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**Phenotypic and functional characteristics of highly differentiated CD57<sup>+</sup>NKG2C<sup>+</sup> NK cells in HIV-1-infected individuals**

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## Abstract

Natural killer (NK) cells are important anti-viral effector cells. The function and phenotype of the NK cells that constitute an individual's NK cell repertoire can be influenced by ongoing and/or previous viral infections. Indeed, infection with human cytomegalovirus (HCMV) drives the expansion of a highly differentiated NK cell population characterized by expression of CD57 and the activating NKG2C receptor. This NK cell population has also been noted to occur in HIV-1-infected individuals. We evaluated the NK cells of HIV-1-infected and –uninfected individuals to determine the relative frequency of highly differentiated CD57<sup>+</sup>NKG2C<sup>+</sup> NK cells and characterize these cells for their receptor expression and responsiveness to diverse stimuli. Highly differentiated CD57<sup>+</sup>NKG2C<sup>+</sup> NK cells occurred at higher frequencies in HCMV-infected donors relative to HCMV-uninfected donors and were dramatically expanded in HIV-1/HCMV co-infected donors. The expanded CD57<sup>+</sup>NKG2C<sup>+</sup> NK cell population in HIV-1-infected donors remained stable following antiretroviral therapy. CD57<sup>+</sup>NKG2C<sup>+</sup> NK cells derived from HIV-1-infected individuals were robustly activated by antibody-dependent stimuli that contained anti-HIV-1 antibodies or therapeutic anti-CD20 antibody, and these NK cells mediated cytolysis through NKG2C. Lastly, CD57<sup>+</sup>NKG2C<sup>+</sup> NK cells from HIV-1-infected donors were characterized by reduced expression of the inhibitory NKG2A receptor. The abundance of highly functional CD57<sup>+</sup>NKG2C<sup>+</sup> NK cells in HIV-1-infected individuals raises the possibility that these NK cells could play a role in HIV-1 pathogenesis or serve as effector cells for therapeutic/cure strategies.

Keywords: Natural Killer Cells; Human Immunodeficiency Virus; Human Cytomegalovirus; Cytolysis; Differentiation

## Abbreviations

ADCC – Antibody-dependent cellular cytotoxicity (ADCC)

ART – Antiretroviral therapy

DARTS - Dual-affinity re-targeting molecules

ELISA – Enzyme-linked immunosorbent assay

HCMV – Human cytomegalovirus

HIVIG – HIV immunoglobulin

IVRN - Immunovirology Research Network

KIR – Killer immunoglobulin-like receptor

LDH – Lactate dehydrogenase

MHC-I or HLA-I – Major histocompatibility complex class I

MSHC – Melbourne Sexual Health Center

NK – Natural killer cell

PBMC- Peripheral blood mononuclear cells

VIDRL - Victorian Infectious Disease Reference Laboratory

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## Introduction

Natural killer (NK) cells are key mediators of anti-viral immune responses (Waggoner et al., 2016). Interaction with virus-infected target cells can trigger NK cell activation, resulting in cytolysis of target cells and/or the release of cytokines. NK cells also modulate adaptive antiviral immune responses. Activation of NK cells by target cells is achieved through direct recognition of ligands for activating NK cell receptors, or via crosslinking of Fc $\gamma$ RIIIa/CD16 expressed on NK cells (Biron et al., 1999). NK cells also express numerous inhibitory receptors that recognize various self-ligands. These inhibitory receptors regulate NK cell responsiveness to cellular stimuli. NK cell activation is triggered when a cumulative activating signal is received following interaction with a putative target cell (Lanier, 2005). NK cells can respond to HIV-1-infected target cells and mediate an array of anti-viral functions, including direct cytotoxicity, antibody-dependent cellular cytotoxicity (ADCC) and the release of beta chemokines that inhibit viral replication by occluding the HIV-1 co-receptor, CCR5 (Bonaparte and Barker, 2004; Ward et al., 2004; Song et al., 2014; Boudreau et al., 2016; Bruel et al., 2016; Tomescu et al., 2021). As such, there is much interest in designing HIV-1 vaccines and therapeutics that engage and utilize NK cell-mediated immune responses. A deeper understanding of how NK cells are altered during HIV-1 infection will benefit the design of NK cell-engaging HIV-1 therapeutics.

Numerous phenotypic and functional changes in NK cells have been noted during active HIV-1 infection, including a decreased capacity to mediate cytotoxicity, lower expression of activating natural cytotoxicity receptors and increased expression of inhibitory killer immunoglobulin-like receptors (KIR) (De Maria et al., 2003; Mavilio et al., 2003; Mavilio et al., 2005). These alterations are largely reversed by suppression of viremia by antiretroviral therapy (ART) (Mavilio et al., 2003). In addition to these changes in NK cell function and phenotype, altered frequencies of NK cells with

differentiated phenotypes have been observed in HIV-1-infected donors and persist after the initiation of ART (Heath et al., 2016;Peppia et al., 2018). Such long-lasting changes are linked to the high prevalence of human cytomegalovirus (HCMV) co-infection in HIV-1-infected individuals (>80%) and the capacity of HCMV to drive NK cell differentiation (Guma et al., 2004;Lopez-Verges et al., 2011;Barrett et al., 2014).

In general, the differentiation of NK cells is reflected by the phenotypic transition from  $CD56^{\text{bright}}CD16^{-/\text{dim}}CD57^{-}$  to  $CD56^{\text{dim}}CD16^{+}CD57^{-}$  and finally  $CD56^{\text{dim}}CD16^{++}CD57^{+}$  (Bjorkstrom et al., 2010;Lopez-Verges et al., 2010;Solana et al., 2012). Throughout these stages NK cells gradually gain more cytotoxic potential, reflected in increased intracellular levels of perforin and granzyme B. Furthermore, the cell surface inhibitory NK cell receptors that regulate NK cell function are modified throughout the differentiation process. Initially, the  $CD56^{\text{bright}}$  NK cells are characterized by low expression of KIR, which recognize classical major histocompatibility complex class I (MHC-I or HLA-I; i.e. HLA-A, B and C) ligands, and high expression of the inhibitory NKG2A receptor, which recognizes the non-classical HLA-E ligand (Braud et al., 1998;Beziat et al., 2010). As NK cells differentiate, the percentage of cells expressing NKG2A decreases and the percentage of cells expressing KIR increases (Beziat et al., 2010;Bjorkstrom et al., 2010). Infection with HCMV has been shown to drive the expansion of a highly differentiated population of  $CD56^{\text{dim}}CD57^{+}$  NK cells, which additionally express the NKG2C activating receptor that recognizes HLA-E (Guma et al., 2004;Lopez-Verges et al., 2011). These cells are highly responsive to stimulation through CD16 and mediate robust functions through the gained NKG2C receptor (Wu et al., 2013). An HCMV-dependent expansion of these highly differentiated  $CD57^{+}NKG2C^{+}$  NK cells has also been reported in HIV-1-infected donors (Heath et al., 2016;Peppia et al., 2018). Indeed, the expansion of the highly differentiated NK cell subset is exaggerated by HIV-1/HCMV co-infection.

