Response to reviewer's comments

Reviewer 1

1. The experiments were performed using an HIV envelope-coated T cell line as targets. This is a major limitation as this system lacks interactions that would be expected between the multitude of NK cell inhibitory and activating receptors in an autologous system. The authors acknowledge this limitation in the discussion, but it does greatly diminish the weight of the conclusions that can be drawn.

Response: The reviewer has raised an interesting issue regarding the data presented in our manuscript. Since the initial submission we have performed additional experiments to assess the ability of educated KIR2DL1^{+} NK cells to respond to anti-HIV-1 antibody-dependent activation against autologous target cells. The new Figure 3 displays data obtained from a whole blood anti-HIV-1 antibody-dependent NK cell activation assay, which measures the activation of NK cells by autologous CD4^{+} targets coated with gp140 and anti-HIV-1 antibodies. As shown in the new Figure 3, we now demonstrate that in 4/5 HLA-C2 carriers the activation advantage of KIR2DL1^{+} NK cells is sustained, despite the presence of the HLA-C2 ligand for the inhibitory KIR2DL1 receptor, against autologous targets. (see Figure 3; Methods Pages 7-8; Results pages 11-12; discussion page 14).

2. The authors cite several studies suggesting a role for ADCC in protection/control of HIV. The submitted manuscript only addresses the ability of antibodies to activate NK cells (observed as intracellular IFN accumulation). ADCC is not investigated and thus the study feels incomplete.

Response: In a recent manuscript cited within our paper we have demonstrated that the target cells used in the current study are susceptible to ADCC (Gooneratne et al. 2015; JVirol). We agree with the reviewer that it would be ideal to be able to assess the impact of NK education upon the ability of NK cells to mediate ADCC. Such studies, however, would be terribly difficult to interpret. These experiments would require purified NK cells to exclude other ADCC effector cells (i.e., monocytes and granulocytes). Although this is not difficult to achieve, further sorting would be required to distinguish cells expressing all known educating KIRs (i.e., 3DL1, 2DL1, 2DL2 and 2DL3), as well as NKG2A and CD57^{+} and CD57^{-} NK cells. While such a sort is possible, we worry loading NK cells with such an array of antibodies might make the results of cytolysis assays difficult to interpret. Indeed, any inadvertent in vitro cross-linking of antibody-coated receptors during sorting could alter the results obtained. Given the potential uncertainties about cytolysis assay readouts from such cells, we feel that these experiments would not add much in terms of concrete data.

3. The gating strategy used to identify IFN accumulation in the presence of HIV seropositive plasma appears to select the total CD56+ NK cell population. It is most likely that only the CD16+ NK cells are responding to the presence of antibody, and there are likely different frequencies of CD16+ NK present in the different donor PBMC, therefore, it is possible that these potential differences in

the size of the CD16+ NK populations might unintentionally impact the results given the small size of the study cohort. Can the gating be modified to look specifically at CD16+ NK, or can the authors demonstrate no significant difference in the frequency of CD16+ NK between the study groups?

Response: The reviewer is correct to point out that differences in CD16 expression could drive differences in antibody-dependent NK cell activation. Although our manuscript does not direct compare between HLA-C2 carriers and HLA-C2- donors, it is possible that differences in CD16 expression between the KIR2DL1+ and KIR2DL1- NK cell subsets within these two donor groups could account for trends observed in each group. As such, we assessed CD16 expression on KIR2DL1+ and KIR2DL1- NK cells in both HLA-C2+ and HLA-C2- donors. In both groups we observed a higher CD16 median fluorescence intensity on the KIR2DL1+ NK cell subset. As such, if CD16 expression was driving the observed trends, it would be expected that both HLA-C2+ and HLA-C2- donors would have higher antibody-dependent NK cell activation in their KIR2DL1+ NK cell subset. Alternatively, NK cell activation is only preferentially higher in the KIR2DL1+ NK cell subset in HLA-C2+ donors. This suggests that NK cell education and not CD16 expression levels are responsible for the reported differences in NK cell activation. (see Methods Page 8; Results Page 10).

