Clinical and Experimental Immunology, 2022, **210**, 163–174
https://doi.org/10.1093/cei/uxac082
Advance access publication 2 September 2022
Research Article

Clinical & Experimental IMMUNOLOGY

OXFORD

Research Article

Phenotypic and functional characteristics of highly differentiated CD57⁺NKG2C⁺ NK cells in HIV-1-infected individuals

Anne B. Kristensen[1], Kathleen M. Wragg[1], Hillary A. Vanderven[1,2], Wen Shi Lee[1], Julie Silvers[3], Helen E. Kent[3], Michael D. Grant[4], Anthony D. Kelleher[5], Jennifer A. Juno[1], Stephen J. Kent[1,3,6] and Matthew S. Parsons[1,7,8,9,*]

[1]Department of Microbiology and Immunology, Peter Doherty Institute for Infection and Immunity, University of Melbourne, Melbourne, Victoria, Australia
[2]Biomedicine, College of Public Health, Medical and Veterinary Sciences, James Cook University, Douglas, Queensland, Australia
[3]Melbourne Sexual Health Centre and Department of Infectious Diseases, Alfred Hospital and Central Clinical School, Monash University, Melbourne, Victoria, Australia
[4]Immunology and Infectious Diseases Program, Division of BioMedical Sciences, Faculty of Medicine, Memorial University of Newfoundland, St. John's, Newfoundland and Labrador, Canada
[5]Kirby Institute, University of New South Wales, Sydney, New South Wales, Australia
[6]ARC Centre for Excellence in Convergent Bio-Nano Science and Technology, University of Melbourne, Parkville, Victoria, Australia
[7]Division of Microbiology and Immunology, Emory National Primate Research Center, Emory University, Atlanta, Georgia, USA
[8]Department of Pathology and Laboratory Medicine, Emory University School of Medicine, Atlanta, Georgia, USA
[9]Present affiliation: Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand; U.S. Military HIV Research Program, Walter Reed Army Institute of Research, Silver Spring, Maryland, USA; and Henry M. Jackson Foundation for the Advancement of Military Medicine, Bethesda, Maryland, USA

*Correspondence: Matthew S. Parsons, Department of Retrovirology, United States Army Medical Directorate - Armed Forces Research Institute of Medical Sciences (USAMD-AFRIMS), 315/6 Rajvithi Road, Bangkok 10400, Thailand. Email: parsonsm@hiv-th.org

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 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 HIV-1-uninfected individuals to determine the relative frequency of highly differentiated CD57⁺NKG2C⁺ NK cells and characterize these cells for their receptor expression and responsiveness to diverse stimuli. Highly differentiated CD57⁺NKG2C⁺ 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⁺NKG2C⁺ NK cell population in HIV-1-infected donors remained stable following antiretroviral therapy. CD57⁺NKG2C⁺ 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⁺NKG2C⁺ NK cells from HIV-1-infected donors were characterized by reduced expression of the inhibitory NKG2A receptor. The abundance of highly functional CD57⁺NKG2C⁺ 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; ART: antiretroviral therapy; DARTs: dual-affinity re-targeting molecules; HCMV: human cytomegalovirus; HIVIG: HIV immunoglobulin; IVRN: Immunovirology Research Network; KIR: killer immunoglobulin-like receptor; LDH: lactate dehydrogenase; MSHC: Melbourne Sexual Health Centre; MHC-I or HLA-I: major histocompatibility complex class I; NK: natural killer cell; PBMC: peripheral blood mononuclear cells; UD: undetectable.

Introduction

Natural killer (NK) cells are key mediators of anti-viral immune responses [1]. 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 FcγRIIIa/CD16 expressed on NK cells [2,3]. NK cells also express numerous inhibitory receptors that recognize various

Received 22 March 2022; Revised 7 August 2022; Accepted for publication 1 September 2022

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 [3]. 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 chemokines that inhibit viral replication by occluding the HIV-1 co-receptor, CCR5 [4–9]. 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 viremic 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) [10–12]. These alterations are largely reversed by suppression of viremia by antiretroviral therapy (ART) [11]. 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 [13, 14]. Such changes in NK cell differentiation are linked to the high prevalence of human cytomegalovirus (HCMV) co-infection in HIV-1-infected individuals (>80%) and the capacity of HCMV to shape the NK cell repertoire and drive NK cell differentiation [15–17].

In general, the differentiation of NK cells is reflected by the phenotypic transition from $CD56^{bright}CD16^{-/dim}CD57^{-}$ to $CD56^{dim}CD16^{+}CD57^{-}$ and finally $CD56^{dim}CD16^{++}CD57^{+}$ [18–20]. 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 $CD56^{bright}$ 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 [21, 22]. As NK cells differentiate, the percentage of cells expressing NKG2A decreases and the percentage of cells expressing KIR increases [18, 21]. Infection with HCMV has been shown to drive the expansion of a highly differentiated population of $CD56^{dim}CD57^{+}$ NK cells, which additionally express the NKG2C activating receptor that recognizes HLA-E [17]. These cells are highly responsive to stimulation through CD16 and mediate function through the gained NKG2C receptor [23]. An HCMV-dependent expansion of highly differentiated $CD57^{+}NKG2C^{+}$ NK cells has also been reported in HIV-1-infected donors [13]. 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 $CD57^{+}NKG2C^{+}$ 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 cytolysis through NKG2C and that highly differentiated $CD57^{+}NKG2C^{+}$ 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 $CD57^{+}NKG2C^{+}$ 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

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[a] 35[b]
Range	21–51	21–84	28–55[a] 30–58[b]
Male gender – no. (%)	9 (50)	51 (94.4)	n/a
Viral load – HIV RNA copies/ml plasma			
Median	n/a	UD[c]	45 898[a] UD[b]
Range	n/a	UD-22,445[c]	5400–194 949[a] UD-145[b]
HCMV seropositive – no.	13 of 18 tested	29 of 30 tested	10 of 10 tested
Months on ART			
Median	n/a	n/a	29[b]
Range	n/a	n/a	21–43[b]

IVRN: Immunovirology Research Network; MSHC: Melbourne Sexual Health Centre; n/a: not applicable or not available; UD: undetectable.
[a]At pre-ART blood collection
[b]At post-ART blood collection
[c]Five subjects had detectable viral loads (32, 75, 446, 3850, and 22 445 RNA copies/ml plasma)

HIV-1-uninfected donors by forearm venipuncture into sodium heparin containing Vacuettes. Characteristics of the subjects are given in Table 1. The Victorian Infectious Disease Reference Laboratory 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 given 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$^+$ 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 characterization of NK cells

