Could natural killer cells compensate for impaired CD4\(^+\) T-cell responses to CMV in HIV patients responding to antiretroviral therapy?

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**KEYWORDS**

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**Abstract** We evaluated NK cell subsets and functions in previously immunodeficient HIV patients responding to ART. Cytokine receptor mRNA was quantitated in purified CD56\(^+\) cells. Data were correlated with CD4\(^+\) T-cell counts and IFN\(\gamma\) responses to CMV. NK cell IFN\(\gamma\) responses to K562 cells and proportions of CD56\(^lo\) NK cells were correlated in patients (p\(<\)0.001) and both were lower than in controls (p\(<\)0.001 and p\(=\)0.008, respectively), so all patients had poor NK cell function. Proportions of CD56\(^hi\) NK cells correlated inversely with current CD4\(^+\) T-cell counts (p\(=\)0.006) and perforin expression in CD56\(^hi\) NK cells was higher in patients than controls (p\(<\)0.05). Hence increased proportions and cytolytic function of CD56\(^hi\) NK cells may partially compensate for CD4\(^+\) T-cell deficiency. NK cell IFN\(\gamma\) responses correlated inversely with expression of IL-10 and IL-12 receptor mRNA. Expression of these transcripts is reduced by exposure to the cytokines, which may reflect immune activation in immunodeficient patients.

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**Introduction**

Natural killer (NK) cells are large granular lymphocytes which can recognize and respond to bacteria, parasites, virus-infected cells and neoplastic target cells. NK cells exhibit cytotoxicity and secretion of cytokines without prior sensitization. Hence they contribute to innate responses against infection and to tumor surveillance [1,2].

Untreated HIV infection is associated with abnormalities of natural killer (NK) cell phenotypes and function assessed by cytotoxicity and production of cytokines such as interferon-gamma (IFN\(\gamma\)) [3–7]. Antiretroviral therapy (ART) suppresses viral replication and thus promotes recovery of CD4\(^+\) T-cell numbers, but some patients retain poor antigen-specific CD4\(^+\) T-cell responses [8,9]. Restoration of NK cell functions after ART varies among studies [3].

Levels of CD56 expression define functionally distinct populations of NK cells. CD56\(^lo\) NK cells represent about 90\% of the CD56\(^+\) NK population. In contrast to CD56\(^hi\) NK cells, CD56\(^lo\) NK cells express high levels of Fc\(\gamma\)RIII (CD16), killer cell Ig-like receptors (KIR) and perforin, which makes them effective mediators of natural cytotoxicity and antibody-dependent cellular cytotoxicity (ADCC) but they secrete...
cytokines at lower levels [10]. They respond better to NK-sensitive target cells than cytokines or mitogens [11–13].

NK cells can produce pro-inflammatory (GM-CSF, TNFα), type 1 (IFNγ, TNFβ) and type 2 (IL-10, IL-13) cytokines. CD56hi NK cells produce more of these cytokines than CD56lo NK cells following stimulation by other cytokines (e.g. IL-12 and IL-15) or PMA plus ionomycin but express low levels of perforin and CD16. Therefore, CD56hi NK cells have an important immuno-regulatory role but exert weak cytoltoxicity [13–15].

HIV infection causes early loss of CD56hi NK cells, followed by the loss of CD56lo NK cells and an increase in CD56−CD16+ NK cells [16]. These changes are partially restored after >6 months of successful ART [3,7,17–19]. CD56−CD16+ NK cells are relatively inert, displaying more inhibitory NK cell receptors and less natural cytotoxicity receptors [5,16,17]. The baseline CD4+ T-cell count was not defined in these studies and may affect recovery of NK cells on ART.

Cytotoxicity and IFNγ production by IL-2-activated NK cells responding to a pan-NK target cell line (K562 cells) were lower in viremic HIV patients (both treated and untreated) than healthy controls [5,6]. However, the effects of successful ART are not clear. In one study, aviremic patients (>24 months on ART) showed a persistently low cytotoxicity against K562 cells [5], whilst IFNγ production recovered. In another study, cytotoxicity recovered and IFNγ production increased in aviremic patients (>24 months on ART) [5].

