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Endogenous IL-32 Controls Cytokine and HIV-1 Production

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IL-32, a proinflammatory cytokine that activates the p38MAPK and NF-κB pathways, induces other cytokines, for example, IL-1β, IL-6, and TNF-α. This study investigated the role of endogenous IL-32 in HIV-1 infection by reducing IL-32 with small interfering (si)RNA in freshly infected PBMC and in the latently infected U1 macrophage cell line. When PBMC were pretreated with siRNA to IL-32 (siIL-32), IL-6, IFN-γ, and TNF-α were reduced by 57, 51, and 36%, respectively, compared with scrambled siRNA. Cotransfection of NF-κb and AP-1 reporter constructs with siIL-32 decreased DNA binding of these transcription factors by 42 and 46%, respectively. Cytokine protein array analysis revealed that the inhibitory activity of siIL-32 primarily targeted Th1 and proinflammatory cytokines and chemokines, e.g., MIP-1α/β. Unexpectedly, HIV-1 production (as measured by p24) increased 4-fold in these same PBMC when endogenous IL-32 was reduced. Because IFN-γ was lower in siIL-32-treated PBMC, we blocked IFN-γ bioactivity, which enhanced the augmentation of p24 by siIL-32. Furthermore, siIL-32 reduced the natural ligands of the HIV-1 coreceptors CCR5 (MIP-1α/β and RANTES) and CXCR4 (SDF-1). Inhibition of endogenous IL-32 in U1 macrophages also increased HIV-1. When rhIL-32γ was added to these cells, p24 levels fell by 72%; however, in the same cultures IFN-α increased 4-fold. Blockade of IFN-α/β bioactivity in IL-32γ-stimulated U1 cells revealed that IFN-α conveys the anti-HIV-1 effect of rhIL-32γ. In summary, depletion of endogenous IL-32 reduced the levels of Th1 and proinflammatory cytokines but paradoxically increased p24, proposing IL-32 as a natural inhibitor of HIV-1. The Journal of Immunology, 2008, 181: 557–565.

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The introduction of highly active antiretroviral therapy (HAART), as HAART, for HIV-1 infection, morbidity and mortality have declined dramatically (13). However, advances in antiretroviral therapy with cytokines or endogenous cytokines that induce antiviral responses, for example, may have a role in controlling disease progression. IL-32 affects NF-κB and p38MAPK in macrophages and T cells, the target cells of HIV-1. In addition, IL-32-induced IL-1β, TNF-α, IL-6, and chemokines (IL-8 and MIP-2) are relevant to the pathogenesis of HIV-1 (14, 15). We therefore investigated the role of this proinflammatory cytokine in infection with HIV-1.

To study the role of endogenous IL-32 in vivo, we used a PBMC as well as a U1 cell model, respectively. U1 is a subclone of the promonocytic U937 cell line, which has two copies of HIV-1 integrated into its genome (16). Resembling latently infected cells in vivo, U1 cells release few HIV-1 virions under resting conditions but synthesize large amounts of the virus when stimulated by PMA (17), IL-1β (18), and possibly other cytokines.
TNF-α (19), or IL-18 (20). U1 cells can thus be used as an in vitro model resembling latently infected cells, as is the case with chronic HIV infection. PBMC, in contrast, can be rendered susceptible to infection with HIV-1 in vitro by a conditioning regimen of IL-2 and PHA as in Ref. 15, allowing for acute infection. Similar to the acute phase of the HIV infection, freshly infected PBMC produce considerable quantities of HIV-1 without exogenous stimulation.

Materials and Methods

Reagents

RPMI 1640, PBS, FCS, and penicillin/streptomycin (P/S) (50 U/ml and 50 µg/ml, respectively) were purchased from Cellgro. LPS (O55:B5), PHA, and PMCA were obtained from Sigma-Aldrich. The Dual Luciferase kit was purchased from Promega. The Nucleofector II electroporation device and reagents were obtained from Amfion. Small interfering RNA (siRNA) to IL-32 (si-IL-32) (antisense sequence: 5′-UCAUCA GAGGACCUUCGUU-3′) was an ON-TARGETplus duplex supplied by Thermo Fisher Scientific. Scrambled siRNA (Silencer negative control no.1) was purchased from Ambion. The lactate dehydrogenase (LDH) detection kit was from BioVision. Recombinant human IL-1β, recombinant human IL-32γ, the IFN-α multisubtype ELISA, and the human cytokine Ab arrays (proteome profiles) were purchased from R&D Systems. The IFN-α multisubtype ELISA was from R&D Systems.

