Influence of interleukin-15 on CD8\(^+\) natural killer cells in human immunodeficiency virus type 1-infected chimpanzees

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Chimpanzees are susceptible to human immunodeficiency virus type-1 (HIV-1) and develop persistent infection but generally do not progress to full-blown AIDS. Several host and immunological factors have been implicated in mediating resistance to disease progression. Chimpanzees have a higher prevalence of circulating natural killer (NK) cells than humans; however, their role in mediating resistance to disease progression is not well understood. Furthermore, NK cell survival and activity have been shown to be dependent on interleukin-15 (IL-15). Accordingly, the influence of IL-15 on NK cell activity and gamma interferon (IFN-\(\gamma\)) production was evaluated in naive and HIV-1-infected chimpanzees. In vitro stimulation of whole-blood cultures with recombinant gp120 (rgp120) resulted in enhanced IFN-\(\gamma\) production predominantly by the CD3\(^-\)CD8\(^+\) subset of NK cells, and addition of anti-IL-15 to the system decreased IFN-\(\gamma\) production. Moreover, in vitro stimulation with recombinant IL-15 (rIL-15) augmented IFN-\(\gamma\) production from this subset of NK cells and increased NK cell cytotoxic activity. Stimulation with rgp120 also resulted in a 2- to 7-fold increase in IL-15 production. These findings suggest that chimpanzee CD3\(^-\)CD8\(^+\) NK cells play a vital role in controlling HIV-1 infection by producing high levels of IFN-\(\gamma\), and that IL-15 elicits IFN-\(\gamma\) production in this subpopulation of NK cells in HIV-1-infected chimpanzees.

INTRODUCTION

Natural killer (NK) cell numbers and activity have been shown to correlate inversely with human immunodeficiency viral loads (Kestens \textit{et al.}, 1995; Kottill \textit{et al.}, 2003). Consequently, studies have evaluated cytokines and cellular interactions that may account for this NK cell antiviral activity (Azzoni \textit{et al.}, 2002; Ondoa \textit{et al.}, 2002, 2003). NK cells perform an important role in surveillance in conjunction with activation of the adaptive immune system. NK cells are potent producers of \(\beta\)-chemokines, tumour necrosis factor alpha (TNF-\(\alpha\)) (Alter \textit{et al.}, 2004) and gamma interferon (IFN-\(\gamma\)) (Alter \textit{et al.}, 2004; Pien \textit{et al.}, 2000; Vitale \textit{et al.}, 2000; Siren \textit{et al.}, 2004; Vankayalapati \textit{et al.}, 2004) and have also been shown to express toll-like receptor 3 (Schmidt \textit{et al.}, 2004) and alpha-defensin-1 mRNA (Obata-Onai \textit{et al.}, 2002).

NK cell survival has been shown to be dependent on interleukin-15 (IL-15). IL-15 is produced by several cell populations including monocytes/macrophages (Grabstein \textit{et al.}, 1994; Waldmann & Tagaya, 1999) and dendritic cells (Kuniyoshi \textit{et al.}, 1999), whereas contradicting information has been generated on production by T cells (Grabstein \textit{et al.}, 1994; Bamford \textit{et al.}, 1996). IL-15 has been shown to stimulate memory CD8\(^+\) T cells and is a chemoattractant for T cells (Waldmann & Tagaya, 1999). In addition, gene-knockout studies have demonstrated that IL-15 is required for the development and homing of NK cells, NKT cells and \(\gamma\)\(\delta\) T cells (Kennedy \textit{et al.}, 2000; Ohteki \textit{et al.}, 2001; Lodolce \textit{et al.}, 2002). IL-15 mRNA has been shown to be increased in human immunodeficiency virus type 1 (HIV-1)-infected humans, though IL-15 secretion is reduced (Ahmad \textit{et al.}, 2003). IL-15 upregulates CD40 ligand (CD40L) (Kuniyoshi \textit{et al.}, 1999) and functions synergistically with IL-12, resulting in the production of IFN-\(\gamma\), TNF-\(\alpha\) and granulocyte–macrophage colony-stimulating factor (GM-CSF) from NK cells (Fehniger \textit{et al.}, 1999).

Interestingly, surface-bound IL-15 has been reported to be expressed on purified human monocytes, with upregulation following IFN-\(\gamma\) stimulation (Musso \textit{et al.}, 1999).

