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Natural killer cell inhibits human immunodeficiency virus replication in chronically infected immune cells

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Abstract

Natural killer (NK) cells are a crucial component of the host innate immune system. We investigated the noncytolytic anti-human immunodeficiency virus (HIV) activity of NK cells in chronically HIV-infected immune cells. Supernatants collected from NK cell cultures (both primary NK cells and NK cell lines, YTS and NK 92) inhibited HIV activation in peripheral blood mononuclear cells (PBMCs) from HIV-infected subjects. NK supernatants (NK SN) also suppressed tumor necrosis factor (TNF)-α-induced HIV activation in chronically infected cell lines (U1 and ACH-2 cells). The antibody to interferon (IFN)-γ blocked NK SN-mediated anti-HIV effect, while the antibodies to CC-chemokines had no impact on NK SN-mediated HIV inhibition in U1 and ACH-2 cells. Investigation of mechanism(s) responsible for the NK action showed that NK SN inhibited TNF-α-mediated activation of HIV-long-terminal repeat (LTR), and upregulated the expression of signal transducer and activator of transcription (STAT)-1 and phosphorylated P38 mitogen-activated protein kinase (MAPK). The P38 MAPK inhibitor (SB 203580) blocked NK SN-mediated HIV inhibition. These data provide compelling evidence that NK cells have a critical role in controlling HIV activation in the reservoirs.
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Keywords: T lymphocytes; Innate immunity; Interferon-γ

1. Introduction

Human immunodeficiency virus (HIV) infection is characterized by a period of long clinical latency before the development of symptoms and HIV-related disease (Fauci, 1988). The chronical period of HIV infection is influenced by factors that either suppress or activate HIV replication in different cell types. One of the puzzling aspects in the immunopathogenesis of HIV infection in vivo is its prolonged incubation period during which the virus replication occurs preferentially and continuously in lymphoreticular tissues (lymph node, spleen, gut-associated lymphoid cells, and macrophages) (Embretson et al., 1993; Levy, 1993; Pantaleo et al., 1991; Pantaleo et al., 1993; Wei et al., 1995). HIV establishes a state of latent infection in resting in vivo (Finzi et al., 1999; Finzi et al., 1997; Hammer et al., 1997; Wong et al., 1997). Although combination antiretroviral therapy is effective in controlling HIV replication in many infected individuals (Finzi et al., 1999; Finzi et al., 1997; Gulick et al., 1997; Hammer et al., 1997; Wong et al., 1997), discontinuation of antiretroviral therapy in such individuals could allow reactivation of HIV from chronically infected cells and renewed active replication (Finzi et al., 1999; Finzi et al., 1997; Wong et al., 1997). The persistence of chronically infected immune cells have been demonstrated as the majority obstacle in preventing the eradication of HIV (Chun and Fauci, 1999; Chun et al., 1997; Finzi et al., 1997; Ho, 1998; Wong et al., 1997).

Host innate immune response plays a key role in host defense against viral infections (Koup et al., 1994). Natural killer (NK) cells, as a central component of the innate immune system, are crucial in host defense against certain viruses. The importance of NK cell-mediated innate immunity for the control of HIV replication in infected individuals is now widely recognized (Azzoni et al., 2002; d'Ettorre et al., 2005; Fauci et al., 2005; Kottilil, 2003; Kottilil et al., 2003; Kottilil et al.,

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2004). NK-mediated suppression of HIV replication is as potent as that of $CD8^+$ T cells (Kottilil, 2003; Kottilil et al., 2004). Although direct NK cell-mediated cytotoxicity has a significant role in controlling viral infections including HIV, NK cells also mediate noncytolytic suppression of viral replication (Kottilil, 2003; Kottilil et al., 2004). The noncytolytic anti-HIV activity of NK cells is mediated predominantly through secretion of CC-chemokines (Kottilil, 2003). CC-chemokines, particularly regulated on activation, normal T expressed and secreted (RANTES), macrophage inflammatory protein (MIP)-1α, and MIP-1β, block the entry of HIV strains that use the CCR5 coreceptor by competitive inhibition (Alkhatib et al., 1996; Choe et al., 1996; Dragic et al., 1996). Since NK cells are the major source of CC-chemokines, NK cells have a key role in suppressing HIV R5 strain infection of the immune cells (Kottilil et al., 2004). However, the role of NK cells in suppressing HIV activation in chronically infected immune cells remains to be determined. The present study examined the noncytolytic anti-HIV activity of NK cells in HIV chronically infected immune cells and the mechanisms involved in the NK cell action.

2. Materials and methods

2.1. Cell lines

Peripheral blood samples were obtained from three healthy donors and three asymptomatic, HIV-infected adult subjects with $CD4^+$ T cell counts ranging from 400 to 1200/mm^3. The Institutional Review Board of the Children's Hospital of Philadelphia approved this investigation. Informed consent was obtained from the subjects. Peripheral blood mononuclear cells (PBMCs) from three HIV-infected subjects were processed as described previously (Hassan et al., 1986) using lymphocyte separation medium (Amersham Pharmacia Biotech, Uppsala, Sweden). PBMCs were subjected to $CD8^+$ T lymphocyte depletion using magnetic cell sorter CD8 microbeads, according to the manufacturer's instructions (Miltenyi Biotec, Auburnm, CA). $CD8^+$ T cell-depleted PBMCs were stimulated with 1% phytohemagglutinin (PHA) for 72 h and then seeded into 48-well plates (10^6 cells/well), precoated anti-CD3 (1 μg/ml) and cultures in the presence of human interleukin (IL)-2 (50 U/ml) (Hoffmann-LaRoche, Nutley, NJ). Primary NK cells were isolated from peripheral blood of three adult healthy donors lacking antibodies to HIV. NK cells were enriched by immunomagnetic negative selection (Miltenyi Biotec) (Li et al., 2004). The purity (% of $CD56^+CD3^-$) of primary NK cells measured by fluorescence-activated cell sorting (FACS) analysis was greater than 98%. The human NK cell line (NK 92 obtained from H.G. Klingermann, Rush University, Chicago, IL) (Gong et al., 1994) was cultured in Myelocult H5100 medium (StemCell Technologies, Vancouver, Canada) supplemented with 50 U/ml of IL-2. The human YTS cells, a subclone of the YT lymphoid cell line, derived from a patient with NK cell leukemia and is IL-2-independent (Cohen et al., 1999), were cultured in IMDM Iscove's modified Dulbecco's medium supplemented with 10% fetal calf serum and L-glutamine.