The mAbs to the IFN-γ and the IFN-α/β receptors, anti-IFN-γR and anti-IFN-α/βR, as well as to soluble IFN-α/βR were provided by Dr. D. Novick, Weizmann Institute, Rehovot, Israel. The affinity-purified goat anti-human-IL-32 Ab used for Western blotting was prepared with the same Ag as that used for the immunization of rabbits. Affinity purification of polyclonal anti-human-IL-32 Ab used for Western blotting was prepared with the same Ag as that used for the immunization of rabbits. Affinity purification of polyclonal anti-human-IL-32 Ab used for Western blotting was prepared with the same Ag as that used for the immunization of rabbits.

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U1 cells

U1 cells were purchased from American Tissue Culture Collection and cultured in RPMI 1640 with 10% FCS and P/S. For experiments requiring transfection, cells were counted with trypan blue, centrifuged at 120 × g, and resuspended in prewarmed cell line Nucleofector solution V (Amfion). Immediately thereafter, siRNA was added and electroporation was performed according to manufacturer’s instructions (Amfion). We tested four different sequences for siRNA-2 and demonstrated that these to be effective before selecting the most efficient one for further experimentation. Cells were then transferred to tubes containing 500 µl of serum-free RPMI 1640 and incubated at 37°C for 20 min, followed by another transfer to 2 ml of prewarmed RPMI 1640 with FCS and P/S in 6-well polystyrene plates. After an overnight recovery period, cells were counted with trypan blue, placed in 24-well polystyrene plates at 1 × 10⁶ cells/ml, and either stimulated or left untreated for controls for 21 h. The harvest is described in the next paragraph under the heading PBMC. In addition to the experiments described, we performed control electroporations without siRNA that did not show significant differences compared with scrambled siRNA-transfected conditions.

PBMC

These studies were approved by the Colorado Multiple Institutional Review Board (Auraria, CO). After informed consent was obtained, PBMC were isolated from peripheral venous blood of healthy volunteers who had abstained from taking any medication at least 2 wk before the blood draw as described previously (21). Cells (3 × 10⁹/ml) were suspended in R3 medium (RPMI 1640 with 10% FCS, P/S, IL-2 (25 ng/ml), and PHA (3 µg/ml). After a 48 h incubation at 37°C in 5% CO₂ in polystyrene tissue culture flasks, cells were detached, counted, and examined for viability by trypan blue exclusion. For experiments requiring transfection, cells were centrifuged at 120 × g and resuspended in 100 µl of prewarmed human T cell Nucleofector solution (Amfion) per condition. Immediately thereafter, siRNA was added and electroporation was performed according to manufacturer’s instructions (Amfion). Following the procedure, cells were transfected to tubes containing 500 µl of serum-free human T cell culture medium (Amfion) and incubated at 37°C. After 20 min, the cells were centrifuged, the cultures were supplemented with 2 ml of human T cell culture medium with FCS and P/S, as well as IL-2/PHA, respectively, and

allowed to recover overnight. On the next morning, cells were again counted with trypan blue, transferred to a 15-ml polystyrene tube, and centrifuged at 400 × g for 10 min and the pellet was discarded.

Electrochemiluminescence (ECL) assays

Cytokines and p24 were measured using specific Abs immobilized on magnetic beads. Biotinylated Abs were mixed with streptavidin-coated beads and then incubated with the samples. A second cytokine-specific Ab labeled with ruthenium (Wellstat Diagnostics) was added and the amount of chemiluminescence was determined by using an Origin analyzer (Bio-Versis). Abs pairs for IL-1α, IL-1β, IL-6, IL-8, and TNF-α were from R&D Systems. Abs pairs for IFN-γ were purchased from Fitzgerald Industries International. The ECL assay for IL-32 has been described previously (2, 4). For comparison, Western blotting was performed on the IL-32 protein standard used in this assay. According to the results shown in Fig. 1, U1 cell lysates contain constitutive IL-32 protein in the 5- to 25-kDa range. This contrasts with the ECL readings that measured IL-32 levels in U1 as well as PBMC samples in the 5- to 500-pg range. One reason for this discrepancy may be that during the preparation of the samples for Western blotting, boiling may have resulted in the uncovering of epitopes (e.g., by