HIV-1-infected chimpanzees do not generally progress to AIDS (Heeney \textit{et al.}, 1993), although a few chimpanzees exposed to multiple isolates of HIV have been reported to

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have declining CD4 counts and progressive disease (Novembre et al., 1997; Davis et al., 1998). Mutations that have been associated with resistance to infection or delayed disease progression in humans such as the A32 mutation in CCR5 coreceptor have not been identified in chimpanzees (ten Haaf et al., 2001; Voevodin et al., 1998), although they have recently been shown to exhibit multiple copies of the CCL3L1 (macrophage inflammatory protein-1α-P) gene (Gonzalez et al., 2005). Various hypotheses have been developed to account for control of HIV infection in chimpanzees. It has been shown that chimpanzees develop cytotoxic T lymphocyte (CTL) responses to HIV-1 conserved epitopes (Balla-Jhagjhoorsingh et al., 1999; Nehete et al., 1998), acquire a high level of suppressor CD8^+ T-cell antiviral activity (Castro et al., 1992), normally lack CD4^+ T-cell depletion (Castro et al., 1992; Gougeon et al., 1993; Droge et al., 1993; O’Neil et al., 2000) and have been suggested to have an absence of immune activation (Di Rienzo et al., 1994). Human CD8^+ T cells have also been shown to play a significant role in the control of HIV infection (Matano et al., 1998; Ogg et al., 1999; Edwards et al., 2002). Additional studies have demonstrated that CD8^+ T-cell activity plays a vital role, although the activity is not sufficient to control infection and does not correlate with virus inhibition (Addo et al., 2003; Betts et al., 2001).

Chimpanzees have been reported to have a higher prevalence of NK cells than humans, and viral loads have been shown to correlate inversely with NK cells (Kestens et al., 1995; Kottlitz et al., 2003). This and other differences in human and non-human primate immunological systems indicate that the role of host factors and mechanisms of resistance require further investigation. This study was undertaken to address NK-cell activity in HIV-1-infected chimpanzees and to determine the role of IL-15 in mediating NK-cell activity via induction of IFN-γ production. It is hypothesized that adequate and consistent IL-15 production is likely to result in robust NK-cell activity and viral suppression in chimpanzees. To test this hypothesis, IFN-γ production and NK cytotoxic levels were examined in relation to IL-15 and plasma viral load in HIV-1-infected chimpanzees.

**METHODS**

**Chimpanzees.** Three chimpanzees (4X0124, 4X0149 and 4X0190), chronically infected with HIV-1 for more than 10 years, were utilized in this study. Chimpanzee 4X0124 was initially infected in 1986 with lymphadenopathy-associated virus (LAV) and then infected in 1995 with IIIB, and has been consistently positive for virus isolation by coculture methods since 1992; 4X0149 was infected in 1986 with LAV and has been positive for virus isolation by coculture methods since 2000; and 4X0190 was infected with SF2 in 1994 and has been sporadically positive for virus isolation. Whole-blood samples (heparinized and EDTA) and clotted blood samples were collected at 4-week intervals from the HIV-1-infected chimpanzees. Heparinized blood and clotted blood samples were also collected from three to six naïve chimpanzees that served as controls during routine physicals.

Chimpanzees were housed at the Southwest Foundation for Biomedical Research facility and maintained in accordance with protocols and procedures approved by the Institutional Animal Care and Use Committee.

**Intracellular staining (ICS).** Whole-blood aliquots were removed from heparinized blood samples for ICS. ICS was conducted utilizing a Cytofix/Cytoperm Plus kit with GolgiPlug (BD Biosciences Pharmingen) following the manufacturer’s protocol with modifications. Briefly, whole blood was incubated with PBS for controls or stimulated with 10 μg recombinant gp120 (rgp120) ml^-1 (generously provided by VarGen) or recombinant IL-15 (rIL-15) (20 ng ml^-1; R&D Systems) and incubated at 37 °C with 5 % CO2 for 1 h. GolgiPlug (1 μl) was added to stimulated and unstimulated samples and incubated for an additional 14–15 h. After incubation, samples were processed for flow cytometry. Each sample was resuspended in red-blood-cell lysis buffer (EL buffer; Qiagen) followed by washing and resuspended in 500 μl 1 % stain wash (1 % fetal bovine serum in 1 x PBS with 0.9 % sodium azide) for addition to conjugated antibodies. The conjugated antibodies included: CD3–fluorescein isothiocyanate (FITC) (clone SK7; BD Biosciences Pharmingen), CD69–phycocerythrin (PE) (clone CH/4; Caltag), CD4–PC5 (phycocerythrin–cyanin 5.1) (clone 13B8.2; Beckman Coulter), CD8–PC5 (clone B9.11; Beckman Coulter) and CD56–PC5 (clone N901; Beckman Coulter) for surface staining and IFN-γ–PE (clone 4S.B3; BD Biosciences Pharmingen) or IL-15–PE (clone 4559; R&D Systems) for ICS. Cell suspensions with the conjugated antibodies were incubated for 30 min at 4 °C in the dark for surface staining. Cells were then washed and fixed with Cytofix and incubated in the dark at room temperature for 20 min. Samples were washed once in Perm Wash (BD Biosciences Pharmingen), resuspended in 50 μl and incubated with conjugated antibodies for ICS at room temperature in the dark for 1 h. Samples were washed once in Perm Wash and resuspended in 200–300 μl fixative, placed at 4 °C and acquired by FACS immediately. Twenty thousand events within the gated lymphocyte population were collected for IFN-γ analysis and 50,000–100,000 events were collected for IL-15 analysis. Flow cytometry analysis was performed on the total lymphocyte populations. CD8^+ and CD56^+ populations within the lymphocyte gate were further analysed by using forward scatter vs CD8 and drawing a gate around the CD8 population for CD8 analysis and forward scatter vs CD56 for CD56 population analysis. The CD56 population was analysed for CD3^+CD56^+ IFN-γ^+ NK cells and CD8^+ cells were analysed for CD3^+CD8^+ IFN-γ^+ NK cells and CD3^+CD56^+ IFN-γ^+ T cells. Additional flow cytometry included four-colour analysis using IFN-γ–FITC (clone 4S.B3; BD Biosciences Pharmingen), CD3–Alexa 700 (clone Sp34-2; BD Biosciences Pharmingen), CD56–PE (clone N901; Beckman Coulter) and CD8–APC (clone 3B5; Caltag). Antibody isotype controls were used in all assays.