To assess the expression of CD57, NKG2A and NKG2C on NK cells from HIV-1-infected and uninfected donors, 200 µl whole blood or 10^6 PBMC were stained with the following antibodies for 30 minutes at room temperature: anti-CD3 PerCP (clone SK7; BD Biosciences) or anti-CD3 BV785 (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$^-$, CD57$^+$NKG2C$^-$ and CD57$^+$NKG2C$^+$ NK cell subsets, donors with less than 100 total 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 [24, 25]. Briefly, CEM.NKr-CCR5 target cells were prepared by coating with HIV-1_{BaL} gp120 (3 µg/ml/10^6 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 (10 µg/ml/10^6 cells; Roche) for 60 minutes at 4°C. Freshly isolated PBMC (1×10^6) from HIV-1-infected donors were combined at a 5:1 ratio with either CEM.NKr-CCR5 cells (2×10^5) in the absence or presence of a 1:1000 dilution of pooled HIV immunoglobulin (HIVIG; NIH AIDS Reagent Program) or 721.221 cells (2×10^5) 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 HIV-1-uninfected donors and demonstrated NK cell activation to only occur in the presence of anti-HIV-1 antibodies [26]. Cells were incubated for 5 hours at 37°C. As a control, 10^6 PBMC were incubated alone. All incubations included brefeldin A (5 µg/ml; Sigma) and monensin (6 µg/ml; BD Biosciences). After incubation, cells were surface stained with anti-CD3 BV785 (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-IFNγ 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$^-$, CD57$^+$NKG2C$^-$ and CD57$^+$NKG2C$^+$ NK cell subsets, donors with less than 100 events in any of the three NK cell subsets were excluded from analysis.

Depletion of CD57$^+$ cells

Depletion of CD57$^+$ 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 [27], 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® Non-Radioactive Cytotoxicity Assay kit (Promega), as previously described [25]. Briefly, freshly isolated PBMC (2×10^5) from HIV-1-infected donors were combined with P815 target cells (1×10^4) at a 20:1 ratio and in the absence or presence of 5µg/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$^+$ cells, the number of CD57$^-$ PBMC that would be present in whole PBMC at 20:1 E:T ratio was combined with 1×10^4 P815 target cells. For experiments assessing the cytotoxicity of enriched NK cells, NK cells (5×10^4) were combined at a 5:1 ratio with P815 target cells (1×10^4). 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 × *g* for 4 minutes and incubated for 4 hours at 37°C. After incubation, plates were spun at 250 × *g* for 4 minutes and 50 µl/well of supernatant was transferred to an enzyme-linked immunosorbent assay plate (Thermo Fisher Scientific). Next, 50 µl 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 50 µl/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 by Dunn's post-test. Unpaired data were analyzed 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$^+$NKG2C$^+$ NK cell frequency in HIV-1-infected and HIV-1-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$^+$NKG2C$^+$ NK cells by flow cytometry. A flow cytometry panel allowed for the identification of CD3$^-$CD56^{dim} NK cells and determination of the relative frequency of CD57$^{+/-}$ NKG2C$^{+/-}$ subsets within each donor's NK cell population (Fig. 1A). Within both HIV-1-infected and HIV-1-uninfected donors a large range was noted in the percentage of NK cells that were CD57$^+$NKG2C$^+$. A significantly higher percentage of NK cells exhibited the CD57$^+$NKG2C$^+$ phenotype in HIV-1-infected than HIV-1-uninfected donors (median 33.6% [14.2–53.6%] vs. 4.4% [1.4–11.2%], $P < 0.0001$, Fig. 1B).

HCMV has a known major impact on the constitution and function of NK cell populations [16, 17, 23]. Among HIV-1-uninfected donors, serology revealed 13 HCMV-infected donors and 5 HCMV-uninfected donors. Consistent with previous publications [13, 17], we noted a significantly higher frequency of CD57$^+$NKG2C$^+$ 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%], $P = 0.0009$, Fig. 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$^+$NKG2C$^+$ NK cells than HIV-1-uninfected HCMV-infected donors (median 43.7% [15.1–57.5%] vs. 7.3% [3.7–16.9%], $P < 0.0001$, Fig. 1D). Comparison of the frequency of CD57$^+$NKG2C$^+$ NK cells in HIV-1-infected HCMV-infected donors to the frequency of CD57$^+$NKG2C$^+$ 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$^+$NKG2C$^+$ NK cells during HIV-1 infection [13]. Indeed, the frequency of CD57$^+$NKG2C$^+$ 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*. [13] 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$^+$NKG2C$^+$ NK cells. These data reaffirm that HCMV infection expands CD57$^+$NKG2C$^+$ NK cells and illustrate that an exaggeration of this expansion occurs in HIV-1/HCMV co-infected donors.

Impact of ART on CD57$^+$NKG2C$^+$ frequency in HIV-1-infected donors

As suppression of viremia by ART is linked to restored NK cell cytotoxicity and normalized expression of natural cytotoxicity receptors and KIR [11], we next investigated if the frequency of CD57$^+$NKG2C$^+$ NK cells was changed after prolonged ART. The cohort screened in Fig. 1 for CD57$^+$NKG2C$^+$ NK cells were sampled at a single time point and contained mostly ART-treated donors. As such, the data from donors in Fig. 1 provides no information regarding the impact of ART on CD57$^+$NKG2C$^+$ 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$^+$NKG2C$^+$ NK cells were noted between pre- and post-ART samples (median 17.5% [12.9–37.8%] vs. 35.7% [14.4–54.6%], $P = 0.24$, Fig. 2). These data demonstrate that expanded CD57$^+$NKG2C$^+$ NK cells persist in HIV-1-infected donors, even after prolonged suppression of viremia by ART.

Antibody-dependent functions of CD57$^+$NKG2C$^+$ NK cells from HIV-1-infected donors

NK cells can be activated by diverse stimuli [2, 3]. 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$^+$ NK cells mediate more robust functions through CD16 than CD57$^-$ NK cells [19, 28]. Furthermore, CD57$^+$NKG2C$^+$ NK cells from HCMV-infected individuals mediate robust function following stimulation through CD16 [23]. To determine if NK cell subsets (i.e. CD57$^-$ vs. CD57$^+$NKG2C$^-$ vs. CD57$^+$NKG2C$^+$) within HIV-1-infected individuals respond distinctly to antibody-dependent stimuli, we assessed the functions of NK cells from

