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Shared Alterations in NK Cell Frequency, Phenotype, and Function in Chronic Human Immunodeficiency Virus and Hepatitis C Virus Infections

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Human immunodeficiency virus (HIV) and hepatitis C virus (HCV) cause clinically important persistent infections. The effects of virus persistence on innate immunity, including NK cell responses, and the underlying mechanisms are not fully understood. We examined the frequency, phenotype, and function of peripheral blood $CD3^-$ $CD56^+$ NK subsets in HIV^+ and HCV^+ patients and identified significantly reduced numbers of total NK cells and a striking shift in NK subsets, with a marked decrease in the $CD56^{dim}$ cell fraction compared to $CD56^{bright}$ cells, in both infections. This shift influenced the phenotype and functional capacity (gamma interferon production, killing) of the total NK pool. In addition, abnormalities in the functional capacity of the $CD56^{dim}$ NK subset were observed in HIV^+ patients. The shared NK alterations were found to be associated with a significant reduction in serum levels of the innate cytokine interleukin 15 (IL-15). In vitro stimulation with IL-15 rescued NK cells of HIV^+ and HCV^+ patients from apoptosis and enhanced proliferation and functional activity. We hypothesize that the reduced levels of IL-15 present in the serum during HIV and HCV infections might impact NK cell homeostasis, contributing to the common alterations of the NK pool observed in these unrelated infections.

Persistent viral infections constitute a major health burden worldwide. Human immunodeficiency virus type 1 (HIV-1) causes a persistent infection in humans that is ultimately associated with the development of AIDS. Around 40 million people are infected with this virus worldwide, with approximately 3 million deaths occurring per year due to AIDS (73). Hepatitis C virus (HCV) affects more than 170 million people and causes an estimated 460,000 deaths per year (24). A small proportion of patients are able to clear this infection, but most become carriers and may eventually develop chronic liver diseases, including chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma. There is a pressing need to develop both prophylactic vaccines and effective therapeutic strategies for these infections. To facilitate this, a better understanding of mechanisms involved in the initiation and maintenance of persistence and of alterations in host immune function induced in the context of viral persistence is urgently required.

The importance of adaptive immune responses in combating virus infections is well known. Much work has focused on the roles played by humoral and T-cell responses in control of HIV and HCV infections, and a variety of immune evasion strategies have been identified (42, 44, 57, 66). By contrast, innate immune responses remain relatively poorly characterized (11, 47, 50, 70). It is increasingly recognized that optimal control of persistent infections requires interaction between multiple arms of the immune response (48). Innate immune responses play an important role in early effector functions and in the activation and maintenance of adaptive immune responses (13, 28) and also participate in containment of persistent viral infections (34). Defects in innate responses may thus impact directly or indirectly on the control of viral replication and are, therefore, important to characterize.

NK cells contribute to innate host defense by cytolysis and production of cytokines such as gamma interferon (IFN-γ). $CD56^+$ $CD3^-$ NK cells constitute ~13% of human peripheral blood mononuclear cells. Two subpopulations of NK cells expressing different levels of CD56 have been described. Most NK cells (90%) are $CD56^{dim}$. These cells are the main mediators of NK cytotoxicity, contain high levels of perforin, and express CD16 (FcγRIII) (21). The $CD56^{dim}$ subset expresses a unique repertoire of natural killer receptors distinct from those found on $CD56^{bright}$ NK cells (21). A minority of NK cells (10%) are $CD56^{bright}$. They exert only weak cytotoxic activity but act as an important source of immunoregulatory cytokines (21). Interleukin 12 (IL-12) and IL-18 have been shown to be strong inducers of NK cell production of IFN-γ (19).

NK cell development and maturation are strongly influenced by the innate cytokine IL-15 (49, 51, 77, 79). IL-15 is produced by multiple cell types, including fibroblasts, epithelial cells, and

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stromal cells. It is also produced by mature dendritic cells (DCs) in response to IFN-α/β and danger signals, such as double-stranded RNA or lipopolysaccharide, and promotes DC activation in an autocrine manner (55). IL-15 is involved in the activation and homeostatic maintenance of cells of both the innate and adaptive immune systems. It plays an important role in CD8 T-cell homeostasis by promoting survival or proliferation of naive and memory phenotype CD8 T cells (5, 10, 36, 76, 80) and influences NK survival (27, 58). The defects observed in NK and memory T-cell lineages in IL-15-deficient mice attest to the obligatory role played by this cytokine in their generation and survival (46, 68, 78).

In this study, we addressed the frequency, phenotype, and functional capacity of $CD56^+$ $CD3^-$ NK cells and the subsets, $CD56^{dim}$ and $CD56^{bright}$, in HIV^+ and HCV^+ patients. In both infections we found a reduced frequency of NK cells and an altered subset distribution, which affected the functional capacity of the total NK pool. Given the involvement of IL-15 in NK cell homeostasis, we then addressed whether there were changes in serum levels of IL-15 and observed a significant reduction in serum IL-15 levels of both HIV^+ and HCV^+ patients. We show that IL-15 rescued NK cells of HIV^+ and HCV^+ patients from apoptosis and promoted proliferation and NK cell function. Reduced serum levels of IL-15 may constitute a common mechanism underlying the NK cell abnormalities in these two unrelated chronic virus infections.

MATERIALS AND METHODS

Patients. HIV^+ and HCV^+ patients were recruited from the Mortimer Market Centre, London, United Kingdom, the Institute of Hepatology, University College London, United Kingdom, and the Gastroenterology clinic at the John Radcliffe Hospital, Oxford, United Kingdom. Ethical approval was obtained from local institutional review boards, and blood was drawn following written informed consent. HCV^+ patients were consistently HCV RNA^+ and had median alanine aminotransferase levels of 80 IU (range, 17 to 187); none of these individuals were currently or recently (within the last 6 months) on IFN-α treatment. HIV^+ patients were not receiving antiretroviral therapy and were all clinically asymptomatic, with median CD4 counts of 480 cells/mm^3 (range, 260 to 1,130) and median viral loads of 28,800 RNA copies/ml (range, 1,900 to 275,600). None of the patients included in this study were coinfected with HIV and HCV. The controls were healthy age-matched HIV^-, HCV^- individuals.

Phenotypic and functional studies. For direct phenotypic studies, CD3/56 staining was combined with CD57-fluorescent isothiocyanate (FITC) (BD/Pharmingen, Oxford, United Kingdom). Intracellular perforin and Ki67 staining was performed with CD3/56 and perforin-FITC or Ki67-FITC (BD/Pharmingen) or a negative control antibody. Annexin V staining was done using Annexin V-FITC (BD/Pharmingen). To determine the capacity of NK cells to produce IFN-γ, frozen peripheral blood mononuclear cells (PBMC) (HIV^+ patients) or whole blood (HCV^+ patients) were incubated overnight with IL-12 (R&D Systems, Minneapolis, Minn.) and IL-18 (MBL, Nagoya, Japan) (each at 1.25 μg/ml). GolgiStop (BD/Pharmingen) was added for 3 h and cells permeabilized before staining with IFN-γ–FITC (BD/Pharmingen), CD56-PC5 (Coulter/Immunotech, Marseille, France), and CD3-phycoerythrin (BD/Pharmingen) or an immunoglobulin G control antibody (BD/Pharmingen). IFN-γ production was compared to that of matched frozen or fresh cells from control individuals. All samples were acquired with a FACSCalibur (Becton Dickinson, Oxford, United Kingdom) and analyzed using CellQuest software (Becton Dickinson).

