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IL-21 augments NK effector functions in chronically HIV-infected individuals

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Abstract

Objective—This study addresses the interleukin (IL)-21 effects on resting peripheral blood NK cells in chronically HIV-infected individuals.

Design—The effects of IL-21 on perforin expression, proliferation, degranulation, IFN- γ production, cytotoxicity and induction of STAT phosphorylation in NK cells were determined *in vitro*.

Methods—Peripheral blood mononuclear cells from HIV-infected and healthy individuals were incubated *in vitro* for 6h, 24h or 5 days with IL-21 or IL-15. Percentages of perforin, IFN- γ , CD107a, NKG2D and STAT3-5 positive cells were determined within NK cell populations. K562 cells were used as target cells in NK cytotoxicity assay.

Results—Frequency of CD56^{dim} cells in chronically HIV-infected individuals was diminished. Perforin expression in CD56^{dim} and CD56^{bright} was comparable in healthy and HIV-infected individuals. IL-15 up-regulated perforin expression primarily in CD56^{bright} NK cells while IL-21 up-regulated perforin in both NK subsets. IL-21 and IL-15 up-regulated CD107a and IFN- γ as well as NK cytotoxicity. IL-15 predominantly activated STAT5, while IL-21 activated STAT5 and STAT3. IL-15, but not IL-21 increased NK cell proliferation in uninfected and HIV-infected individuals.

Conclusion—IL-21 augments NK effector functions in chronically HIV-infected individuals and due to its perforin enhancing properties it has potential for immunotherapy or as a vaccine adjuvant.

Keywords

NK cells; IL-21; IL-15; HIV; perforin; cytotoxicity

Introduction

Natural killer (NK) lymphocytes are important components of innate immunity, and can lyse tumor- and virally transformed target cells without prior sensitization [1]. In HIV infection, some studies have implicated reduced NK cell numbers and activity [2,3] with progression to AIDS, while others have not found this association [4]. HIV-exposed but uninfected individuals

have strong NK activity, arguing for a possible protective role in preventing HIV infection [5].

In HIV-infected subjects, the functions of NK cells such as cytotoxicity and production of interferon- γ (IFN- γ) are augmented with cytokines, e.g. IFN- α and common γ -chain family cytokines [6–10]. Among the latter, IL-21 is a more recently identified cytokine, and is produced almost exclusively by activated CD4⁺ T cells. IL-21 stimulates secretion of IFN- γ by NK and T cells [11], induces their proliferation and cytotoxic activity, [12] and has shown promising antitumor activity in a variety of murine models [13–16]. In the B16 melanoma model, the antitumor effect of IL-21 was found to be dependent on NK cells, but not T cells, as depletion of NK cells prevented tumor regression following IL-21 administration [13,14, 16]. Given the stimulatory effects of IL-21 on NK cells and antitumor effects in vivo [17], we hypothesized that this cytokine would uniquely augment NK cell effector molecules such as perforin.

The majority of human peripheral NK cells display a CD3^{neg}CD56⁺CD16⁺ phenotype [18]. According to expression density of CD56, NK cells can be divided into CD56^{dim}, representing the vast majority of human NK cells, and a small distinct population of CD56^{bright} NK cells [19]. In HIV-infected persons with active viral replication, the CD56^{dim} NK cell subset is decreased and CD56 negative cells are expanded [2,16,20]. Following virological control with antiretroviral therapy the CD56 negative cells normalize [2], but the deficiency of CD56^{dim} NK cell subset persists [2,21]. We examined whether IL-21 and IL-15, a potent activator of NK cells [8,9], have differential effects on effector functions of CD56^{dim} versus CD56^{bright} populations of NK cells.

Materials and Methods

Study Subjects

Peripheral venous blood was collected from healthy donors (n=6) and HIV-infected individuals (n=10) with the following criteria: a) Age, 18–60 years (median, 46); b) CD4 cell counts > 200 (range 267–1198/mm³; median 772); c) Viral load (VL) < 50 HIV RNA copies/ml in plasma and d) highly active anti-retroviral therapy (HAART) with ≥ 3 anti-retrovirals for ≥ 12 months. All individuals signed informed consents approved by the Institutional Review Board of University of Miami, FL.