We now provide further evidence that the expansion of highly differentiated CD57<sup>+</sup>NKG2C<sup>+</sup> NK cells in HIV-1/HCMV co-infected donors exceeds the expansion observed in HIV-1-uninfected HCMV-infected controls. Furthermore, we demonstrate that NK cells from HIV-1-infected donors mediate robust cytotoxicity through NKG2C and that highly differentiated CD57<sup>+</sup>NKG2C<sup>+</sup> NK cells mediate potent antibody-dependent functions utilizing either polyclonal anti-HIV-1 antibodies or a therapeutic anti-CD20 antibody, Rituximab. Lastly, we demonstrate that the expansion of CD57<sup>+</sup>NKG2C<sup>+</sup> NK cells coincides with a reduction in the NK cell population regulated through NKG2A. These results are discussed in terms of their potential relevance for understanding HIV-1 pathogenesis and therapy design.

## Materials and Methods

### Participants

Whole blood was collected from 54 HIV-1-infected donors at the Melbourne Sexual Health Centre (MSHC) and from 18 HIV-1-uninfected donors by forearm venipuncture into sodium heparin containing Vacuette. Characteristics of the subjects are shown in Table 1. The Victorian Infectious Disease Reference Laboratory (VIDRL) screened 18 HIV-1-uninfected and 30 HIV-1-infected donors for HCMV seroconversion. Among HIV-1-infected and HIV-1-uninfected donors, 29 and 13, respectively, were seropositive for HCMV. For assessments of NK cell function and phenotype, whole blood or isolated peripheral blood mononuclear cells (PBMC) were utilized. PBMC were isolated from whole blood by ficoll density gradient prior to use.

Cryopreserved PBMC from pre- and post-ART time points were obtained from an additional 10 HIV-1-infected donors. All 10 donors were confirmed to be HCMV seropositive. The Immunovirology

Research Network (IVRN) provided these samples. The characteristics of these subjects are shown in Table 1.

All donors provided informed consent prior to sample collection, and the ethics committee of all participating institutions approved the conducted studies.

### **Cell lines**

The CEM.NKr-CCR5 CD4<sup>+</sup> human T cell line was obtained from the NIH AIDS Reagent Program. The 721.221 human B cell line and the murine Fc-receptor-bearing mastocytoma P815 cell line were kind gifts from Dr. Andrew Brooks (University of Melbourne).

### **Phenotypic characterisation of NK cells**

To assess the expression of CD57, NKG2A and NKG2C on NK cells from HIV-1-infected and uninfected donors, 200µl whole blood or 10<sup>6</sup> PBMC were stained with the following antibodies for 30 minutes at room temperature: anti-CD3 PerCP (clone SK7; BD Biosciences) or anti-CD3 BV786 (clone SK7; Biolegend), anti-CD56 PE-Cy7 (clone NCAM16.2; BD Biosciences), anti-CD57 Pacific Blue (clone HCD57; Biolegend), anti-NKG2C PE (clone 134591; R&D Systems) and anti-NKG2A APC (clone Z199; Beckman Coulter). After whole blood staining, red blood cells were lysed in BD FACS lysis solution (BD Biosciences) and cells were washed and fixed in 1% formaldehyde. After PBMC staining, cells were washed and fixed. Samples were acquired on an LSR Fortessa flow cytometer (BD Biosciences). Flow cytometry data were analyzed using FlowJo software (FlowJo LLC). For analysis of the expression of NKG2A on CD57<sup>-</sup>, CD57<sup>+</sup>NKG2C<sup>-</sup> and CD57<sup>+</sup>NKG2C<sup>+</sup> NK cell subsets, donors with less than 100 events in any of the three NK cell subsets were excluded from analysis.

Staining of pre- and post-ART cryopreserved samples from the IVRN was performed as described above for fresh samples, with the additional inclusion of a Live/Dead blue viability dye (Thermo Fisher).

### **NK cell activation intracellular cytokine staining assay**

The functional importance of NK cell differentiation in antibody-dependent NK cell activation was assessed using a NK cell activation intracellular cytokine staining assay, as previously described (Selva et al., 2017; Kristensen et al., 2018). Briefly, CEM.NKr-CCR5 target cells were prepared by coating with HIV-1<sub>BaL</sub> gp120 (3µg/ml/10<sup>6</sup> cells; NIH AIDS Reagent Program) for 30 minutes at 37°C. 721.221 target cells were prepared by coating with anti-CD20 monoclonal antibody Rituximab (10µg/ml/10<sup>6</sup> cells; Roche) for 60 minutes at 4°C. Freshly isolated PBMC (1 × 10<sup>6</sup>) from HIV-1-infected donors were combined at a 5:1 ratio with either CEM.NKr-CCR5 cells (2 × 10<sup>5</sup>) in the absence or presence of a 1:1,000 dilution of pooled HIV immunoglobulin (HIVIG; NIH AIDS Reagent Program) or 721.221 cells (2 × 10<sup>5</sup>) alone or pre-coated with Rituximab. We have previously performed the anti-HIV-1 antibody-dependent NK cell activation assay using plasma samples from both HIV-1-infected and -uninfected donors and demonstrated NK cell activation to only occur in the presence of anti-HIV-1 antibodies (Gooneratne et al., 2016). Cells were incubated for five hours at 37°C. As a control, 10<sup>6</sup> PBMC were incubated alone. All incubations included brefeldin A (5µg/ml; Sigma) and monensin (6µg/ml; BD Biosciences). After incubation, cells were surface stained with anti-CD3 BV786 (clone SK7; Biolegend), anti-CD56 PE-Cy7 (clone NCAM16.2; BD Biosciences), anti-CD57 Pacific Blue (clone HCD57; Biolegend) and anti-NKG2C PE (clone 134591; R&D Systems) antibodies for 30 minutes at room temperature. Next, cells were fixed in 1% formaldehyde, permeabilized with permeabilization buffer (BD Biosciences) and stained with anti-IFN $\gamma$  Alexa Fluor

700 antibody (Clone B27; BD Biosciences) for 60 minutes at room temperature. Lastly, cells were acquired on an LSR Fortessa flow cytometer (BD Biosciences). Flow cytometry data were analyzed using FlowJo software (FlowJo LLC). For analysis of the activation of the CD57<sup>-</sup>, CD57<sup>+</sup>NKG2C<sup>-</sup> and CD57<sup>+</sup>NKG2C<sup>+</sup> NK cell subsets, donors with less than 100 events in any of the three NK cell subsets were excluded from analysis.