Preparation of LIL. Liver-infiltrating lymphocytes (LIL) were isolated from a surplus part of a liver biopsy specimen, which was obtained as part of a routine diagnostic procedure for HCV^+ patients. The liver tissue was dissociated and the cell suspension passed through a nylon mesh (Becton Dickinson Labware, Oxford, United Kingdom) and centrifuged to pellet the hepatocytes. The supernatant was centrifuged to pellet the LIL. The yield of LIL from each specimen was 3×10^5 to 5×10^5, with viability of >95%. Both we and others have previously shown that LIL isolated in this way are a distinct population of cells, with a higher frequency of virus-specific T cells than PBMC (1, 2, 38, 39). PBMC were obtained from each patient at the time of liver biopsy. Paired samples of LIL and PBMC from HCV^+ patients were cryopreserved and stored in liquid nitrogen.

Chromium release assay. NK cell cytolytic activity was assessed in a chromium release assay. K562 target cells (10^6) were labeled with 100 μCi of $Na^{51}CrO_4$ (Amersham Pharmacia Biotech Ltd., Little Chalfont, United Kingdom) for 1 h at 37°C. Effector cells were frozen PBMC which had been incubated overnight with or without IL-15 (1 ng/ml) (R&D Systems, Minneapolis, Minn.) and used as effectors at different effector/target (E:T) ratios. The supernatant was harvested after a 5-h incubation at 37°C. The percent lysis was calculated as (mean test counts − mean spontaneous counts)/(mean maximum counts − mean spontaneous counts) × 100.

CD107 (lysosome-associated membrane protein 1) expression assay. CD107a expression was used to measure NK cell degranulation, as recently described (4, 12). PBMC were stimulated with or without IL-15 (1 ng/ml) overnight and then incubated with K562 target cells at E:T ratio of 5:1. CD107a-FITC antibody (BD Biosciences) was added directly to the cultures. Following 1 h of stimulation with K562 cells, GolgiStop was added for another 5 h at 37°C, and cells were then stained with CD56/3 antibody prior to fluorescence-activated cell sorting analysis.

IL-15 assay. Serum IL-15 concentrations were determined using the Quanti-Glo chemiluminescence immunoassay (R&D Systems, Minneapolis, Minn.).

IL-15 studies. To study the effect of IL-15 on NK cells, PBMC or sorted NK cells (the $CD56^+$ $CD3^-$ fraction of PBMC, sorted using a MoFlo cytometer (DakoCytomation, Fort Collins, Colorado) were cultured overnight or for up to 1 week in graded concentrations of IL-15 before phenotypic and functional analysis.

Statistical analysis. Analysis was performed using an unpaired *t* test, using Prism software (version 3.03; GraphPad Software Inc., San Diego, Calif.).

RESULTS

Reduced frequencies of NK cells in HIV and HCV infection and shift between subsets. To gain insight into NK cell abnormalities induced in the context of persistent viral infections, we initially addressed the number and subset composition of NK cells in the peripheral blood of HIV-1^+ or HCV^+ patients. The clinical profiles of the patient groups studied are outlined in the methods section. The frequency of NK cells ($CD56^+$ $CD3^-$) in the PBMC pool was analyzed in 18 HIV^+ and 36 HCV^+ patients and 30 healthy age-matched individuals. The total percentage of NK cells ($CD56^+$ $CD3^-$) in the PBMC population was significantly decreased in both HIV^+ and HCV^+ individuals compared to controls (controls, 13.95% ± 1.4%; HIV^+, 5.7% ± 0.99%; $P = 0.0002$; HCV^+, 9.3% ± 1.16%; $P = 0.0132$) (Fig. 1a). Absolute numbers were similarly reduced (controls, 272.1 ± 27.5 NK cells per microliter of blood; HIV, 90.4 ± 21.4 [$P = 0.0008$]; HCV, 185.8 ± 23.22 [$P = 0.019$]; data not shown). Within the NK pool, the proportion of $CD56^{bright}$ NK cells was significantly increased in HIV^+ and HCV^+ patients (controls, 5.7% ± 0.9%; HIV^+, 10.0% ± 1.45%; $P = 0.0115$; HCV^+, 13.2% ± 1.4%; $P = 0.0001$) (Fig. 1b). As a result, the $CD56^{bright}/CD56^{dim}$ NK cell ratio was significantly altered for HIV^+ and HCV^+ patients relative to that for controls (controls, 0.06 ± 0.02; HIV^+, 0.12± 0.02; $P = 0.04$; HCV^+, 0.15 ± 0.02; $P = 0.0034$). This analysis thus identified that a significant decrease in NK cells and a significant shift of NK subsets had occurred during HIV and HCV infection.

Decreased number of peripheral NK cells in HCV^+ patients is not due to accumulation in liver. To address whether the altered subset composition of NK cells in the blood of HCV^+ patients was due to selective accumulation of the $CD56^{dim}$ NK cells in the liver, five pairs of LIL and matched PBMC from HCV^+ patients were stained for CD56/CD3. As shown in Fig.