Antibodies and Reagents

The following anti-human monoclonal antibody (mAb) reagents were obtained from BD Pharmingen, San Diego, CA: anti-CD3 [allophycocyanin (APC), Pacific Blue, (PacBlue) fluorescein isothiocyanate (FITC)], anti-CD8 [peridinin chlorophyll protein (PerCP)], APC-Cy7], anti-CD56 (APC, PE-Cy7), anti-CD16 [PacBlue, phycoerythrin (PE)], anti-CD107a (FITC, PE-Cy5), anti-IFN- γ (PE, APC), anti-Perforin (FITC, PE), anti-pSTAT3 (PE), anti-pSTAT4 (PE) and anti-pSTAT5 (PE). Recombinant human (rh) IL-21 was a gift from ZymoGenetics, Seattle, WA. RhIL-15 and IL-2 were obtained from R&D Systems, Minneapolis, MN.

Processing of Blood Samples

Fresh peripheral blood mononuclear cells (PBMC) were isolated from peripheral venous blood using Hypaque-Ficoll (Accuprep, Norway) density centrifugation. For some studies, CD56 cells were enriched with RosetteSep NK cell cocktail (StemCell Technologies, Vancouver, Canada) with greater than 95% purity.

Immunofluorescent staining/analysis

PBMC, freshly isolated or following stimulation, were stained with mAb for surface immunophenotyping. For intracellular staining, the cells were washed once, fixed/permeabilized with Cytotfix/Cytoperm (BD), washed twice with a Saponin-containing buffer, and stained with specific mAb for perforin or IFN- γ . For IFN- γ staining, Brefeldin A, 10 μ g/ml was added for the last 4 hrs of culture to block exocytosis. Cells were fixed in 1% paraformaldehyde (PFA, EM Science, Darmstadt, Germany) prior to acquisition on FACSCalibur for 4 color analyses or on LSR IITM (both from Becton Dickinson Biosciences) for 6 and 7 color analyses. Data was analyzed using FlowJo software (Tree Star, San Carlos, CA). The CD3negCD56+ cells were gated as live scatter-gated lymphocytes. Between 100,000 and 500,000 events were collected for each sample.

Analysis of NK cell division by CFSE dye dilution

PBMC were labeled with 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE, Molecular Probes, Eugene, OR) at 4 μ M/ 5 $\times$ 10⁶ cells for 10 min at 37°C. Labeling was terminated by addition of an equal volume of 100% fetal bovine serum (FBS). After 4 washes in complete media [RPMI 1640 (Invitrogen) containing 10% FBS (Atlanta Biologicals, Norcross, GA), 2 mM L-glutamine (Mediatech), and 50IU/ml penicillin (Mediatech)] cells were cultured with appropriate stimuli for 5 days at 37°C/5% CO₂ and stained for CD3 and CD56 expression. Cell division was analyzed in gated CD3negCD56+ cells based on decrease in CFSE, resulting from dilution of the dye with each division. Cells cultured in medium alone did not proliferate and had less than 1% CFSE dim cells. The division index (defined by the number of cells entering cell division and the average number of cell divisions they undergo) was calculated after gating on CD3negCD56+ cells and presented as mean $\pm$ SEM from healthy controls and HIV-infected individuals.

CD107a [lysosome-associated membrane protein (LAMP)-1] expression assay

Degranulation of intracellular vesicles by lymphocytes can be measured using CD107a, as described for CD8⁺ T cells [22]. The frequency of degranulating NK cells following stimulation of 10⁶ PBMC/ml with the MHC-devoid target, K562 cell line (ATCC), at an E:T ratio of 10:1 was determined. CD107a mAb was added to the test cells at a dilution of 20 μ l/ml, followed by incubation for 1h at 37°C in 5% CO₂. Monensin (Golgi-block; BD Biosciences) was added for a final concentration of 6 μ g/ml and incubated for an additional 5h at 37°C in 5% CO₂. Surface staining for NK markers (CD3, CD16, CD8, NKG2D) was performed for 30 min. Cells were stained for intracellular markers (perforin and IFN- γ) as described above. The cells were washed and resuspended in 1% PFA and analyzed on LSR II where 50,000 to 200,000 events were acquired. The background degranulation activity ranged from 0.05–3.8% of NK cells, with a mean of 2.3% and a standard deviation (SD) of 1.26%. Thus, a positive response was defined as the percentage of cells expressing CD107a that were three SD's above mean background activity, which was then subtracted from the experimental value.