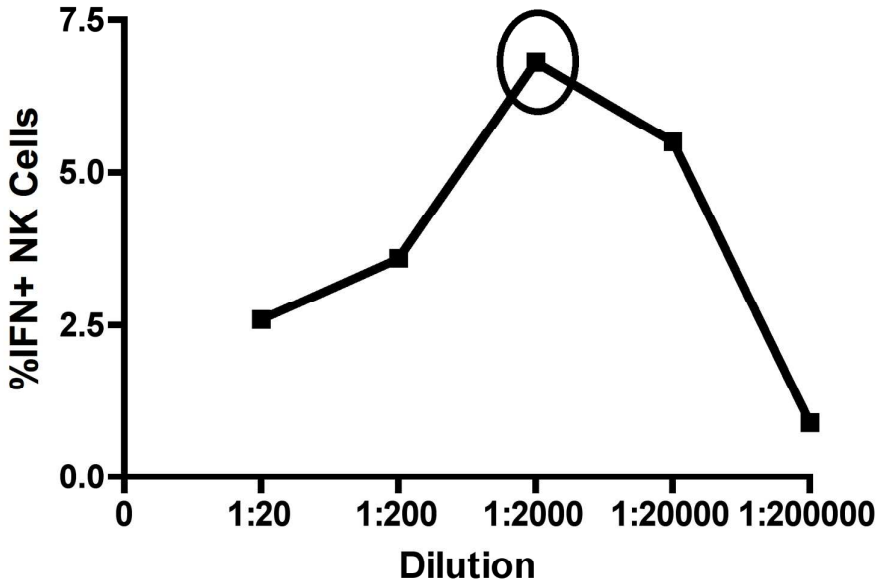
4. The methods describe use of a single dilution of HIV seropositive plasma for NK activation experiments. Please clarify why this dilution was selected, optimal activation, low background? Also, the background IFN accumulation in the absence of plasma is only shown in one example. It does not appear that the potential for non-specific activity was evaluated using plasma from a non-infected healthy donor. These controls would help to demonstrate that the activity observed is restricted to activation of the NK cells by HIV-specific antibody and not just due to the presence of allogenic target cells, or nonspecific activity of plasma.

Response: The reviewer raises two key issues that are addressed in the amended manuscript.

First, we choose the single dilution of HIV-infected plasma due to previously reported prozone effects for anti-HIV-1 ADCC assays. It has been shown that 1:1000 or 1:2000 dilutions generally works best for detecting anti-HIV-1 antibody-dependent NK cell responses (Pollara et al. 2011; Cytometry Part A and Gooneratne et al. 2015; JVirol) Indeed, we provide the reviewer with an example of this prozone effect in the graph pasted below. Additionally, we have amended the manuscript to mention previously reported prozone effects as the reason for utilizing the 1:2000 plasma dilution. (see Methods Page 7).

Secondly, in the newly added supplementary figure 1 we now present data demonstrating the utilized assay detects only HIV-1 specific antibody-dependent NK cell activation. Indeed, the new supplementary figure 1 demonstrates that HIV-uninfected plasma does not induce NK cell activation against gp120-coated

CEM.NKr-CCR5 target cells. (see Methods Page 7; Results page 9; Supplementary Figure 1).



Reviewer 2

The manuscript by Gooneratne et al describes differences in the antibody-dependent activation of NK cells depending on expression of KIR2DL1+/- and HLA-C2+/- status. The manuscript is generally well-written and the data presented in appropriate complete and well-controlled.

The findings would be of greater interest if they were generalized to extend to other antibody samples or antigenic targets. The authors should make note of these limitations as well as add discussion as to whether they think this difference in activity would be expected to be observed across antibody samples and antigen specificities. Alternatively, if they have data which generalizes their observation, they should include it in this paper and broaden the introduction and discussion sections.

Response: As per the reviewer's request, we have now added a section to our discussion highlighting a need for future studies to assess if our results extend to other infectious disease targets, such as influenza. Additionally, we state that research is needed to investigate a role for education in antibody-dependent responses triggered by monoclonal antibodies used to treat malignancies (see Discussion Page 15).

I question the use of the word "implying" in the abstract, where the authors suggest their data "imply" that education matters to NK activity. The title of the manuscript states that education grants an advantage - therefore it seems the authors believe their data does more than implicate eduction and they should strengthen their word choice. Alternatively, if the data only provides an implication, I'm not sure it warrants being in the title.

Response: We have changed the word implying to demonstrating (see abstract Page 2).

In the introduction, the authors discuss "roles" for KIR2DL1 in HIV progression and pathogenesis, but the citations appear to simply describe associations.

Response: We have softened the language used to discuss these studies in the introduction (see Introduction pages 4-5).

Lastly, the authors state that their observations are relevant for optimizing antibody-based HIV-1 vaccines. I fail to see how that is the case, and despite making the statement several times (abstract, into, conclusion), the means whereby this observation could contribute to vaccine optimization is not described. If the authors cannot support this argument they should reframe the significance of their study.

Response: Suggestions to optimizing vaccines have been removed throughout the text and replaced with statements regarding the role of the data in improving our understanding of regulation of anti-HIV-1 antibody-dependent NK cell responses (see pages 2. 5 and 16).