HIV chronically infected U1 and ACH-2 cell lines were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institutes of Health. U1 is a cloned cell line derived from U937 cells surviving acute HIV infection (LAV-1 strain) (Folks et al., 1987). ACH-2 is a cloned cell line derived from a human T cell line that survived acute HIV-1 infection. Restriction enzyme analysis indicated the presence of a single integrated copy of proviral DNA (Folks et al., 1989). 1G5 is a Jurkat T cell line that harbors two copies of a stable transfected plasmid, containing the luciferase reporter gene downstream of the HIV long-terminal repeat (LTR) (Aguilar-Cordova et al., 1994). U1, ACH-2, and 1G5 cells were cultured as described (Aguilar-Cordova et al., 1994; Folks et al., 1987; Perez et al., 1991). The cellular and molecular mechanisms of activation of chronically integrated HIV provirus in these cells have been well delineated (Folks et al., 1987; Folks et al., 1988; Perez et al., 1991). Both U1 and ACH-2 cells have been used as in vitro models for viral latency (Chen et al., 1994).

2.2. Reagents

Tumor necrosis factor (TNF)-α, monoclonal anti-human interferon (IFN)-γ antibody, monoclonal anti-MIP-1α antibody, monoclonal anti-RANTES antibody, recombinant IFN-γ were purchased from R&D Systems Inc. (Minneapolis, MN). Rabbit polyclonal antibody against signal transducer and activator of transcription (STAT)-1 was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Mouse monoclonal antibody against phosphor-P38 mitogen-activated protein kinase (MAPK) was purchased from Cell Signaling Technology, Inc. (Beverly, MA). SB2003580 and rabbit polyclonal antibody against actin were purchased from Sigma–Aldrich Co. (St. Louis, MO).

2.3. Preparation of NK cell culture supernatants (NK SN)

NK SN was prepared as follows: supernatants from NK 92 and YTS cell cultures were collected 48 h after the cells were plated. For primary NK cell cultures, supernatants were collected 48 h after IL-2 stimulation. All NK SN were filtered through 0.22-μm filters and stored at −70 °C in aliquots (Li et al., 2004). The IFN-γ protein level in primary NK cells and NK cell lines (NK 92 and YTS) ranges from 900–1600 pg/ml based on our previous data (Li et al., 2004).

2.4. NK SN treatment of the target cells

U1 and ACH-2 cells plated in triplicate (5×10^5 cells/well) in 48-well culture plate were incubated with or without NK SN (25%, v/v) for 24–72 h. HIV in these cells was activated by TNF-α (2 ng/ml). HIV reverse transcriptase (RT) activity was measured in culture SN collected 24–72 h after-TNF-α treatment. For the experiments using the antibodies to IFN-γ, MIP-1α and RANTES, NK SN was first incubated with the antibodies for 30 min, and then added into U1 or ACH-2 cell cultures. For the experiments using the p38 MAPK inihibitor (SB203580), the cells were first treated with SB203580 (10 μM)

for 30 min prior to the addition of NK SN. 1G5 cells in 48-well culture plates (2×10^5 cells/well) stimulated with TNF-α (2 ng/ml) were treated with IFN-γ or NK SN and/or anti-IFNγ antibody for 48 h. The lysed 1G5 cells were then subjected to luciferase assay.

2.5. Western blot analysis

For STAT-1 protein detection, total cell protein extracted from U1 and ACH-2 cells treated with or without NK SN (25%, v/v) were prepared using lysis buffer (Promega, Madison, WI). For phosphorylated-P38 MAPK protein detection, total cell lysates from the U1 and ACH-2 cells were prepared using the lysis buffer described previously (Zhang et al., 2005). Protein concentration was determined using the Bio-Rad DC Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA). Proteins were resuspended in NuPAGE LDS Sample Buffer (NOVEX, San Diego, CA), heated for 5 min at 95 °C and then equal amounts of protein for each sample were separated in a 10% Bis-Tris Gel in a NuPAGE Running Buffer with 0.25% Running Buffer Antioxidant for 50 min at 200 V. Proteins were transferred to the nitrocellulose membranes (Bio-Rad) in NuPAGE Transfer Buffer at 100 V for 1 h. The membranes were blocked by 5% fat-free milk in PBST (0.05% Tween 20 in PBS) for 1 h and incubated with appropriate optimal diluted antibodies in 5% fat-free milk in PBST for 1 h at room temperature. After washing four times for 5 min each with PBST, the membrane was incubated with appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies in 5% fat-free milk in PBST for 1 h at room temperature and washed four times as described above. The bound antibodies were developed using SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) according to the manufacturer's instruction. Pre-stained molecular markers (Bio-Rad) were used to determine molecular weight of immunoreactive bands.

2.6. HIV RT assay

HIV RT activity was determined based on the technique of Willey et al. (1988) with modifications (Ho et al., 1992). In brief, 10 μl of supernatants collected from U1, and ACH-2 cell cultures were added to a cocktail containing poly (A), oligo (dT) (Pharmacia Inc., Piscataway, NJ), $MgCl_2$ and ^{32}P dTTP (Amersham Corp., Arlington Heights, IL) and incubated for 20 h at 37 °C. Then 30 μl of the cocktail were spotted onto DE81