In a separate experiment, whole-blood samples were incubated with anti-IL-15 or anti-IL-12 monoclonal antibodies (mAbs) (R&D Systems) at 10 μg ml^-1 or PBS (control) and incubated for 1 h at room temperature. rgp120 was then added to each sample at 10 μg ml^-1, mixed well and incubated for 1 h at 37 °C before the addition of GolgiPlug (1 μl). The ICS procedure was then followed as described above.

**Isolation and storage of PBMCs.** EDTA and heparinized blood samples were processed by centrifugation at 700 g for 15 min to separate plasma. Plasma aliquots were frozen at −80 °C for RNA extraction and RT-PCR analysis. A density gradient (Histopaque 1077) was used for recovery of peripheral blood mononuclear cells (PBMCs). PBMCs recovered from EDTA blood were cryopreserved.
and stored at −130 °C for DNA PCR analysis. PBMCs recovered from heparinized blood were utilized for virus isolation and NK cytotoxic analysis.

**Viral load analysis**

**TaqMan real-time RT-PCR.** A standard curve was developed by using gag primers G0 (5′-GACTAGCCGAGGTCTAGAC-3′) and G0 (5′-AAGGCTGCTTGGCCCAAAGA-3′) (Sanders-Buell et al., 1995) to amplify the gag region of plasmid pBKHB105 (provided by the AIDS Research and Reference Reagent Program) by PCR and inserting the PCR product into TOPO TA cloning vector pCRII-TOPO (Invitrogen). The plasmid was linearized followed by in vitro transcription with Megascript (Ambion) and treated with DNase I.

**Viral load analysis**

**TaqMan DNA PCR.** DNA extraction from frozen PBMCs was performed utilizing the QIAGEN Flexigene kit according to the manufacturer’s protocol. Samples were adjusted to 1 μg per 10 μl for each 50 μl QIAGEN reaction. Perkin Elmer HIV-1 DNA standards were utilized at 1, 10, 100 and 10,000 copies per 50 μl reaction. Samples were prepared at room temperature with the Platinum Quantitative PCR SuperMix-UDG kit (Invitrogen) in conjunction with primers and probe as described previously (Palmer et al., 2003). Specimen DNA and standards were tested in triplicate. The 7700 Thermocycler conditions were as follows: reverse transcription at 45 °C for 45 min, Platinum Taq activation at 95 °C for 10 min and 40 amplification cycles at 95 °C for 15 s and 60 °C for 1 min.

**Virus isolation.** PBMCs (10⁶) derived from HIV-1-infected chimpanzees were placed in a T-25 culture flask with 10% FBS RPMI-C with IL-2 and phytohaemagglutinin (PHA-P; 5 μg ml⁻¹). Human PHA-P-stimulated blasts (10⁶) were then added and cultures were incubated for 3 days at 37 °C with 5% CO₂. The cells were centrifuged and the supernatant was removed and replaced with fresh 10% FBS RPMI-C with IL-2. Supernatant (5 ml) was collected on days 7, 14, 21 and 28 and aliquots were frozen at −20 °C for p24 antigen assay. The Beckman Coulter p24 antigen-capture ELISA was performed on supernatants according to manufacturer’s protocol.

**Enzyme-linked immunoassay test for antibody to HIV-1.** Clotted blood samples were collected at each time point and serum was removed and frozen at −20 °C. Antibody testing was performed according to manufacturer’s protocol utilizing an enzyme immunoassay kit (Detect-HIV; Adalitis) with 96-well microtitre plates coated with p24 peptide.