NK cytotoxicity assay

Purified CD56+ cells were cultured in medium with IL-21, IL-15, or no cytokine for 24h and then used as a source of effector cells. K562 target cells were labeled with red fluorescent cell linker PKH-26 Red (Sigma Aldrich), washed 3 times in complete medium and adjusted to 10⁵ cells/ml. 100 μ l target cells were mixed in 12 $\times$ 75 mm round-bottom polystyrene tubes (Falcon) with CD56+ effector cells at E:T ratios of 6:1, 12.5:1 and 25:1, centrifuged at 25°C for 4 min at 300 rpm (25 xg) and incubated at 37°C for 4h. 7-AAD (BD Pharmingen) was added to the cells 10–15 min before data acquisition. A total of 10,000 events were collected per sample. Target cells were gated by side scatter and fluorescence (FL2) and analyzed for 7-

AAD uptake. Percent lysis was determined as [(percent 7-AAD staining in sample – percent 7-AAD staining of negative control)/(100-percent 7-AAD staining of negative control)] × 100.

Simultaneous phosphospecific and surface mAb staining

Phosphospecific flow cytometry was performed as previously described [23]. Briefly, PBMC in RPMI 1640 with 10% FBS were cultured with IL-21 (50ng/ml), IL-15 (50ng/ml) or IL-2 (1000 IU/ml) for 15 min at 37°C before fixation with 1.6% formaldehyde for 10 min. Then cells were pelleted, resuspended in ice-cold methanol, and incubated for 30 min at 4°C. The cells were washed twice with staining buffer (PBS containing 0.5% BSA and 0.02% sodium azide), resuspended at 10⁶ cells/ml, and stained with mAbs including CD3, CD56 and phospho-Stat (pStat3) (Y705), or CD3, CD56 and pStat4 or CD3, CD56 and pStat5 (Y694). Cells were stained for 30 min, washed, and resuspended before acquisition by flow cytometry. Typically, 250,000–300,000 events were collected per experiment to give >1,000 cells in all populations analyzed.

RT-PCR

Total RNA was extracted from fresh and cultured purified CD56+ cells using the RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The first-strand cDNA was synthesized using the Omniscript Reverse Transcription kit (Qiagen, Valencia, CA) with random hexaprimers. Perforin mRNA relative expression levels were quantified by real-time PCR with the ABI/PRISM 7700 sequence detection system (Applied Biosystems, Foster City, CA), and primers and probes for perforin and the housekeeping gene, human hypoxanthine–guanine phosphoribosyl-transferase (*HPRT*) were obtained as assays on demand from Applied Biosystems. Each sample was examined for both perforin and *HPRT* in a final reaction volume of 25µl using TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA), and amplification was carried out over 15 min at 95°C (denaturation step) followed by 40 cycles of 15 s at 94°C and 60 s at 60°C. The relative quantitation of perforin mRNA was carried out using the comparative threshold cycle (C_T) method ($2^{-\Delta\Delta C_T}$) [24].

Statistical Analysis

Non-parametric Mann-Whitney *U* test was used to evaluate differences between 2 groups. One-way ANOVA was used for analysis of differences between unstimulated and various stimulated cultures. Spearman's correlation coefficient was utilized to determine relationships between examined parameters. Values of $p < 0.05$ were considered statistically significant.

Results

Frequency of CD56^{dim} NK cells is diminished in HIV-infected individuals compared to HIV negative controls

Numbers of CD3negCD56+ NK cells in peripheral blood of HIV-infected subjects although not significantly reduced from HIV-negative controls (Fig. 1a), were found to be positively correlated with CD4 counts (Fig. 1b). The CD56^{dim} subset of NK cells in HIV-infected patients was decreased in comparison with uninfected controls (Fig. 1c). The percentage of CD56^{bright} cells was maintained or even increased, but the overall difference from uninfected controls was not significant (data not shown).

The main killing mechanism of NK cells is mediated through the perforin pathway, therefore intracellular perforin was examined. The percentage of perforin+ cells and the mean fluorescence intensity (MFI) of perforin staining was lower in CD56^{bright} cells than in CD56^{dim} cells in both, patients and control subjects. Although it was heterogeneous in its

distribution, perforin expression was equivalent between patients and controls in the CD56^{dim} and CD56^{bright} NK cell subsets (data not shown).