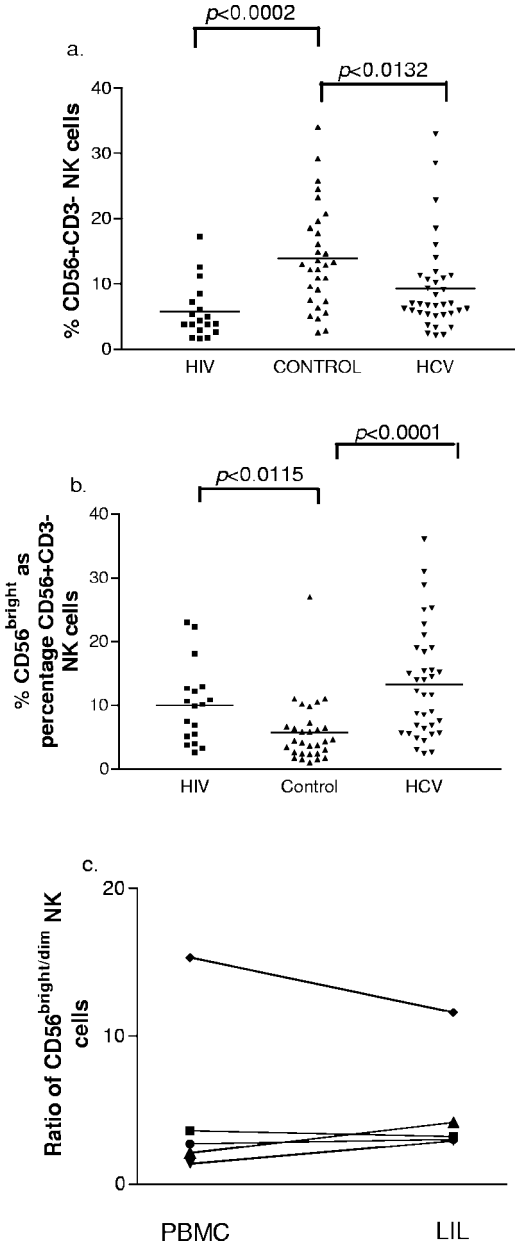

FIG. 1. Reduced frequency of $CD56^+$ $CD3^-$ NK cells in HIV^+ and HCV^+ patients and shift between NK subsets. (a) Reduced frequency of $CD56^+$ $CD3^-$ NK cells in HIV^+ and HCV^+ patients. Each symbol represents the percentage of $CD56^+$ $CD3^-$ NK cells within the PBMC population in a single individual. The mean values for each group (18 HIV^+, 36 HCV^+, and 30 control individuals) are indicated by a horizontal bar, and the significance of the differences between groups is shown. (b) Shift between NK cell subsets for HIV^+ and HCV^+ patients. Each symbol represents the proportion of $CD56^{bright}$ NK cells in a single subject, expressed as a percentage of their $CD56^+$ $CD3^-$ NK cells. The means of each group (18 HIV^+, 36 HCV^+, and 30 control individuals) are indicated by a horizontal bar, and the significance of the difference between the groups is shown. (c) Ratio of $CD56^{bright}$/$CD56^{dim}$ NK cells in the peripheral blood and a matched LIL population of five HCV^+ patients.

1c, we found similar ratios of NK subsets in the liver and blood. Hence, the reduced proportion of $CD56^{dim}$ NK cells in the blood of HCV^+ individuals was not due to their preferential accumulation in the liver.

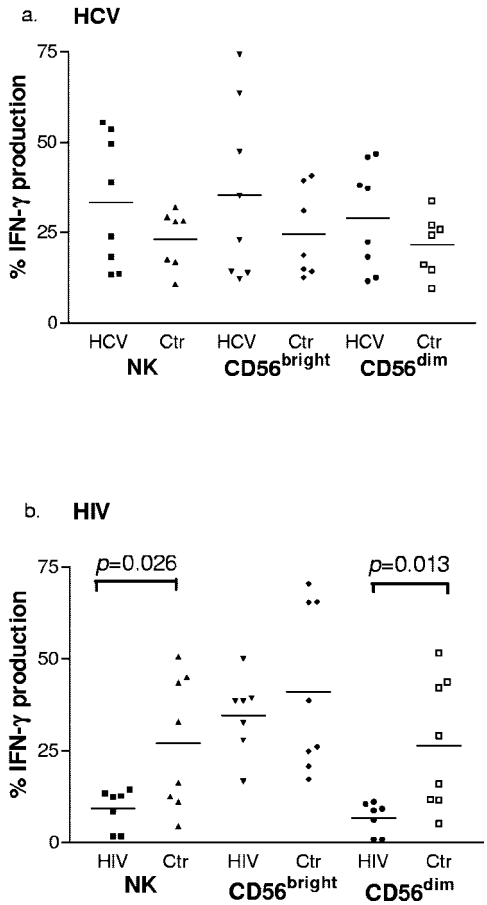

FIG. 2. Reduced IFN-γ production by NK cells from HIV^+ patients but not HCV^+ patients following stimulation with IL-12 IL-18. Comparison of IFN-γ production by $CD56^+$ $CD3^-$ NK cells and the $CD56^{bright}$ or $CD56^{dim}$ subset from control individuals and (a) HCV^+ and (b) HIV^+ patients. Each symbol represents the percentage of IFN-γ-producing cells within the indicated phenotypic subset in a single individual. The mean values for each group (eight HCV^+, seven HIV^+, and seven to eight control individuals) are indicated by a horizontal bar, and where there are significant differences between control and patient groups, these are shown.

Reduced IFN-γ production by NK cells from HIV^+ patients but not HCV^+ patients following stimulation with IL-12 plus IL-18. To gain insight into the functional capacity of NK cells in HIV^+ and HCV^+ patients, we assessed their ability to produce IFN-γ, a cytokine that promotes the induction of adaptive immune responses (14, 67) and plays a key role in control of hepatotropic viruses (41). We compared IFN-γ production by $CD56^{bright}$ and $CD56^{dim}$ NK cells from HIV^+, HCV^+, and control individuals following stimulation with IL-12 plus IL-18 (Fig. 2). This stimulus elicited IFN-γ production from around 25% of the NK cells ($CD56^+$ $CD3^-$) of healthy control individuals. IFN-γ was produced by both NK subsets, although the numerical superiority of the $CD56^{dim}$ NK cells meant that they comprised the majority of the IFN-γ-producing cells in the total NK pool ($CD56^{dim}$ $IFN\gamma^+$, 92%).

NK cells from HCV^+ patients were also readily responsive to IL-12 plus IL-18, with a slightly but not significantly higher percentage of NK cells producing IFN-γ (33.4% ± 6.4%) than

in normal individuals (23.3% ± 3.0%) (Fig. 2a). However, whereas less than 10% of total IFN-γ producing NK cells in control individuals were derived from the CD56^{bright} pool, in HCV$^+$ patients the CD56^{bright} pool made a greater contribution to the overall IFN-γ production (CD56^{bright} IFN-γ$^+$, 15.4%).

A similar phenomenon was observed for HIV$^+$ patients, where 36.3% of total IFN-γ-producing NK cells were derived from the CD56^{bright} subset (Fig. 2b). Notably, however, there was also a reduction in the total number of NK cells induced to produce IFN-γ upon stimulation with IL-12 plus IL-18 compared to results for control individuals (controls, 27.11% ± 6.4%; HIV, 9.3% ± 2.0%; $P = 0.026$). The CD56^{dim} subset from HIV$^+$ patients produced significantly less IFN-γ than cells from control individuals (controls, 26.43% ± 6.2%; HIV$^+$, 6.76% ± 1.6%; $P = 0.013$). By contrast, the proportion of CD56^{bright} NK cells producing IFN-γ was not significantly reduced (controls, 41.1% ± 4%; HIV, 34.8% ± 7.9%). There was thus a selective defect in the IFN-γ-producing capacity of CD56^{dim} NK cells in HIV$^+$ patients.