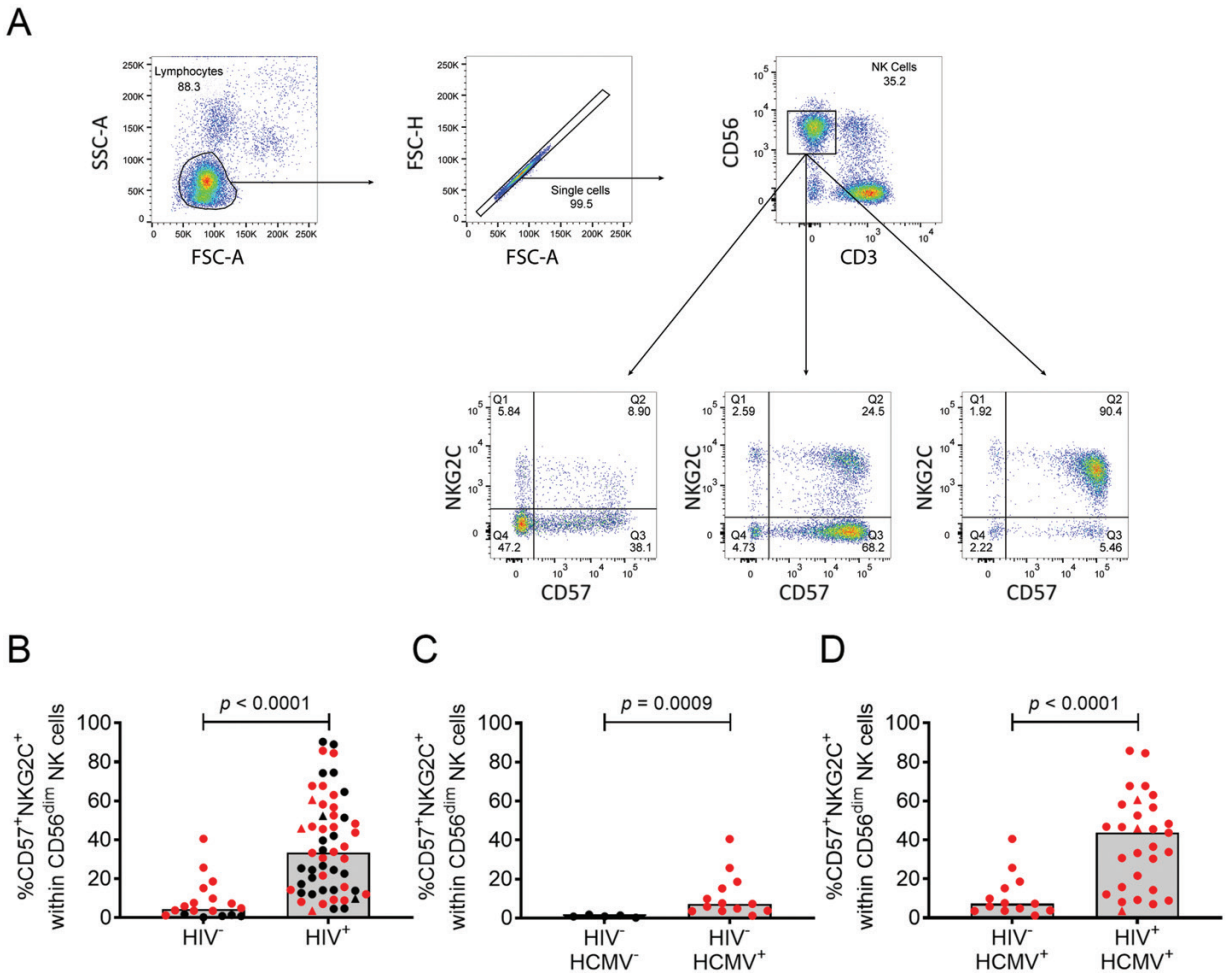

Figure 1: Expansion of highly differentiated CD57⁺NKG2C⁺ NK cells in donors infected with HIV-1, HCMV, or both viruses. Co-expression of CD57 and NKG2C on NK cells from 54 HIV-1-infected and 18 HIV-1-uninfected donors, with and without HCMV infection, was assessed by flow cytometry. (A) Gating strategy used to identify the expression of CD57 and NKG2C on $CD56^{dim}$ 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 $CD56^{dim}$ NK cells that co-express CD57 and NKG2C in (B) HIV-1-infected and HIV-1-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.

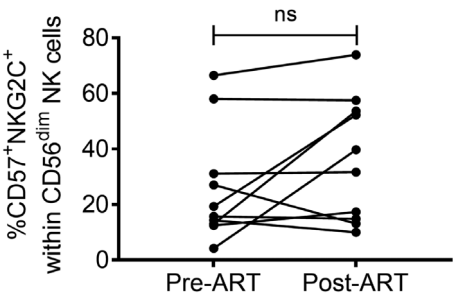

Figure 2: Impact of ART on the frequency of highly differentiated CD57⁺NKG2C⁺ NK cells in HIV-1-infected subjects. The frequency of highly differentiated NK cells in pre- and post-ART samples from 10 subjects infected with both HIV-1 and HCMV was evaluated by flow cytometry. Data were compared with a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

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 IFNγ. 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 (3850 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 IFNγ 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⁺NKG2C⁺ subset was the most activated subset. CD57⁺NKG2C⁺ NK cells were significantly more activated than $CD57^-$ NK cells following anti-HIV-1 antibody-dependent stimulation ($P < 0.0001$) and Rituximab-dependent