### **Depletion of CD57<sup>+</sup> NK cells**

Depletion of CD57<sup>+</sup> cells was performed per manufacturer's protocol using human anti-CD57 MicroBeads (Miltenyi Biotec). The efficacy of the depletion was assessed by flow cytometry using CD3 PerCP (clone SK7; BD Biosciences), CD56 PE-Cy7 (clone NCAM16.2; BD Biosciences) and CD57 FITC (clone TB03; Miltenyi Biotec) antibodies. Cells were acquired on an LSR Fortessa flow cytometer (BD Biosciences) and data were analyzed using FlowJo software (FlowJo LLC).

### **Enrichment of NK cells**

As previously described (Parsons et al., 2016), NK cells were enriched, following the manufacturer's protocol, using an NK cell enrichment kit (StemCell Technologies).

### **Lactate dehydrogenase release cytotoxicity assay**

The redirected lysis of P815 target cells was measured with the CytoTox 96<sup>®</sup> Non-Radioactive Cytotoxicity Assay kit (Promega), as previously described (Selva et al., 2017). Briefly, freshly isolated PBMC ( $2 \times 10^5$ ) from HIV-1-infected donors were combined with P815 target cells ( $1 \times 10^4$ ) at a 20:1 ratio and in the absence and presence of 5 $\mu$ g/ml anti-NKG2C (clone 134591; R&D Systems), anti-NKG2A (clone Z199; Beckman Coulter) or anti-CD16 (clone 3G8; Biolegend) antibodies. In

experiments assessing the cytotoxicity potential of PBMC depleted of CD57<sup>+</sup> cells, the number of CD57<sup>-</sup> PBMC that would be present in whole PBMC at 20:1 E:T ratio was combined with  $1 \times 10^4$  P815 target cells. For experiments assessing the cytotoxicity of enriched NK cells, NK cells ( $5 \times 10^4$ ) were combined at a 5:1 ratio with P815 target cells ( $1 \times 10^4$ ). Both target and effector cells were incubated alone to determine their spontaneous lactate dehydrogenase (LDH) release. Maximum LDH release from target cells was assessed by the addition of lysis solution (Promega) to wells containing only target cells. Additional wells containing culture medium alone were also included. All assay conditions were conducted in triplicate in 96-well round-bottom tissue culture plates. The plates were spun at  $250 \times g$  for four minutes and incubated for four hours at 37°C. After incubation, plates were spun at  $250 \times g$  for four minutes and 50µl/well of supernatant was transferred to an enzyme-linked immunosorbent assay (ELISA) plate (Thermo Fisher Scientific). Next, 50µl of substrate (Promega) was added to each well containing supernatant and incubated for 30 minutes at room temperature in the dark. Lastly, the reaction was stopped by the addition of 50µl/well stop solution (Promega), and absorbance was recorded at 492 nm. The optical density values for wells containing media only were subtracted from all other optical density values. The remaining values were used to calculate the percentage of cytotoxicity with this formula: % cytotoxicity = [(experimental – effector spontaneous – target spontaneous)/(target maximum – target spontaneous)] × 100. For analysis of redirected lysis, donors mediating greater than 10% cytolysis in both the negative control conditions (i.e., no antibody and anti-NKG2A) were excluded from analysis.

### **Data analyses**

Statistical analyses were performed with GraphPad PRISM (GraphPad Software). Paired data were compared using a non-parametric Wilcoxon matched-pairs signed rank test or a Friedman test followed with Dunn's multiple comparisons post-test. Unpaired data were analysed using a non-parametric Mann-Whitney test. Correlations were assessed using the non-parametric Spearman

correlation co-efficient. Data throughout are reported in the format of (Median [Interquartile range]). Differences were considered statistically significant at two tailed  $p < 0.05$ .

## Results

### CD57<sup>+</sup>NKG2C<sup>+</sup> NK cell frequency in HIV-1-infected and –uninfected donors

To determine if HIV-1 infection coincides with altered NK cell differentiation, we screened 54 HIV-1-infected and 18 HIV-1-uninfected donors (as described in Table 1) for CD57<sup>+</sup>NKG2C<sup>+</sup> NK cells by flow cytometry. A flow cytometry panel allowed for the identification of CD3<sup>-</sup>CD56<sup>dim</sup> NK cells and determination of the relative frequency of CD57<sup>+/–</sup>NKG2C<sup>+/–</sup> subsets within each donor's NK cell population (Figure 1A). Within both HIV-1-infected and –uninfected donors a large range was noted in the percentage of NK cells that were CD57<sup>+</sup>NKG2C<sup>+</sup>. A significantly higher percentage of NK cells exhibited the CD57<sup>+</sup>NKG2C<sup>+</sup> phenotype in HIV-1-infected than HIV-1-uninfected donors (median 33.6% [14.2-53.6%] vs 4.4% [1.4-11.2%],  $p < 0.0001$ , Figure 1B).

HCMV has a known major impact on the constitution and function of NK cell populations (Guma et al., 2004;Lopez-Verges et al., 2011). Amongst HIV-1-uninfected donors, serology revealed 13 HCMV-infected donors and five HCMV-uninfected donors. Consistent with previous publications (Guma et al., 2004;Lopez-Verges et al., 2011;Heath et al., 2016;Peppia et al., 2018), we noted a significantly higher frequency of CD57<sup>+</sup>NKG2C<sup>+</sup> NK cells within the NK cell population of HCMV-infected donors than HCMV-uninfected donors (median 7.3% [3.7-16.9%] vs 0.9% [0.5-1.6%],  $p = 0.0009$ , Figure 1C). Within the HIV-1-infected donors, 29 were confirmed seropositive for HCMV. These HIV-1/HCMV co-infected donors exhibited significantly higher frequencies of CD57<sup>+</sup>NKG2C<sup>+</sup> NK cells than HIV-1-uninfected HCMV-infected donors (median 43.7% [15.1-57.5%] vs 7.3% [3.7-16.9%],  $p < 0.0001$ , Figure 1D). Comparison of the frequency of CD57<sup>+</sup>NKG2C<sup>+</sup> NK cells in HIV-1-infected HCMV-infected

donors to the frequency of CD57<sup>+</sup>NKG2C<sup>+</sup> NK cells reported in an historical cohort of HIV-1-infected HCMV-uninfected controls (median 1.6%) reveals that HCMV is a major driver of the expansion of CD57<sup>+</sup>NKG2C<sup>+</sup> NK cells during HIV-1-infection (Heath et al., 2016). Indeed, the frequency of CD57<sup>+</sup>NKG2C<sup>+</sup> NK cells is similar between HIV-1-infected HCMV-uninfected donors and HIV-1-uninfected HCMV-uninfected donors. It is important to note that Heath et al. employed a distinct panel of fluorochrome conjugated antibodies to those used in the current study. However, the same anti-NKG2C clone was used, and all the antibodies were well-characterized and commercially available. As such, there is little reason to suspect differences between the Heath et al. study and the current study in terms of the ability to identify CD57<sup>+</sup>NKG2C<sup>+</sup> NK cells. These data reaffirm that HCMV infection expands CD57<sup>+</sup>NKG2C<sup>+</sup> NK cells and illustrate that an exaggeration of this expansion occurs in HIV-1/HCMV co-infected donors.