IL-21 increases perforin in both, CD56^{bright} and CD56^{dim} NK cells of HIV-infected subjects, but does not induce NK cell proliferation

IL-15 is known to induce proliferation, differentiation and perforin up-regulation in resting peripheral blood NK cells [8]. We compared the effects of IL-15 and IL-21 on CD56+ cell subset frequency, perforin expression, and proliferation. We observed dose-dependent effects on perforin expression by IL-15 and IL-21 with a peak response at 50 ng/ml (data not shown). Addition of IL-15, but not IL-21 to PBMC cultures for 5 days resulted in increased frequency of CD56^{bright} NK cells (Fig. 2a, $p<0.05$) with a concomitant decrease in CD56^{dim} subset (Figs. 2b and 2c, $p<0.05$). In contrast, cells cultured with IL-21 had a marginal increase in the numbers of the CD56^{dim} NK cell subset (Fig. 2b). Both IL-15 and IL-21 were potent inducers of perforin in the two subsets. At the protein level, only IL-21 increased the perforin expression in CD56^{dim} NK cells (Fig. 2e) while both IL-15 and IL-21 induced statistically significant perforin expression in the CD56^{bright} cells (Fig. 2d, $p<0.05$). In purified CD56+ NK cells cultured for 5 hours with IL-21, a 5-fold higher perforin mRNA expression was noted by quantitative RT-PCR compared to cultures containing medium alone (Fig. 2f).

In CFSE-labeled PBMC cultures, IL-15 induced significant proliferation of NK cells in both, HIV-infected individuals and healthy control donors (Fig. 3). Notably, the division index of IL-15 stimulated NK cells in HIV-infected individuals was higher than in uninfected controls, suggesting that NK cells from HIV-infected individuals were more responsive to IL-15 induced proliferation than cells of healthy donors. IL-21 did not induce proliferation of NK cells in CFSE-labeled PBMC cultures of healthy controls, however cells from some HIV-infected individuals manifested marginal proliferation.

IL-21 or IL-15 treated NK cells manifest rapid induction of degranulation marker, CD107a, and intracytoplasmic IFN- γ upon coculture with K562 cells

We investigated expression of CD107a [22,25], as well as intracellular content of perforin and IFN- γ in NK cells following 24h culture with or without cytokines and a 6h stimulation with MHC-devoid NK-sensitive K562 cells. Similar to the 5h effect on perforin expression, 24h culture of PBMC with IL-15 or IL-21 increased the MFI of perforin in gated CD3negCD56+ cells. There was no effect on CD107a expression (Fig. 4a). After 6h co-culture of medium treated PBMC with K562 cells, there was a decrease in perforin expression in CD56+ cells of HIV-infected and uninfected individuals (Fig. 4b, left), with a slight increase in CD107a which was significant in patients (Fig. 4b, middle). Further, there was no change in IFN- γ expression in control NK cells following incubation with K562 cells, but a significant increase was noted in patient NK cells (Fig. 4b, right). In cytokine treated cells, coculture with K562 cells results in a slight decrease in perforin (Fig. 4c,d, left) concomitantly with significant increase in CD107a (Fig 4c,d, middle) and increase in IFN- γ (Fig 4c,d, right) compared to PBMC not cultured with K562 cells. The enhancement of perforin expression occurs in the presence of IL-15 or IL-21 due to accumulation of perforin inside the cells (Fig 4c,d). The effect of IL-21 was consistently less than that of IL-15, and the response of HIV infected subjects was equal to or greater than that of uninfected subjects.

Since both IL-15 and IL-21 induced significant expression of surface CD107a, the effector function of CD3negCD56+ cells was examined in NK cytotoxicity assay. Purified CD3negCD56+ NK cells cultured with IL-21 or IL-15 for 24h demonstrated significantly increased perforin content (Fig 4e) and cytotoxicity against K562 cells, compared to unstimulated cells (Fig. 4f). Again, the effect of IL-15 was more pronounced than that of IL-21. These results suggest that both cytokines have a direct effect on NK cells. IL-21 and IL-15 not

only increased perforin and IFN- γ content, but they also enhance the release of cytotoxic granules and subsequent killing of target cells.