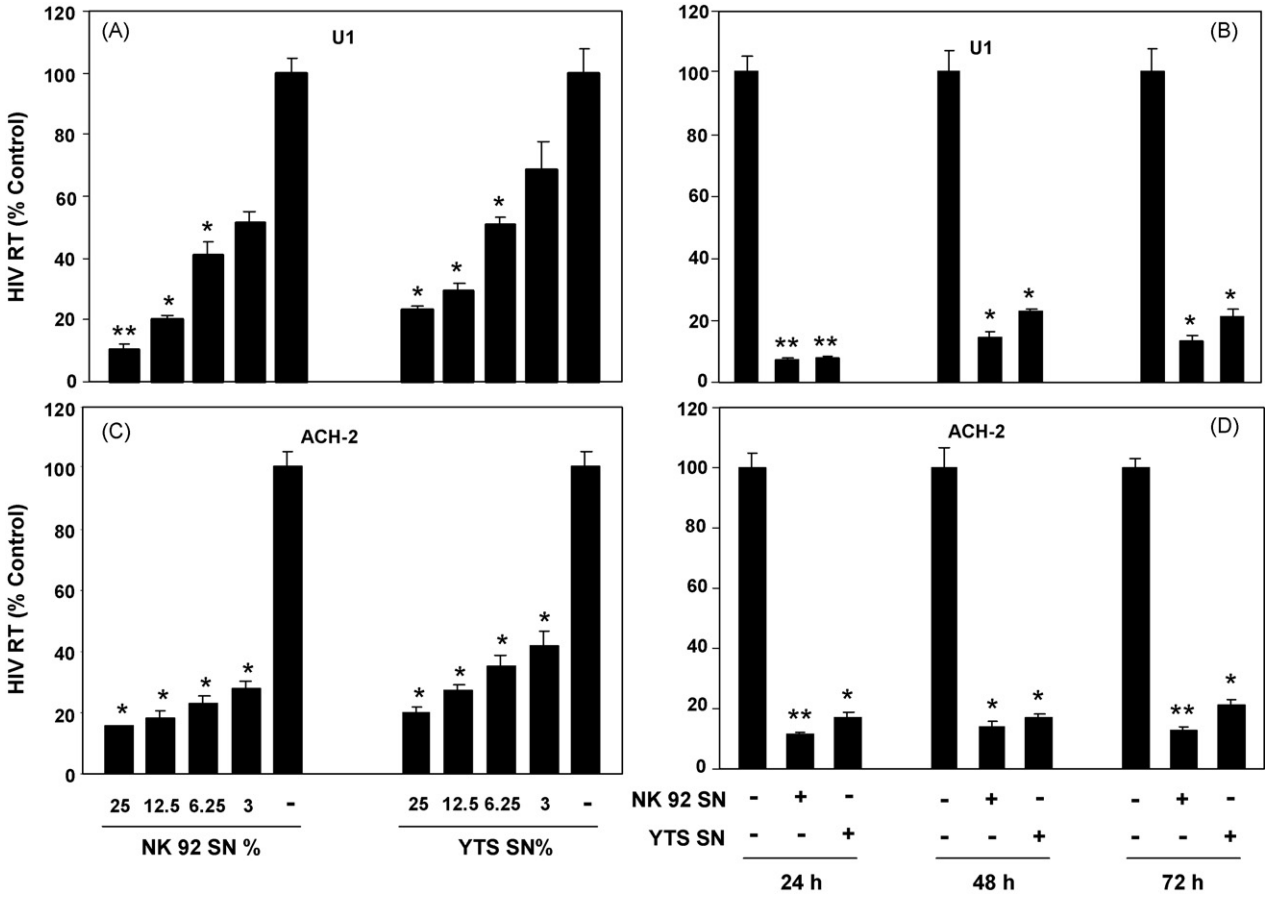

Fig. 1. Dose dependent and time course inhibition of HIV replication by NK SN. (A and B) U1 and ACH-2 cells stimulated with TNF-α (2 ng/ml) were cultured in the presence or absence of NK SN (from NK 92 and YTS cell lines) at indicated concentrations. (C and D) U1 and ACH-2 cells stimulated with TNF-α (2 ng/ml) were cultured in the presence or absence of NK SN (from NK 92 and YTS cell lines) at the concentration of 25% (v/v). HIV RT activity was determined at 24 h post NK SN treatment. The data are expressed as HIV RT activity in NK SN-treated cultures (% of control) to control cultures (without NK SN treatment, which is defined as 100%). The results shown are mean ± S.D. of triplicate cultures, representative of three separate experiments. "+", in the presence; "−", in the absence. $^{*}P < 0.05$; $^{**}P < 0.01$.

paper, dried and washed five times with 2× saline-sodium citrate buffer and once with 95% ethanol. The filter paper was then air-dried. Radioactivity was counted in a liquid scintillation counter (Packard Instrument Inc., Palo Alto, CA).

2.7. Luciferase assay

The luciferase activity was determined using a Luciferase Assay Kit (Promega Biotec, Madsion, WI). 1G5 cells were lysed in 150 μl of Reporter Lysis Buffer (Promega Biotec). Lysates (50 μl) were mixed with an equal volume of luciferase substrate (Promega Biotec). Emitted light in each well was measured by a luminescence counter (PerkinElmer Wallac Inc. Gaithersburg, MD) over a 0.5-s period and designated as relative light units (RLU).

2.8. Statistical analysis

Where appropriate, data were expressed as mean ± S.D. For comparison of the mean of the two groups, statistical significance was assessed by ANOVA with the appropriate post hoc test. Calculations were performed with the use of Stata Statistical Software (StataCorp., College Station, TX). Statistical significance was defined as $P < 0.05$.

3. Results

3.1. NK SN suppresses HIV replication in chronically infected immune cells

NK SN collected from NK cell cultures (NK 92 and YTS) was examined for the anti-HIV activity in TNF-α-stimulated U1 and ACH-2 cells. As demonstrated in Fig. 1, NK SN was capable of suppressing TNF-α-induced HIV replication in both U1 and ACH-2 cells in a concentration dependent fashion, and the maximum inhibition was observed at 24 h post-treatment. Similar inhibitory effects on TNF-α-induced HIV activation in U1 and ACH-2 cells were observed in the experiments using NK SN collected from primary NK cell cultures (Fig. 2). In addition, primary NK SN also suppressed HIV activation in CD8 T cell-depleted PBMCs isolated from infected subjects (Fig. 2).

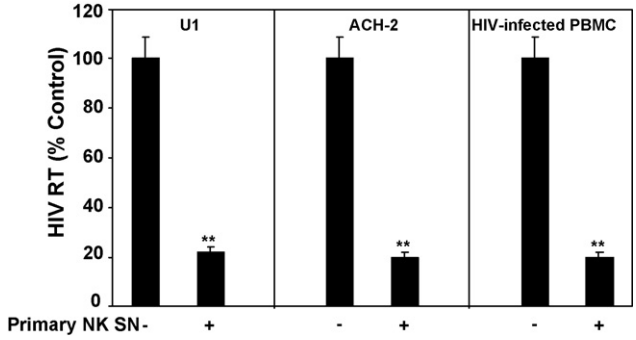

Fig. 2. Primary NK SN inhibition of HIV activation in U1 and ACH-2 cells and PBMCs from HIV-infected subjects. U1 and ACH-2 cells stimulated with TNF-α (2 ng/ml) were cultured in the presence or absence of pooled NK SN (from primary NK cell cultures of three healthy donors) at the concentration of 25% (v/v). CD8 T cell-depleted PBMCs from three HIV-infected subjects were stimulated with 1% PHA for 72 h, and incubated with or without the primary NK SN. Cell cultures were refed with fresh medium containing primary NK SN every 3 days. Day 9 culture SN was collected for measurement of HIV RT activity. The data are expressed as HIV RT activity in NK SN-treated cultures (% of control) to control cultures (without treatment with NK SN, which is defined as 100%). The results shown are mean ± S.D. of triplicate cultures, representative of three separate experiments. "+", in the presence; "−", in the absence. $^{**}P < 0.01$.