**NK cell cytotoxicity assay.** Target cells included K562 cells, which lack MHC class I expression (NK-susceptible), and MOLT-4 (NK-resistant). Target cells were cultured in 10% FBS RPMI-C and cells in the exponential phase of growth were utilized for the assay. Effector cells consisted of whole PBMC populations. NK-cell activity was tested in a 4 h calcein-acetoxyethyl (calcein-AM) cytotoxicity assay as described previously (Neri et al., 2001) with modifications. rIL-15 was added to cultures for a final concentration of 2 or 20 ng ml⁻¹ to determine its influence on NK-cell activity. Labelled target cells were added to the 96-well plate in 50 μl volumes (5000 per well) and mixed with effector cells serially diluted in 2.5% FBS RPMI-C to attain effector: target ratios of 100:1, 50:1, 25:1 and 12.5:1 in a total volume of 200 μl. Spontaneous and maximum release control wells were prepared by addition of 2.5% FBS RPMI-C to six wells (spontaneous release) and 4% Triton-X to six wells (maximum release). Plates were incubated for 4 h at 37 °C and then centrifuged at 45 g for 3 min. A volume of 100 μl was removed from each well and transferred to a black 96-well plate for analysis. A microfluorimeter with excitation filter set at 485 nm and emission filter set at 535 nm was utilized to perform fluorescence analysis. Specific lysis was calculated as 100 × [(mean specific fluorescence − mean spontaneous fluorescence)/(mean maximum fluorescence − mean spontaneous fluorescence)].

**Statistical analysis.** Data were analysed by t-test utilizing GraphPad Prism 4 software; values of P ≤ 0.05 were considered as significant. Associations between IFN-γ + lymphocyte subpopulations and plasma RNA viral load were determined by simple linear correlation (Pearson’s r). The value of −1.00 represents a perfect negative correlation.

**RESULTS**

**Viral load analysis**

Plasma samples from 4X0124 exhibited varying viral loads during the study, which ranged from <7000 to >14 000 copies ml⁻¹; however, the RNA viral load was below detectable levels for chimpanzees 4X0149 and 4X0190. In contrast, proviral DNA was present in all infected animals and ranged from <10 to 48 copies (4X0149), <10 to 112 copies (4X0190) and 454 to 3500 copies (4X0124) per million PBMCs. Virus isolation by coculture was positive for 4X0124 throughout the study and positive for 4X0149 at four of the five time points. Chimpanzee 4X0190 was positive at only one out of the five time points. Antibody titres for 4X0124 were greater than 1:204 800 and greater than 1:12 800 for 4X0149 and 4X0190 throughout the study (Table 1). The above data indicate that chimpanzees 4X0124 and 4X0149 have persistently, chronic, active infections, whereas the other infected animal appears to have a chronic, silent infection.

NK-cell cytotoxicity was evaluated against K562 and MOLT-4 target cells, with addition of 2 or 20 ng rIL-15 ml⁻¹ to determine the influence of IL-15 on NK cytotoxic activity in HIV-1-infected chimpanzees compared with control chimpanzees (Fig. 1). Cytotoxic activity for all three HIV-1-infected chimpanzees varied throughout the study and was shown to increase 2.5-fold with 2 ng ml⁻¹ and 3- to 5-fold with addition of 20 ng rIL-15 ml⁻¹. No correlation between NK-cell cytotoxic activity and the percentage of CD56⁺ NK cells was observed. Cytotoxic activity for two of the three naive controls was negligible without addition of rIL-15; however, activity for naive
In chimpanzee 4X0149, the CD3+ IFN-γ production was evaluated in CD4+ and CD8+ T cells and CD56+ NK-cell populations in relation to rIL-15 and rgp120 stimulation. Whole-blood stimulation was employed to determine the IFN-γ production of the different subpopulations within the total lymphocyte gate using forward vs side scatter (Fig. 2a) and further gating for CD8+ cells within the lymphocyte gate (Fig. 2b). Representative flow cytometry analysis of unstimulated controls and cultures stimulated with rgp120 (10 μg ml⁻¹) and rIL-15 (20 ng ml⁻¹) are shown for the CD8+ populations (Fig. 2c, d). For chimpanzee 4X0124, the CD3+ CD8+ IFN-γ+ cell population increased from 1.0% for unstimulated to 5.7 and 5.0%, respectively, for rgp120- and rIL-15-stimulated cultures. CD8+ T cells increased from 0.5% to 1.2 and 1.7%, respectively. In chimpanzee 4X0149, the CD3+ CD8+ IFN-γ+ NK-cell population increased from 0.7% for unstimulated to 5.7 and 2.9%, respectively, for rgp120- and rIL-15-stimulated cultures. The CD3+ CD8+ IFN-γ+ T-cell population increased from 0.6 to 1.0% from rgp120 and remained at 0.6% for rIL-15. For chimpanzee 4X0190, the CD3+ CD8+ IFN-γ+ T-cell population increased from 1.4% for the unstimulated control to 8.4% for both rgp120- and rIL-15-stimulated cultures. The CD3+ CD8+ IFN-γ+ T-cell population increased from 0.4% to 1.0 and 1.1%, respectively, for rgp120- and rIL-15-stimulated cultures. Cultures for control animals did not exhibit an increase in IFN-γ+ cells with rgp120 stimulation. However, rIL-15-stimulated cultures for control animals demonstrated an increase in CD3+ CD8+ IFN-γ+ cells and CD3+ CD8+ IFN-γ+ T cells ranging from 2- to 4-fold and 2- to 8-fold, respectively. A representative dot plot for control animals is shown in Fig. 2(d).

