoculation with heat-inactivated human immunodeficiency virus; +HIV-1), total cellular RNA was isolated and analyzed by reverse transcription polymerase chain reaction (RT-PCR) using primers specific for iNOS and  $\alpha$ -tubulin. Control cells (-HIV-1) were cultured under the same conditions, but without addition of the virus. iNOS, inducible nitric oxide synthase; PBMC, peripheral blood mononuclear cell; h.p.i, hours post inoculation.

a time-dependent induction of iNOS RNA in these cultures. Expression of iNOS RNA reached maximum at day 3 after infection and decreased thereafter. Therefore, nonproductive interaction with HIV-1 induced iNOS expression, both in macrophages and nonadherent PBMC.

#### *Inhibitors of NOS Decreased MIP-1 $\alpha$ Production*

NO is a second-messenger molecule that mediates signal transduction and activation in a variety of cells (20,21). We, therefore, investigated the possibility that NO could regulate expression of  $\beta$  chemokine peptides in HIV-1-infected macrophages, where these molecules were induced by infection alone and in the absence of any additional stimulation (4). Results presented in Figure 2A demonstrate that NOS inhibitor L-NMMA, but not its inactive control D-NMMA, significantly reduced levels of MIP-1 $\alpha$  secreted by HIV-infected macrophages. Similar results were obtained with a different inhibitor of NOS, aminoguanidin (not shown). Interestingly, levels of two other  $\beta$  chemokines implicated in anti-HIV response, MIP-1 $\beta$  and RANTES, were not up-regulated significantly by HIV-1 infection and were not affected by NOS inhibitors (Fig. 2A). This result is in seemed contradiction with our previous observation that expression of both MIP-1 $\alpha$  and MIP-1 $\beta$  were up-regulated during HIV-1 infection of macrophages (4). A likely explanation for this difference is that the time course of MIP-1 $\beta$  expression was much shorter than that of MIP-1 $\alpha$  (4); therefore, we may have missed the peak of production in this experiment.



**Fig. 2. Effects of NOS inhibitors on  $\beta$  chemokine production.** (A) Monocyte cultures (uninfected or infected with human immunodeficiency virus-type 1<sub>ADA</sub> (HIV-1<sub>ADA</sub>)) were cultivated with N<sup>G</sup>-monomethyl-L-arginine or N<sup>G</sup>-monomethyl-D-arginine (L- or D-NMMA; 2 mM). Eleven days after infection, culture supernatants were withdrawn and tested for HIV-1 reverse transcription (RT) activity (4) and chemokine content by ELISA. RT activity was similar in all supernatants and equaled  $1.5 \times 10^6$  cpm/ml. (B) HIV-1-infected and uninfected (control) monocyte cultures were stimulated with 10 ng/ml lipopolysaccharide (LPS) in the presence or absence

of L-NMMA (2 mM) or aminoguanidine (AG; 0.5 mM). 24 hr after stimulation, chemokines were assayed by ELISA. (C) Phytohemmagglutinin (PHA)-activated peripheral blood mononuclear cells (PBMCs); depleted of monocytes by adherence to plastic were cultured in the presence of 2 mM L- or D-NMMA. At the indicated intervals, chemokines in the culture supernatants were assayed by ELISA. All data are presented as mean  $\pm$  SEM of the triplicate cultures, and \* indicates  $p$ -value  $\leq$  0.05 calculated using the two-tailed  $t$ -test. MIP-1 $\alpha$ ; MIP-1 $\beta$ .

HIV-1-infected macrophage cultures stimulated with LPS produced significantly higher levels of MIP-1 $\alpha$  peptide than similarly stimulated uninfected cells (Fig. 2B). Again, this effect was much less evident for MIP-1 $\beta$  and RANTES. NOS inhibitors, aminoguanidine and L-NMMA, but not D-NMMA, significantly reduced HIV-1-specific enhancement of LPS-induced MIP-1 $\alpha$  (Fig. 2B), indicating a role of NO in this process. Inhibitors of NOS did not affect LPS-induced production of chemokines in uninfected monocyte cultures, in agreement with the previously demonstrated lack of NOS activity in LPS-stimulated human macrophages (7,22).