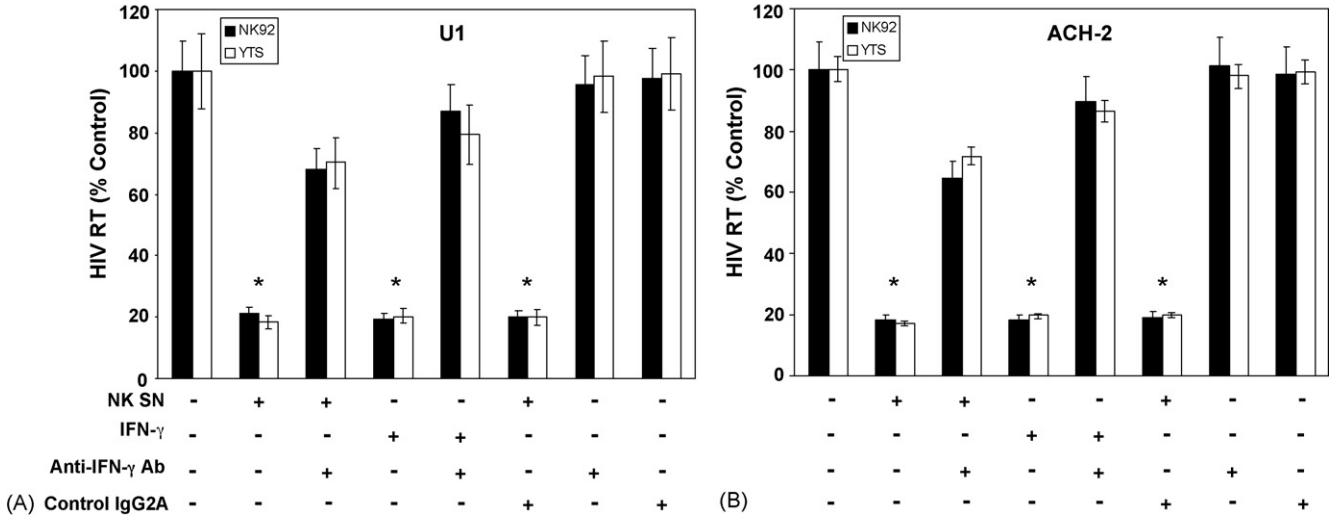

Fig. 3. Anti-IFN-γ antibody blocks NK SN-mediated anti-HIV activity. U1 and ACH-2 cells stimulated with TNF-α (2 ng/ml) were cultured in the presence or absence of NK SN (25%, v/v, from NK 92 and YTS cell lines) with or without antibody against IFN-γ (10 μg/ml) or control IgG 2A (10 μg/ml). For the cultures in the presence of NK SN and the antibody against IFN-γ, NK SN were pre-incubated with the antibody against IFN-γ for 30 min before added to U1 and ACH-2 cell cultures. IFN-γ (1000 U/ml) alone was added to the cell culture as a positive control. HIV RT activity was determined 24 h post exposure to NK SN. The data are expressed as HIV RT activity in NK SN-treated cultures (% of control) to control cultures (without treatment with NK SN, which is defined as 100%). The results shown are mean ± S.D. of triplicate cultures, representative of three separate experiments. "+", in the presence; "−", in the absence; Ab, antibody. (A) U1 cell; (B) ACH-2 cell. $^{*}P < 0.05$.

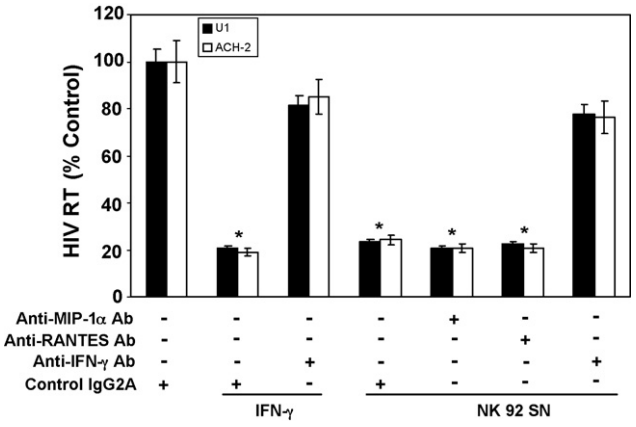

Fig. 4. Effects of antibodies to CC-chemokines and IFN-γ on NK SN-mediated anti-HIV activity. U1 and ACH-2 cells stimulated with TNF-α (2 ng/ml) were cultured in the presence or absence of NK 92 SN (25%, v/v) or IFN-γ (10 μg/ml) and/or antibodies as indicated. For the cultures in the presence of NK 92 SN or IFN-γ and the indicated antibodies, NK 92 SN or IFN-γ was pre-incubated with these antibodies for 30 min before added to U1 and ACH-2 cell cultures. HIV RT activity was determined 24 h post exposure to NK SN or IFN-γ. The data are expressed as HIV RT activity in NK SN-treated cultures (% of control) to control cultures (with treatment of control IgG2A, which is defined as 100%). The results shown are mean ± S.D. of triplicate cultures, representative of three separate experiments. "+", in the presence; "−", in the absence. $^{*}P < 0.05$.

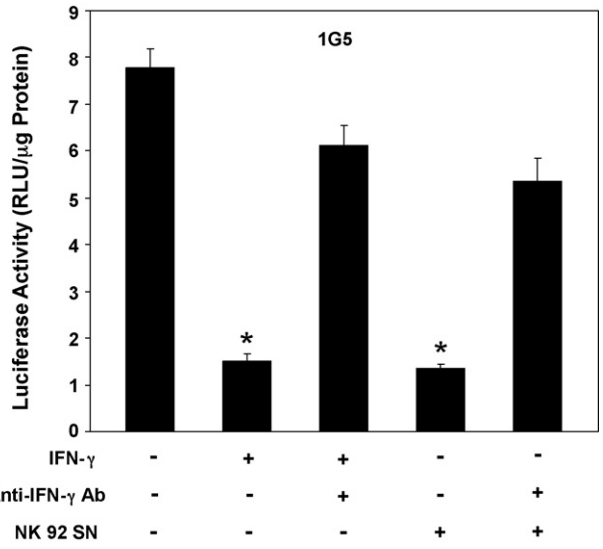

Fig. 5. Effect of NK SN on TNF-α-mediated HIV LTR activation. 1G5 cells were incubated in the presence or absence of NK 92 SN (25%, v/v) with or without antibody against IFN-γ (10 μg/ml) for 48 h. LTR-directed luciferase expression was induced by treatment of 1G5 cells with TNF-α (2 ng/ml). Cell lysates were collected for measurement of luciferase activity 24 h post-NK SN treatment. The data are expressed as luciferase activity normalized with total protein content (RLU/μg protein). The results shown are mean ± S.D. of triplicate cultures, representative of three separate experiments. "+", in the presence; "−", in the absence. $^{*}P < 0.05$.