#### **Impact of ART on CD57<sup>+</sup>NKG2C<sup>+</sup> frequency in HIV-1-infected donors**

As suppression of viremia by ART restores NK cell cytotoxicity and normalizes the expression of natural cytotoxicity receptors and KIR (Mavilio et al., 2003), we next investigated if the frequency of CD57<sup>+</sup>NKG2C<sup>+</sup> NK cells was changed after prolonged ART. The cohort screened in Figure 1 for CD57<sup>+</sup>NKG2C<sup>+</sup> NK cells were sampled at a single time point and contained mostly ART-treated donors. As such, the data from donors in Figure 1 provides no information regarding the impact of ART on CD57<sup>+</sup>NKG2C<sup>+</sup> NK cell frequency. Cryopreserved PBMC samples from 10 HIV-1-infected donors were obtained from times pre- and post-ART. All 10 donors were confirmed HCMV-infected by serology on stored samples from the pre-ART date. The post-ART samples were obtained a median of 29 months (range 21-43 months) after initiation of therapy. No significant differences in CD57<sup>+</sup>NKG2C<sup>+</sup> NK cells were noted between pre- and post-ART samples (median 17.5% [12.9-37.8%] vs 35.7% [14.4-54.6%],  $p=0.24$  Figure 2). These data demonstrate that expanded CD57<sup>+</sup>NKG2C<sup>+</sup> NK cells persist in HIV-1-infected donors, even after prolonged suppression of viremia by ART.

### Antibody-dependent functions of CD57<sup>+</sup>NKG2C<sup>+</sup> NK cells from HIV-1-infected donors

NK cells can be activated by diverse stimuli (Biron et al., 1999). Activation can result from direct recognition of target cell surface expressed ligands for activating receptors, or recognition of the constant region of IgG bound to target cell antigens. The state of NK cell differentiation can influence the capacity of the cell to respond to certain stimuli. Indeed, CD57<sup>+</sup> NK cells mediate more robust functions through CD16 than CD57<sup>-</sup> NK cells (Lopez-Verges et al., 2010; Parsons et al., 2014). Furthermore, CD57<sup>+</sup>NKG2C<sup>+</sup> NK cells from HCMV-infected individuals mediate robust function following stimulation through CD16 (Wu et al., 2013). To determine if NK cell subsets (i.e., CD57<sup>-</sup> vs. CD57<sup>+</sup>NKG2C<sup>-</sup> vs. CD57<sup>+</sup>NKG2C<sup>+</sup>) within HIV-1-infected individuals respond differentially to antibody-dependent stimuli, we assessed the functions of NK cells from 12 HIV-1-infected donors following: (i) antibody-dependent stimulation with a transformed B cell line coated with the anti-CD20 antibody, Rituximab (721.221+RTX); and (ii) antibody-dependent stimulation with HIV-1 gp120-coated CEM.NKr-CCR5 cells in the presence of HIVIG (CEM.NKr+gp120+HIVIG). The level of NK cell activation was detected by intracellular cytokine staining for IFN $\gamma$ . Among the 12 donors, five were confirmed HCMV seropositive and seven were not tested for HCMV serostatus. Nine of the donors were treated with ART and had undetectable viremia (i.e., <20 copies/mL). Two donors were not treated with ART at the time of sampling and had detectable viremia (3,850 and 22,445 copies/mL). The last donor was receiving ART but experiencing adherence issues. This donor also had detectable viremia (446 copies/mL). Figure 3A depicts the gating implemented to assess IFN $\gamma$  production in the absence of stimulation or following exposure to each of the cellular stimuli in the presence or absence of antibody. Following antibody-dependent stimulation with anti-HIV-1 antibodies or Rituximab, the CD57<sup>+</sup>NKG2C<sup>+</sup> subset was the most activated subset. CD57<sup>+</sup>NKG2C<sup>+</sup> NK cells were significantly more activated than CD57<sup>-</sup> NK cells following anti-HIV-1 antibody-dependent stimulation ( $p < 0.0001$ ) and Rituximab-dependent stimulation ( $p < 0.0001$ , Figure 3B). Although the

data presented in Figure 3B did not undergo subtraction of background NK cell activation, it is important to note that 721.221 and CEM.NKr-CCR5 cells might activate NK cells through antibody-independent mechanisms. As such, we performed additional analyses in which direct NK cell activation (i.e., activation in the absence of antibody) was subtracted from the activation observed in conditions containing antibodies. For both the anti-HIV-1 antibody-dependent and Rituximab-dependent stimulations CD57<sup>+</sup>NKG2C<sup>+</sup> NK cells were significantly more activated than CD57<sup>-</sup> NK cells following background subtraction ( $p < 0.0001$ ). Collectively, our data imply that within HIV-1-infected individuals CD57<sup>+</sup>NKG2C<sup>+</sup> NK cells are potent mediators of antibody-dependent functions.

#### **NKG2C-dependent redirected cytotoxicity by CD57<sup>+</sup>NKG2C<sup>+</sup> NK cells from HIV-1-infected donors**

The NKG2C receptor is an activating receptor with specificity for HLA-E (Braud et al., 1998). The HLA-E ligand represents a potentially interesting target on HIV-1-infected cells, as some HIV-1 strains have been demonstrated to upregulate HLA-E expression (Martini et al., 2005; Nattermann et al., 2005). Expanded CD57<sup>+</sup>NKG2C<sup>+</sup> NK cells from HCMV-infected donors can become activated through NKG2C ligation (Wu et al., 2013). To determine if CD57<sup>+</sup>NKG2C<sup>+</sup> NK cells from HIV-1-infected donors could mediate cytotoxicity through NKG2C, we performed redirected cytotoxicity assays. Briefly, freshly isolated PBMC from HIV-1-infected donors were co-cultured with the murine P815 cell line in the absence of antibody or the presence of an anti-NKG2C antibody. As a positive control for redirected lysis, anti-CD16 antibody was implemented. An antibody against the inhibitory NKG2A receptor was used as a negative control for redirected lysis. As depicted in Figure 4A, we observed a range of cytotoxicity of P815 target cells through NKG2C (4.8-99.9%) and CD16 (7.2-91.6%), but not through NKG2A or in the absence of antibody, across 18 HIV-1-infected donors. The 18 donors were all treated with ART and had undetectable viremia (i.e., <20 copies/mL). Among the donors, 10 were seropositive for HCMV, one was seronegative, and seven had unknown serostatus. The capacity of effector cells from HIV-1-infected donors to mediate NKG2C-specific redirected lysis of P815 target

cells strongly correlated with the percentage of NK cells exhibiting the CD57<sup>+</sup>NKG2C<sup>+</sup> phenotype ( $r=0.8$ ,  $p<0.0001$ , Figure 4B).