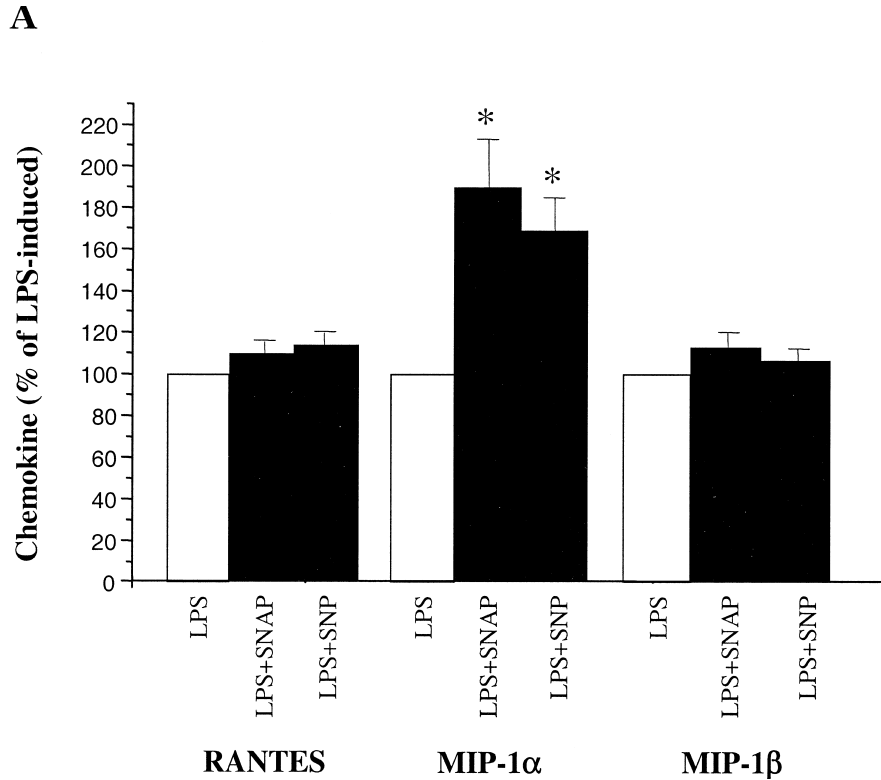
T lymphocytes are the other major producers of  $\beta$  chemokines in the body, including MIP-1 $\alpha$ , MIP-1 $\beta$ , and RANTES (3). Expression of MIP-1 $\alpha$ , MIP-1 $\beta$ , and RANTES was induced in monocyte-depleted PBMC by stimulation with PHA or anti-CD3 antibodies (not shown). This induction was suppressed significantly by L-NMMA, as demonstrated in Figure 2C for MIP-1 $\alpha$  and MIP-1 $\beta$ . Cultures treated with D-NMMA served as controls. This result suggested that NO amplified  $\beta$  chemokine expression in PHA-stimulated T lymphocytes. We did not observe any HIV-1-dependent increase in  $\beta$

chemokine secretion by nonadherent PBMC cultures (not shown), presumably because, unlike the situation with macrophages, HIV-1 infection did not alter NO production in activated T cells.

#### *Chemical Generators of NO Amplified LPS-induced Chemokine $\beta$ Production by Macrophage Cultures*

To directly investigate the effects of NO on  $\beta$  chemokine production by human monocytes, we employed two different chemical generators of NO: sodium nitroprusside (SNP) and s-nitroso-N-acetyl-penicillamine (SNAP). Both compounds amplified LPS-mediated induction of MIP-1 $\alpha$  (Fig. 3A). The effect of these compounds on RANTES and MIP-1 $\beta$  expression was significantly smaller, thus, reproducing results obtained with HIV-1-infected cells. No induction of chemokines was observed by NO-generating compounds alone.