FIGURE 1. Comparison of the IL-32 ECL assay with Western blotting. A. A serial dilution of the rIL-32α protein used in the ECL assay as the standard was subjected to Western blot analysis (lanes a–h). In lanes j and k, U1 cell lysates were included for comparison. Lane i is an empty lane. The numbers above the blot indicate the readings of the same samples in the ECL assay; the numbers below the blot indicate the concentration of the standard as calculated from the stock preparation. The scientific expressions for lanes f, g, and h are for 0.2, 0.04, and 0.008 ng/ml, respectively. One representative of three independently performed experiments is shown. B. The concentrations of the ECL assay (x-axis) are plotted against the concentrations calculated from the stock preparation (y-axis) as determined by the Western blot shown in A. The high values of IL-32 in the ECL shown on the x-axis were measured in lysates from human endothelial cells stimulated with IL-1β (data not shown).

3 Abbreviations used in this paper: P/S, penicillin/streptomycin; ECL, electrochemiluminescence; LDH, lactate dehydrogenase; RLU, relative light unit; siRNA to IL-32, small interfering RNA; TCID₅₀, 50% tissue culture infective dose.
deoligomerization and/or unfolding), rendering the protein more readily detectable. Moreover, the rIL-32 we used as the ECL standard consists of the α isof orm, whereas cell culture lysates highly probably comprise a mixture of IL-32 isoforms. However, as there are no tools available at this time that can differentiate between these isoforms on the protein level, this aspect remains to be clarified by a future study. The fact that IL-32 runs slightly higher on the gel than the recombinant protein is highly probably due to glycosylation.

The biotinylated Ab for p24 determination was provided by L. Lambricht, University of Massachusetts, Worcester, MA and the secondary Ab was obtained from the National Institutes of Health AIDS Research and Repository, Germantown, MD. The results from this assay were verified by comparison to a p24 ELISA from Beckman Coulter.

Western blotting
Twenty-five micrograms of total protein were solubilized in SDS sample buffer with 2-ME and separated by electrophoresis on 10, 13, or 15% SDS-polyacrylamide gels. Proteins were then transferred to nitrocellulose membranes (0.2 μm). IL-32 was detected using an affinity-purified goat primary polyclonal Ab and a HRP-labeled rabbit anti-goat secondary Ab. For visualization, we used the ECL technology by Pierce. Equal loading of proteins was ascertained by β-actin staining.

Cytokine protein array
Equal volumes of cell culture supernatants were incubated with the pre-coated Proteome Profiler array membrane (R&D Systems) according to the manufacturer’s instructions. Densitometric analysis of the dot blot was performed using the AIDA software from Raytest. IL-2 is not shown, as it was added to the PBMC cultures exogenously.

NF-κB and AP-1 DNA-binding assays
For determination of DNA-bound NF-κB and AP-1, PBMC were simultaneously transfected with siIL-32 or scrambled siRNA, a firefly luciferase-labeled NF-κB or AP-1 construct, and a Renilla luciferase-labeled construct (for control of transfection efficiency). After the recovery period, cells were aliquoted in 24-well plates, stimulated as indicated in Results, and then lysed in a buffer containing 1% Triton X-100, 50 mM Tris (pH 7.5), 100 mM NaCl, 50 mM NaF, 40 mM N H4 phosphate, and 5 mM EDTA. Firefly and Renilla luciferase activities were analyzed with a Dual luciferase kit (Promega), according to the manufacturer’s instructions. Arbitrary units were calculated for each sample as follows: relative light units (RLU) of target × average RLU all controls/RLU individual control.

FACS analysis
After the 3-day incubation period, PBMC were washed in PBS containing 1% BSA. Cells were then stained with Abs directed against CD3, CD4, and CXC4R5 or CD14 and CCR5, followed by two more wash steps and fixation in 1% formaldehyde. Fluorescence was measured using a FACSCalibur cytometer (BD Biosciences).

Statistical analysis
Data were analyzed by paired or unpaired Student’s t test and/or by the Mann-Whitney rank sum or the Wilcoxon signed rank tests on raw data. Arbitrary units were calculated by setting scrambled siRNA at 100% for each concentration of siIL-32, the same concentrations of scrambled siRNA was tested. Cells were then either stimulated with IL-1β (10 ng/ml) or left untreated. After harvest, IL-32 protein levels in the lysates were determined by Western blotting (A) or ECL (B and C). A, Twenty-five microtiter plates of the cell lysates were subjected to Western blot analysis with staining for IL-32 (upper panel) and β-actin (lower panel). One representative of three independently performed experiments is shown, B and C. The percentage changes in IL-32 protein levels were calculated by setting scrambled siRNA at 100% for each concentration (B); range in scrambled siRNA lysates for unstimulated cells, 19-63 pg/ml; for cells stimulated with IL-1β, 18-63 pg/ml or time point (C). B, Data are presented as mean ± SEM, n = 7; * p < 0.05; ** p < 0.01 for siIL-32 compared with concentration-matched scrambled siRNA. C, After transfection with 500 nM siRNA, U1 cells were harvested at the indicated time points and the lysates were assayed for IL-32. One representative of four independently performed experiments is shown.