To assess if CD57<sup>+</sup>NKG2C<sup>+</sup> cells were responsible for at least a proportion of the NKG2C-specific redirected lysis of P815 target cells, we performed anti-CD57 antibody-dependent magnetic bead-based depletions of CD57<sup>+</sup> cells across a subset of six HIV-1-infected donors (five HCMV seropositive and one with an unknown serostatus). Patient PBMC were assessed pre- and post-depletion by flow cytometry to determine the effectiveness of the depletions (Figure 4C). The depletions were robust, eliminating a majority of CD57<sup>+</sup> cells across the six donors (median 93.8%, Figure 4D). Depletion of CD57<sup>+</sup> cells dramatically reduced redirected lysis of P815 through NKG2C (median 91.3% reduction in cytotoxicity [82.7-97.2%];  $p=0.03$ , Figure 4E), highlighting an important role for CD57<sup>+</sup>NKG2C<sup>+</sup> cells in the cytotoxic response.

As NKG2C can also be expressed on CD8<sup>+</sup> T cells (Guma et al., 2005), we also performed redirected cytotoxicity assays with enriched NK cells from six HIV-1-infected donors to confirm that the lysis was mediated by NK cells. Among the six donors, four were HCMV seropositive and two had unknown serostatuses. All donors were treated with ART and had undetectable viremia (i.e., <20 copies/mL). As depicted in Figure 4F, NK cell enrichments were successful across all six donors (pre-enrichment: median 12.8% [8.2-17.8%] versus post enrichment: median 82.3% [79.6-91.3%],  $p=0.03$ ). As shown in Figure 4G, enriched NK cells mediated robust NKG2C-dependent redirected lysis of P815 cells that was significantly higher than NK cell-mediated lysis of P815 cells in the absence of antibody ( $p=0.03$ ).

## **NKG2A expression profile of CD57<sup>+</sup>NKG2C<sup>+</sup> NK cells from HIV-1-infected donors**

In HIV-1-uninfected donors the differentiation of NK cells has been shown to not only involve the transition of cells from CD57<sup>-</sup> to CD57<sup>+</sup>, but to involve the loss of NKG2A and a gain of KIR expression in a stepwise pattern (Beziat et al., 2010; Bjorkstrom et al., 2010). As such, we assessed if the expansion of CD57<sup>+</sup>NKG2C<sup>+</sup> NK cells within HIV-1-infected donors was accompanied by a decrease in NK cells regulated through NKG2A. We assessed the surface phenotype of NK cells within samples from 54 HIV-1-infected donors by flow cytometry. As shown in Figure 5A, we note that NKG2A was expressed on a low proportion of the CD57<sup>+</sup>NKG2C<sup>+</sup> NK cell subset, which contained significantly fewer NKG2A<sup>+</sup> NK cells than CD57<sup>-</sup> and CD57<sup>+</sup>NKG2C<sup>-</sup> NK cells ( $p < 0.0001$  for both). Consistent with the observation that the CD57<sup>+</sup>NKG2C<sup>+</sup> NK cell subset contained the fewest NKG2A<sup>+</sup> NK cells, we noted a robust inverse correlation between the expansion of CD57<sup>+</sup>NKG2C<sup>+</sup> NK cells and the percentage of total NK cells that expressed NKG2A in all 54 HIV-1-infected donors ( $p < 0.0001$ , Figure 5B). Furthermore, we observed significantly fewer NKG2A<sup>+</sup> NK cells in HIV-1-infected donors than in HIV-1-uninfected donors ( $p < 0.01$ , data not shown). Collectively, these data imply that the expansion of CD57<sup>+</sup>NKG2C<sup>+</sup> NK cells in HIV-1-infected donors could facilitate a shift away from NKG2A-driven NK cell regulation.

## **Discussion**

In the presented data we report higher CD57<sup>+</sup>NKG2C<sup>+</sup> NK cells in HCMV infected donors, which is significantly exaggerated in HCMV-seropositive HIV-1-infected donors. Within HIV-1-infected individuals the CD57<sup>+</sup>NKG2C<sup>+</sup> NK cell subset remained consistent in frequency throughout ART and exhibited a unique phenotypic and functional profile. Indeed, CD57<sup>+</sup>NKG2C<sup>+</sup> NK cells mediated robust antibody-dependent functions, and CD57<sup>+</sup>NKG2C<sup>+</sup> NK cells were also able to mediate cytotoxicity following ligation of NKG2C. Lastly, significantly fewer CD57<sup>+</sup>NKG2C<sup>+</sup> NK cells expressed NKG2A than the CD57<sup>-</sup> and CD57<sup>+</sup>NKG2C<sup>-</sup> NK cell subsets.

In the current report, we have broadened the characterization of expanded CD57<sup>+</sup>NKG2C<sup>+</sup> NK cells from HIV-1-infected donors. We demonstrate that this highly differentiated NK cell population is highly functional through CD16, in the context of HIV-1-specific antibodies and the anti-CD20 therapeutic monoclonal antibody, Rituximab. A limitation to the functional data in the current manuscript is the unknown HCMV serostatus of some donors. Analyses based on HCMV serostatus could be useful, as HCMV might induce changes to NK cells unrelated to differentiation and NKG2C expression. We also report data that CD57<sup>+</sup>NKG2C<sup>+</sup> NK cells persist during ART. This observation is consistent with previous studies assessing the frequency of NKG2C<sup>+</sup> NK cells in HIV-1-infected donors prior to and following ART (Mela et al., 2005; Brunetta et al., 2010). For a small proportion of donors, we noted that CD57<sup>+</sup>NKG2C<sup>+</sup> NK cells increased in frequency following ART. Post-ART expansion of CD57<sup>+</sup>NKG2C<sup>+</sup> NK cells was previously reported by (Kared et al., 2018). The clear conclusion from the accumulated data is that many HIV-1-infected donors have an expanded CD57<sup>+</sup>NKG2C<sup>+</sup> population. Since this expanded NK cell subset is highly functional, it might be of importance for understanding HIV-1 pathogenesis and designing therapeutics and/or cure strategies.