Interestingly, the CD3− CD8+ NK-cell population was shown to be the predominant IFN-γ+ population throughout the study (Fig. 3). This population was significantly greater than the CD3+ CD8+ IFN-γ+ T-cell population for all three HIV-1-infected animals when stimulated with rgp120 (P=0.02, 0.003 and 0.02 for 4X0124, 4X0149 and 4X0190, respectively). The CD3− CD8+ IFN-γ+ population was also shown to be significantly greater than the CD3− CD56+ IFN-γ+ NK-cell population for 4X0124 and 4X0190 (P=0.03 and 0.01, respectively); the values were not significantly different for 4X0149 (P=0.07). The mean percentage values of IFN-γ+ subpopulations from the five collection points for the HIV-1-infected chimpanzees are compared with those for the control chimpanzees in Fig. 3.

### Intracellular staining

IFN-γ production was evaluated in CD4+ and CD8+ T cells and CD56+ NK-cell populations in relation to rIL-15 and rgp120 stimulation. Whole-blood stimulation was employed to determine the IFN-γ production of the different subpopulations within the total lymphocyte gate using forward vs side scatter (Fig. 2a) and further gating for CD8+ cells within the lymphocyte gate (Fig. 2b). Representative flow cytometry analysis of unstimulated controls and cultures stimulated with rgp120 (10 μg ml⁻¹) and rIL-15 (20 ng ml⁻¹) are shown for the CD8+ populations (Fig. 2c, d). For chimpanzee 4X0124, the CD3+ CD8+ IFN-γ+ cell population increased from 1.0% for unstimulated to 5.7 and 5.0%, respectively, for rgp120- and rIL-15-stimulated cultures. CD8+ T cells increased from 0.5% to 1.2 and 1.7%, respectively. In chimpanzee 4X0149, the CD3+ CD8+ IFN-γ+ NK-cell population increased from 0.7% for unstimulated to 5.7 and 2.9%, respectively, for rgp120- and rIL-15-stimulated cultures. The CD3+ CD8+ IFN-γ+ T-cell population increased from 0.6 to 1.0% from rgp120 and remained at 0.6% for rIL-15. For chimpanzee 4X0190, the CD3+ CD8+ IFN-γ+ T-cell population increased from 1.4% for the unstimulated control to 8.4% for both rgp120- and rIL-15-stimulated cultures. The CD3+ CD8+ IFN-γ+ T-cell population increased from 0.4% to 1.0 and 1.1%, respectively, for rgp120- and rIL-15-stimulated cultures. Cultures for control animals did not exhibit an increase in IFN-γ+ cells with rgp120 stimulation. However, rIL-15-stimulated cultures for control animals demonstrated an increase in CD3+ CD8+ IFN-γ+ cells and CD3+ CD8+ IFN-γ+ T cells ranging from 2- to 4-fold and 2- to 8-fold, respectively. A representative dot plot for control animals is shown in Fig. 2(d).

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The expression of activation marker CD69 was found to be enhanced on CD3− CD8+ NK cells, CD3− CD56+ NK cells and CD3+ CD8+ T cells in all three HIV-1-infected chimpanzees when stimulated with rgp120 or rIL-15 (Table 2). The CD3− CD8+ CD69+ population increased from 43 to 54% following stimulation with rgp120 and 56% with rIL-15 stimulation for 4X0124. In animal 4X0149, the