**FIGURE 2.** siIL-32 reduces IL-32 protein levels in U1 cells. Scrambled siRNA (scr) and siIL-32 (si32) were transfected into U1 cells. For each concentration of siIL-32, the same concentration of scrambled siRNA was tested. Cells were then either stimulated with IL-1β (10 ng/ml) or left untreated. After harvest, IL-32 protein levels in the lysates were determined by Western blotting (A) or ECL (B and C). A, Twenty-five microtiter plates of the cell lysates were subjected to Western blot analysis with staining for IL-32 (upper panel) and β-actin (lower panel). One representative of three independently performed experiments is shown, B and C. The percentage changes in IL-32 protein levels were calculated by setting scrambled siRNA at 100% for each concentration (B); range in scrambled siRNA lysates for unstimulated cells, 19-63 pg/ml; for cells stimulated with IL-1β, 18-63 pg/ml or time point (C). B, Data are presented as mean ± SEM, n = 7; * p < 0.05; ** p < 0.01 for siIL-32 compared with concentration-matched scrambled siRNA. C, After transfection with 500 nM siRNA, U1 cells were harvested at the indicated time points and the lysates were assayed for IL-32. One representative of four independently performed experiments is shown.

Results
Reduction of endogenous IL-32 by siRNA up-regulates HIV-1 in U1 cells
Under steady-state culture conditions, U1 cells express both IL-32 mRNA (data not shown) and protein (see Fig. 2). IL-32 protein is primarily cell associated in U1 cells. To study the role of endogenous IL-32 in latent HIV-1 infection, we used specific siRNA to IL-32, which targets each of the IL-32 isoforms as confirmed by a BLAST (basic local alignment search tool) alignment. U1 macrophages were transfected with increasing concentrations of siIL-32 and matching concentrations of scrambled siRNA were used as controls. siIL-32 was tested in U1 under both resting and IL-1β-stimulated conditions. As shown in Fig. 2B, constitutive production of IL-32 protein was dose-dependently reduced in resting cells transfected with siIL-32 (reductions of 40% at 200 nM and 64% at 500 nM; p < 0.05 and <0.01, respectively). These reductions were also observed by Western blotting of identically treated lysates. As shown in Fig. 2A, the same concentrations of scrambled siRNA did not affect IL-32 levels whereas IL-32 was progressively reduced by increasing concentrations of siIL-32. Also shown in Fig. 2B is the effect of siIL-32 in U1 cells stimulated with IL-1β. Stimulation...
with IL-1β did not affect the constitutive expression of IL-32 protein levels but the reductions in IL-32 by siIL-32 were comparable to those in resting U1 cells.

The reductions in constitutive IL-32 protein levels by siIL-32 were greatest within the first 24 h after transfection (Fig. 2C). Thereafter, IL-32 levels slowly recovered but remained 30% below baseline 4 days after electroporation. To exclude the possibility that the effects of siIL-32 on endogenous IL-32 protein levels were due to cell death, we performed cell counts and measured LDH levels in the supernatants. Both assessments showed no differences between siIL-32 and scrambled siRNA (data not shown).

As shown in Fig. 3A, we observed a progressive increase in constitutive HIV-1 p24 production in U1 cells transfected with siIL-32 compared with scrambled siRNA controls (2.6-fold increase at 500 nM siIL-32; \( p < 0.01 \)). Stimulation with IL-1β enhanced the production of p24 2.3-fold in U1 cells transfected with scrambled siRNA compared with constitutive levels. However, similar to the siIL-32-induced increases in constitutive p24 levels, comparable increases were observed in IL-1β-stimulated cells (2.1-fold increase in IL-1β-stimulated siIL-32 vs IL-1β-stimulated scrambled siRNA (Fig. 3A)). The increases in HIV-1 p24 production by siIL-32 were paralleled by comparable increases in constitutive IL-8 (Fig. 3B). In contrast, constitutive as well as IL-1β-induced intracellular IL-1α protein levels were reduced by siIL-32 (Fig. 3C).