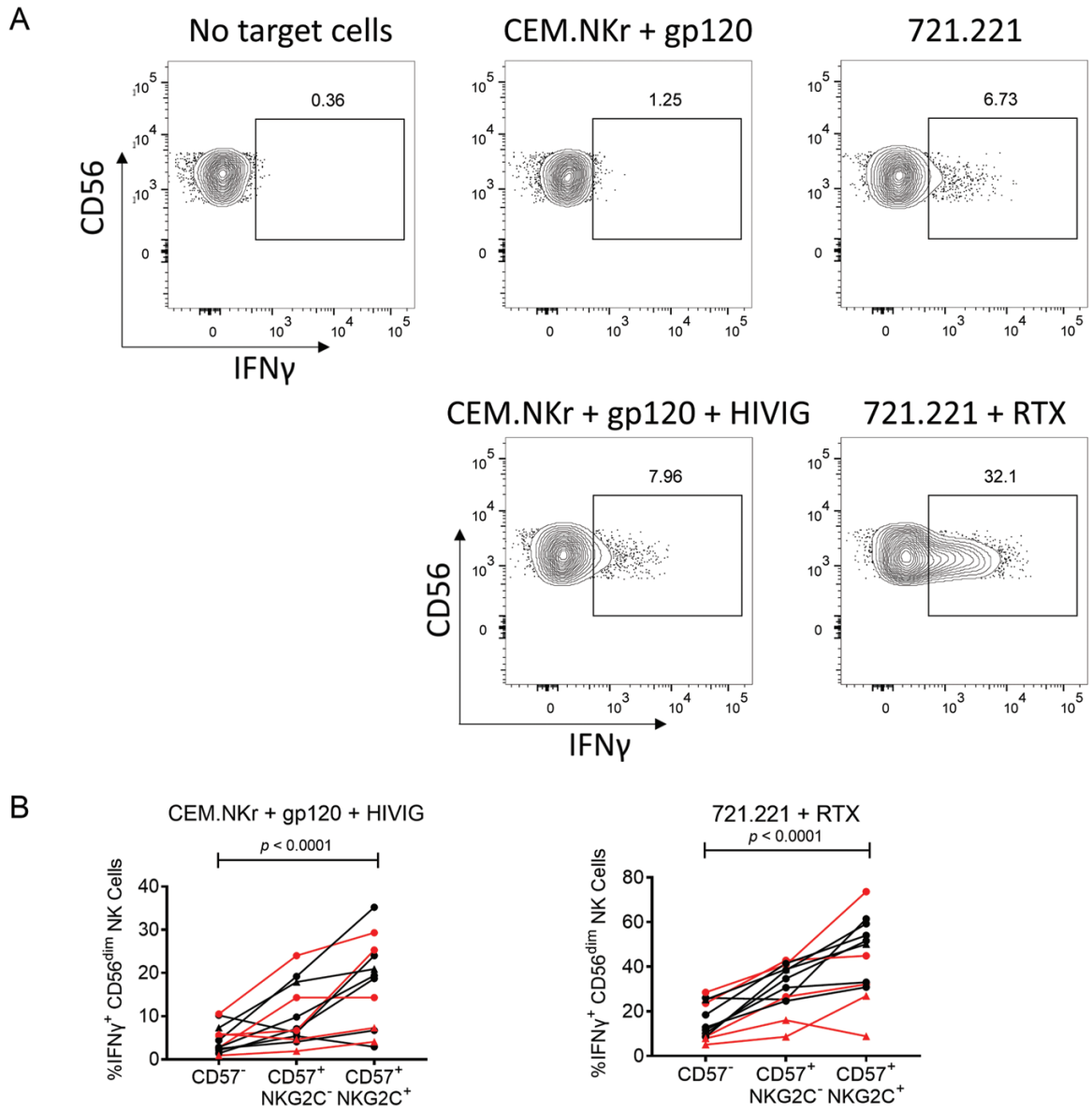

Figure 3: Functional characteristics of highly differentiated CD57⁺NKG2C⁺ NK cells from HIV-1-infected donors. NK cell activation was measured by flow cytometry as the percentage of $CD56^{dim}$ NK cells expressing IFNγ after antibody-dependent stimulation. (A) FACS plots showing the gating on $IFN\gamma^{+}$ $CD56^{dim}$ NK cells in PBMC incubated 5 hours in the absence of target cells, with $HIV\text{-}1_{BaL}$ 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 $IFN\gamma^{+}$ cells within $CD56^{dim}CD57^{-}$, $CD56^{dim}CD57^{+}NKG2C^{-}$ and $CD56^{dim}CD57^{+}NKG2C^{+}$ 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 by Dunn's post-tests to assess differences between $CD56^{dim}CD57^{+}NKG2C^{+}$ cells and $CD56^{dim}CD57^{-}$ or $CD56^{dim}CD57^{+}NKG2C^{-}$ NK cells. $P < 0.05$ was considered significant.

stimulation ($P < 0.0001$, Fig. 3B). Although the data presented in Fig. 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, $CD57^{+}NKG2C^{+}$ NK cells were significantly more activated than $CD57^{-}$ NK cells following background subtraction ($P < 0.0001$). Collectively, our data imply that within HIV-1-infected individuals $CD57^{+}NKG2C^{+}$ NK cells are potent mediators of antibody-dependent functions.

NKG2C-dependent redirected cytolysis by $CD57^{+}NKG2C^{+}$ NK cells from HIV-1-infected donors

The NKG2C receptor is an activating receptor with specificity for HLA-E [22]. The HLA-E ligand represents a potentially interesting target on HIV-1-infected cells, as some HIV-1 strains upregulate HLA-E expression [29, 30]. Expanded $CD57^{+}NKG2C^{+}$ NK cells from HCMV-infected donors are activated following NKG2C crosslinking [23]. To determine if $CD57^{+}NKG2C^{+}$ NK cells from HIV-1-infected donors could

mediate cytolysis through NKG2C, we performed redirected cytolysis 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 Fig. 4A, we observed a range of cytolysis 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$^+$NKG2C$^+$ phenotype (r = 0.8, P < 0.0001, Fig. 4B).

To assess if CD57$^+$NKG2C$^+$ 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$^+$ 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 (Fig. 4C). The depletions were robust, eliminating a majority of CD57$^+$ NK cells across the six donors (median 93.8%, Fig. 4D). Depletion of CD57$^+$ cells dramatically reduced redirected lysis of P815 through NKG2C (median 91.3% reduction in cytotoxicity [82.7–97.2%]; P = 0.03, Fig. 4E), highlighting an important role for CD57$^+$NKG2C$^+$ cells in the cytotoxic response.

As NKG2C can also be expressed on T cells [31], we also performed redirected cytolysis 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 Fig. 4F, NK cell enrichments were successful across all six donors (pre-enrichment: median 12.8% [8.2–17.8%] vs. post-enrichment: median 82.3% [79.6–91.3%], P = 0.03). As shown in Fig. 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$^+$NKG2C$^+$ 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$^-$ to CD57$^+$ but to involve the loss of NKG2A expression and the gain of KIR expression [18, 21]. As such, we assessed if the expansion of CD57$^+$NKG2C$^+$ 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 the MSHC cohort of HIV-1-infected donors by flow cytometry. As shown in Fig. 5A, we noted that NKG2A was expressed on a low proportion of the CD57$^+$NKG2C$^+$ NK cell subset, which contained significantly fewer NKG2A$^+$ NK cells than CD57$^-$ and CD57$^+$NKG2C$^-$ NK cells (P < 0.0001 for both). Consistent with the observation that the CD57$^+$NKG2C$^+$ NK cell subset contained the fewest NKG2A$^+$ NK cells, we noted a robust inverse correlation between the frequency of CD57$^+$NKG2C$^+$ NK cells and the percentage of total NK cells that expressed NKG2A (P < 0.0001, Fig. 5B). Furthermore, we observed significantly fewer NKG2A$^+$ 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$^+$NKG2C$^+$ 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$^+$NKG2C$^+$ NK cell frequencies in HCMV infected donors, which are significantly exaggerated in HCMV-seropositive HIV-1-infected donors. Within HIV-1-infected individuals, the CD57$^+$NKG2C$^+$ NK cell subset remained consistent in frequency throughout ART and exhibited a unique phenotypic and functional profile. Indeed, CD57$^+$NKG2C$^+$ NK cells mediated robust antibody-dependent functions, and CD57$^+$NKG2C$^+$ NK cells were also able to mediate cytotoxicity following ligation of NKG2C. Lastly, significantly fewer CD57$^+$NKG2C$^+$ NK cells expressed NKG2A than the CD57$^-$ and CD57$^+$NKG2C$^-$ NK cell subsets.