<table>
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<tr>
<th>Table 1</th>
<th>Demographics of study cohort.</th>
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<tr>
<td></td>
<td>CMV-lo</td>
</tr>
<tr>
<td>n</td>
<td>8</td>
</tr>
<tr>
<td>Age (years)</td>
<td>54 (42–65)</td>
</tr>
<tr>
<td>Nadir CD4+ T-cell count (cells/μl)</td>
<td>8 (0–48)b</td>
</tr>
<tr>
<td>Current CD4+ T-cell count (cells/μl)</td>
<td>594 (120–961)</td>
</tr>
<tr>
<td>Time on ART (months)</td>
<td>102 (17–112)</td>
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<tr>
<td>IFNγ response to CMV (mediated by CD4+ T-cells)a</td>
<td>47 (19–145)c</td>
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<tr>
<td>IFNγ response to K562 cells (mediated by NK cells)a</td>
<td>451 (190–655)d</td>
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Values are presented as median (range), NA=Not applicable.

a ELISpots per 200,000 PBMC.
b Lower than CMV-hi (p=0.05).
c Lower than CMV-hi and controls (p<0.001).
d Lower than controls (p<0.01).

Figure 1 Gating strategies based on expression of (A) CD56 and CD16, and (B) CD56 and Perforin. CD56lo NK cells expressed more CD16 and perforin than CD56hi NK cells.

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remained low [6]. IFN-γ production by NK cells from peripheral blood mononuclear cells (PBMC) activated with IL-2/IL-12 and IL-15/IL-12 remained lower in aviremic patients on ART for >12 months, when compared to healthy controls [19]. In contrast, elevated NK cell cytotoxicity and IFN-γ secretion are reported in viremic HIV patients compared with healthy controls. Cytotoxicity against K562 cells was higher in PBMC from viremic patients and normalized when patients became aviremic after 24 weeks of ART [20]. Expression of CD107a (a marker of lysosomal granule exocytosis) and intracellular IFN-γ in NK cells stimulated with K562 cells were higher in treated and untreated viremic patients, but similar in aviremic patients on ART (>6 months) and healthy controls [18]. The opposing results from different studies may reflect characteristics of their patients, such as baseline CD4+ T-cell counts and recovery of CD4+ T-cell function. However, these parameters are not uniformly reported.

Following ART, the recovery of NK cell IFN-γ responses might parallel the recovery of CD4+ T-cell IFN-γ responses. Alternatively NK cells may compensate for inadequate CD4+ T-cell function. To address this, we undertook a cross-sectional study of NK cell numbers and function in HIV patients with good recovery of CD4+ T-cell numbers after long-term ART (median of 8.5 years) who were stratified by CMV-specific IFN-γ CD4+ T-cell responses. We measured the proportions of NK subsets, FcγRIII (CD16) and perforin expression, as well as IFN-γ responses to K562 cells. To elucidate mechanisms underlying the observed deficiencies, we also measured mRNA for IFN-γ, IL-10R1, IL-12Rβ1 and IL-12Rβ2 in NK cells enriched from PBMC.

Figure 2  (A) Proportions of each NK cell subset as a percentage of lymphocytes. Expression of perforin (B) and CD16 (C) as a percentage of each NK cell subset (left panel) and MFI (right panel).
Methods and materials

Study subjects

Eighteen male CMV-seropositive, HIV patients attending clinics at Royal Perth Hospital (Perth, Western Australia) and nine age-matched, male, CMV-seropositive healthy volunteers were recruited for this study. All patients had started ART with CD4+ T-cell counts of <50/μl and maintained undetectable plasma HIV RNA (<50 copies/ml) for more than 12 months after (median = 8.5, range = 1.4–9.4 years) on treatment. HIV patients were divided into low (n = 8) and high (n = 10) IFNγ responders based on CD4+ T-cell responses to CMV assessed by ELISpot (denoted CMV-lo and CMV-hi, respectively; See Table 1). All patients had been assayed approximately 19 months previously. Responses were stable, with all patients remaining in the same group. This enabled us to use samples collected earlier or later from five patients for mRNA studies, as the original samples were exhausted. Institutional ethics approval was obtained for the study and for mRNA studies, as the original samples were exhausted.