3.2. IFN-γ plays a major role in NK SN-mediated anti-HIV activity

Since NK cells play a significant role in antiviral defense, in part, through their ability to secrete cytokines such as IFN-γ (Biron, 1997), which plays a crucial role in suppressing HIV transcription from chronically infected cell lines (Sarol et al., 2002; Wang et al., 2005), macrophages (Kornbluth et al., 1989) and human colon epithelial cells (Yahi et al., 1992), we examined whether IFN-γ is responsible for the observed NK-cell-mediated anti-HIV activity in U1 and ACH-2 cells. Recombinant IFN-γ, when added to U1 and ACH-2 cell cultures, significantly inhibited (up to 70%) TNF-α-induced HIV replication (Fig. 3), while NK SN pre-incubated with the antibody to IFN-γ showed diminished anti-HIV activity (Figs. 3 and 4). However, the antibodies to MIP-1α and RANTES could not block NK SN-mediated anti-HIV effects in both U1 and ACH-2 cells.

3.3. NK SN inhibits HIV LTR activation

To investigate the mechanism(s) responsible for the anti-HIV activity of NK SN, we selected 1G5 cells that contain a stably transfected luciferase gene under the transcriptional control of the HIV LTR. The levels of TNF-α-induced luciferase expression were reduced by 80% in the 1G5 cells treated with NK SN (NK 92). This inhibitory effect was similar to that of exogenous IFN-γ (Fig. 5). Antibody against IFN-γ blocked NK SN-mediated suppression of HIV-LTR-driven luciferase activity (Fig. 5). The specificity of IFN-γ antibody was evidenced by its blocking effect on exogenous IFN-γ's action (Fig. 5).

3.4. NK SN enhances the expression of STAT-1 and phosphorylated P38 MAPK

Since IFN-γ is involved in NK SN-mediated anti-HIV action, we hypothesized that IFN-γ-mediated signaling pathways are also involved in the NK SN-mediated anti-HIV action. We examined the effect of NK SN on the expression of STAT-1, the major component of the IFN signaling cascade (Darnell, 1997; Darnell et al., 1994; Fu et al., 1992). NK SN enhanced STAT-1 expression in both U1 and ACH-2 cells as determined by Western blot (Fig. 6). To further determine whether NK SN, through enhancing IFN signaling pathway, inhibits HIV replication, we examined the effect of NK SN on the expression of P38 MAPK, an important element required

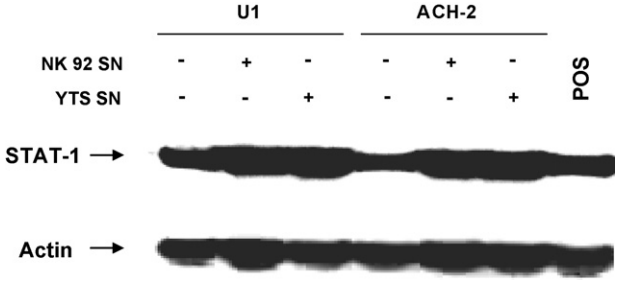

Fig. 6. NK SN enhances STAT-1 expression in U1 and ACH-2 cells. Equal amounts (30 μg) of total proteins extracted from U1 and ACH-2 cells treated with or without 25% NK SN (either NK 92 or YTS SN) for 24 h were subjected to Western blot assay using the antibodies against STAT-1 (1:500) and actin (1:3000). Pos. (positive control): $CD8^{+}$ T cells. Arrowheads indicate the position of STAT-1 and actin. Results shown are representative of two independent experiments. "+", in the presence; "−", in the absence.

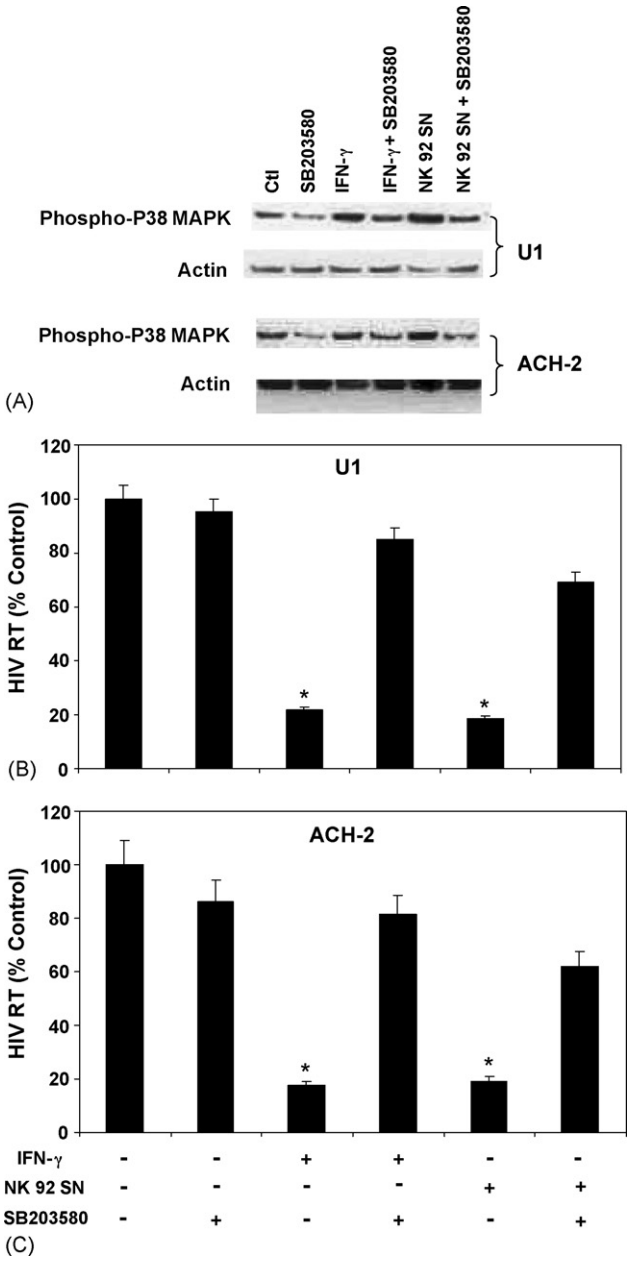

Fig. 7. Effect of SB203580 on NK SN-induced P38 MAPK phosphorylation and NK SN-mediated anti-HIV activity in U1 and ACH-2 cells. (A) Effect of SB203580 on NK 92 SN-induced P38 MAPK phosphorylation in U1 and ACH-2 cells. Equal amounts (10 μg) of total proteins extracted from U1 and ACH-2 cells treated with or without IFN-γ (1000 U/ml) or NK 92 SN (25%, v/v) and/or SB 203580 (10 μM) for 24 h were subjected to Western blot assay using the antibodies against phosphorylated P38 MAPK (1:500) and actin (1:3000). Results shown are representative of two independent experiments. (B and C) Effect of SB203580 on NK 92 SN-mediated anti-HIV activity. U1 and ACH-2 cells were cultured in the presence or absence of IFN-γ (1000 U/ml) or NK 92 SN (25%, v/v) and/or SB203580 (10 μM). HIV RT activity was determined 24 h post NK 92 SN and/or SB203580 treatment. The data are expressed as RT activity in NK SN-treated cultures (% of control) to control cultures (without treatment, which is defined as 100%). The results shown are mean ± S.D. of triplicate cultures, representative of three separate experiments. "+", in the presence; "−", in the absence. (B) U1 cell; (C) ACH-2 cell. $^{*}P < 0.05$.