Additional research is needed to build on the observations in the current report. It was recently reported that CD57<sup>+</sup> NK cells expressing high or low levels of NKG2C from ART-treated HIV-1-infected individuals mediate robust ADCC of HIV-1-infected cells coated with broadly neutralizing antibodies (Tomescu et al., 2021). Consistent with this observation, CD57<sup>+</sup>NKG2C<sup>+</sup> NK cells from HIV-1-infected donors exhibit transcriptional profiles linked to ADCC (Costanzo et al., 2018). However, more research is required to understand the full scope of anti-viral functions mediated by CD57<sup>+</sup>NKG2C<sup>+</sup> NK cells. It will be important to establish the capacity of CD57<sup>+</sup>NKG2C<sup>+</sup> NK cells stimulated via direct and antibody-dependent pathways to both lyse virus infected cells and inhibit viral replication through non-cytolytic mechanisms. Another area of potential interest for future

research is to determine if the accumulation of CD57<sup>+</sup>NKG2C<sup>+</sup> NK cells in HIV-1-infected individuals contributes to other phenotypical or functional changes to NK cells that occur during HIV-1 infection. This would include determining the relationship between the accumulation of CD57<sup>+</sup>NKG2C<sup>+</sup> NK cells and the development of dysfunctional CD56<sup>-</sup>CD16<sup>+</sup> NK cells (Mavilio et al., 2005). Finally, it will be important to determine if CD57<sup>+</sup>NKG2C<sup>+</sup> NK cells from HIV-1-infected individuals exhibit similar functional profiles to cells with similar phenotypes from HIV-1-uninfected donors.

The expansion of CD57<sup>+</sup>NKG2C<sup>+</sup> NK cells, which have lost NKG2A expression, in HIV-1-infected donors interjects an interesting factor for consideration with regards to a recently proposed mechanism of HIV-1 pathogenesis. Ramsuran et al (2018) recently noted that HLA-A alleles that correspond to enhanced HLA-A surface expression are linked with higher viral loads and poorer CD4 counts (Ramsuran et al., 2018). The authors demonstrated that these alleles enhance HLA-E expression, due to the ability of the HLA-A signal peptide to bind and stabilize HLA-E. Furthermore, using NK cells from HIV-1-uninfected donors, they demonstrated that HIV-1-infected target cells that contain highly expressed HLA-A alleles inhibit NK cell degranulation via ligation of NKG2A by HLA-E. The authors disqualified a role for NKG2C, citing previous literature showing that NKG2A signaling dominates if both NKG2A and NKG2C are expressed on the same cell (Beziat et al., 2011). We now provide evidence, however, that many HIV-1-infected individuals gain CD57<sup>+</sup>NKG2C<sup>+</sup> NK cells and lose NKG2A<sup>+</sup> NK cells (Figure 1 and 5). Indeed, amongst the participants of the current study, HIV-1-infected donors had fewer NKG2A<sup>+</sup> total NK cells than HIV-1-uninfected donors ( $p < 0.01$ ). In HIV-1-infected donors that have lost NKG2A<sup>+</sup> NK cells, it is difficult to foresee a role for NKG2A/HLA-E interactions in advancing HIV-1 disease progression. In fact, expanded CD57<sup>+</sup>NKG2C<sup>+</sup>NKG2A<sup>-</sup> NK cells from HIV-1-infected individuals might serve as better effector cells than NKG2A<sup>+</sup> NK cells for responding to HIV-1-infected target cells. Interestingly, the presence of NKG2C<sup>+</sup>NKG2A<sup>-</sup> NK cells has previously been linked to lower viral load set point (Ma et al., 2017). Furthermore, Gondois-Rey et al

(2017) observed an association between the presence of CD57<sup>+</sup>NKG2C<sup>+</sup> NK cells and control of viremia in primary HIV-1 infection (Gondois-Rey et al., 2017). In contrast, Alsulami et al (2021) noted no relationship between the frequency of NKG2C<sup>+</sup> NK cells and viral load setpoint (Alsulami et al., 2021). We propose that future studies are required to determine if highly expressed HLA-A alleles contribute to HIV-1 disease progression differentially in individuals with NK cell populations consisting primarily of NKG2A<sup>+</sup> cells versus individuals with NK cell populations consisting largely of CD57<sup>+</sup>NKG2C<sup>+</sup>NKG2A<sup>-</sup> cells.

The potential negative contribution of NKG2A to HIV-1 disease progression might partly explain the exaggerated expansion of CD57<sup>+</sup>NKG2C<sup>+</sup> NK cells in HIV-1-infected donors. We hypothesize that the enhanced expansion of CD57<sup>+</sup>NKG2C<sup>+</sup> NK cells in HIV-1-infected individuals occurs through a multistep process. Initially, individuals infected with HCMV generate populations of less differentiated CD57<sup>+</sup>NKG2C<sup>+</sup> and more differentiated CD57<sup>+</sup>NKG2C<sup>+</sup> NK cells. Next, the same individuals become infected with HIV-1, which drives the expansion of NK cells readily responsive to HIV-1-infected cells. Given that NKG2C<sup>+</sup> NK cells are less likely to express NKG2A (Figure 5) and NKG2A negatively contributes to NK cell responsiveness to HIV-1-infected cells (Ramsuran et al., 2018), we predict NKG2C<sup>+</sup> NK cells with proliferation potential would be likely to expand following HIV-1 infection and ultimately contribute to the CD57<sup>+</sup>NKG2C<sup>+</sup> NK cell population. This hypothetical pathway for the exaggerated expansion of CD57<sup>+</sup>NKG2C<sup>+</sup> NK cells during HIV-1 infection would be testable with longitudinal samples from HCMV donors from dates prior to and following HIV-1 infection. Furthermore, non-human primates infected with CMV and SIV could be useful to test this hypothesis, as Ram et al (2018) demonstrated NK cell expression of NKG2A and NKG2C can be distinguish in rhesus macaques using RNA flow (Ram et al., 2018).

Attempts to design immune-based therapeutics and/or cure strategies for HIV-1 might benefit from the utilization of expanded CD57<sup>+</sup>NKG2C<sup>+</sup> NK cells. Indeed, the enhanced antibody-dependent functions of highly differentiated NK cells might confer passively infused therapeutic broadly neutralizing antibodies with greater anti-viral potential (Caskey et al., 2015; Caskey et al., 2017). Another putative therapeutic/cure strategy under consideration for HIV-1 are dual-affinity re-targeting (DART) molecules. To date, DARTs have contained an anti-HIV-1 binding arm linked to an arm capable of engaging a receptor on an effector cell that can trigger cytotoxicity (i.e. T cell receptor/CD3) (Sloan et al., 2015). Given the potent cytotoxic potential of CD57<sup>+</sup>NKG2C<sup>+</sup> NK cells to kill through NKG2C, we propose that DARTs containing an HIV-1-specific arm and an NKG2C-engaging arm should be evaluated for anti-viral potential.