Sample collection, plasma HIV RNA level and CD4+ T-cell counts

Whole blood was collected into lithium heparin tubes. PBMC were obtained by Ficoll gradient centrifugation and separated using magnetic bead technology or cryopreserved in liquid nitrogen. Plasma HIV-1 RNA was measured using the COBAS Amplicor HIV-1 Monitor Test, v1.5 (Roche Diagnostics, Indianapolis, IN, USA). CD4+ T-cell counts were performed by routine flow cytometric methods.

ELISpot assay

Nitrocellulose plates (Millipore, MA, USA) were coated with 90 μl anti-human IFNγ antibody (15 μg/ml; Mabtech, Stockholm, Sweden) overnight at 4 °C. PBMC were added in RPMI 1640 with 10% fetal calf serum at 1.0 ×10^5 to 2.0 ×10^5 cells per well and stimulated for 20 h with CMV antigen [21] or K562 cells at effector to target ratio of 10:1. Spots were detected with biotinylated anti-human IFNγ antibody (100 μl/well; Mabtech, Stockholm, Sweden), streptavidin horseradish peroxidase conjugate (100 μl/well; BD Pharmingen, San Jose, CA, USA) and tetramethylbenzidine (TMB) substrate (1–2 min), and counted using AID ELISpot Reader v2.9 software (Autoimmun Diagnostika GmbH, Strassberg, Germany).

Flow cytometry

Thawed PBMC were washed in flow buffer (1% BSA/PBS) and 5 ×10^5 cells were surface stained with the following monoclonal antibodies: CD3-APC, CD16-PECy5 and CD56-PE (Coulter Immunotech, Marseille, France). For the staining of intracellular perforin, cells were permeabilized using FAC-Slyse and incubated with Perforin-FITC (BD Pharmingen, San Jose, CA, USA). Data were acquired on the same day using a BD FACSCanto cytometer for 4-colour protocols. 50,000-100,000 events were recorded per tube and analyzed using the FlowJo program v5.7.2 (Tree Star, Ashland, OR, USA).

IFNγ, IL-10R and IL-12R mRNA expression from purified CD56+ NK cells

CD56+ cells were purified from fresh PBMC using conjugated magnetic bead kits (Miltenyi Biotec, Bergisch Gladbach, Germany). Two samples from each group were excluded because the purity was <70%. For the remainder, the median purity was 80% (range 70–93) based on the phenotype CD3-CD56+. Most contaminants were CD56+CD8+ T-cells (data not shown). Real-time PCR were used to quantify mRNA for β-actin, IFNγ, IL-10R1, IL-12Rβ1 and IL-12Rβ2. The PCR protocol comprised 95 °C for 300 s, followed by 40 cycles of denaturation (95 °C for 10 s), annealing (62 °C for β-actin and 112Rβ1; 68 °C for 12Rβ1 for 15 s) and extension (72 °C for 25 s). Primer sequences for β-actin were 5’-GATGACCCAGATCATGTTTGA-3’ and 5’-GACTCCATGCCAGGAAGAA-3’, for IL-12Rβ1 were 5’-CTTCCAGAGGCTGTCAAG-3’ and 5’-GATGTTGAGCATGCACAGGACAGAA-3’, for IL-12Rβ2 were 5’-GGATGCTCATTGGCATTTAT-3’ and 5’-GATGTTGAGCATGCACAGGACAGAA-3’. [22]. IFNγ and IL-10R1 mRNA levels were determined using Quantitect® sequence specific probes and Hs_IL10RA_SG_1 Quantitect® Primer Assay (Qiagen, CA, USA). Real-time PCR was performed on a Rotorgene™ (Corbett, Sydney, Australia). Quantitation utilized standard curves generated from amplification of serial 10-fold dilution of pooled PCR products and results were

Figure 3  (A) Numbers of NK cells producing IFNγ after stimulation with K562 cells was low in CMV-lo and CMV-hi patients. (B) IFNγ production by NK cells was directly related to the proportion of CD56lo NK cells in HIV patients.

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expressed relative to β-actin mRNA. The lower limit of detection was a ratio of 0.00001.

Statistical analyses

Mann–Whitney tests were used to compare groups of individuals. Spearman’s test was used to calculate the significance of non-parametric correlation coefficients. For all comparisons, p-values below 0.05 were considered to be statistically significant.