In the current report, we have broadened the characterization of expanded CD57$^+$NKG2C$^+$ 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 showing that CD57$^+$NKG2C$^+$ NK cells persist during ART. This observation is consistent with a previous study assessing the frequency of NKG2C$^+$ NK cells in HIV-1-infected donors prior to and following ART, as well as a study comparing NKG2C$^+$ NK cell frequency between ART-treated and -untreated HIV-1-infected donors [32, 33]. For a small proportion of donors, we noted that CD57$^+$NKG2C$^+$ NK cells increased in frequency following ART. Post-ART expansion of CD57$^+$NKG2C$^+$ NK cells has been previously reported [34]. The clear conclusion from the accumulated data is that many HIV-1-infected donors have an expanded CD57$^+$NKG2C$^+$ NK cell 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$^+$ NK cells expressing high or low levels of NKG2C from ART-treated HIV-1-infected individuals exhibit robust antibody-dependent activation following stimulation with gp120-pulsed target cells coated with anti-HIV-1 antibodies [8]. Consistent with this observation, CD57$^+$NKG2C$^+$ NK cells from HIV-1-infected donors exhibit transcriptional profiles linked to ADCC [35]. However, more research is required to understand the full scope of anti-viral functions mediated by CD57$^+$NKG2C$^+$ NK cells. It will be important to establish the capacity of CD57$^+$NKG2C$^+$ NK cells stimulated via direct

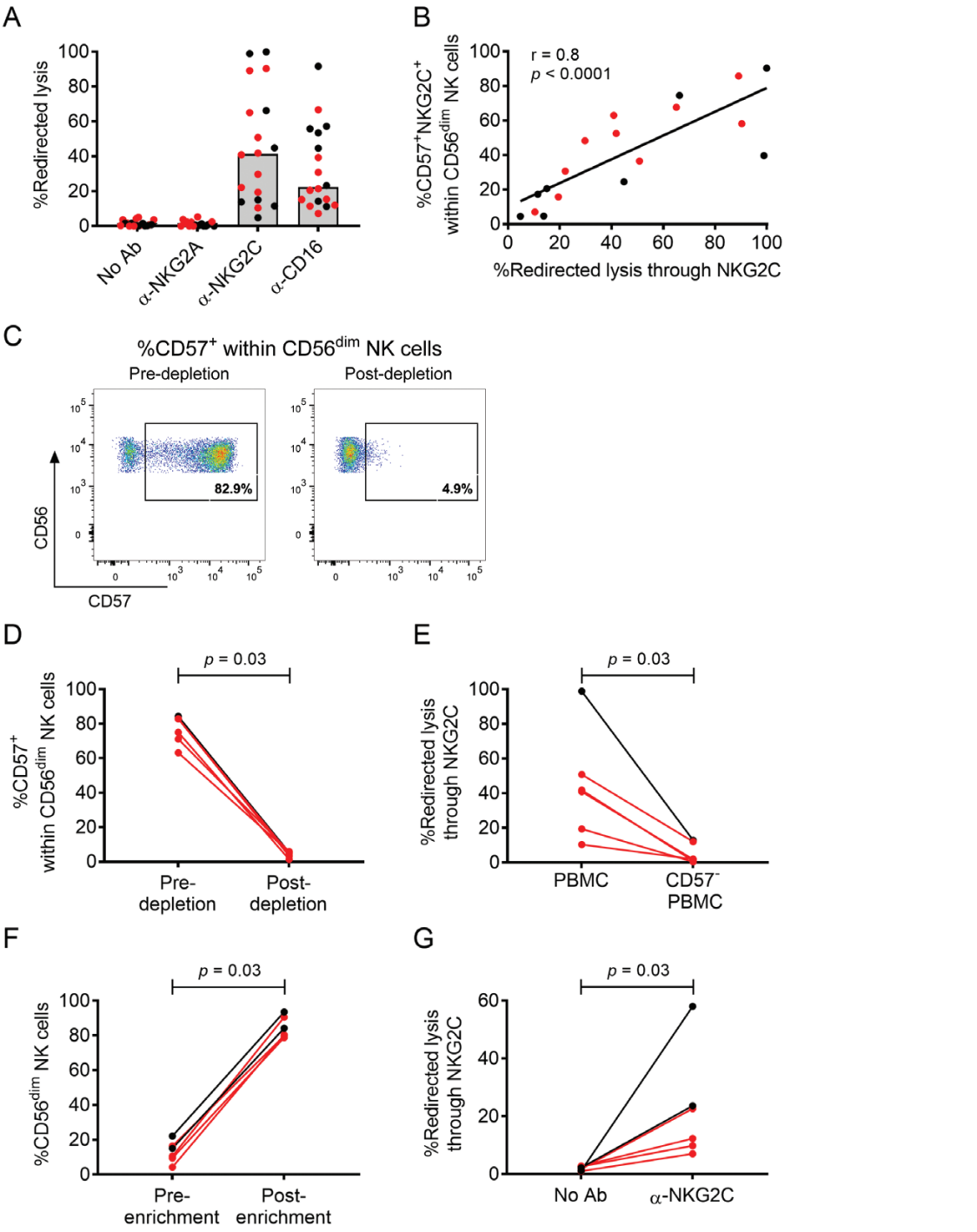

Figure 4: NKG2C-mediated redirected lysis of target cells by highly differentiated NK cells. PBMC from HIV-1-infected donors were incubated with Fc-receptor-bearing P815 target cells for four hours in the absence or presence of anti-NKG2A, anti-NKG2C or anti-CD16 antibodies. (A) The graph depicts the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

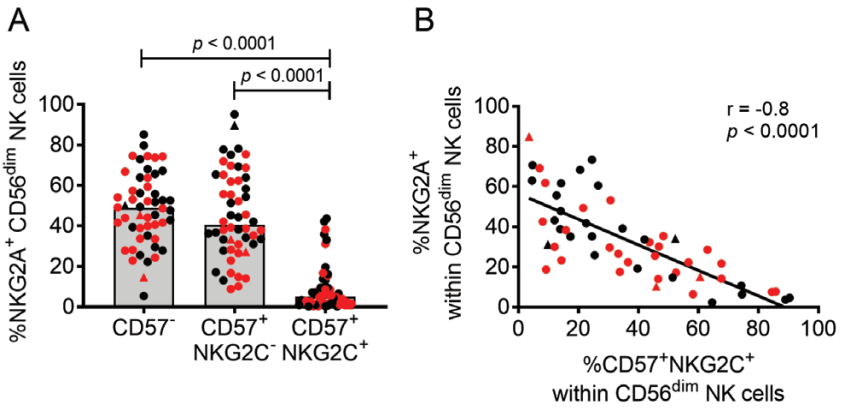

Figure 5: Phenotypic characterization of highly differentiated CD57⁺NKG2C⁺ NK cells in HIV-1-infected donors. Flow cytometry was used to assess the expression of NKG2A on $CD56^{dim}$ NK cells that were $CD57^-$, $CD57^+NKG2C^-$ and $CD57^+NKG2C^+$. (A) The graph depicts the percentage of NKG2A⁺ NK cells within $CD57^-$ and $CD57^+NKG2C^{+/-}$ $CD56^{dim}$ NK cells. Columns represent medians. Data were compared with a Friedman test followed by Dunn's post-tests, which assessed differences between $CD57^+NKG2C^+$ and $CD57^-$ or $CD57^+NKG2C^-$ NK cells. (B) The graph shows the correlation between NKG2A expression and CD57/NKG2C co-expression on $CD56^{dim}$ 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 analyzed using a non-parametric Spearman correlation. $P < 0.05$ was considered significant.