To determine mechanisms of chemokine regulation by NO, levels of MIP-1 $\alpha$  and MIP-1 $\beta$  RNA expression in macrophages were assayed using an RNase protection method. This analysis demonstrated that MIP-1 $\alpha$ , but not MIP-1 $\beta$ , RNA was up-regulated by HIV-1 infection (Fig. 3B). Levels of RNA encoding MIP-1 $\alpha$  in HIV-1-



**Fig. 3. Effects of nitric oxide (NO) generators on  $\beta$  chemokine production.** (A) Analysis of chemokine  $\beta$  peptides in the supernatants of monocyte cultures. Monocyte cultures were stimulated with 0.5 ng/ml lipopolysaccharide (LPS) alone or in combination with NO donors, S-nitroso-N-acetyl-penicillamine (SNAP; 40  $\mu$ M) or sodium nitroprusside (SNP; 100  $\mu$ M). 18 hr after the stimulation, chemokine  $\beta$  peptides were assayed by ELISA. Data shown are mean  $\pm$  SEM of the triplicate cultures, and \* indicates  $p$ -value  $\leq$  0.05 calculated using the two-tailed t-test. (B) RNase protection analysis of human immunodeficiency virus-type 1 (HIV-1)-infected and NO-treated

monocytes. HIV-1-infected and control monocyte cultures were treated with 0.5 ng/ml LPS. To some cultures, donors of NO were added together with LPS at the following concentrations: SNP at 100  $\mu$ M, spermine bis(nitric oxide) adduct (NONOate) at 10  $\mu$ M, SNAP at 500  $\mu$ M. 2 hr after stimulation, cytoplasmic RNA was extracted and assayed by RNase protection method. Bands corresponding to protected fragments of MIP-1 $\alpha$ , MIP-1 $\beta$ , and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are marked. Intensity of the bands is directly proportional to the amount of corresponding RNA.

infected macrophages stimulated with LPS were significantly higher than in similarly stimulated uninfected cells. Chemical generators of NO (spermine NONOate, SNAP, and SNP) increased LPS-induced RNA levels of chemokines (Fig. 3B). Some discrepancy between the effects of NO on chemokine-specific RNA expression and chemokine levels in the culture medium might indicate either a slow induction of this chemokine, or an additional level of its regulation. In particular, there was a noticeable enhancement of MIP-1 $\beta$  RNA by NO donors in Fig. 3B, whereas, the levels of this chemokine in the culture medium were not significantly changed (Fig. 3A). Taken together, these results suggested that HIV-1 and NO regulated  $\beta$  chemokine expression at the level of mRNA, most likely by enhancing transcription of  $\beta$  chemokine genes.

## Discussion

Results presented in this report demonstrate a novel mechanism of  $\beta$  chemokine regulation. We show that NO, which is induced in macrophages upon infection with HIV-1 (7), amplifies both HIV-1- and LPS-induced expression of MIP-1 $\alpha$  in these cells. No stimulatory effect of HIV-1 infection on  $\beta$  chemokine production is observed in monocyte-depleted PBMC cultures. Nevertheless, exogenous NO amplifies  $\beta$  chemokine secretion by monocyte-depleted activated PBMC, similar to its effect on LPS-stimulated macrophage cultures. These results are consistent with the previously demonstrated synergism between NO and LPS in the stimulation of TNF $\alpha$  expression in macrophages (16). We observed certain differences with regard to NO contribution to chemokine