Results

Proportions of CD56lo NK cells were lower in HIV patients than controls

Patients with a long-term virological response to ART were divided into CMV-lo and CMV-hi groups based on CD4+ T-cell IFNγ responses to CMV. The groups had similar durations of treatment and current CD4+ T-cell counts, but the CMV-lo group had a lower median nadir CD4+ T-cell count (see Table 1). Proportions of NK cell subsets were calculated as a percentage of lymphocytes and correlated with the parameters indicated. NK cells were defined as CD3− lymphocytes which were CD56− CD16+, CD56lo or CD56hi.

Patients from CMV-lo and CMV-hi groups had a lower proportion of CD56lo NK cells than controls (p < 0.03; Fig. 2A), so the major NK cell population was deficient in all HIV patients after long-term ART (1.4–9.2 years) with prolonged viral suppression. The proportions of CD56hi and CD56− CD16+ NK cells were similar in all groups.

Patient cohorts were then combined and proportions of each subset of NK cells were correlated with CD4+ T-cell IFNγ responses to CMV and current or nadir CD4+ T-cell counts. Proportions of CD56lo NK cells correlated inversely with current CD4+ T-cell counts (r = −0.62, p = 0.006). Hence CD56lo cells may compensate for CD4+ T-cell deficiency.
Expression of perforin by CD56_{hi} NK cells was elevated in HIV patients compared to healthy controls

Perforin and CD16 were analyzed in CD56_{lo} and CD56_{hi} NK cells as a percentage of each NK subset and as mean fluorescence intensity (MFI). CD56_{lo} NK cells have a high expression of perforin and CD16 while CD56_{hi} NK cells have a low or no expression of perforin and CD16 (Figs. 1A and B). Hence, perforin and CD16 expression was higher in CD56_{lo} NK cells than CD56_{hi} NK cells in all subjects (Figs. 2B and C).

CD16 expression by CD56_{lo} and CD56_{hi} NK cells was similar in all groups (Fig. 2C). When the patient groups were combined, expression of CD16 by CD56_{lo} NK cells correlated with NK IFNγ responses (r=0.55, p=0.028) and CMV-specific IFNγ CD4+ T-cell responses (r=0.48, p=0.043).

Perforin expression by CD56_{lo} NK cells was similar across all groups (Figs. 2B and C). Perforin expression by CD56_{hi} NK cells analyzed by MFI was higher in CMV-lo and CMV-hi patients than healthy controls (p=0.015 and p=0.006 respectively). When the CMV-lo and CMV-hi groups were combined, expression of perforin by CD56_{hi} NK cells was higher in patients than controls (p=0.048 by percentages, p=0.003 by MFI).

NK cell IFNγ responses were lower than healthy controls in all HIV patients

NK cell IFNγ responses were lower in the CMV-lo (p=0.003) and CMV-hi (p=0.002) groups than healthy controls (Fig. 3A). Amongst patients, NK cell IFNγ responses did not correlate with CD4+ T-cell IFNγ responses to CMV (r=0.21, p=0.44), nadir CD4+ T-cell counts (r=-0.34, p=0.20) or current CD4+ T-cell count (r=0.41, p=0.11). However, they correlated directly with the proportion of CD56_{lo} NK cells (r=0.92, p<0.001; Fig. 3B) and with expression of CD16 in this NK subset (r=0.55, p=0.028; data not shown). Therefore, NK cell IFNγ responses to K562 cells are attributed to CD56_{lo} NK cells. This was confirmed using 4 non-HIV and 2 HIV samples assessed by flow cytometry. CD56_{lo} NK cells are the major producers of IFNγ after stimulation with K562 cells (Fig. 1C).

Levels of mRNA for IL-10 and IL-12 receptors in CD56+ cells do not explain poor NK cell IFNγ responses in HIV patients

We hypothesized that impaired NK cell IFNγ responses in HIV patients may reflect a low expression of receptors for IL-10 or IL-12 since these cytokines are important in NK cell activation. This was assessed in cells purified using CD56-coated magnetic beads. IFNγ mRNA was assessed in parallel. This and expression of perforin and CD16 reflect NK cell activity without in vitro stimulation.