Analysis of perforin expression by NK subsets from control, HIV$^+$, and HCV$^+$ individuals. We next analyzed perforin expression in NK cells from HIV$^+$ and HCV$^+$ patients. Figure 3a shows perforin staining in representative control, HIV$^+$, and HCV$^+$ individuals. CD56^{dim} and CD56^{bright} subsets differ in their perforin content. CD56^{bright} cells are low in perforin, while CD56^{dim} cells express higher levels of perforin. This staining pattern was preserved in NK cells from HCV$^+$ and HIV$^+$ patients. However, in HIV$^+$ and HCV$^+$ individuals, who had an altered subset distribution, the percentage of CD56^{dim} perforin^{high} NK cells was diminished (Fig. 3b), resulting in a lower level of perforin expression in the total NK pool. In line with this, the cytotoxic capacity of the overall NK pool was decreased in both HCV$^+$ patients and HIV$^+$ patients (Fig. 3c).

Greater replicative senescence of the CD56^{dim} subset, which is underrepresented in HIV$^+$ and HCV$^+$ individuals. The impact of the shift of NK cell subsets in HIV$^+$ and HCV$^+$ patients on the properties of the overall NK cell pool was also apparent when we examined expression of CD57, a marker associated with NK cell replicative senescence (16). CD57 expression was exclusively observed on the CD56^{dim} subset in control, HIV$^+$, and HCV$^+$ individuals (Fig. 4a). Although the proportion of CD56^{dim} NK cells expressing CD57 was not significantly different for HIV$^+$, HCV$^+$, and control individuals, the percentage of all NK cells expressing CD57 was reduced in HIV$^+$ and HCV$^+$ patients with an altered subset ratio (data not shown). The staining pattern for CD57 was in concordance with ex vivo staining for Annexin V, a marker for apoptosis. As illustrated by the example in Fig. 4b, CD56^{bright} cells (in this case derived from a control individual) are negative for Annexin V and CD57, whereas the CD56^{dim} population is more prone to spontaneous apoptosis ex vivo (9). After 1 week of culture, it was also predominantly the CD56^{dim} population of NK cells from control, HIV$^+$, and HCV$^+$ individuals that underwent apoptosis (Fig. 4c). Hence, the more senescent CD56^{dim} NK subset has a higher susceptibility to apoptosis than the CD56^{bright} subset.

In summary, we saw decreased frequencies of NK cells in HCV$^+$ and HIV$^+$ patients and an altered subset distribution,

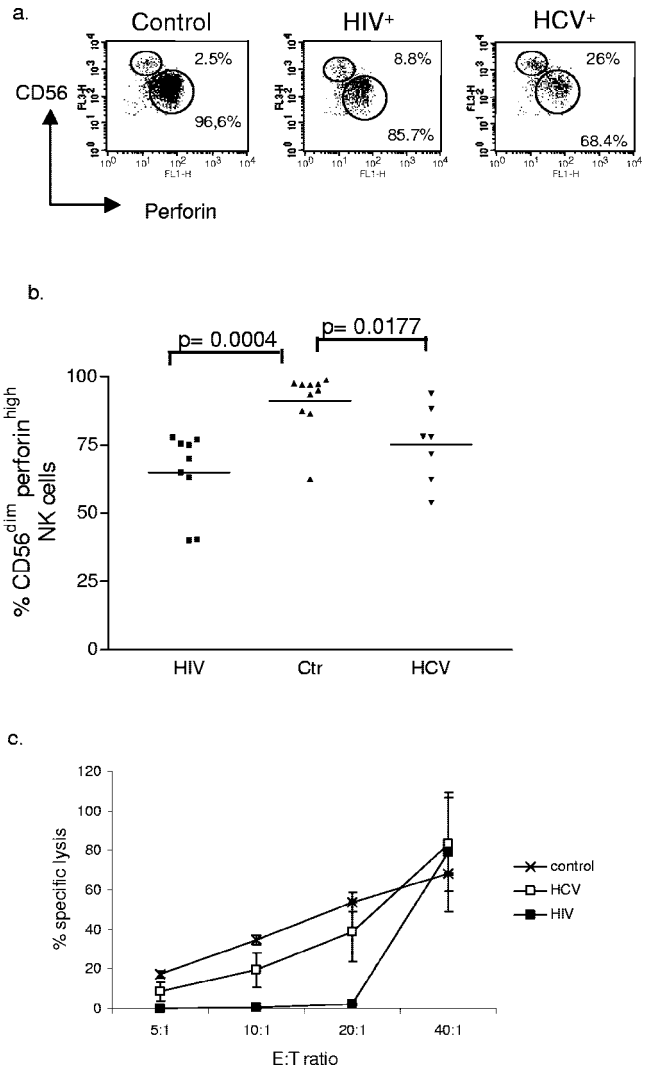

FIG. 3. Perforin content and cytolytic capacity of NK cells from HIV$^+$ and HCV$^+$ patients. (a) Dot plots showing perforin staining of CD56^{bright} and CD56^{dim} NK subpopulations in a representative control individual, HIV$^+$ patient, and HCV$^+$ patient. Gates were set for the CD56$^+$ CD3$^-$ population, and staining for CD56 and perforin is shown. Percentages indicate the numbers of cells in the CD56^{bright} perforin^{low} and CD56^{dim} perforin^{high} pools. (b) Reduced proportion of CD56^{dim} perforin^{high} NK cells in HIV$^+$ and HCV$^+$ patients. Each symbol represents the proportion of CD56^{dim} perforin^{high} NK cells in a single individual, expressed as a percentage of their CD56$^+$ CD3$^-$ NK cells. The means of each group (9 HIV$^+$, 7 HCV$^+$, and 10 control individuals) are indicated by a horizontal bar, and the significance of the differences between groups is shown. (c) Lysis of the NK target cell line K562 by PBMC from HIV$^+$, HCV$^+$, and control individuals. The percentage of specific lysis of K562 target cells by PBMC from two HIV$^+$, HCV$^+$, and control individuals was determined at different E:T ratios. The values plotted are the mean percentages of specific lysis mediated by the effector cells from the individuals of each group.

which affected the phenotype and the functional capacity of the overall NK pool. In both infections we also found an accumulation of "early" NK forms (perforin^{low} CD57$^-$) and a decrease in "mature" forms (perforin^{high} CD57$^+$), as has been reported for CD8$^+$ T cells in HIV$^+$ and HCV$^+$ patients (7, 40).