### Table 1. Virological and serological status of HIV-1-infected chimpanzees

<table>
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<th>Collection time point (weeks)</th>
<th>Plasma RNA viral load (copies ml⁻¹)</th>
<th>Proviral DNA (copies per 10⁶ PBMCs)</th>
<th>Virus isolation (day 7)</th>
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ND, No data.
expression on CD3+ T cells. The expression of CD69 on CD3+ CD8+ T cells increased from 6% to 14% for 4X0149 and from 5% to 10% for 4X0190 when stimulated with rgp120 or rIL-15. CD69 expression on CD3− CD56+ NK cells increased from 9% to 39% for 4X0149 when stimulated with anti-CD16 mAbs. All three subpopulations exhibited an increase in CD69 expression following stimulation with rgp120 or rIL-15; however, CD3− CD8+ and CD3− CD56+ NK-cell populations had high levels of expression of CD69 before stimulation, and the expression of CD69 was increased markedly following stimulation with rgp120 or rIL-15. The total percentages of CD8+ or CD56+ subpopulations did not demonstrate an increase between the controls (PBS) and rgp120- or rIL-15-stimulated cultures that would account for the increase in the percentage of IFN-γ+ cells (Table 2).

In order to determine whether rgp120 induces the production of IL-15, whole-blood cultures were incubated with rgp120 (10 μg ml−1). An increase of more than 2-fold in IL-15+ cells was observed with rgp120 stimulation for 4X0124 and 4X0149 and an increase of more than 3-fold was observed for 4X0190. In contrast, control animals did not exhibit an increase in IL-15+ cells when stimulated with rgp120 (Fig. 4). To confirm that the induction of IFN-γ was in response to rIL-15, anti-IL-15 or anti-IL-12 mAbs were added to cultures stimulated with rgp120. Addition of either anti-IL-15 or anti-IL-12 resulted in a marked decrease in the percentage of IFN-γ+ cells (Fig. 5), thus confirming the role of IL-15 in inducing IFN-γ production by NK-cell populations.

The disparity between CD3− CD8+ NK cells and CD3+ CD8+ T cells with respect to IFN-γ production was demonstrated throughout the study. To investigate the potential role of CD3− CD8+ NK cells further in HIV-1-infected chimpanzees, RNA plasma viral load was plotted against IFN-γ+ lymphocyte subpopulations as shown in Fig. 6 for 4X0124 (PBS controls shown). A similar investigation was not possible with the other two HIV-1+ chimpanzees because their viral RNA loads were below the detectable level throughout the study. A strong inverse correlation was observed between the viral load and CD3− CD8+ IFN-γ+ cells (r = −0.97), while CD56+ NK cells (r = −0.34) and CD8+ T cells (r = −0.44) had low inverse correlation values. The significant inverse correlation observed between the viral load and the total IFN-γ+ cells (r = −0.99) and CD8− cells (r = −0.98) appeared to be due predominantly to the CD3− CD8− subpopulation of NK cells (r = −0.97).

**DISCUSSION**

These results demonstrate that CD3− CD8+ NK cells are the primary source of IFN-γ in HIV-1-infected chimpanzees, and this subpopulation of NK cells had a significant (r = −0.97) inverse correlation to plasma viral load in one of the infected chimpanzees. Addition of rgp120 to *in vitro* cultures was shown to increase IL-15 production in all three chimpanzees. rIL-15 was also shown to upregulate IFN-γ production in NK cells within hours of stimulation, and addition of anti-IL-15 antibodies markedly decreased the total percentage of IFN-γ+ cells, confirming the role of IL-15 in eliciting IFN-γ production by NK cells. Addition of anti-IL-12 monoclonal antibody had a similar effect, resulting in a decrease in the percentage of IFN-γ+ cells. These *in vitro* observations are in agreement with an *in vivo* chimpanzee study which demonstrated that systemic administration of rIL-12 induces IL-15, followed by an increase in IFN-γ production (Lauw *et al.*, 1999). Furthermore, CD8− cell cultures supplemented with rIL15 or rIL12 have also been shown to suppress HIV...
In addition, the present study demonstrated that NK-cell-mediated cytotoxic activity against K562 targets was enhanced by the addition of rIL-15, and activity could be further enhanced by increasing the concentration of rIL-15 from 2 to 20 ng ml\(^{-1}\). The significant increase in cytotoxic activity demonstrated by control animals following rIL-15 stimulation may be considered a normal response. IL-15 mRNA has been shown to be constitutively expressed in many cell popula-

Fig. 2. Flow cytometry analysis of CD8\(^+\) cells: ICS for IFN-\(\gamma\). (a) Forward scatter vs side scatter plot with lymphocyte gate. (b) Forward scatter vs CD8 with CD8 gate. (c) Dot plots illustrating percentages of CD3\(^+\) CD8\(^+\) IFN-\(\gamma\)^+ and CD3\(^-\) CD8\(^+\) IFN-\(\gamma\)^+ populations: control (PBS), rgp120 (10 \(\mu\)g ml\(^{-1}\)) and rIL-15 (20 ng ml\(^{-1}\)) stimulation. (d) Naive control 1 [control (PBS): rgp120 (10 \(\mu\)g ml\(^{-1}\))] and naive control 2 [control (PBS), rIL-15 (20 ng ml\(^{-1}\))]. Percentages of CD8\(^+\) cells are given in (c) and (d).