The supernatants of U1 cells treated with siIL-32 were tested for IL-1β, IL-6, and TNF-α protein levels but were below the detection limit of the ECL assay. As expected, IFN-γ was not detected in U1 cells (data not shown).

Exogenous IL-32γ suppresses HIV-1 in U1 cells via an IFN-dependent mechanism

After the observation that reducing endogenous IL-32 in U1 cells resulted in increased levels of p24, we next assessed the effect of exogenous rIL-32γ. IL-32γ suppressed p24 in unstimulated U1 cells (reduction by 55% at 10 ng/ml IL-32γ, \( n = 5; p < 0.01 \)). However, cytokine-induced HIV expression is often the result of infection, i.e., bacterial, viral, or fungal, in which TLRs on macrophages directly stimulate HIV expression as well as the release of proinflammatory cytokines, which then also contribute to the production of HIV. Therefore, we induced HIV expression with LPS in U1 cells in the presence of IL-32γ. As expected, LPS induced a 3-fold increase in p24 in the absence of IL-32γ (\( n = 5; p < 0.01 \)). As shown in Fig. 4A, the combination of IL-32γ plus LPS reduced LPS-induced HIV expression by 72% at 1 ng/ml of IL-32γ (\( p = 0.022 \)) and by 65% at 10 ng/ml (\( p = 0.003 \)). Hence, exogenous IL-32 reduces HIV-1 production consistent with an antiviral effect as well as with the increase of HIV expression by blocking endogenous IL-32 with siRNA.

We next determined whether the reduction in p24 by exogenous IL-32γ was due to type I IFN. We blocked the bioactivity of type I IFNs using either anti-IFN-α/βR mAbs or soluble IFN-α/βR. As shown in Fig. 4A, blocking the IFN-α/β receptors reversed the reduction in p24 induced by the addition of IL-32γ to LPS-stimulated cultures. At 10 ng/ml IL-32γ, blocking the IFN-αR with a mAb or a soluble receptor completely reversed the antiviral activity of IL-32.

As neutralization of type I IFNs abrogated the antiviral activity of IL-32, we next measured type I IFN protein levels in U1 cells using a multitype subtype IFN-α ELISA to unveil a possible effect of exogenous IL-32γ on IFN-α. Whereas the induction of IFN-α protein by LPS was minimal, we found a 4-fold increase with IL-32γ in the presence and absence of LPS (Fig. 4B).

Infection with HIV-1 reduces IL-32 in PBMC

To render freshly obtained PBMC susceptible for infection with HIV-1 in vitro, pretreatment with IL-2 and PHA is required. To determine the effect of this regimen on IL-32, we compared the
levels of the cytokine in lysates from cells cultured for 2 days with or without IL-2/PHA. Although IL-32 protein levels in freshly obtained PBMC from HIV-1 negative donors are often below the detection limit, IL-32 protein levels were consistently present after IL-2/PHA treatment (data not shown). Next, we sought to investigate whether infection with HIV-1 after 2 days of IL-2/PHA would affect IL-32 protein expression. As shown in Fig. 5, 24 h after infection with 100 or 500 TCID<sub>50</sub> of the T cell-tropic A018A strain of HIV-1, there was a moderate but significant inhibition (by 35%; p = 0.018) of IL-32 in PBMC from HIV-1-infected PBMC. This pattern of inhibition was also observed in unstimulated PBMC infected with A018A (reduction by 34%, n = 9; p = 0.024 for 500 TCID<sub>50</sub> vs noninfected). To investigate whether the suppression of IL-32 was also conveyed by a macrophage-tropic strain of HIV-1, we infected PBMC with MR011 virus under the conditions described above. These experiments yielded results similar to the T cell-tropic infection (18 and 21% decrease, n = 5; p = 0.011 and 0.014 for 500 TCID<sub>50</sub> vs noninfected PBMC in unstimulated and PMA-stimulated PBMC, respectively).