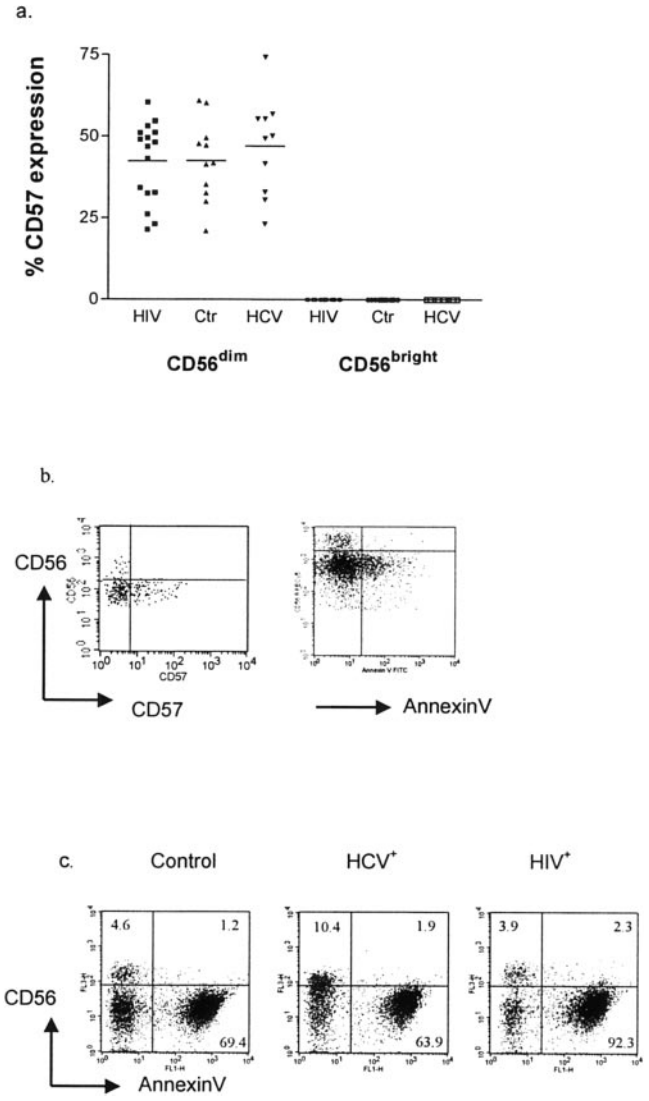

FIG. 4. Replicative senescence and apoptotic susceptibility of $CD56^{dim}$ NK cells in healthy controls and HIV^+ and HCV^+ patients. (a) Expression of CD57 by $CD56^+$ $CD3^-$ NK cells from HIV^+, HCV^+, and control subjects. Each symbol represents the percentage of $CD57^+$ $CD56^{dim}$ or $CD56^{bright}$ NK cells in a single individual. The mean values for each group (16 HIV^+, 10 HCV^+, and 11 control individuals) are indicated by a horizontal bar. (b) Dot plots showing CD57 expression by $CD56^{bright}$ and $CD56^{dim}$ NK cell subsets in a representative healthy control individual and the relative ex vivo apoptotic susceptibility of these NK subsets (assessed by Annexin V staining). (c) Dot plots showing Annexin V staining of NK cells from representative HIV^+, HCV^+, and control individuals after 1 week of culture.

Reduced serum levels of IL-15 in patients with HCV and HIV infection. The observation of common alterations in NK numbers, subset composition, and maturation state in both HIV and HCV infections raised the question of whether the underlying mechanisms may be similar in the two infections. We focused our attention on the innate cytokine IL-15, which is known to play an important role in lymphocyte homeostasis. We thus went on to address whether serum IL-15 levels were altered during HIV and HCV infection. A sensitive luciferase-based enzyme-linked immunosorbent assay was used to measure serum levels of IL-15. We found significantly reduced levels of IL-15 in the sera of HCV^+ and HIV^+ patients (Fig. 5a) compared to those of healthy controls (controls, 3.3 pg/ml $\pm$ 0.31; $n = 8$; HIV^+, 1.88 pg/ml $\pm$ 0.3; $P = 0.0056$; $n = 10$; HCV^+, 2.4 pg/ml $\pm$ 0.23; $P = 0.036$; $n = 11$).

IL-15 promotes proliferation and survival of NK cells from control, HCV^+, and HIV^+ individuals. We next addressed the effects of IL-15 on the proliferation and survival of NK cells to gain insight into its potential role in the control of the size of the peripheral NK pool. To test the ability of IL-15 to stimulate proliferation of NK cells, we cultured fresh PBMC from control, HCV^+, and HIV^+ individuals with graded concentrations of IL-15 for 48 h. Staining for Ki67 was used to assess the proliferative response of NK cells. Serum-level concentrations of IL-15 did not stimulate overt proliferation of NK cells, but 1 ng/ml of IL-15 was sufficient. NK cells from HIV^+ and HCV^+ patients were equally responsive to IL-15 as cells from control individuals (Fig. 5b). To test the effect of IL-15 on NK cell survival, we isolated NK cells from a control individual by sorting and cultured them in medium containing picomolar concentrations of IL-15 (3 pg/ml; 30 pg/ml; 300 pg/ml). After 1 week of culture, the cells were stained with Annexin V to identify apoptotic cells. The proportion of Annexin V^- NK cells increased as the cells were cultured with increasing concentrations of IL-15, indicating a direct effect of this cytokine on NK cell survival (Fig. 5c).

We then addressed the ex vivo survival of NK cells from HIV^+ and HCV^+ patients in the presence or absence of IL-15. Cells from both patient groups remained responsive to IL-15, with a higher percentage of Annexin V^- cells being observed after culture with IL-15 than in its absence (Fig. 5d). Hence, IL-15 can act directly on NK cells and promotes their survival, even in the context of HCV and HIV infection.

Further experiments dissected the effects of IL-15 on the $CD56^{dim}$ and $CD56^{bright}$ subsets of NK cells from healthy individuals. IL-15 was found to stimulate the proliferation of both $CD56^{dim}$ and $CD56^{bright}$ NK cells in a dose-dependent fashion; however, the proportion of $CD56^{bright}$ cells induced to proliferate (as measured by Ki67 staining) was much higher than that of $CD56^{dim}$ cells (Fig. 5e). $CD56^{bright}$ NK cells thus proliferate more readily than $CD56^{dim}$ NK cells in response to IL-15. Importantly, the effects of IL-15 on the survival of the two NK cell subsets also differed. When cultured in vitro, $CD56^{bright}$ NK cells survived relatively well even in the absence of IL-15, and only a slight increase in the percentage of Annexin V^- cells was observed in the presence of IL-15. By contrast, IL-15 had a much more striking effect on the survival of the $CD56^{dim}$ NK cell subset, with the percentage of live (Annexin V^-) cells recovered after 1 week of culture in the presence of IL-15 being almost double that recovered in the absence of this cytokine (59% versus 34%) (Fig. 5f).