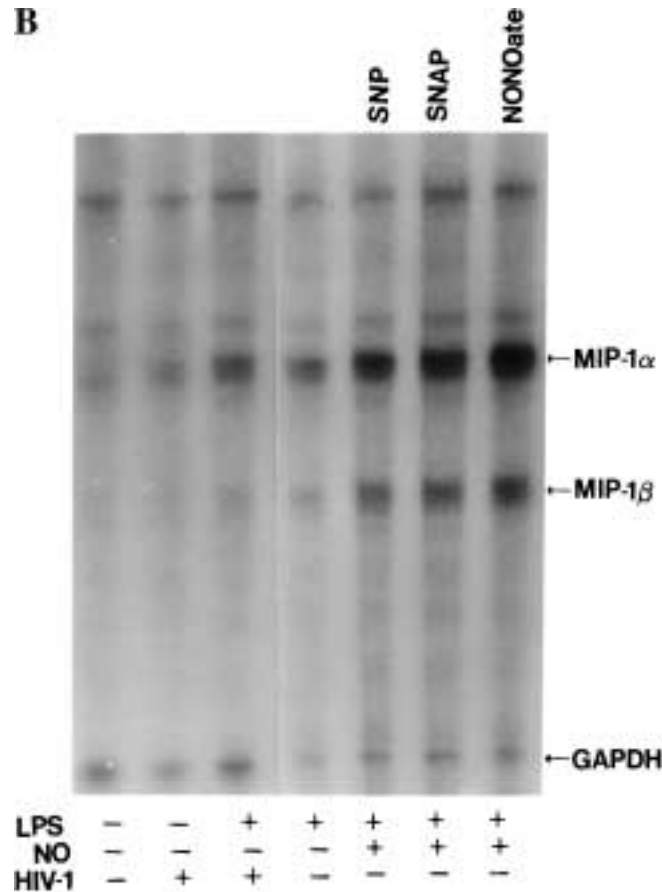


Fig. 3. (Continued)

production by uninfected mononuclear cells. Uninfected monocyte cultures do not produce NO, even after stimulation with LPS, and NOS inhibitors, therefore, do not affect chemokine expression in LPS-stimulated uninfected monocytes. However, NOS inhibitors have a significant effect on chemokine production by PHA-stimulated uninfected lymphocytes (Fig. 2C). This result suggests that PHA-stimulated lymphocytes produce NO. Our results also indicate that RNA encoding the inducible form of NOS [the one that is activated in HIV-1-infected monocytes (7)] is expressed in HIV-1-infected PBMCs, but is undetectable in uninfected cells. This leaves the possibility that a constitutive form of NOS (cNOS) is responsible for NO-mediated effects in uninfected lymphocytes and is involved in the regulation of chemokine expression in these cells. Expression of cNOS has been demonstrated in T lymphocytes, together with a complex cross-regulation of cNOS and iNOS mRNA expression during cell activation (23). Taken together, these results indicate that, in both macrophages

and T lymphocytes, HIV-1 infection induces iNOS expression, which may participate in the regulation of these cells' response to stimulating factors.

NO modulates MIP-1 $\alpha$  levels both in unstimulated and LPS-stimulated HIV-1-infected monocytes. However, by itself, NO does not induce chemokine expression. These results imply that HIV-1 affects MIP-1 $\alpha$  expression in monocytes by a bimodal mechanism, inducing chemokine production directly and amplifying activation-induced MIP-1 $\alpha$  secretion through NO-mediated mechanism. The latter mechanism appears to act at the transcriptional level, but there is a certain selectivity with its effects. Production of MIP-1 $\beta$ , readily induced by LPS, is affected much less by HIV-1 infection and NO-mediated enhancement than is MIP-1 $\alpha$ . This result indicates that the ratio of  $\beta$  chemokine peptides secreted by an immune cell can be affected by HIV-1 infection, and underscores the importance of the studies aimed to determine the effect of this ratio on anti-HIV activity of  $\beta$  chemokines.

The finding that HIV-1 infection affects expression of  $\beta$  chemokines via induction of NO reveals a novel mechanism of NO-mediated anti-viral activity of immune cells. Previous studies demonstrated protective effects of NO against several viruses (23–26), including the murine retrovirus Friend leukemia virus (27) and HIV-1 (10,28). Our study reveals an additional possible mechanism for this activity, at least in the case of HIV-1 infection, through enhancement of chemokine  $\beta$  expression. Three peptides from this group of chemokines (i.e. MIP-1 $\alpha$ , MIP-1 $\beta$ , and RANTES) have been shown to exert a powerful anti-HIV activity (3) and the ability of NO to affect levels of these peptides could be an important factor in the development of anti-HIV response. T lymphocytes and monocytes/macrophages are major producers of these chemokines (1) and, in both these cell populations, chemokine production is up-regulated by NO, thus, suggesting that NO-chemokine link is engaged as a general response of immune cells to HIV-1 infection.

Although up-regulation of  $\beta$  chemokines may well be a protective response of monocytes to HIV-1 infection, one can not exclude the possibility that their release may contribute also to the pathologic sequelae associated with this infection, in part due to attraction of uninfected monocytes and T lymphocytes to an infected cell. This provides new targets for the virus and also results in amplification of potentially harmful inflammatory reactions, as recently demonstrated for Coxsackie- and influenza-infected mice (5). Which one of these effects of chemokines prevails could be an important factor determining the outcome of the fight between the immune system and the virus.

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