A concentration of 5 nM PMA did not induce a change in IL-32 levels (data not shown). Twenty-four hours after HIV-1 infection, LDH levels in the supernatants were not affected, indicating that the increase in IL-32 was not due to cell death (for noninfected, 100 TCID<sub>50</sub>, and 500 TCID<sub>50</sub>; 239 ± 59, 243 ± 51, and 247 ± 52 arbitrary units in unstimulated cells and 340 ± 64, 387 ± 81, and 317 ± 67 arbitrary units for PMA-stimulated cells). To control for insufficient infection of any individual donor’s PBMC as a possible reason for differences in IL-32 levels, we measured p24 levels in the supernatants of each experiment described in this section and observed that p24 levels fell into similar range in each donor’s PBMC.

siIL-32 augments HIV-1 in PBMC but reduces IL-6, IFN-γ, and TNF-α

To investigate the role of IL-32 in acute HIV-1 infection, PBMC from healthy volunteers were transfected with siIL-32 or scrambled siRNA and infected with HIV-1. As shown in Fig. 6A, in accord with siIL-32 in U1 cells, we observed a concentration-dependent reduction in the constitutive IL-32 protein levels. Although the largest reduction in IL-32 protein (83% compared with scrambled siRNA) was measured 24 h after transfection with 250 nM siIL-32, levels were still significantly reduced by 39 and 53% 96 h after electroporation with 100 and 250 nM siIL-32, respectively.

Similar to U1 cells, PBMC produced increased levels of p24 with siIL-32. As shown in Fig. 6B, there was a 2.4-fold increase in p24 at 100 nM and a 4-fold increase at 250 nM. However, in the same cultures, IL-6, IFN-γ, and TNF-α protein levels were reduced by 57% (Fig. 6C), 52% (Fig. 6D), and 37% (Fig. 6E), respectively. At 250 nM siIL-32, IL-1α in PBMC lysates did not change (data not shown).

In addition to quantification of cytokine levels in the supernatants of PBMC by specific immunoassays, we performed an array of 36 cytokines based on a protein dot blot method (Proteome Profiler cytokine arrays; see Materials and Methods). As shown in Fig. 7, with the exception of I-309, each cytokine was decreased in the supernatants from infected PBMC treated with siIL-32. IL-6, IFN-γ and TNF-α were among the cytokines reduced by siIL-32 on the array (by 44, 39, and 61%, respectively) confirming the data from specific immunoassays. In addition, IL-1β was also decreased in the dot blot by 47%. The sole increase (by 46%; n = 3) was in I-309, a chemokine highly active on regulatory T cells.
Flow cytometry analysis revealed that siRNA to IL-32 did not affect surface expression of CXCR4 and CCR5 on PBMC. In addition, siIL-32 did not induce any changes in the distribution of CD3+ and CD14+ cells within the total PBMC population (data not shown).

A reduction in endogenous IL-32 is associated with reduced DNA binding of NF-κB and AP-1 in PBMC

We next investigated the activity levels of NF-κB and AP-1 in PBMC in which endogenous IL-32 was reduced by siRNA. PBMC were simultaneously transfected with siIL-32 or scrambled siRNA and a luciferase-containing plasmid linked to either AP-1 or NF-κB. As shown in Fig. 8, A and B, significantly lower DNA-binding activities of both AP-1 and NF-κB were measured in LPS-stimulated cells transfected with siIL-32. The mean reduction in AP-1 binding was 46% (*, p < 0.05) and that of NF-κB was 42% (**, p < 0.01).

Modulation of the bioactivity of IFN-γ in PMBC cultures affects p24 production

Having demonstrated that the production of IFN-γ was reduced in freshly infected PBMC treated with siIL-32, we considered that decreased IFN-γ results in a state of reduced endogenous antiviral activity. In HIV-1-infected PBMC treated with exogenous IL-18, a decrease in p24 production was linked to increased endogenous IFN-γ activity as IL-18 induces IFN-γ (21). Therefore, we blocked IFN-γ activity using a mAb to the IFN-γ receptor (anti-IFN-γR, previously described in Ref. 23). As shown in Fig. 9, adding exogenous IFN-γ to infected PBMC reduced HIV-1 production by 59% at 50 ng/ml. The far right bars of Fig. 9 shows that anti-IFN-γR increased p24 levels by 65%. These studies are consistent with the concept that in
PBMC, endogenous IFN-γ provides an antiviral state and that the ability of siIL-32 to reduce constitutive IFN-γ accounts, in part, for the increase in p24.

**FIGURE 8.** siIL-32 inhibits DNA binding of NF-κB and AP-1 in PBMC. PBMC were transfected with 250 nM scrambled siRNA or siIL-32, as well as with either 1 μg of an NF-κB (A) or 2 μg of an AP-1 (B) firefly luciferase-labeled reporter construct and 1 μg of a Renilla luciferase-labeled construct for control of transfection efficiency. After recovery, cells were plated, plated into 24-well plates, and stimulated with 100 ng/ml LPS for 30 min (NF-κB) or 1 h (AP-1). Luciferase activities were then measured in the lysates. Data are shown as mean ± SEM, n = 3; *, p < 0.05; **, p < 0.01 for siIL-32 compared with scrambled siRNA.