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 $CD57^+NKG2C^+$ 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 $CD57^+NKG2C^+$ NK cells and the development of dysfunctional $CD56^-CD16^+$ NK cells [12]. Finally, it will be important to determine if $CD57^+NKG2C^+$ NK cells from HIV-1-infected individuals exhibit similar functional profiles to cells with similar phenotypes from HIV-1-uninfected donors.

The expansion of $CD57^+NKG2C^+$ 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.* recently noted that enhanced HLA-A surface expression is linked with higher viral loads and poorer CD4 counts [36]. The authors demonstrated that enhanced HLA-A expression increases 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 exhibiting increased HLA-A expression can inhibit the degranulation of $NKG2A^+$ NK cells - presumably due to enhanced HLA-E expression. The authors downplayed a role for NKG2C, citing previous literature showing that NKG2A signaling dominates if both NKG2A and NKG2C are expressed on the same cell [37]. We now provide evidence, however, that many HIV-1-infected individuals gain $CD57^+NKG2C^+$ NK cells and lose $NKG2A^+$ NK cells (Figs 1 and 5). Indeed, among the participants of the current study, HIV-1-infected donors had fewer $NKG2A^+$ total NK cells than HIV-1-uninfected donors ($P < 0.01$). In HIV-1-infected donors that have lost $NKG2A^+$ NK cells, it is difficult to foresee a role for NKG2A/HLA-E interactions in advancing HIV-1 disease progression. In fact, expanded $CD57^+NKG2C^+NKG2A^-$ NK cells from HIV-1-infected individuals might serve as better effector cells than $NKG2A^+$ NK cells for responding to HIV-1-infected target cells. Interestingly, higher frequencies of $NKG2C^+NKG2A^-$ NK cells has previously been linked to lower viral load set point [38]. Furthermore, Gondois-Rey *et al.* observed an inverse association between the frequency of $CD57^+NKG2C^+$ NK cells and viral load in primary HIV-1 infection [39]. In contrast, Alsulami *et al.* noted no relationship between the frequency of $NKG2C^+$ NK cells and viral load setpoint [40]. 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 $NKG2A^+$ cells versus individuals with NK cell populations consisting largely of $CD57^+NKG2C^+NKG2A^-$ cells.

The potential negative contribution of NKG2A to HIV-1 disease progression might partly explain the exaggerated expansion of $CD57^+NKG2C^+$ NK cells in HIV-1-infected donors. We hypothesize that the enhanced expansion of $CD57^+NKG2C^+$ NK cells in HIV-1-infected individuals occurs through a multistep process. Initially, individuals infected with HCMV generate populations of less differentiated $CD57^-NKG2C^+$ and more differentiated $CD57^+NKG2C^+$ 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 $NKG2C^+$ NK cells are less likely to express NKG2A (Fig. 5) and NKG2A negatively contributes to NK cell responsiveness to HIV-1-infected cells [36], we predict $NKG2C^+$ NK cells with proliferation potential would be likely to expand following HIV-1 infection and ultimately contribute to the $CD57^+NKG2C^+$ NK cell population. This hypothetical pathway for the exaggerated expansion of $CD57^+NKG2C^+$ 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.* demonstrated NK cell expression of NKG2A and NKG2C can be distinguish in rhesus macaques using RNA-based flow cytometry [41].

Attempts to design immune-based therapeutics and/or cure strategies for HIV-1 might benefit from the utilization of expanded $CD57^+NKG2C^+$ NK cells. Indeed, the enhanced antibody-dependent functions of highly differentiated NK cells could be engaged to enhance the anti-viral potential of

passively infused therapeutic broadly neutralizing antibodies [42, 43]. Another putative therapeutic/cure strategy under consideration for HIV-1 are dual-affinity re-targeting molecules (DARTs). DARTs contain an anti-HIV-1 binding arm linked to an arm capable of engaging a receptor on an effector cell that can trigger cytolysis (i.e. T-cell receptor/CD3) [44]. Given the potent cytolytic potential of CD57$^+$NKG2C$^+$ 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, the accumulation of terminally differentiated T cells and selective expansion of a distinct NK cell subset. While these processes occur in parallel in people living with HIV-1, driven by similar features [13], they may have dichotomous effects. The terminal differentiation of HCMV-specific T cells is associated with shortened telomeres and progress toward replicative senescence [45]. Accumulation of senescent cells is a potential contributor to persistent inflammation and reduced resilience in HIV-1-infected individuals co-infected with HCMV [15, 46, 47]. In contrast, the expanded subset of CD57$^+$NKG2C$^+$ NK cells in HIV-1-infected individuals co-infected with HCMV shows no selective evidence of extensive replication and progress toward replicative senescence [45]. 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$^+$NKG2C$^+$ NK cell subset in HIV-1 infection represents a potentially powerful effector cell population for monoclonal anti-HIV-1 broadly neutralizing antibody therapies [42, 43]. 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$^+$NKG2C$^+$ NK cells.

Acknowledgements

We want to acknowledge the contribution of blood samples by the study participants. We thank Dr. Andrew G Brooks (University of Melbourne) for providing the 721.221 and P815 cell lines.

Funding

This work was supported by a National Health and Medical Research Council Program Grant to Stephen J Kent (APP1149990). This work was also supported by the National Institutes of Health's Office of the Director, Office of Research Infrastructure Programs (P51OD011132). Lastly, the work was supported by a K01 Award to Matthew Sidney Parsons from the National Institutes of Health's Office of the Director (K01OD031900). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Conflict of interest

None declared.

Author contributions

Conception: S.J.K. and M.S.P. Experimental work and data analysis: A.B.K., K.M.W., H.A.V., W.S.L., J.S., H.E.K., A.D.K., and J.A.J. Manuscript preparation, editing, and review: A.B.K., M.D.G., S.J.K., and M.S.P.