IL-2 and IL-15 predominantly activate STAT5 in both healthy and HIV-infected CD3negCD56⁺ NK cells, while IL-21 activates STAT5 and STAT3

IL-15 utilizes STAT5 for downstream signaling, whereas IL-21 preferentially activates STAT3 [26–28]. Perforin gene activation has been linked to STAT3 and STAT5 activation in NK cells [29] and to STAT5 activation in CTL [30]. PBMC isolated from uninfected (Fig. 5a,c) and HIV-infected (Fig. 5b,d) individuals cultured with IL-2 or IL-15 for 15 minutes strongly induced STAT5 phosphorylation in HIV-infected and uninfected individuals. Interestingly, STAT5 phosphorylation induced by IL-2 and IL-15 was weaker in healthy volunteers than in HIV-infected individuals. Furthermore, treatment of PBMC with IL-21 for 15 minutes stimulated phosphorylation of STAT3 and STAT5, which was higher in NK cells of HIV-infected individuals than uninfected subjects (Fig. 5 c, d). IL-2-, IL-15-, and IL-21-induced STAT4 activation was very weak in all study subjects.

Discussion

Reduced NK cell activity and a decrease in NK cell numbers have been implicated in HIV disease progression [2,3,20] and their function is known to be augmented by exogenous cytokines, particularly IL-15 [31,32]. We have recently demonstrated that IL-21 is a potent inducer of perforin in CD8⁺ T cells and that this activity is independent of CD8⁺ T cell proliferation [33]. We tested the hypothesis that the cytokine IL-21 would have similar effects on NK cells as on T cells in HIV-infected patients. Our data shows that IL-21 increases expression of perforin in NK cells, enhances degranulation and induces NK cell cytotoxicity without augmenting NK cell proliferation.

Among NK cell subsets, the CD56 negative subset is expanded in viremic HIV-infected subjects, but following control of viremia with ART, it decreases to a low frequency that is the norm in healthy HIV-uninfected persons [2,16,20,34]. In aviremic HIV-infected people on ART, the CD56^{dim} NK subset is described to be defective [2,35]. As all subjects in this study were on potent antiretroviral therapy and were virologically controlled (plasma HIV RNA <50 copies/mL) and immunologically stable (CD4 counts >200), we focused our attention on CD56^{dim} NK subset. This NK cell subset was significantly decreased in peripheral blood of patients as compared to healthy control subjects. The CD56^{bright} NK cell subset was well preserved, and even increased in some patients. One potential explanation for the maintenance of CD56^{bright} and decrease of CD56^{dim} NK cell subset could be that these two distinct subsets of mature NK cells are differentially affected by HIV-1, possibly due to alterations in the cytokine milieu. The development of CD56^{bright} NK cells is dependent on IL-15 [12], which is mainly produced by monocytes. On the contrary, the development of CD56^{dim} NK cells is dependent upon IL-21 [12], which is produced by CD4⁺ T lymphocytes. It could be hypothesized that CD4 T cell deficiency negatively influences IL-21 production whereas IL-15 activity is sustained in HIV-infected patients. The positive effect of IL-15 on the CD56^{bright} NK cell population was evident in our study. There was an increase in CD56^{bright} cells and a concomitant decrease in CD56^{dim} NK cells following culture of PBMC with IL-15. This effect of IL-15 may represent a maturation induced transition of CD56^{dim} phenotype to CD56^{bright} NK phenotype as described for IL-2 [16] or might result from proliferation of CD56^{bright} cells, as IL-15 induces NK cell proliferation. The IL-21 effect on NK cells was distinctly different from that of IL-15. Cell cultures with IL-21 led to minimal changes in frequency of CD56^{dim} and CD56^{bright} NK cells without significant cellular proliferation. As the CD56^{dim} subset is the primary NK cell subset with cytolytic properties, we examined perforin expression in both NK subsets. Perforin content was higher in the CD56^{dim} subset as compared to the