for STAT-1 function (Goh et al., 1999). Similar to the effect of exogenous IFN-γ, NK SN activated phosphorylated P38 protein expression in U1 and ACH-2 cells (Fig. 7A), while SB203580, a specific inhibitor of P38 MAPK, abolished NK SN-induced phosphorylated P38 MAPK protein expression (Fig. 7A). The role of phosphorylated P38 in NK SN-mediated anti-HIV ability was further demonstrated in the experiments showing that SB203580 abolished the NK SN-mediated anti-HIV ability in U1 and ACH-2 cells (Fig. 7B and C).

4. Discussion

In this study, we demonstrated that reactivation of viral latency in U1 and ACH-2 cells by TNF-α and HIV-infected PBMCs could be suppressed by NK cell-released noncytolytic factor(s), primarily IFN-γ. Although it has been demonstrated that NK cells through secretion of CC-chemokines inhibit HIV R5 strain entry into macrophages (Kottilil, 2003; Kottilil et al., 2004; Oliva et al., 1998), little is known about the role of NK cell-mediated noncytolytic anti-HIV effects in chronically infected immune cells. We used chronically HIV-infected cell systems to examine NK cell-mediated noncytolytic anti-HIV activity. NK SN from both NK cell lines and primary NK cell cultures suppressed HIV replication in U1 and ACH-2 cells. This anti-HIV activity of NK SN differs from that observed in acute infection system where CC-chemokines contribute almost entirely to viral suppression (Kottilil, 2003), since the antibodies to CC-chemokines (MIP-1α and RANTES) failed to neutralize NK SN-mediated anti-HIV effects in U1 and ACH-2 cells. This NK cell-mediated anti-HIV activity is innate in nature, as the primary NK cells isolated from HIV seronegative subjects also inhibited HIV activation in the chronically infected cells. Most importantly, we demonstrated that primary NK SN not only suppressed TNF-α-induced HIV activation in the infected cell lines, but also inhibited HIV activation in PBMCs isolated from HIV-infected subjects (Fig. 2). Since IL-2 was used to culture primary NK cells, the involvement of IL-2 in NK SN-mediated HIV inhibition was analyzed. IL-2 did not inhibit TNF-α-mediated HIV replication in both U1 and ACH-2 cells (data not shown). In addition, NK SN from IL-2-independent NK cell line cultures (YTS) also suppressed HIV replication (Figs. 1 and 3), indicating that IL-2 is not involved in the NK SN action. Thus, our data in conjunction with the studies by others (d'Ettorre et al., 2005; Fauci et al., 2005; Kottilil, 2003; Kottilil et al., 2003; Poli et al., 1994) provide compelling evidence that NK cells have an important role in inhibiting both acute and chronical HIV infection.

Although direct NK-mediated cytotoxicity is critical in controlling certain viral infections, the antiviral cytokines such as IFN-γ produced by NK cells, also play a key role in the control of viral infections, including HCV and CMV (Li et al., 2004; Orange et al., 1995). IFN-γ also is a powerful anti-HIV cytokine (Azzoni et al., 2002; d'Ettorre et al., 2005). The anti-HIV ability of IFN-γ is also demonstrated in our experiments showing that recombinant IFN-γ suppressed HIV replication in chronically infected cells (Figs. 3 and 4). IFN-γ and other unknown factor(s) appear to play a major role in NK cell-mediated anti-HIV activity in chronically infected cells. The role of IFN-γ in NK SN-

mediated anti-HIV effect is evidenced by the observations that antibody to IFN-γ significantly blocked the NK SN action in U1 and ACH-2 cells (Fig. 3), while antibodies to CC-chemokines had little effect on the NK SN-mediated HIV inhibition (Fig. 4). In addition, antibody to IFN-γ diminished NK SN-mediated suppression of HIV-LTR promoter activation in 1G5 cells (Fig. 5). The important role of NK-released IFN-γ in controlling HIV infection is further supported by a recent study showing that exposure of NK cells to HIV envelop proteins suppressed the ability of NK cells to secrete IFN-γ (Kottilil et al., 2006). In more advanced patients with HIV infection, fewer cells produce IFN-γ in response to HIV. However, the number of IFN-γ-producing cells initially increases with HAART (Bailer et al., 1999; Huang et al., 2000; Lieberman et al., 2001; Ullum et al., 1997). Therefore, as a major source of IFN-γ, NK cells are critical in the host innate defense mechanism(s) against persistent HIV infection.

Our data showing that NK SN enhanced STAT-1 protein expression in both U1 and ACH-2 cells (Fig. 6) provide a plausible mechanism responsible for the NK cell action on HIV. STAT-1 is essential for the innate immune response and plays a dedicated role in signaling for IFN-mediated biologic effects including antiviral activity (Bach et al., 1997). STAT-1 activation is directly involved in the inhibition of HIV transcription mediated by $CD8^+$ T-lymphocyte antiviral factor (Chang et al., 2002). Although STAT-1 is the primary components of the IFN signaling cascade, other signaling molecules such as P38 MAPK also are involved in the biological effects of IFNs (Platanias, 2003). P38 MAPK is required for STAT-1 serine phosphorylation and transcriptional activation induced by both IFN-α and IFN-γ (Platanias, 2003). Therefore, it is of importance to determine whether NK SN modulates P38 MAPK expression. Our observations that NK SN enhanced the expression of phosphorylated P38 MAPK protein and that NK SN-mediated anti-HIV ability was inhibited by the P38 inhibitor (SB203580) (Fig. 7) provide an additional mechanism involved in the NK SN action on HIV in chronically infected cells. Collectively, these data suggest that NK SN, through enhancing IFN signaling pathway in chronically HIV-infected cells, inhibit HIV replication in the target cells.