Ethical approval

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

Data availability

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

References

1. Waggoner SN, Reighard SD, Gyurova IE, Cranert SA, Mahl SE, Karmele EP, et al. Roles of natural killer cells in antiviral immunity. *Curr Opin Virol* 2016, 16, 15–23. doi:10.1016/j.coviro.2015.10.008
2. Biron CA, Nguyen KB, Pien GC, Cousens LP, Salazar-Mather TP. Natural killer cells in antiviral defense: function and regulation by innate cytokines. *Annu Rev Immunol* 1999, 17, 189–220. doi:10.1146/annurev.immunol.17.1.189
3. Lanier LL. NK cell recognition. *Annu Rev Immunol* 2005, 23, 225–74. doi:10.1146/annurev.immunol.23.021704.115526
4. Bonaparte MI, Barker E. Killing of human immunodeficiency virus-infected primary T-cell blasts by autologous natural killer cells is dependent on the ability of the virus to alter the expression of major histocompatibility complex class I molecules. *Blood* 2004, 104, 2087–94. doi:10.1182/blood-2004-02-0696
5. Boudreau JE, Mulrooney TJ, Le Luduec JB, Barker E, Hsu KC. KIR3DL1 and HLA-B density and binding calibrate NK education and response to HIV. *J Immunol* 2016, 196, 3398–410. doi:10.4049/jimmunol.1502469
6. Bruel T, Guivel-Benhassine F, Amraoui S, Malbec M, Richard L, Bourdic K, et al. Elimination of HIV-1-infected cells by broadly neutralizing antibodies. *Nat Commun* 2016, 7, 10844. doi:10.1038/ncomms10844
7. Song R, Lisovsky I, Lebouche B, Routy JP, Bruneau J, Bernard NF. HIV protective KIR3DL1/S1-HLA-B genotypes influence NK cell-mediated inhibition of HIV replication in autologous CD4 targets. *PLoS Pathog* 2014, 10, e1003867. doi:10.1371/journal.ppat.1003867
8. Tomescu C, Kroll K, Colon K, Papasavvas E, Frank I, Tebas P, et al. Identification of the predominant human NK cell effector subset mediating ADCC against HIV-infected targets coated with BNAbs or plasma from PLWH. *Eur J Immunol* 2021, 51, 2051–61. doi:10.1002/eji.202149188
9. Ward JP, Bonaparte MI, Barker E. HLA-C and HLA-E reduce antibody-dependent natural killer cell-mediated cytotoxicity of HIV-infected primary T cell blasts. *AIDS* 2004, 18, 1769–79. doi:10.1097/00002030-200409030-00005
10. De Maria A, Fogli M, Costa P, Murdaca G, Puppo F, Mavilio D, et al. The impaired NK cell cytolytic function in viremic HIV-1 infection is associated with a reduced surface expression of natural cytotoxicity receptors (NKp46, NKp30 and NKp44). *Eur J Immunol* 2003, 33, 2410–8. doi:10.1002/eji.200324141
11. Mavilio D, Benjamin J, Daucher M, Lombardo G, Kottilil S, Planta MA, et al. Natural killer cells in HIV-1 infection: dichotomous