Expression of mRNA for IFNγ, IL-10R1, IL-12Rβ1 and IL-12Rβ2 was similar in CMV-lo, CMV-hi and healthy control groups (Fig. 4A). Hence, a low constitutive expression of these transcripts does not explain the poor NK cell IFNγ responses of patients. Expression of the receptors did not correlate with expression of IFNγ mRNA by unstimulated CD56+ NK cells in patients (Fig. 4B), so their baseline expression does not limit NK responses.

Rather, we observed several inverse correlations. Co-expression of perforin and CD16 correlated inversely with the expression of mRNA for IL-10R1 (r=-0.56, p=0.016) and IL-12Rβ1 (r=-0.63, p=0.005) but not IL-12Rβ2 (r=-0.22, p=0.46). NK cell IFNγ responses correlated inversely with the expression of mRNA for IL-10R1 (r=-0.76, p=0.002), IL-12Rβ1 (r=-0.74, p=0.004) and IL-12Rβ2 (r=-0.64, p=0.024) in the combined patient group (Fig. 4B).

Discussion

Our study establishes that there are persistent defects in NK cell IFNγ responses to a pan-NK target (K562 cells) in HIV patients who had undergone long-term ART (median=8.5 years) compared to healthy controls. NK cell IFNγ responses in patients were attributed to lower proportions of the major CD56_{lo} NK cell subset but not correlated with the CD4+ T-cell IFNγ responses. As reviewed in [1], NK cell production of IFNγ is important in controlling various viral, parasitic and bacterial infections. For example, the inhibition of human CMV viral replication is dependent on IFNγ production by infected cells induced by NK cell IFNγ [23]. Therefore, HIV patients with stable virological responses to ART and recovery of CD4+ T-cell numbers might have an increased risk of infection because of impaired NK cell function. Furthermore, although the incidence of AIDS-related malignancies has declined in HIV patients receiving ART, there has been an increase in non-AIDS-related malignancies compared with the general population [24]. Immune surveillance may be limited by impaired NK cell IFNγ production.

Here, the proportion of CD56_{hi} NK cells showed a significant inverse correlation with current CD4+ T-cell counts and elevated expression of perforin by CD56_{hi} NK cells compared to healthy controls. CD56_{hi} NK cells can acquire the phenotype of CD56_{lo} NK cells and increased perforin and CD16 expression upon stimulation with IL-2 [12] and peripheral fibroblasts [25], so persistent immune activation may explain the observed elevation. The cytotoxic capability of NK cells highly depends on the levels of perforin content in their granules [11]. CD16 is an activatory receptor on NK cells and its ligation stimulates NK cell proliferation, cytotoxicity and cytokine secretion [26]. Hence, increased proportion and cytotoxic capacity of CD56_{hi} NK cells may reflect the activation of this NK subset to compensate for poor CD4+ T-cell recovery in HIV patients on ART.

Proportions of CD56_{lo} NK cells and IFNγ responses to a pan-NK target (K562 cells) were significantly lower in HIV patients than controls and were not correlated with the CD4+ T-cell IFNγ responses. NK cell IFNγ responses in patients were attributed to CD56_{lo} NK cells. Expression of CD16 directly correlates with NK cell IFNγ responses in patients and CMV-specific CD4+ T-cell IFNγ responses. This probably reflects the activated or functional level of CD56_{lo} NK cells rather than the direct role of CD16 in IFNγ production by NK cells or CD4+ T-cells as CD16 is not important in the interaction of NK cells and K562 cells [27–29].

Our data establishes that there is a persistent defect in IFNγ responses of CD56_{lo} NK cells, irrespective of the
Natural killer cells in HIV patients responding to ART

patients’ ability to mount a CD4 T-cell response to CMV. This makes it unlikely that CD4+ T-cell IFN\(\gamma\) responses are limited by NK cell IFN\(\gamma\) responses or vice versa. IL-10 production by NK cells can suppress antigen-specific CD4+ T-cell proliferation and IFN\(\gamma\) production [30], so higher IL-10 secretion by NK cells could affect CD4+ T-cell responses. Here IL-10 mRNA was not detectable in unstimulated CD56+ cells from the HIV patients (data not shown), but IL-10 production by stimulated NK cells was not assessed.