**FIGURE 9.** Effect of IFN-γ on HIV-1-infected PBMC. PBMC were transfected with 250 nM siIL-32. After infection, cells were plated and treated as indicated, followed by a 3-day incubation. Thereafter, p24 protein levels in the supernatants were determined by ECL (range in control supernatants, 3–19 ng/ml). Percentage changes in p24 levels are depicted as mean ± SEM, n = 7; *, p < 0.05; **, p < 0.01 for IFN-γ or anti-IFN-γR compared with control. The difference between 2 and 10 μg/ml anti-IFN-γR is not significant.

**Discussion**

Several viruses and bacteria are known to skew cytokine production in host cells to create an environment favorable for their proliferation. HIV-1 is an example of a virus using this survival strategy (24, 25). In the present study, the use of siIL-32 in IL-2/PHA-primed PBMC suggested that IL-32 inhibits HIV-1. Indeed, when constitutive IL-32 was reduced by siIL-32 in both U1 macrophages and freshly infected PBMC, HIV-1 production increased. In addition, IL-8 levels were also increased by siIL-32. The latter effect may be secondary to the increase in p24, as IL-8 is elevated in HIV-positive individuals and known to be induced by the virion-derived protein R (Vpr) of HIV-1 in various cells (26, 27). After having observed the augmentation of HIV-1 induced by silencing IL-32, we decided to investigate the effects of exogenous stimulation with this cytokine. Indeed, the addition of exogenous rhIL-32γ strongly suppressed HIV-1 production in U1 macrophages (Fig. 4).

In PBMC, the increase in p24 protein associated with lowered levels of endogenous IL-32 (shown in Fig. 6B) was accompanied by a reduction in several cytokines; IFN-γ, IL-6, and TNF-α protein were decreased by 51, 57, and 36%, respectively (Figs. 6, C–E). The same was observed for IL-1α in U1 cell lysates (reduction by 32%; Fig. 3C). The extent of this inhibition is remarkable and underscores the importance of IL-32 in the production of these cytokines. The decrease in IFN-γ, IL-6, TNF-α, and IL-1α was achieved by a knockdown of IL-32 of 83% at 24 h and 53% at 96 h. Moreover, when contrasted with the increase in p24 these observations were unexpected, because HIV-1 replication is well known to be up-regulated by TNF-α and IL-6 (28, 29) and inhibited by blocking these and other cytokines. Hence, the increase in p24 in association with decreased IL-32 is likely independent of the reduction in TNF-α and IL-6. The decrease in IFN-γR, in contrast, may explain, in part, the increase in HIV-1 expression, as discussed below.

As HIV uses the chemokine receptors CXCR4 and CCR5 as coreceptors for binding to monocytes/macrophages and T cells (30, 31), we hypothesized that modulation of surface expression of these receptors could be a mechanism by which siIL-32 promotes production of HIV-1 while reducing the levels of several cytokines. However, there were no differences in CXCR4 and CCR5 surface expressions between siIL-32- and scrambled siRNA-transfected cells.

We also studied the effects of siIL-32-induced decreases in levels of endogenous IL-32 on the DNA-binding activities of NF-κB and AP-1. By binding to their respective sites within the long-terminal repeat promoter of HIV-1 (32), both transcription factors induce strong boosts of gene expression and release of HIV-1 from acutely as well as latently infected cells (33). Furthermore, the expressions of IFN-γ (34), IL-6, and TNF-α (35) are, in part, driven by the activity of NF-κB and/or AP-1. IL-32 activates NF-κB in cell lines (2). In fact, as shown in the present study, in primary PBMC, siIL-32 reduced DNA binding of NF-κB and AP-1 at the same concentrations which reduced IL-32 and increased p24. The reduction in NF-κB and AP-1 binding helps explain the decrease of IL-6, IFN-γ, and TNF-α in PBMC and of IL-1α in U1 cells associated with reduced endogenous IL-32. However, the inhibition of the binding of both transcription factors fails to provide a sufficient explanation for the paradoxical increase in p24.