Chronic viral infections can alter the function and phenotype of both T and NK cells. In the case of HCMV infection, this leads to T cell memory inflation and accumulation of terminally differentiated T cells and to selective expansion of a distinct NK cell subset. While these processes occur in parallel in people living with HIV-1, driven by similar features (Heath et al., 2016), they may have dichotomous effects. The terminal differentiation of HCMV-specific T cells is associated with shortened telomeres and progress towards replicative senescence (Heath et al., 2018). Accumulation of senescent cells is a potential contributor to persistent inflammation and reduced resilience in HIV-1-infected individuals co-infected with HCMV (Barrett et al., 2014; Barrett et al., 2016; Freeman et al., 2016). In contrast, the expanded subset of CD57<sup>+</sup>NKG2C<sup>+</sup> NK cells in HIV-1-infected individuals co-infected with HCMV shows no selective evidence of extensive replication and progress towards replicative senescence (Heath et al., 2018). Such changes nonetheless leave a lasting imprint on NK cell function/phenotype even after successful treatment of viremia and have the potential to impact disease progression. It is important to characterize lasting modifications of NK cells from HIV-1-infected donors, whether induced by HIV-1, HCMV or a combination of the two

infections, to inform the design of potential therapeutic and/or cure strategies that might best exploit these changes. The expanded highly differentiated CD57<sup>+</sup>NKG2C<sup>+</sup> NK cell subset in HIV-1 infection represents a potentially powerful effector cell population for monoclonal anti-HIV-1 broadly neutralizing antibody therapies (Caskey et al., 2015; Caskey et al., 2017). Similarly, linkage of NKG2C functionality to therapeutic molecules, such as DARTs, could confer significant benefit. Future research should assess the potential advantages of directly targeting anti-HIV-1 immune-based therapies to stimulate the functions of CD57<sup>+</sup>NKG2C<sup>+</sup> NK cells.

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### **Animal Statement**

Not applicable.

### **Data Availability Statement**

The data underlying this article will be shared on reasonable request to the corresponding author.

### **Competing Interests Statement**

The authors declare no conflicts of interest.

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## Author Contributions

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Manuscript preparation, editing, and review: ABK, MDG, SJK, and MSP

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## Figure legends

**Figure 1. Expansion of highly differentiated CD57<sup>+</sup>NKG2C<sup>+</sup> NK cells in donors infected with HIV-1, HCMV or both viruses.** Co-expression of CD57 and NKG2C on NK cells from 54 HIV-1-infected and 18 -uninfected donors, with and without HCMV infection, was assessed by flow cytometry. (A) Gating strategy used to identify the expression of CD57 and NKG2C on CD56<sup>dim</sup> NK cells in three representative HIV-1-infected donors. Two donors had undetermined HCMV serostatuses and one was HCMV seropositive. The graphs depict the percentage of CD56<sup>dim</sup> NK cells that co-express CD57 and NKG2C in (B) HIV-1-infected and -uninfected subjects, regardless of HCMV infection, (C) HIV-1-uninfected subjects with and without HCMV infection and (D) HCMV-infected subjects with and without HIV-1 co-infection. Donors with confirmed HCMV seropositivity are depicted in red. Donors with suppressed viremia are represented with circles, and those with detectable viremia are represented with triangles. Data were compared with Mann-Whitney tests.  $p < 0.05$  was considered significant. Columns represent medians.

**Figure 2. Effect of ART on the frequency of highly differentiated CD57<sup>+</sup>NKG2C<sup>+</sup> NK cells in HIV-1-infected subjects.** The frequency of highly differentiated NK cells in pre- and post-ART samples from 10 subjects infected with both HIV-1 and HCMV was evaluated by flow cytometry. Data were compared with a Wilcoxon matched-pairs signed rank test.  $p < 0.05$  was considered significant.

**Figure 3. Functional characteristics of highly differentiated CD57<sup>+</sup>NKG2C<sup>+</sup> NK cells from HIV-1-infected donors.** NK cell activation was measured by flow cytometry as the percentage of CD56<sup>dim</sup> NK cells expressing IFN $\gamma$  after antibody-dependent stimulation. (A) FACS plots showing the gating on IFN $\gamma$ <sup>+</sup> CD56<sup>dim</sup> NK cells in PBMC incubated five hours in the absence of target cells, with HIV-1<sub>BaL</sub>

gp120-coated CEM.NKr-CCR5 target cells alone (no antibody control), 721.221 target cells alone (no antibody control), gp120-coated CEM.NKr-CCR5 cells in the presence of pooled HIV-1 immunoglobulin (HIVIG) or 721.221 cells pre-coated with the anti-CD20 antibody Rituximab (RTX). (B) The graphs depict the percentage of IFN $\gamma$ <sup>+</sup> cells within CD56<sup>dim</sup>CD57<sup>-</sup>, CD56<sup>dim</sup>CD57<sup>+</sup>NKG2C<sup>-</sup> and CD56<sup>dim</sup>CD57<sup>+</sup>NKG2C<sup>+</sup> NK cells stimulated with CEM.NKr-CCR5 cells with HIVIG (left) or 721.221 cells with RTX (right) for 12 HIV-1-infected donors. Donors with confirmed HCMV seropositivity are depicted in red. Donors with suppressed viremia are represented with circles, and those with detectable viremia are represented with triangles. Data were compared with Friedman tests followed with Dunn's multiple comparisons post-tests to assess differences between CD56<sup>dim</sup>CD57<sup>+</sup>NKG2C<sup>+</sup> cells and CD56<sup>dim</sup>CD57<sup>-</sup> or CD56<sup>dim</sup>CD57<sup>+</sup>NKG2C<sup>-</sup> NK cells.  $p < 0.05$  was considered significant.

**Figure 4. NKG2C-mediated lysis of target cells by highly differentiated NK cells.** PBMC from HIV-1-infected donors were incubated with Fc-receptor-bearing P815 target cells for four hours in the absence and presence of anti-NKG2A, anti-NKG2C or anti-CD16 antibodies. (A) The graph depicts the redirected lysis of P815 cells induced by the engagement of the inhibitory receptor NKG2A and the activating receptors NKG2C and CD16. Columns represent medians. (B) The correlation between NKG2C-mediated redirected lysis and co-expression of CD57 and NKG2C within CD56<sup>dim</sup> NK cells is shown in the graph. Data was analysed with a non-parametric Spearman correlation.  $p < 0.05$  was considered significant. (C) PBMC from six HIV-1-infected donors were depleted of CD57<sup>+</sup> cells. The FACS plots depict the percentage of CD57<sup>+</sup> NK cells within the CD56<sup>dim</sup> NK cell population before and after CD57 depletion in a representative donor. (D) The graph shows the percentage of CD57<sup>+</sup> NK cells within the CD56<sup>dim</sup> NK cell population pre- and post-CD57-depletion in all six donors. (E) The graph depicts the redirected lysis of P815 cells through NKG2C in whole PBMC and CD57-depleted PBMC. (F) NK cells were enriched from PBMC from six HIV-1-infected donors. The graph shows the

percentage of NK cells within the lymphocyte population pre- and post-enrichment. (G) The graph depicts the lysis of P815 target cells by enriched NK cells in the absence of antibody or the presence of anti-NKG2C antibody. Donors with confirmed HCMV seropositivity are depicted in red. All donors had suppressed viremia. Data were compared with Wilcoxon matched-pairs signed rank tests.  $p < 0.05$  was considered significant.