CD56^{bright} subset, supporting its increased cytolytic potential. Although perforin-expressing CD56^{dim} NK cells from HIV-infected individuals and uninfected subjects were comparable, short-term culture with IL-21, but not IL-15, augmented perforin expression in this subset. Both, IL-21 and IL-15 were found to upregulate perforin expression in the CD56^{bright} subset of NK cells. Ex vivo culture with IL-15 and IL-21 resulted in modulation of effector functions of NK cells as tested by coculturing them with target MHC-devoid K562 cells. This was manifested by an increase in surface CD107a expression, intracellular content of perforin and IFN- γ , and cytotoxicity in NK cells. CD107a is located within membrane-bound lytic lysosomal vesicles containing proteins such as granzymes and perforin, and up-regulation of CD107a has been shown to occur in synchrony with secretion of perforin [36]. We have shown that NK cells from HIV-infected individuals express more CD107a on their surface following stimulation with K562 than uninfected individuals. This finding is in agreement with Alter *et al* [37] who found that CD107a on NK cells is elevated in viremic HIV-infected individuals and represents a practical marker of NK activity in HIV infection. Furthermore, we found that both IL-15 and IL-21 induced CD107a expression in NK cells mediating cytotoxicity in healthy and HIV-infected individuals. These results suggest that both cytokines not only increase perforin content, but they also induce efficient release of cytotoxic granules and subsequent target cell killing. However, IL-15 induced cytotoxicity was more potent and was accompanied by selective induction of NKG2D, a well-known activating receptor on NK and CD8+ T cells (data not shown).

As was true with CD8+ T cells, IL-21 had a minimal effect in inducing proliferation whereas IL-15 had a potent effect on NK cell proliferation. This observation is consistent with the known proliferative effect that IL-15 has on NK cells [8,31]. Notably, proliferation of NK cells of HIV-infected individuals was higher in comparison to uninfected controls. Moreover, cell division patterns for HIV-infected samples were different from uninfected samples. This finding is reminiscent of common γ -chain cytokine effects on CD8+ T cells [32] of HIV-infected subjects which are more responsive to cytokine-induced proliferation than cells of healthy donors.

Perforin gene activation has been linked to STAT3 and STAT5 activation in NK cells [29] and to STAT5 activation in CTL [30] via upstream enhancers of the perforin gene. Binding of IL-21 to the IL-21 receptor (IL-21R) results in the activation of STAT proteins, which translocate to the nucleus and initiate transcription of IL-21-responsive genes. IL-21-induced expression of IFN- γ in NK cells and T cells has been shown to be dependent upon the activation of STATs [11]. In agreement with Roda *et al* [38] we found that IL-21 induced activation of STAT3 and STAT5, but not STAT4 as reported by Strengell *et al.* [11]. Interestingly, as was true for T cells, IL-15 predominantly activated STAT5 in CD3^{neg}CD56⁺ NK cells from healthy and HIV-infected individuals, while IL-21 activated STAT5 and STAT3 [33]. Furthermore, IL-21-stimulated NK cells from HIV-infected individuals demonstrated higher phosphorylation of STAT3 than uninfected subjects. STAT5 phosphorylation was also higher in HIV-infected individuals, correlating with greater perforin induction by IL-21 in patient cells compared to control cells.

IL-21 is currently proposed as an adjuvant in cancer immunotherapy [39–41]. Our results suggest that the IL-21 needs to be investigated further for its ability to modulate immune responses in HIV-infected individuals.

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Figure 1. In chronically HIV-infected individuals frequency of CD56^{dim} cells is diminished compared to healthy controls

(a) Freshly isolated PBMC from healthy controls (n=6) and HIV-infected individuals (n=9) were analyzed for the frequency of CD56⁺ NK cells within gated CD3negative cells. Mean values for each group are indicated by a horizontal bar; (b) Scatter diagram representing the relationship between the CD4 counts and percentage of CD3^{neg}CD56⁺ cells in HIV-infected individuals; (c) Freshly isolated PBMC from healthy controls (n=6) and HIV infected individuals (n=9) were analyzed for the frequency of CD56^{dim} and CD56^{bright} NK cells. Mean values for each group are indicated by a horizontal bar. Spearman's correlation coefficient was utilized to determine relationships between CD4 counts and percentage of CD3negative CD56⁺ cells. Non-parametric Mann-Whitney *U* test was used to evaluate differences between 2 groups. Differences were considered significant when $p < 0.05$.