IL-15 increases IFN-γ production by NK cells from HIV^+ and HCV^+ patients and normal controls. Since we had shown that NK cells from patients responded normally to IL-15 with respect to proliferation and survival, we wanted to see whether they also responded normally to IL-15 with respect to increase in function. Fresh PBMC from HIV^+, HCV^+, and control individuals were stimulated overnight with IL-15 either alone or in combination with IL-12 plus IL-18. IL-15 alone (3 pg/ml, 1 ng/ml, and 10 ng/ml) did not lead to IFN-γ production by NK

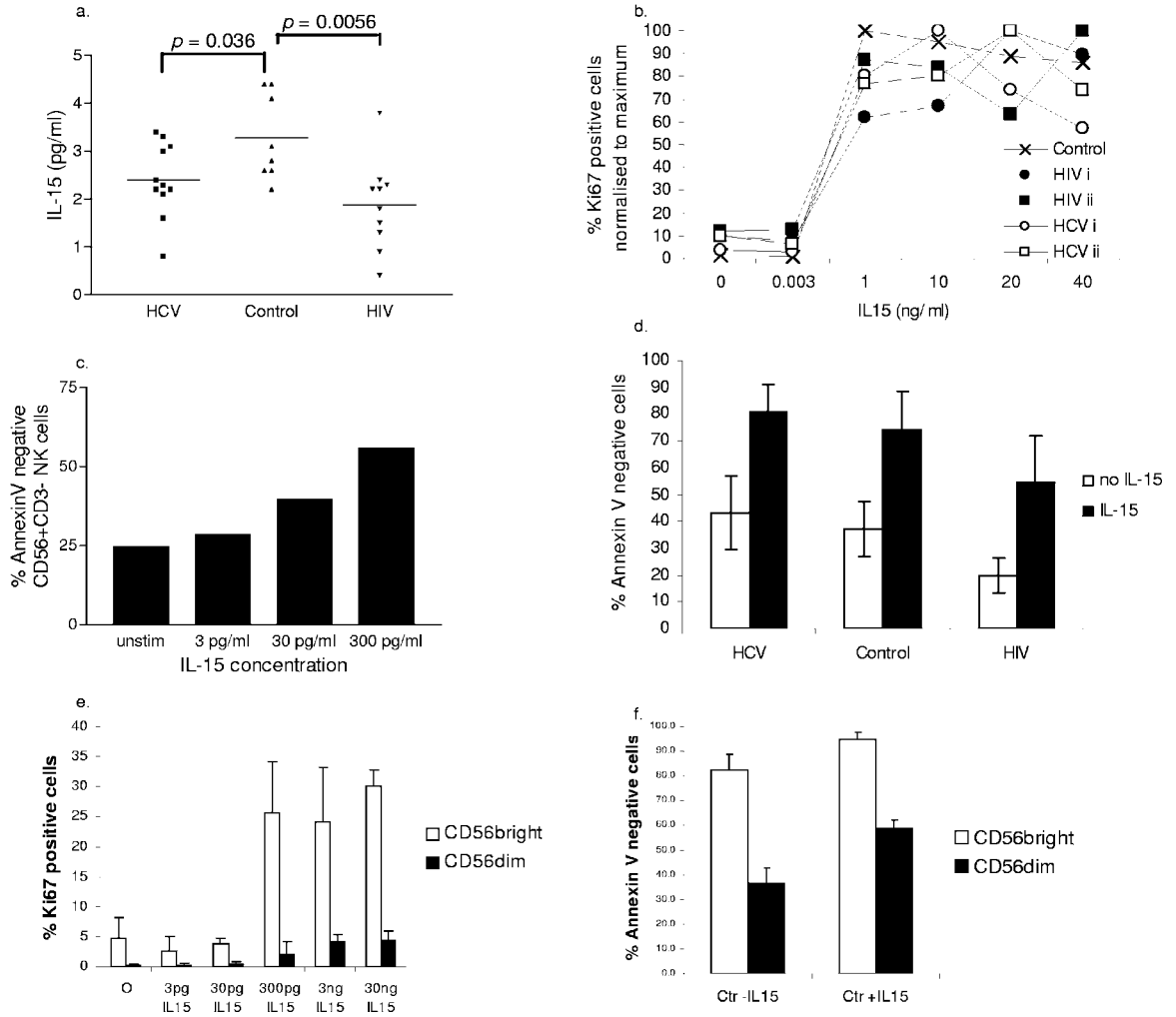

FIG. 5. Serum IL-15 levels for control, HIV$^+$, and HCV$^+$ individuals and analysis of the effects of IL-15 on NK cell proliferation and survival. (a) Serum levels of IL-15 in HIV$^+$ and HCV$^+$ patients. Each symbol represents the serum level (pg/ml) of IL-15 in a single HIV$^+$, HCV$^+$, or control individual, as determined by Quantiglow enzyme-linked immunosorbent assay. The mean values for each group (10 HIV$^+$, 11 HCV$^+$, and 8 control individuals) are shown by a horizontal bar. The statistical significance of differences between each patient group and the control group is indicated. (b) IL-15 promotes proliferation of NK cells from HIV$^+$, HCV$^+$, and control individuals. The graph shows Ki67 expression (normalized to maximum) upon culture of NK cells from HIV$^+$ and HCV$^+$ patients and a control individual with graded amounts of IL-15 (0 pg/ml, 3 pg/ml, 1 ng/ml, 10 ng/ml, and 40 ng/ml). (c) IL-15 promotes survival of NK cells from a control individual. The graph shows the percentage of Annexin V$^-$ NK cells remaining after in vitro culture of NK cells isolated from a control individual for 1 week with graded concentrations of IL-15. (d) Ex vivo survival of sorted NK cells from HIV$^+$, HCV$^+$, and control individuals in the absence or presence of IL-15. The graph shows the mean percentage of Annexin V$^-$ NK cells remaining after in vitro culture of NK cells isolated from control individuals and HIV$^+$ and HCV$^+$ patients (two to four individuals/group) for 1 week with IL-15 (300 pg/ml). (e) Proliferation of CD56^{bright} and CD56^{dim} NK cells from healthy individuals upon stimulation with different amounts of IL-15. The graph shows the mean (of results from two healthy individuals) percent Ki67-positive NK cells after stimulation of PBMC for 48 h with graded amounts of IL-15 (3 pg, 30 pg, 300 pg, 3 ng, and 30 ng). (f) Ex vivo survival of CD56^{bright} and CD56^{dim} NK cells within sorted NK populations from control individuals in the absence and presence of IL-15. The graph shows the mean percentages of Annexin V$^-$ CD56^{bright} and CD56^{dim} NK cells after 1 week of culture of cells from four healthy individuals in the presence or absence of IL-15 (300 pg/ml).

cells; however, when given together with IL-12 plus IL-18, IFN-γ production could be increased. As shown in Fig. 6a and b, the IFN-γ response of NK cells from HIV$^+$ and HCV$^+$ patients was enhanced by IL-15 to an extent similar to that of control cells.