Fig. 3. IFN-\(\gamma\)^+ cells in HIV-1-infected and control chimpanzees. CD3\(^-\) CD8\(^+\) NK cells exhibited significant increases in IFN-\(\gamma\)^+ cells for all three HIV-1-infected animals with rgp120 stimulation. The control chimpanzees did not exhibit an increase in IFN-\(\gamma\)^+ cells. Results for HIV-1-infected chimpanzees represent the means ± SEM of five experiments; \(P<0.05\). The control chimpanzees were analysed at one time point. Percentage of total lymphocyte population is given. No data were available for the control 1 CD3\(^-\) CD56\(^+\) population.
Addition of rgp120 to cultures resulted in a significant increase in CD3⁻CD8⁺ IFN-γ⁺ NK cells and slight increases were also noted in CD3⁻CD56⁺ IFN-γ⁺ NK cells and CD3⁺CD8⁻ IFN-γ⁺ T cells. This contrast between the subpopulations for all three animals was demonstrated throughout the study. IL-12, IL-15 and IL-18 have previously been shown to induce IFN-γ production in NK cells (Fehniger et al., 1999; Pien et al., 2000; Siren et al., 2004). Monocytes and dendritic cells have both been shown to produce IL-15 and to elicit IFN-γ production from NK cells (Waldmann & Tagaya, 1999; Kuniyoshi et al., 1999).

CD3⁻CD56⁺ NK cells have been shown to exhibit increased IFN-γ production in HIV-viraemic individuals (Alter et al., 2004); CD3⁻CD8⁺CD56⁺ NK cells have also been shown to exhibit IFN-γ production in humans (Vitale et al., 2000). Notably, IL-18 in combination with IL-12 has been shown to induce higher IFN-γ production in CD56⁺ NK cells compared with a combination of IL-12 and IL-15 (Fehniger et al., 1999). The current study demonstrated that IFN-γ⁺ cells were present at higher frequency in CD3⁻CD8⁺ NK cells and at lower levels in CD3⁻CD56⁺ NK cells and CD3⁻CD8⁻ T cells. The CD3⁻CD8⁺ population has been identified as a subpopulation of NK cells in rhesus macaques, sooty mangabeys, pigtailed macaque (Ibegbu et al., 2001), chimpanzees and humans (Kestens et al., 1995). CD2 has also been demonstrated as a surface marker for this subpopulation (Ibegbu et al., 2001) and was confirmed in this study (data not shown). Since CD56 was absent by flow cytometry surface staining as well as ICS (data not shown) from the CD3⁻CD8⁺ IFN-γ⁺ population, this chimpanzee NK subpopulation is further defined as CD2⁺CD3⁻CD8⁺CD56⁻. It has previously been suggested that IL-15 in

Table 2. Induction of CD69 on T-cell and NK-cell subpopulations by rgp120 and rIL-15

<table>
<thead>
<tr>
<th>Antigen</th>
<th>T cells</th>
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<th>NK cells</th>
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<tbody>
<tr>
<td></td>
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<td>CD3⁺CD8⁺</td>
<td>CD3⁺CD8⁺</td>
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<tr>
<td></td>
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<td></td>
<td>CD69⁺</td>
<td>CD56⁺</td>
</tr>
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</tr>
<tr>
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<td>62.74</td>
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</tr>
<tr>
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<td>62.71</td>
<td>5.39</td>
<td>10.86</td>
</tr>
<tr>
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<td>5.88</td>
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<td></td>
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<td>10.86</td>
</tr>
<tr>
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<td>32.02</td>
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</tr>
<tr>
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<td>20.12</td>
<td>2.88</td>
<td>15.29</td>
</tr>
</tbody>
</table>
humans does not induce or costimulate cytokine production, but selectively expands IFN-γ-producing CD3−CD56+ NK cells (Dunne et al., 2001). However, in this study, whole-blood cultures were incubated for a relatively brief period of 15 h; this time frame is unlikely to result in sufficient proliferation that would account for the 2- to 6-fold increase in IFN-γ+ cells. In addition, the CD3− CD8+ , CD3− CD56+ and CD3+ CD8+ populations did not exhibit an increase with rgp120 or rIL-15 stimulation (Table 2) that would account for the elevated IFN-γ+ subpopulations. The increase in IFN-γ+ cells is probably due to activation or costimulation mediated by rgp120 resulting in the secretion of IL-15, which in turn induced IFN-γ+ production. Furthermore, rIL-15 has been shown to enhance survival of NK cells by maintenance of the anti-apoptotic factor Bcl-2 (Ranson et al., 2003). An in vivo study utilizing rhesus monkeys recently revealed that administration of IL-15 resulted in an increase in CD3− CD8+ NK cells and CD3+ CD8+ T cells within 1 week of treatment (Mueller et al., 2005). In the present study, the percentages of CD8+ T cells and CD8+ NK cells in 4X0124 were shown to be higher than in 4X0149 and 4X0190, while the percentage of CD4+ T cells was considerably lower.