Deficient NK cell IFN\(\gamma\) responses in HIV patients responding to ART may be caused by low levels of cytokines important in the activation of NK cells. IL-10 and IL-12 can stimulate the proliferation, cytotoxicity and IFN\(\gamma\) production of human CD56+ NK cells in the presence of IL-2 [14,15]. Expression of IL-10 mRNA by unstimulated PBMC declines following a virological response to ART [19]. IL-10 production was also lower in PHA-stimulated PBMC from aviremic patients than healthy controls [31]. However the source of IL-10 affecting NK cells and the effects of prolonged ART on its release are unclear.

IL-10 and IL-12 derived from dendritic cells (DC) or monocytes warrant consideration as factors limiting IFN\(\gamma\) production by the patients’ NK cells. Production of IL-10 and IL-12 by myeloid DC was impaired in viremic, but not aviremic HIV patients. This correlated with impaired activation and production of IFN\(\gamma\) by NK cells [32]. Moreover IL-12 mRNA levels were not lower in patients than controls here [33], or in our earlier study which included cells stimulated with PMA [34], so there is no evidence that IL-12 is limiting in patients responding to ART.

NK cell activation and IFN\(\gamma\) production may also be affected by IFN\(\alpha\) secreted by plasmacytoid DC [35]. Aviremic patients in the present study were assessed for proportions of accessory cells and levels of cytokine mRNA in each purified fraction [33]. Proportions of plasmacytoid DC (and not other populations) were directly correlated with CD4+ T-cell IFN\(\gamma\) responses to CMV. However, proportions of DC and levels of IL-10, IL-12 and IFN\(\alpha\) mRNA in DC and monocytes did not correlate with NK cell IFN\(\gamma\) responses here (data not shown).

Here expression of IL-10R1, IL-12R\(\beta\)1 and IL-12R\(\beta\)2 mRNA was similar in both patient groups and controls, so a low constitutive expression of these receptors does not explain the low NK cell IFN\(\gamma\) responses in patients. However, a significant inverse correlation was observed between levels of all three transcripts with induced NK IFN\(\gamma\) responses and the co-expression of perforin and CD16 in the combined patient group (Fig. 4B). We suggest that low IL-10R1, IL-12R\(\beta\)1 and IL-12R\(\beta\)2 mRNA may reflect chronic activation of NK cells. This level of activation may increase NK cell IFN\(\gamma\) responses but not to levels of healthy controls.

Chronic activation can differentially affect the levels of cytokine receptor mRNA and protein. For example; IL-2 reduces levels of IL-10 mRNA but not the binding of labeled IL-10 to NK cells. The authors concluded that expression of just 90 IL-10 receptors per cell yields optimal stimulation, so high levels of IL-10R1 mRNA are redundant [14]. Chronic activation of NK cells by IL-2 is unlikely in HIV patients. Indeed levels of IL-2 mRNA in T-cell fractions from our patients did not correlate with NK IFN\(\gamma\) responses or cytokine mRNA levels in NK cells (data not shown).

Rather chronic immune activation in HIV patients may reflect bacterial products leaking from a gut damaged during periods of extreme immunodeficiency [36]. NK cells express Toll-like receptors (TLR) and can be directly activated by bacterial products [37]. Alternatively, plasmacytoid DC activated via TLR may activate NK cells via IFN\(\alpha\) [35]. In our cohort, nadir CD4+ T-cell count inversely correlated with NK cell IFN\(\gamma\) responses [38] and levels of immune activation of CD4+ T-cells were significantly higher than controls (p=0.01, data not shown), so the degree of previous immunodeficiency and residual immune activation affect NK cell function on ART. We suggest that low cytokine receptor expression (Fig. 4) and small increases in IFN\(\gamma\) production by NK cells are consequences of immune activation in patients with a history of extreme immunodeficiency, however these effects do not compensate for the low proportions of the predominant IFN\(\gamma\)-producing subset (CD56lo NK cells) seen in all patients.

In conclusion; we have identified three mechanisms that may affect NK cell function in HIV patients with a long-term virological response to ART. Firstly, there is increased perforin expression by CD56hi NK cells from HIV patients compared to healthy controls. The second mechanism depresses IFN\(\gamma\) responses in direct proportion to loss of CD56lo NK cells. Finally, we provide indirect evidence that immune activation depresses levels of mRNA for IL-10 and IL-12 receptors. Future studies to dissect mechanisms of persistent defects in NK cell must include TLR and NK receptor signaling and measurement of critical cytokines.

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References