**Figure 5. Phenotypic characterisation of highly differentiated CD57<sup>+</sup>NKG2C<sup>+</sup> NK cells in HIV-1-infected donors.** Flow cytometry was used to assess the phenotypic expression of NKG2A on CD56<sup>dim</sup> NK cells that were CD57<sup>-</sup>, CD57<sup>+</sup>NKG2C<sup>-</sup> and CD57<sup>+</sup>NKG2C<sup>+</sup>. (A) The graph depicts the percentage of NKG2A<sup>+</sup> NK cells within CD57<sup>-</sup> and CD57<sup>+</sup>NKG2C<sup>+/-</sup> CD56<sup>dim</sup> NK cells. Columns represent medians. Data was compared with a Friedman test followed with Dunn's multiple comparisons post-tests, which assessed differences between CD57<sup>+</sup>NKG2C<sup>+</sup> cells and CD57<sup>-</sup> or CD57<sup>+</sup>NKG2C<sup>-</sup> NK cells. (B) The graph shows the correlation between NKG2A expression and CD57/NKG2C co-expression in CD56<sup>dim</sup> NK cells. Donors with confirmed HCMV seropositivity are depicted in red. Donors with suppressed viremia are represented with circles, and those with detectable viremia are represented with triangles. Data were analysed with a non-parametric Spearman correlation.  $p < 0.05$  was considered significant.

**Table 1. Characteristics of study participants**

	HIV-1-uninfected (n=18)	HIV-1-infected (n=54) [MSHC cohort]	HIV-1-infected (n=10) [IVRN cohort]
Age – years			
Median	29.5	47	33 <sup>a</sup>
Range	21-51	21-84	35 <sup>b</sup>
Male gender – no. (%)	9 (50)	51 (94.4)	28-55 <sup>a</sup> 30-58 <sup>b</sup>
Viral load – HIV RNA copies/ml plasma			
Median	n/a	UD <sup>c</sup>	n/a
Range	n/a	UD-22,445 <sup>c</sup>	45,898 <sup>a</sup> UD <sup>b</sup> 5,400-194,949 <sup>a</sup> UD-145 <sup>b</sup>
HCMV seropositive – no.	13 of 18 tested	29 of 30 tested	10 of 10 tested
Months on ART			
Median	n/a	n/a	29 <sup>b</sup>
Range	n/a	n/a	21-43 <sup>b</sup>

UD: undetectable, ART: antiretroviral therapy, n/a: not applicable or not available

<sup>a</sup> At pre-ART blood collection<sup>b</sup> At post-ART blood collection<sup>c</sup> Five subjects had detectable viral loads (32, 75, 446, 3850 and 22,445 RNA copies/ml plasma)

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Figure 1

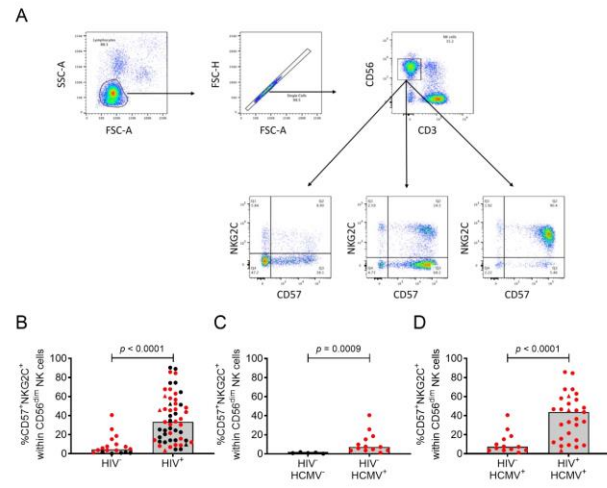


Figure 2

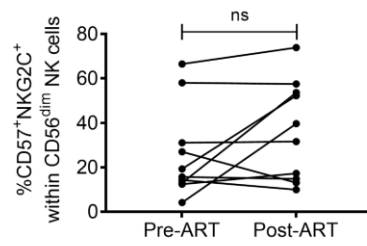


Figure 3

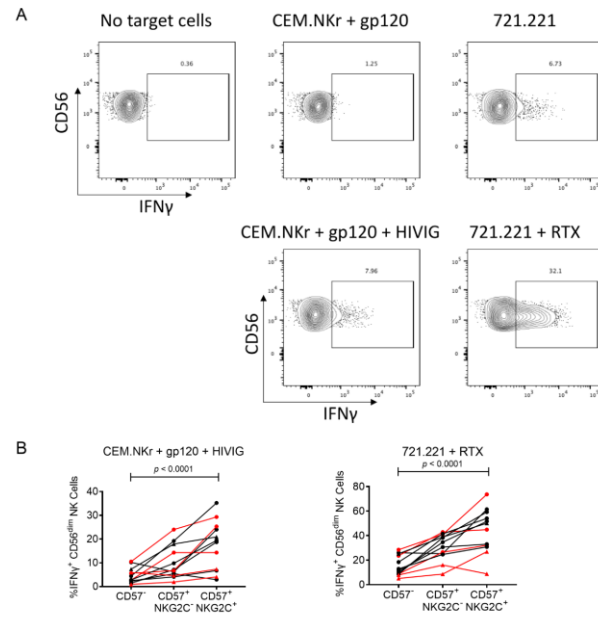


Figure 4

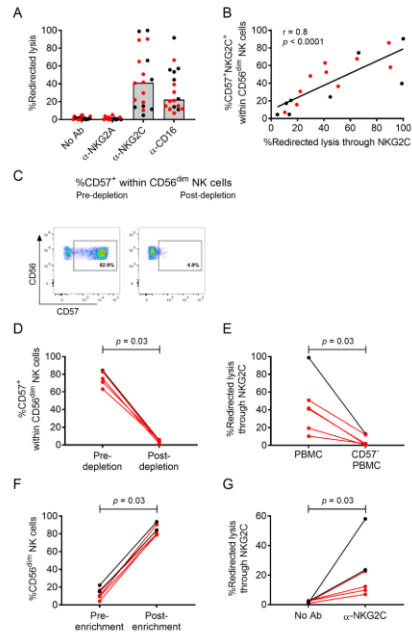


Figure 5