Since HIV latency is the major obstacle in preventing the HIV eradication (Butera, 2000; Chun and Fauci, 1999; Chun et al., 1997; Finzi et al., 1997; Ho, 1998; Pierson et al., 2000; Wong et al., 1997), it is of importance to identify innate immune factors that suppress and eliminate HIV in viral reservoir. NK cells play a key role in the host innate immunity against HIV infection. Our study in conjunction with the findings by others (d'Ettorre et al., 2005; Fauci et al., 2005; Kottilil, 2003; Kottilil et al., 2003; Poli et al., 1994) provide compelling evidence that NK cells not only are crucial in inhibiting HIV infection at entry level, but also may have a role in suppressing HIV activation in the reservoir cells. Further studies are required to validate our in vitro observations and to delineate the precise mechanisms of NK cell-mediated suppression of HIV replication in latently infected cells in vivo. These studies will be critical not only for our basic understanding of HIV immunopathogenesis but also for the design and development of innate immunity-based intervention strategies for eliminating HIV from its reservoirs.

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References

Aguilar-Cordova, E., Chinen, J., Donehower, L., Lewis, D.E., Belmont, J.W., 1994. A sensitive reporter cell line for HIV-1 tat activity, HIV-1 inhibitors, and T cell activation effects. AIDS Res. Hum. Retroviruses 10, 295–301.

Alkhatib, G., Combadiere, C., Broder, C.C., et al., 1996. CC CKR5: a RANTES, MIP-1 alpha, MIP-1beta receptor as a fusion cofactor for macrophage-tropic HIV-1. Science 272, 1955–1958.

Azzoni, L., Papasavvas, E., Chehimi, J., et al., 2002. Sustained impairment of IFN-gamma secretion in suppressed HIV-infected patients despite mature NK cell recovery: evidence for a defective reconstitution of innate immunity. J. Immunol. 168, 5764–5770.

Bach, E.A., Aguet, M., Schreiber, R.D., 1997. The IFN gamma receptor: a paradigm for cytokine receptor signalling. Annu. Rev. Immunol. 15, 563–591.

Bailer, R.T., Holloway, A., Sun, J., et al., 1999. IL-13 and IFN-gamma secretion by activated T cells in HIV-1 infection associated with viral suppression and a lack of disease progression. J. Immunol. 162, 7534–7542.

Biron, C.A., 1997. Activation and function of natural killer cell responses during viral infections. Curr. Opin. Immunol. 9, 24–34.

Butera, S.T., 2000. Therapeutic targeting of human immunodeficiency virus type-1 latency: current clinical realities and future scientific possibilities. Antiviral Res. 48, 143–176.

Chang, T.L., Mosoian, A., Pine, R., Klotman, M.E., Moore, J.P., 2002. A soluble factor(s) secreted from CD8(+) T lymphocytes inhibits human immunodeficiency virus type 1 replication through STAT1 activation. J. Virol. 76, 569–581.

Chen, B.K., Saksela, K., Andino, R., Baltimore, D., 1994. Distinct modes of human immunodeficiency virus type 1 proviral latency revealed by superinfection of nonproductively infected cell lines with recombinant luciferase-encoding viruses. J. Virol. 68, 654–660.

Choe, H., Farzan, M., Sun, Y., et al., 1996. The beta-chemokine receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates. Cell 85, 1135–1148.

Chun, T.W., Fauci, A.S., 1999. Latent reservoirs of HIV: obstacles to the eradication of virus. Proc. Natl. Acad. Sci. U.S.A. 96, 10958–10961.

Chun, T.W., Stuyver, L., Mizell, S.B., et al., 1997. Presence of an inducible HIV-1 latent reservoir during highly active antiretroviral therapy. Proc. Natl. Acad. Sci. U.S.A. 94, 13193–13197.

Cohen, G.B., Gandhi, R.T., Davis, D.M., et al., 1999. The selective downregulation of class I major histocompatibility complex proteins by HIV-1 protects HIV-infected cells from NK cells. Immunity 10, 661–671.

Darnell Jr., J.E., 1997. STATs and gene regulation. Science 277, 1630–1635.

Darnell Jr., J.E., Kerr, I.M., Stark, G.R., 1994. Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. Science 264, 1415–1421.

d'Ettorre, G., Andreotti, M., Carnevalini, M., et al., 2005. Interleukin-15 enhances the secretion of IFN-gamma and CC chemokines by natural killer cells from HIV viremic and aviremic patients. Immunol. Lett. 25, 25.

Dragic, T., Litwin, V., Allaway, G.P., et al., 1996. HIV-1 entry into CD4+ cells is mediated by the chemokine receptor CC-CKR-5. Nature 381, 667–673.

Embretson, J., Zupancic, M., Ribas, J.L., et al., 1993. Massive covert infection of helper T lymphocytes and macrophages by HIV during the incubation period of AIDS. Nature 362, 359–362.

Fauci, A.S., 1988. The human immunodeficiency virus: infectivity and mechanisms of pathogenesis. Science 239, 617–622.

Fauci, A.S., Mavilio, D., Kottilil, S., 2005. NK cells in HIV infection: paradigm for protection or targets for ambush. Nat. Rev. Immunol. 5, 835–843.

Finzi, D., Blankson, J., Siliciano, J.D., et al., 1999. Latent infection of CD4+ T cells provides a mechanism for lifelong persistence of HIV-1, even in patients on effective combination therapy. Nat. Med. 5, 512–517.

Finzi, D., Hermankova, M., Pierson, T., et al., 1997. Identification of a reservoir for HIV-1 in patients on highly active antiretroviral therapy. Science 278, 1295–1300.

Folks, T.M., Clouse, K.A., Justement, J., et al., 1989. Tumor necrosis factor alpha induces expression of human immunodeficiency virus in a chronically infected T-cell clone. Proc. Natl. Acad. Sci. U.S.A. 86, 2365–2368.

Folks, T.M., Justement, J., Kinter, A., Dinarello, C.A., Fauci, A.S., 1987. Cytokine-induced expression of HIV-1 in a chronically infected promonocyte cell line. Science 238, 800–802.

Folks, T.M., Justement, J., Kinter, A., et al., 1988. Characterization of a promonocyte clone chronically infected with HIV and inducible by 13-phorbol-12-myristate acetate. J. Immunol. 140, 1117–1122.

Fu, X.Y., Schindler, C., Improta, T., Aebersold, R., Darnell Jr., J.E., 1992. The proteins of ISGF-3, the interferon alpha-induced transcriptional activator, define a gene family involved in signal transduction. Proc. Natl. Acad. Sci. U.S.A. 89, 7840–7843.

Goh, K.C., Haque, S.J., Williams, B.R., 1999. p38 MAP kinase is required for STAT1 serine phosphorylation and transcriptional activation induced by interferons. EMBO J. 18, 5601.

Gong, J.H., Maki, G., Klingemann, H.G., 1994. Characterization of a human cell line (NK-92) with phenotypical and functional characteristics of activated natural killer cells. Leukemia 8, 652–658.