On the other hand, diminished levels of IFN-γ associated with siIL-32 and thus a reduction in the antiviral activity within the PBMC cultures may explain the increase in p24 (Fig. 6D). IFN-γ is an antiviral cytokine and its range of targets includes HIV-1.
In PBMC infected with HIV-1 in vitro and stimulated with IL-18, the decrease in p24 was due to an increase in IFN-γ (21). In the present study, we show that adding exogenous IFN-γ to PBMC infected in vitro suppresses p24 levels and that blocking endogenous IFN-γ activity increases p24. Thus, the net effect of reducing endogenous IL-32 is in favor of increased HIV-1 production as the antiviral milieu decreases.

Because we were unable to detect IFN-γ in U1 cells, it is unlikely that the increase in p24 in these cultures is due to siIL-32-induced decreases in IFN-γ levels. Hence, we decided to investigate the role of type I IFNs. IFN-α, more specifically at least subtype 1a (37), is produced in U937 cells and inhibits HIV-1 (38, 39). We detected IFN-α protein in the supernatants of the U1 cell cultures and also observed a 4-fold increase in the presence of exogenous siIL-32. Blocking IFN activity with an anti-IFN-α/βR or neutralizing IFN using soluble IFN-α/β completely reversed the antiviral effect of exogenous IL-32. We can thus conclude that the antiviral activity of IL-32 in latently infected U1 cells is, at least in part, mediated by IFN-α.

The chemokine receptors CCR5 (40) and/or CXCR4 (41) are crucial for HIV entry into target cells (31). That discovery laid the foundation for the development of entry inhibitors that block CCR5 or CXCR4. For example, maraviroc occupies CCR5, thus preventing HIV gp120 from binding and using CCR5 as coreceptor (42). In supernatants from freshly infected PBMC analyzed in the present study (Fig. 7), siIL-32 resulted in a decrease of the CXCR4 ligands MIP-1α, MIP-1β, and RANTES as well as a decrease of the CXCR4 ligand stromal cell-derived factor-1 (SDF-1), each of which function as an HIV-1 coreceptor. This reduction in the levels of various chemokines that interfere with HIV-1 fusion is highly relevant, as a greater number of chemokine receptors would remain unoccupied in cultures transfected with siIL-32, leading to a facilitation of HIV-1 entry and enhanced replication. Hence, the reduction in ligands of the HIV-1 chemokine coreceptors may be a mechanism by which siIL-32 increases p24. Furthermore, the CCR2 ligand MCP-1, which was also reduced by siIL-32 on the dot blot, was suggested to slow HIV infection by desensitization of CCR5 and CXCR4 signaling (43, 44).

During the early stages of infection, HIV-1 inhibits the release of IFN-γ, IL-12, and IL-2, as well as the activation of IL-18 (45, 46), thus preventing the mounting of a sufficient Th1 response. Although siRNA-induced reductions in IL-32 were associated with a decrease in nearly all cytokines on the profiler blot, we observed a greater decline of Th1-related cytokines and chemokines (IL-12 and IFN-γ as well as IP-10 and MIP-1αβ) than that of Th2 and anti-inflammatory cytokines. Notably, the single exception from the general inhibition of cytokines by siIL-32 was I-309, a chemokine preferentially affecting regulatory T cells (47). This T cell subset can suppress both Th1 and Th2 responses, thus preventing excessive immune reactions and autoimmunity (48). However, these properties are detrimental in HIV infection as regulatory T cells impair the antiviral activities of HIV-specific T cells (49) and can enhance HIV-1 gene expression (50). Therefore, IL-32 not only induces the production of cytokines in general and proinflammatory mediators in particular but may also play a role in promoting Th1 differentiation of T cells and a role in the inhibition of regulatory T cell chemotaxis. Indeed, IFN-γ and IL-2 induce IL-32 (2).

Knowledge of the role of endogenous IL-32 in HIV-1 is still limited, although it appears that one likely mechanism by which IL-32 suppresses HIV-1 expression is the induction of IFNs. However, the novel findings we report here seem promising, as they suggest that this cytokine may have potential to complement and improve the present control of the disease with highly active antiretroviral therapy (HAART). In general, IL-32 conveys a cytokine environment hostile to HIV-1. More specifically, the influence on both types of IFNs as well as on the production of chemokines that interfere with HIV-1 fusion is likely to be beneficial in any stage of the disease, whereas the promotion of a Th1 response could be important during the early phase of the disease. In summary, the results of this study suggest IL-32 as an antagonist of HIV-1 and as an amplifier of cytokine responses in general and Th1 and proinflammatory subsets in particular.

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Disclosures

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