Functional advantage of educated KIR2DL1^{+} natural killer cells for anti-HIV-1 antibody-dependent activation

Running head: NK cell education and ADCC

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Abstract

Evidence from the RV144 HIV-1 vaccine trial implicates anti-HIV-1 antibody-dependent cellular cytotoxicity (ADCC) in vaccine-conferred protection from infection. Amongst effector cells ~~capable of~~that mediate ADCC are natural killer (NK) cells. The ability of NK cells to be activated in an antibody-dependent manner is reliant upon several factors. In general, NK cell mediated antibody-dependent activation is most robust in terminally differentiated $CD57^+$ NK cells, as well as NK cells educated through ontological interactions between inhibitory killer immunoglobulin-like receptors (KIR) and their major histocompatibility complex class I (MHC-I or HLA-I) ligands. With regards to anti-HIV-1 antibody-dependent NK cell activation, previous research has demonstrated that the epidemiologically relevant KIR3DL1/HLA-Bw4 receptor/ligand combination confers enhanced activation potential. In the present study we assessed the ability of the KIR2DL1/HLA-C2 receptor/ligand combination to confer enhanced activation upon direct stimulation with HLA-I-devoid target cells or antibody-dependent stimulation with HIV-1 gp140-pulsed CEM.NKr-CCR5 target cells in the presence of an anti-HIV-1 antibody source. Amongst donors carrying the HLA-C2 ligand for KIR2DL1, higher IFNγ production was observed within $KIR2DL1^+$ NK cells than in $KIR2DL1^-$ NK cells upon both direct and antibody-dependent stimulation. No differences in $KIR2DL1^+$ and $KIR2DL1^-$ NK cell activation were observed in HLA-C1 homozygous donors. Additionally, higher activation in $KIR2DL1^+$ than $KIR2DL1^-$ NK cells from HLA-C2 carrying donors was observed within less differentiated $CD57^-$ NK cells, ~~implying~~ demonstrating observed differences were due to education and not an overabundance of $KIR2DL1^+$ NK cells within differentiated $CD57^+$ NK cells. These observations are relevant for ~~optimizing antibody-based HIV-1 vaccines~~understanding the regulation of anti-HIV-1 antibody-dependent NK cell responses.

Introduction

A prophylactic vaccine is desired to reduce the number of new HIV-1 infections. Anti-HIV-1 antibodies capable of triggering antibody-dependent cellular cytotoxicity (ADCC) might be important to elicit through vaccination. Following binding of viral epitopes on the surface of HIV-1-infected cells, the constant regions of ADCC antibodies engage the CD16 constant region receptor on innate immune cells, such as NK cells and monocytes. Engagement of CD16 can result in the lysis of the HIV-1-infected target cell [1-3]. Additionally, NK cells activated upon stimulation through CD16 release chemokines and cytokines [4, 5]. Chemokines produced by activated NK cells can directly inhibit HIV-1 replication [6]. In the context of vaccination, ADCC would allow for the elimination of autologous cells that become infected upon HIV-1 exposure, as well as HIV-1-infected allogeneic cells delivered within infected bodily fluids. The modestly successful RV144 vaccine trial has indicated a role for ADCC-competent antibodies in vaccine-conferred protection against HIV-1 infection [7-9]. Indeed, ADCC antibodies were associated with a lower likelihood of infection in vaccinees that carried low levels of anti-envelope IgA that could compete with IgG for antigen binding and block anti-HIV-1 ADCC [7, 9]. This observation highlights the need for further research into the factors regulating the ability of innate immune effector cells to mediate antibody-dependent functions, facilitating utilization of the full potential of ADCC antibodies in HIV-1 vaccine development.

The ability of NK cells to mediate anti-HIV antibody-dependent functions is dependent upon several NK cell factors, including the education status and state of differentiation of the cell [10, 11]. The education status of an NK cell is determined through interactions of activating and inhibitory NK cell receptors with self-major histocompatibility complex class I (MHC-I or HLA-I) ligands, which tunes the functional potential of the NK cell [12-14]. In general, NK cells that express inhibitory receptors capable of binding self-HLA-I are tuned for higher functional potential; whereas, NK cells lacking inhibitory receptors or expressing inhibitory receptors that do not recognize self HLA-I are tuned for reduced functional potential [12, 14]. Furthermore, NK cells expressing activating