IL-15 increases target cell-stimulated degranulation of NK cells from normal controls and HCV$^+$ and HIV$^+$ patients. Finally, we tested whether IL-15 could increase the cytolytic capacity of NK cells. Frozen PBMC were cultured with or without 1 ng/ml of IL-15 overnight, and their ability to degranulate following exposure to the major histocompatibility complex class I low K562 target cell line was assessed by the analysis of expression of CD107a, a marker expressed on the cell surface following activation-induced granule release. As recently described for both NK cells (3) and CD8$^+$ T cells (12), CD107a expression is a good correlate of cytolytic granule release by lytic effector populations. Baseline expression of CD107a on NK cells from control, HIV$^+$, and HCV$^+$ individ-

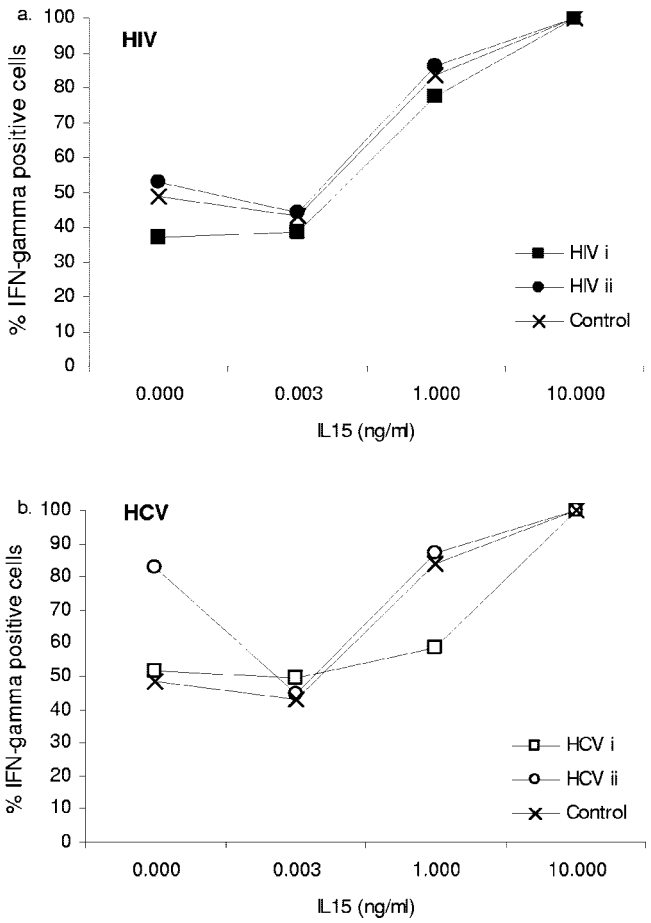

FIG. 6. IL-15 increases IFN-γ production by NK cells from control and (a) HIV^+ or (b) HCV^+ individuals. The graphs show IFN-γ production (percent positive cells normalized to maximum) by NK cells from control, HIV^+, and HCV^+ individuals upon stimulation with IL-12 plus IL-18 and graded amounts of IL-15 (0 pg/ml, 3 pg/ml, 1 ng/ml, and 10 ng/ml).

uals ranged from 0 to 2.5% (mean of 1.2%). The percentage of $CD56^{dim}$ NK cells expressing CD107a increased following incubation of PBMC with K562 cells (Fig. 7); notably, the percentage of NK cells within the $CD56^{dim}$ pool induced to express CD107a following exposure to target cells did not differ for control, HIV^+, or HCV^+ individuals (control, 15.5% ± 3.7%; HIV^+, 17.06% ± 2.9%; HCV^+, 14.73% ± 1.8%), indicating that at least this aspect of NK cell function was not impaired in the patient groups. Overnight incubation with IL-15 dramatically enhanced target cell-stimulated degranulation of NK cells from both control and patient groups (control, 45.3% ± 5.6%; HIV^+, 44.61% ± 4.7%; HCV^+, 32.1% ± 3.6%). Hence, NK cells from HIV^+ and HCV^+ patients stayed responsive to the effects of IL-15 on their functional capacity in addition to proliferation and survival.

DISCUSSION

Analysis of the frequency and function of $CD56^+$ $CD3^-$ NK cells for HIV^+ and HCV^+ patients revealed a significant reduction in NK cell frequency and a quantitative imbalance of the $CD56^{bright}$ and $CD56^{dim}$ subsets within the total NK population. Since the two NK cell subsets exhibit different functional profiles, this perturbation resulted in alterations in the functional capacity of the overall NK pool in both infections. We addressed whether altered levels of the innate cytokine IL-15 could be contributing to the common changes in NK cell numbers and subset composition in these infections and found reduced levels of IL-15 in the serum of HIV^+ and HCV^+ patients. We show that IL-15 was able to increase the survival of NK cells from control, HIV^+, and HCV^+ patients and promoted proliferation and effector functions. The augmenting effect of IL-15 on NK cell survival and functions supports its use as a therapeutic agent in HIV infection, where trials in animal models are already under way (61), and suggests investigation of its use in HCV infection.

We analyzed in detail how the reduction in NK cell numbers and shift of NK subsets may lead to functional deficits within the total NK cell population of HIV^+ and HCV^+ patients. We first looked at the ability of NK cells to produce the cytokine IFN-γ, which has direct antiviral effects and may also affect the downstream adaptive immune response, e.g., promoting a shift of the T-cell functional phenotype towards Th1. We found that the ability of NK cells from HCV^+ patients to make IFN-γ in response to stimulation with IL-12 plus IL-18 was not impaired, although the overall reduction in NK cell numbers that occurs in the context of this infection may result in limited availability of NK cell-derived IFN-γ in vivo. In HIV^+ patients, there was also an additional impairment in the IFN-γ production capacity of the $CD56^{dim}$ NK subset, resulting in a significant reduction in IFN-γ production by the total NK cell pool, as has also been observed in previous studies (56, 69). Superimposed on the alteration in the NK cell subset composition, there thus also appear to be further abnormalities in NK cell functions in HIV^+ individuals.

Second, we looked at the differential perforin expression of the $CD56^{bright}$ and $CD56^{dim}$ subsets. The $CD56^{dim}$ subset acts as the main cytolytic subset and expresses high levels of perforin and natural cytotoxicity receptors (21). In HIV^+ and HCV^+ patients, there was a reduction in the lytic capacity of the overall NK pool, which correlated well with the proportional reduction of the $CD56^{dim}$ NK subset. Degranulation of $CD56^{dim}$ NK cells, a prerequisite for cytolytic activity, seemed functional in HIV^+ and HCV^+ patients, consistent with the hypothesis that the observed reduction in cytolytic activity of the total NK population in HIV^+ and HCV^+ patients may be due in large part to the proportional reduction in the $CD56^{dim}$ cytolytic NK cell subset. Some previous studies have also reported a decrease in NK lytic activity in HCV and HIV infection (18, 23, 52, 72), although others have not seen this (4, 26).

The fact that we identified common alterations in the size and subset composition of the peripheral NK cell pool for patients chronically infected with two unrelated viruses, HIV and HCV, suggested that there may be a common underlying mechanism. Interestingly, similar observations have also been described for patients with postviral fatigue syndrome (60), raising the possibility that this phenomenon may be characteristic of multiple chronic infections.