The results demonstrate that rIL-15 enhanced cytotoxic activity and CD3− CD8+ IFN-γ+ cells. Stimulation with rgp120 also resulted in a significant increase in IFN-γ production by CD3− CD8+ NK cells. CD40−CD40L interactions have been shown to upregulate dendritic cell production of IL-12 (Shu et al., 1995) and IL-15 (Kuniyoshi et al., 1999). IL-15 can also induce CD40L in vitro without antigenic stimulation or addition of IL-2 (Skov et al., 2000). Dendritic cell–NK cell interactions within the lymph node promote NK-cell proliferation when dendritic cell IL-15 expression is high (Ferlazzo et al., 2004). During this study, analysis of whole blood by flow cytometry resulted in IL-15+ cells from several gated regions, including lymphocytes (CD4+ T cells), monocytes (CD3− CD4+ CD16+ cells), granulocytes (CD16+ cells) and dendritic cells (CD83+).

Our initial results have demonstrated surface-bound IL-15 expression on CD4+ T cells and CD4+ CD16+ monocytes (data not shown). Musso et al. (1999) demonstrated upregulation of surface-bound IL-15, but their system used purified monocytes cultured with IFN-γ (500 U ml−1). Recent results by Neely et al. (2004) suggested that surface-bound IL-15 functions in activation of monocytes and increases monocyte adhesion (demonstrated with LPS-, PHA- and GM-CSF-stimulated cultures); however, the soluble form of IL-15 was shown to lack this function. Neely et al. (2004) also proposed that engagement of surface-bound IL-15 results in IL-8 secretion. Characterization of the multiple sources of IL-15 requires additional analysis, although purified monocytes and dendritic cells have been shown to be primary sources. Dendritic cells have been shown to secrete IL-12 and IL-15 in response to viral components, with production enhanced by addition of soluble trimeric CD40L in vitro (Kuniyoshi et al., 1999); further investigation is required to elaborate on the dendritic and CD4+ cellular types that contribute to secretion of these cytokines. In the present study, IFN-γ production by CD3− CD8+ NK cells appeared to require previous exposure of the immune system to the antigen, as CD3− CD8+ NK cells from naive control chimpanzees did not respond to rgp120 stimulation. Analysing IFN-γ+ cells in whole-blood cultures (unstimulated and antigen-stimulated) may mimic in vivo conditions and offer an advantage over purified NK cells. This study has demonstrated that NK-cell activity can also be analysed with specific antigens, as it is the ensuing cytokines such as IL-15 and IL-12, presumably from dendritic cells and CD4+ cells, that...
mediate IFN-γ production from NK-cell subsets. Whole-blood samples from 4X0124 (PBS-treated controls) demonstrated an increasing trend in CD3+CD8+ IFN-γ+ NK cells with a corresponding decrease in plasma viral load. Addition of rgp120 to the whole-blood cultures resulted in an increase in IL-15+ cells and a 2- to 6-fold increase in IFN-γ+producing NK cells. Anti-IL-15 added to cultures resulted in a significant decrease in IFN-γ+ cells. Collectively, these results suggest that CD2+CD3−CD8+CD56− NK cells in HIV-1-infected chimpanzees play a notable antiviral role by secreting high levels of IFN-γ that are positively influenced by IL-15.

IL-15 production is likely to lead to a decrease in viral load by multiple pathways, including increasing IFN-γ production, expanding NK cell populations, enhancing survival and cytotoxic activity of NK cells and enhancing survival of memory CD8+ T cells. Synergy of different combinations of cytokines, including IL-12, IL-18 and IL-21, with IL-15 has been shown to upregulate IFN-γ mRNA (Strengell et al., 2003). The many roles of soluble IL-15 compared with surface-bound IL-15 still require investigation. The present study demonstrates the importance of functional NK cells in maintenance of the asymptomatic state in HIV-1-infected chimpanzees. Investigation of activation pathways, transport mechanisms and genetic variations may provide insights into expression of IL-15 from the various sources and why the main source of IFN-γ production in HIV-1-infected chimpanzees is CD3+CD8+CD56− NK cells rather than CD3−CD8+CD56− NK cells, as exhibited by humans. While the higher prevalence of NK cells and resulting NK-cell activity may contribute to the lack of disease progression in chimpanzees, additional studies are required to define further the resistance mechanisms and genetic variations in this valuable animal model.

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