Figure 2. Effects of IL-21 and IL-15 on NK cell subset frequency and perforin expression

Freshly isolated PBMC from healthy control (n=4) and HIV-infected individuals (n=5) were cultured in medium or with IL-15 (50ng/ml), IL-21 (50ng/ml) for 5 days. The frequency of CD56^{bright} (a) and CD56^{dim} (b) cells within CD3negCD56+ cells is shown. Significance was determined by 1-way ANOVA with Tukey's Multiple Comparison test. (c) Representative dot plots of CD56 expression and matched isotype control (mouse IgG2b) within gated CD3neg cells from one HIV-infected individual are shown. Gates were set for CD56^{dim} population when the intensity of CD56 expression was 10–100 times that of the matched isotype control and for CD56^{bright} populations when the intensity of CD56 expression was 100–1000 times that of the matched isotype control. The percentage of perforin positive cells within (d) CD56^{bright} and (e) CD56^{dim} gates, with mean values for each group are shown. (f) Perforin mRNA expression following 5 hour stimulation with medium, IL-21 or IL-15 in purified CD56 + NK cells from healthy and HIV-infected individual as determined by quantitative RT-PCR.

Figure 3. Differential effects of IL-15 and IL-21 on frequency and proliferation of NK cells. IL-15, but not IL-21 induced proliferation of NK cells in both healthy and HIV-infected individuals
Freshly isolated PBMC from healthy control and HIV infected individuals were stained with CFSE and cultured in medium with IL-15 (50ng/ml), IL-21 (50ng/ml), or without cytokine for 5 days. Average number of cell divisions that the responding cells underwent (Division index) was calculated after gating on CD3negCD56+ cells and presented as mean $\pm$ SEM from healthy (n=5) controls and HIV-infected (n=10) individuals. Significance was determined by 1-way ANOVA with Tukey's Multiple Comparison test.

Figure 4. Rapid induction of degranulation marker, CD107a, perforin and IFN-γ in IL-15 and IL-21 treated NK cells

Freshly isolated PBMC from healthy controls were cultured in medium or with IL-15 (50ng/ml) or IL-21 (50ng/ml) for 24h followed by 6h stimulation with MHC-devoid NK-sensitive K562 cells (a) Representative dot plot of perforin MFI in gated CD3negCD56+ cells after 24h culture with IL-21 or IL-15. (b-d) Expression of perforin, CD107a, and IFN-γ in gated CD3negCD56+ cells after 6h co-culture of medium- or cytokine (IL-21/IL-15) pre-cultured PBMC with K562 cells. Paired t-test was used to evaluate differences between 2 groups. Differences were considered significant when $p < 0.05$. e) Freshly isolated human NK cells (95% purity with <1% each of CD3+, CD20+ and HLA-DR+ cells) from HIV-infected individuals were cultured in medium or with IL-21 (50ng/ml) or IL-15 (50ng/ml) for 24 h and examined for perforin MFI and (f) for cytotoxicity, following coculture of 24 hour cytokine treated NK cells with NK sensitive targets, K562 cells labeled with PKH-26 at the ratios 6:1, 12.5:1, 25:1 for 4 h. Target cells were gated by side scatter and fluorescence (FL2). 7-AAD uptake was determined within gated, target cells. The percent lysis was determined as $[(\text{percent 7-AAD staining sample} - \text{percent 7-AAD staining of negative control}) / (100 - \text{percent 7-AAD staining of negative control})] \times 100$. Significance was determined by 1-way ANOVA with Tukey's Multiple Comparison test.

Figure 5. IL-2 and IL-15 predominantly activate STAT5 in both healthy and HIV-infected CD3negCD56+ NK cells, while IL-21 activates STAT5 and STAT3

PBMCs isolated from (a) healthy and (b) HIV-infected individuals were cultured in medium or stimulated with IL-2 (1000IU/ml), IL-15 (50ng/ml) or IL-21 (50ng/ml) for 15 min and expression of phosphorylated epitopes for STAT3, 4 and 5 was determined on fixed and permeabilized cells. STAT expression depicted as mean $\pm$ SEM from healthy (n=5) controls and HIV-infected (n=3) individuals. Representative histograms of STAT3, STAT4 and STAT5 expression following medium, IL-15 or IL-21 treatment in gated CD3negCD56+ cells from (c) one healthy and (d) one HIV-infected individual are shown.