One potential explanation for the observed changes could be alterations in the tissue localization of NK subsets due to their selective trapping in the liver in the case of HCV infection or

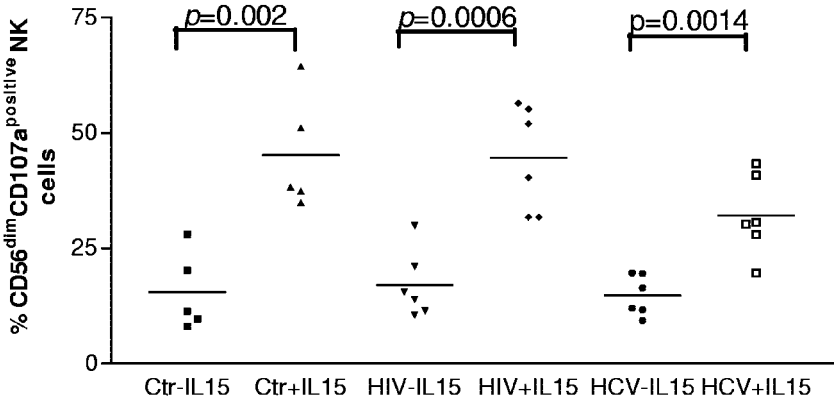

FIG. 7. IL-15 increases the activation of NK cells from control, HIV$^+$, and HCV$^+$ individuals, inducing them to degranulate following target cell recognition. PBMC from control, HIV$^+$, and HCV$^+$ individuals were cultured overnight with or without IL-15 and then were incubated with K562 target cells, and the proportion of NK cells induced to release lytic granules was assessed by CD107a staining. Each symbol represents the percentage of CD56^{dim} NK cells from a single individual expressing CD107a. The means of each group (five HIV$^+$, five HCV$^-$, and five control individuals) are indicated by a horizontal bar, and the significance of the differences between responses of cells incubated with or without IL-15 is shown.

in the lymph nodes in HIV infection (a mechanism suggested to contribute to the reduction in peripheral CD4$^+$ T-cell numbers in HIV infection (17). However, several lines of evidence argue against this. First, previous studies have shown that during HCV$^+$ infection, the total number of NK cells in the liver is normal (25) or decreased (15, 45). Second, we found that the ratios of the CD56^{bright} and CD56^{dim} subsets in the blood and liver of HCV$^+$ patients were very similar. There is thus no evidence to support accumulation of NK cells or selective trapping of CD56^{dim} NK cells in the liver during chronic HCV infection. Accumulation of CD56^{dim} NK cells within the lymph nodes also seems unlikely, based on what is known about their trafficking pattern. It is the CD56^{bright} NK subset, which expresses CCR7 and high levels of L-selectin, that has been found to be present in lymph nodes (29, 74).

Instead, we favor the hypothesis that there may be alterations in NK cell homeostasis in HIV and HCV infection, resulting in a reduction in overall NK cell numbers and a widespread imbalance between the two NK subsets. The NK cell subset ratio could be altered by enhanced production, expansion, or survival of the CD56^{bright} pool, accompanied by decreased generation or survival of CD56^{dim} NK cells. Alternatively, if there were a lineage relationship between these two NK cell subsets (which has been suggested on the basis that CD56^{dim} NK cells appear to be more terminally differentiated than the CD56^{bright} population (21), the balance between these two pools could be altered by impaired maturation of CD56^{bright} cells into CD56^{dim} cells. Biasing of the NK pool in chronic infection towards a potentially less-mature cell subset would have interesting parallels to the enrichment of virus-specific CD8 T cells with an "immature" phenotype (CD27$^+$ CD28$^+$ perforin^{low}) in HIV and HCV infection (7, 8, 40). However, recent evidence suggests that both subsets are in fact products of distinct differentiation lineages (35), making the former hypothesis more likely.

Given the possibility that there may be abnormalities in NK cell homeostasis in HIV$^+$ and HCV$^+$ infections, we addressed whether a deficit in the innate cytokine IL-15 might be partly responsible. IL-15 is known to play an important role in the homeostatic proliferation of naive and memory CD8 T cells (10, 80). Likewise, it is also thought to regulate the homeostasis and functional capacity of NK cells (75) and has been shown to promote NK survival ex vivo (20). We observed that serum levels of IL-15 were reduced during HIV and HCV infections, suggesting that abnormalities in the production of this cytokine may have contributed to the reduction in NK cell numbers and the decrease in the CD56^{dim} population in these infections. Serum IL-15 levels were reduced more dramatically for HIV$^+$ than for HCV$^+$ individuals, and the reduction in overall NK cell numbers was also much greater in the former infection, as would be expected if the two observations were related.

Notably, we found that although the survival of both NK cell subsets was promoted by IL-15 in vitro, the CD56^{dim} NK subset had a greater tendency to undergo apoptosis in the absence of IL-15, and this cytokine had a much more prominent effect on CD56^{dim} cell survival than that of CD56^{bright} NK cells. If IL-15 deficiency also has a greater impact on CD56^{dim} than CD56^{bright} NK cell survival in vivo, this could result in an increase in the CD56^{bright}/CD56^{dim} cell ratio within the peripheral blood NK cell population. Consistent with this, we observed an increase in the CD56^{bright}/CD56^{dim} NK cell subset ratio in the context of reduced serum IL-15 levels in HIV$^+$ and HCV$^+$ individuals. However, the effects seen might not be solely dependent on IL-15; other factors may also be important in determining the phenotype and function of NK cells in these infectious settings. One potential candidate is the recently described cytokine IL-21, which acts in synergy with IL-15 (64).

Given that pathogen components are among the stimuli that elicit production of IL-15, it might have been expected that there would be elevated levels of IL-15 in the serum of patients harboring chronic viral infections. The observed reduction in serum levels might reflect the existence of intrinsic mechanisms for down-regulation of innate responses in the face of chronic stimulation, to reduce associated immunopathological damage. Alternatively, chronic infection might have led to impairment in the number, functional capacity, or responsiveness of IL-15-producing cells. Mature DC constitute one important cellular source of IL-15. A complex cross talk between

DC and NK cells has been proposed (22, 31, 37, 59), whereby DC control functional properties of NK cells and NK cells control DC (30, 32, 65). IFN-α is an important stimulus for the induction of IL-15 production by DCs (63). It is notable that in HIV infection, there is a reduction in the number of plasmacytoid DCs (which produce high levels of IFN-α/β) in the peripheral blood (71). Although in HCV infection, pDC numbers are not dramatically reduced, their IFN-α production capacity may be impaired (6, 62). A recent study also suggested that there may be abnormalities in the ability of DCs from HCV^+ patients to produce IL-15 in response to IFN-α stimulation (43).

IL-15 is currently proposed as adjuvant immunotherapy for HIV^+ individuals (18, 53, 54) and has also been shown to curtail infections by human herpesviruses (33). Our data indicate that it may also be beneficial in HCV infection, where IFN-α treatment is already used, and may potentially be useful in other chronic infections too. A better understanding of the precise nature of the deficits in the innate immune system in chronic infections will be important for the design of appropriate therapeutic strategies.

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