Gulick, R.M., Mellors, J.W., Havlir, D., et al., 1997. Treatment with indinavir, zidovudine, and lamivudine in adults with human immunodeficiency virus infection and prior antiretroviral therapy. N. Engl. J. Med. 337, 734–739.

Hammer, S.M., Squires, K.E., Hughes, M.D., et al., 1997. A controlled trial of two nucleoside analogues plus indinavir in persons with human immunodeficiency virus infection and CD4 cell counts of 200 per cubic millimeter or less. AIDS Clinical Trials Group 320 Study Team. N. Engl. J. Med. 337, 725–733.

Hassan, N.F., Campbell, D.E., Douglas, S.D., 1986. Purification of human monocytes on gelatin-coated surfaces. J. Immunol. Methods 95, 273–276.

Ho, D.D., 1998. Toward HIV eradication or remission: the tasks ahead. Science 280, 1866–1867.

Ho, W.Z., Lioy, J., Song, L., et al., 1992. Infection of cord blood monocyte-derived macrophages with human immunodeficiency virus type 1. J. Virol. 66, 573–579.

Huang, X.L., Fan, Z., Kalinyak, C., Mellors, J.W., Rinaldo Jr., C.R., 2000. CD8(+) T-cell gamma interferon production specific for human immunodeficiency virus type 1 (HIV-1) in HIV-1-infected subjects. Clin. Diagn. Lab. Immunol. 7, 279–287.

Kornbluth, R.S., Oh, P.S., Munis, J.R., Cleveland, P.H., Richman, D.D., 1989. Interferons and bacterial lipopolysaccharide protect macrophages from productive infection by human immunodeficiency virus in vitro. J. Exp. Med. 169, 1137–1151.

Kottilil, S., 2003. Natural killer cells in HIV-1 infection: role of NK cell-mediated non-cytolytic mechanisms in pathogenesis of HIV-1 infection. Indian J. Exp. Biol. 41, 1219–1225.

Kottilil, S., Chun, T.W., Moir, S., et al., 2003. Innate immunity in human immunodeficiency virus infection: effect of viremia on natural killer cell function. J. Infect. Dis. 187, 1038–1045.

Kottilil, S., Shin, K., Jackson, J.O., et al., 2006. Innate immune dysfunction in HIV infection: effect of HIV envelope-NK cell interactions. J. Immunol. 176, 1107–1114.

Kottilil, S., Shin, K., Planta, M., et al., 2004. Expression of chemokine and inhibitory receptors on natural killer cells: effect of immune activation and HIV viremia. J. Infect. Dis. 189, 1193–1198.

Koup, R.A., Safrit, J.T., Cao, Y., et al., 1994. Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. J. Virol. 68, 4650–4655.

Levy, J.A., 1993. Pathogenesis of human immunodeficiency virus infection. Microbiol. Rev. 57, 183–289.

Li, Y., Zhang, T., Ho, C., et al., 2004. Natural killer cells inhibit hepatitis C virus expression. J. Leukoc. Biol. 76, 1171–1179.

Lieberman, J., Shankar, P., Manjunath, N., Andersson, J., 2001. Dressed to kill? A review of why antiviral CD8 T lymphocytes fail to prevent progressive immunodeficiency in HIV-1 infection. Blood 98, 1667–1677.

Oliva, A., Kinter, A.L., Vaccarezza, M., et al., 1998. Natural killer cells from human immunodeficiency virus (HIV)-infected individuals are an important source of CC-chemokines and suppress HIV-1 entry and replication in vitro. J. Clin. Invest. 102, 223–231.

Orange, J.S., Wang, B., Terhorst, C., Biron, C.A., 1995. Requirement for natural killer cell-produced interferon gamma in defense against murine cytomegalovirus infection and enhancement of this defense pathway by interleukin 12 administration. J. Exp. Med. 182, 1045–1056.

Pantaleo, G., Graziosi, C., Butini, L., et al., 1991. Lymphoid organs function as major reservoirs for human immunodeficiency virus. Proc. Natl. Acad. Sci. U.S.A. 88, 9838–9842.

Pantaleo, G., Graziosi, C., Demarest, J.F., et al., 1993. HIV infection is active and progressive in lymphoid tissue during the clinically latent stage of disease. Nature 362, 355–358.

Perez, V.L., Rowe, T., Justement, J.S., et al., 1991. An HIV-1-infected T cell clone defective in IL-2 production and Ca2+ mobilization after CD3 stimulation. J. Immunol. 147, 3145–3148.

Pierson, T., McArthur, J., Siliciano, R.F., 2000. Reservoirs for HIV-1: mechanisms for viral persistence in the presence of antiviral immune responses and antiretroviral therapy. Annu. Rev. Immunol. 18, 665–708.

Platanias, L.C., 2003. The p38 mitogen-activated protein kinase pathway and its role in interferon signalling. Pharmacol. Ther. 98, 129–142.

Poli, G., Biswas, P., Fauci, A.S., 1994. Interferons in the pathogenesis and treatment of human immunodeficiency virus infection. Antiviral Res. 24, 221–233.

Sarol, L.C., Imai, K., Asamitsu, K., et al., 2002. Inhibitory effects of IFN-gamma on HIV-1 replication in latently infected cells. Biochem. Biophys. Res. Commun. 291, 890–896.

Ullum, H., Cozzi Lepri, A., Bendtzen, K., et al., 1997. Low production of interferon gamma is related to disease progression in HIV infection: evidence from a cohort of 347 HIV-infected individuals. AIDS Res. Hum. Retroviruses 13, 1039–1046.

Wang, X., Tan, N., Douglas, S.D., et al., 2005. Morphine inhibits CD8+ T cell-mediated, noncytolytic, anti-HIV activity in latently infected immune cells. J. Leukoc. Biol. 78, 772–776 (Epub 2005 July 6).

Wei, X., Ghosh, S.K., Taylor, M.E., et al., 1995. Viral dynamics in human immunodeficiency virus type 1 infection. Nature 373, 117–122.

Willey, R.L., Smith, D.H., Lasky, L.A., et al., 1988. In vitro mutagenesis identifies a region within the envelope gene of the human immunodeficiency virus that is critical for infectivity. J. Virol. 62, 139–147.

Wong, J.K., Hezareh, M., Gunthard, H.F., et al., 1997. Recovery of replication-competent HIV despite prolonged suppression of plasma viremia. Science 278, 1291–1295.

Yahi, N., Baghdiguian, S., Bolmont, C., Fantini, J., 1992. Inhibition of human immunodeficiency virus infection in human colon epithelial cells by recombinant interferon-gamma. Eur. J. Immunol. 22, 2495–2499.

Zhang, T., Guo, C.J., Douglas, S.D., et al., 2005. Alcohol suppresses IL-2-induced CC chemokine production by natural killer cells. Alcohol Clin. Exp. Res. 29, 1559–1567.