effects of viremia on inhibitory and activating receptors and their functional correlates. *Proc Natl Acad Sci USA* 2003, 100, 15011–6. doi:10.1073/pnas.2336091100
12. Mavilio D, Lombardo G, Benjamin J, Kim D, Follman D, Marcenaro E, et al. Characterization of CD56-/CD16+ natural killer (NK) cells: a highly dysfunctional NK subset expanded in HIV-infected viremic individuals. *Proc Natl Acad Sci USA* 2005, 102, 2886–91. doi:10.1073/pnas.0409872102
13. Heath J, Newhook N, Comeau E, Gallant M, Fudge N, Grant M. NKG2C(+)CD57(+) natural killer cell expansion parallels cytomegalovirus-specific CD8(+) T cell evolution towards senescence. *J Immunol Res* 2016, 2016, 7470124. doi:10.1155/2016/7470124
14. Peppa D, Pedroza-Pacheco I, Pellegrino P, Williams I, Maini MK, Borrow P. Adaptive reconfiguration of natural killer cells in HIV-1 infection. *Front Immunol* 2018, 9, 474. doi:10.3389/fimmu.2018.00474
15. Barrett L, Stapleton SN, Fudge NJ, Grant MD. Immune resilience in HIV-infected individuals seronegative for cytomegalovirus. *AIDS* 2014, 28, 2045–9. doi:10.1097/QAD.0000000000000405
16. Guma M, Angulo A, Vilches C, Gomez-Lozano N, Malats N, Lopez-Botet M. Imprint of human cytomegalovirus infection on the NK cell receptor repertoire. *Blood* 2004, 104, 3664–71. doi:10.1182/blood-2004-05-2058
17. Lopez-Verges S, Milush JM, Schwartz BS, Pando MJ, Jarjoura J, York VA, et al. Expansion of a unique CD57(+)NKG2Chi natural killer cell subset during acute human cytomegalovirus infection. *Proc Natl Acad Sci USA* 2011, 108, 14725–32. doi:10.1073/pnas.1110900108
18. Bjorkstrom NK, Riese P, Heuts F, Andersson S, Fauriat C, Ivarsson MA, et al. Expression patterns of NKG2A, KIR, and CD57 define a process of CD56dim NK-cell differentiation uncoupled from NK-cell education. *Blood* 2010, 116, 3853–64. doi:10.1182/blood-2010-04-281675
19. Lopez-Verges S, Milush JM, Pandey S, York VA, Arakawa-Hoyt J, Pircher H, et al. CD57 defines a functionally distinct population of mature NK cells in the human CD56dimCD16+ NK-cell subset. *Blood* 2010, 116, 3865–74. doi:10.1182/blood-2010-04-282301
20. Solana R, Tarazona R, Gayoso I, Lesur O, Dupuis G, Fulop T. Innate immunosenescence: effect of aging on cells and receptors of the innate immune system in humans. *Semin Immunol* 2012, 24, 331–41. doi:10.1016/j.smim.2012.04.008
21. Beziat V, Descours B, Parizot C, Debre P, Vieillard V. NK cell terminal differentiation: correlated stepwise decrease of NKG2A and acquisition of KIRs. *PLoS One* 2010, 5, e11966. doi:10.1371/journal.pone.0011966
22. Braud VM, Allan DS, O'Callaghan CA, Soderstrom K, D'andrea A, Ogg GS, et al. HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C. *Nature* 1998, 391, 795–9. doi:10.1038/35869
23. Wu Z, Sinzger C, Frascaroli G, Reichel J, Bayer C, Wang L, et al. Human cytomegalovirus-induced NKG2C(hi) CD57(hi) natural killer cells are effectors dependent on humoral antiviral immunity. *J Virol* 2013, 87, 7717–25. doi:10.1128/JVI.01096-13
24. Kristensen AB, Kent SJ, Parsons MS. Contribution of NK cell education to both direct and anti-HIV-1 antibody-dependent NK cell functions. *J Virol* 2018, 92, e02146–17. doi:10.1128/JVI.02146-17
25. Selva KJ, Kent SJ, Parsons MS. Modulation of innate and adaptive cellular immunity relevant to HIV-1 vaccine design by seminal plasma. *AIDS* 2017, 31, 333–42. doi:10.1097/QAD.0000000000001319
26. Gooneratne SL, Center RJ, Kent SJ, Parsons MS. Functional advantage of educated KIR2DL1(+) natural killer cells for anti-HIV-1 antibody-dependent activation. *Clin Exp Immunol* 2016, 184, 101–9. doi:10.1111/cei.12752
27. Parsons MS, Richard J, Lee WS, Vanderven H, Grant MD, Finzi A, et al. NKG2D acts as a co-receptor for natural killer cell-mediated anti-HIV-1 antibody-dependent cellular cytotoxicity. *AIDS Res Hum Retroviruses* 2016, 32, 1089–96. doi:10.1089/AID.2016.0099
28. Parsons MS, Loh L, Gooneratne S, Center RJ, Kent SJ. Role of education and differentiation in determining the potential of natural killer cells to respond to antibody-dependent stimulation. *AIDS* 2014, 28, 2781–6. doi:10.1097/QAD.0000000000000489
29. Martini F, Agrati C, D'Offizi G, Poccia F. HLA-E up-regulation induced by HIV infection may directly contribute to CD94-mediated impairment of NK cells. *Int J Immunopathol Pharmacol* 2005, 18, 269–76. doi:10.1177/039463200501800209
30. Nattermann J, Nischalke HD, Hofmeister V, Kupfer B, Ahlenstiel G, Feldmann G, et al. HIV-1 infection leads to increased HLA-E expression resulting in impaired function of natural killer cells. *Antivir Ther* 2005, 10, 95–107. doi:10.1177/135965350501000107
31. Guma M, Busch LK, Salazar-Fontana LI, Bellosillo B, Morte C, Garcia P, et al. The CD94/NKG2C killer lectin-like receptor constitutes an alternative activation pathway for a subset of CD8+ T cells. *Eur J Immunol* 2005, 35, 2071–80. doi:10.1002/eji.200425843
32. Brunetta E, Fogli M, Varchetta S, Bozzo L, Hudspeth KL, Marcenaro E, et al. Chronic HIV-1 viremia reverses NKG2A/NKG2C ratio on natural killer cells in patients with human cytomegalovirus co-infection. *AIDS* 2010, 24, 27–34. doi:10.1097/QAD.0b013e3283328d1f
33. Mela CM, Burton CT, Imami N, Nelson M, Steel A, Gazzard BG, et al. Switch from inhibitory to activating NKG2 receptor expression in HIV-1 infection: lack of reversion with highly active antiretroviral therapy. *AIDS* 2005, 19, 1761–9. doi:10.1097/01.aids.0000183632.12418.33
34. Kared H, Martelli S, Tan SW, Simoni Y, Chong ML, Yap SH, et al. Adaptive NKG2C(+)CD57(+) natural killer cell and Tim-3 expression during viral infections. *Front Immunol* 2018, 9, 686. doi:10.3389/fimmu.2018.00686
35. Costanzo MC, Kim D, Creegan M, Lal KG, Ake JA, Currier JR, et al. Transcriptomic signatures of NK cells suggest impaired responsiveness in HIV-1 infection and increased activity post-vaccination. *Nat Commun* 2018, 9, 1212. doi:10.1038/s41467-018-03618-w
36. Ramsuran V, Naranbhai V, Horowitz A, Qi Y, Martin MP, Yuki Y, et al. Elevated HLA-A expression impairs HIV control through inhibition of NKG2A-expressing cells. *Science* 2018, 359, 86–90. doi:10.1126/science.aam8825
37. Beziat V, Hervier B, Achour A, Boutolleau D, Marfain-Koka A, Vieillard V. Human NKG2A overrides NKG2C effector functions to prevent autoreactivity of NK cells. *Blood* 2011, 117, 4394–6. doi:10.1182/blood-2010-11-319194
38. Ma M, Wang Z, Chen X, Tao A, He L, Fu S, et al. NKG2C(+) NKG2A(-) natural killer cells are associated with a lower viral set point and may predict disease progression in individuals with primary HIV infection. *Front Immunol* 2017, 8, 1176. doi:10.3389/fimmu.2017.01176
39. Gondois-Rey F, Cheret A, Granjeaud S, Mallet F, Bidaut G, Lecuroux C, et al. NKG2C(+) memory-like NK cells contribute to the control of HIV viremia during primary infection: Optiprim-ANRS 147. *Clin Transl Immunology* 2017, 6, e150. doi:10.1038/cti.2017.22
40. Alsulami K, Bolastig N, Dupuy FP, Mabanga T, Gilbert L, Kiani Z, et al.. Influence of NKG2C genotypes on HIV susceptibility and viral load set point. *J Virol* 2021, 95, e0041721. doi:10.1128/JVI.00417-21
41. Ram DR, Manickam C, Hueber B, Itell HL, Permar SR, Varner V, et al. Tracking KLRC2 (NKG2C)+ memory-like NK cells in SIV+ and rhCMV+ rhesus macaques. *PLoS Pathog* 2018, 14, e1007104. doi:10.1371/journal.ppat.1007104
42. Caskey M, Klein F, Lorenzi JC, Seaman MS, West AP Jr., Buckley N, et al. Viraemia suppressed in HIV-1-infected humans by broadly neutralizing antibody 3BNC117. *Nature* 2015, 522, 487–91. doi:10.1038/nature14411
43. Caskey M, Schoofs T, Gruell H, Settler A, Karagounis T, Kreider EF, et al. Antibody 10-1074 suppresses viremia in HIV-1-infected individuals. *Nat Med* 2017, 23, 185–91. doi:10.1038/nm.4268

44. Sloan DD, Lam CY, Irrinki A, Liu L, Tsai A, Pace CS, et al. Targeting HIV reservoir in infected CD4 T cells by dual-affinity re-targeting molecules (DARTs) that bind HIV envelope and recruit cytotoxic T cells. *PLoS Pathog* 2015, 11, e1005233. doi:10.1371/journal.ppat.1005233
45. Heath JJ, Fudge NJ, Gallant ME, Grant MD. Proximity of cytomegalovirus-specific CD8(+) T cells to replicative senescence in human immunodeficiency virus-infected individuals. *Front Immunol* 2018, 9, 201. doi:10.3389/fimmu.2018.00201
46. Barrett L, Fudge NJ, Heath JJ, Grant MD. Cytomegalovirus immunity and exhaustive CD8+ T cell proliferation in treated human immunodeficiency virus infection. *Clin Infect Dis* 2016, 62, 1467–8. doi:10.1093/cid/ciw148
47. Freeman ML, Mudd JC, Shive CL, Younes SA, Panigrahi S, Sieg SF, et al. CD8 T-cell expansion and inflammation linked to CMV coinfection in ART-treated HIV infection. *Clin Infect Dis* 2016, 62, 392–6. doi:10.1093/cid/civ840