receptors capable of binding self HLA-I are tuned for lower functional potential [13]. The education status of NK cells is linked to their ability to become activated by antibody-dependent and independent stimuli [12]. With regards to anti-HIV antibody-dependent NK cell activation, our group has previously demonstrated that educated NK cells expressing the inhibitory killer immunoglobulin-like receptor 3DL1 (KIR3DL1), derived from donors carrying the HLA-Bw4 ligand, exhibit a functional advantage over autologous KIR3DL1$^-$ NK cells and allogeneic KIR3DL1$^+$ NK cells, derived from donors lacking the HLA-Bw4 ligand [11]. In addition to education, NK cells independently undergo a differentiation process whereby they phenotypically progress from CD56^{bright}CD16$^-$CD57$^-$ to CD56^{dim}CD16$^+$CD57$^-$, and finally develop into CD56^{dim}CD16$^+$CD57$^+$NK cells [15]. Along with changes in phenotype, the functional profile of NK cells is also altered by differentiation. Indeed, NK cells expressing the CD57 differentiation marker exhibit more robust activation upon stimulation through CD16 [10, 16]. Differentiated CD57$^+$ NK cells are also more likely to express inhibitory KIRs [16]. Although differentiated NK cells expressing inhibitory KIR for self HLA-I would be educated for higher functional potential, the contributions of NK cell education and differentiation to NK cell antibody-dependent functional potential appear to be at least partially distinct [10].

Our previous research regarding the contribution of NK cell education to the antibody-dependent functional potential of NK cells focused upon the KIR3DL1/HLA-Bw4 receptor/ligand combination [10, 11, 17]. The results of those KIR3DL1/HLA-Bw4 studies corroborate epidemiological studies that have linked allelic combinations of this receptor/ligand pair to protection from HIV-1 infection and progression to AIDS [18, 19]. Several recent studies have now suggested that the inhibitory KIR2DL1 receptor might also be important for understanding susceptibility to HIV-1 infection and HIV-1 pathogenesis. The KIR2DL1 receptor binds a subset of HLA-C alleles, termed HLA-C2, which are characterized by the presence of lysine at amino acid position 80 [20]. Non-HLA-C2 alleles are termed HLA-C1 and are characterized by the presence of asparagine at amino acid position 80. ~~Evidence for a potential role of~~An association between the KIR2DL1/HLA-C2 receptor/ligand combination and ~~in~~ protection from HIV-1 infection has been provided by the

observation that exposed but uninfected Senegalese carry the education-competent KIR2DL1/HLA-C2 combination, while their infected partners lack HLA-C2 ligands that could inhibit the recognition and cytolysis of HIV-1-infected allogeneic leukocytes [21]. ~~Suggestive of a role for~~A potential link has also been noted between KIR2DL1 ~~in~~ and HIV-1 disease progression ~~is~~ through the demonstration by Korner et al. that KIR2DL1$^+$ NK cells are expanded in primary HIV-1 infection in individuals carrying the HLA-C2 ligand [22]. These expanded KIR2DL1$^+$ NK cells also exhibited a functional advantage over KIR2DL1$^-$ NK cells upon stimulation with HLA-I-devoid target cells. Interestingly, the frequency of KIR2DL1$^+$ NK cells appears to wane during chronic HIV-1 infection, further indicating a role in HIV-1 pathogenesis. Indeed, this phenomenon has been demonstrated in HIV-1 clade C-infected South Africans and clade A and D-infected Ugandans [23, 24]. Additional research has demonstrated that while the functional advantage of NK cells expressing the HLA-C binding KIR2DL1/2/3 receptors upon stimulation with HLA-I-devoid target cells is observed in HIV-1-uninfected donors, this advantage is not present in chronically HIV-1-infected donors [25].

Given that recent evidence has implied that educated KIR2DL1$^+$ NK cells might play a role in protection from HIV-1 infection and suppressing viral replication during primary HIV-1 infection, we sought to determine if education of NK cells through KIR2DL1/HLA-C2 interactions enhanced the ability of NK cells to become activated in an anti-HIV-1 antibody-dependent manner. We now present data demonstrating that education of NK cells through KIR2DL1/HLA-C2 interactions enhances NK cell activation upon exposure to antibody-dependent and antibody-independent stimuli. Furthermore, we demonstrate, through assessing CD57$^-$ NK cells, that the functional advantage of educated KIR2DL1$^+$ NK cells upon antibody-dependent stimulation is not a bystander effect of NK cell differentiation. These results enhance our understanding of the regulation of ~~ADCC effector cells and will be important for understanding how to optimize vaccines that induce ADCC-competent antibodies~~anti-HIV-1 antibody-dependent NK cell responses.

Materials and methods

Participants

Blood was collected from 13 HIV-1-uninfected donors by forearm venepuncture into vacuettes containing sodium heparin anti-coagulant. Ficoll Paque PLUS (GE Healthcare Life Sciences) density gradient centrifugation was employed to obtain PBMCs from whole blood. These PBMCs were utilized as effector cells in NK cell activation assays. As a source of anti-HIV-1 antibodies, plasma was obtained from an HIV-1-infected client of the Melbourne Sexual Health Centre. This HIV-1-infected donor's plasma has previously been shown to carry antibodies capable of activating NK cells in an anti-HIV-1-dependent manner [26]. All donors provided informed consent prior to collection of biological samples and the ethics committees of the participating institutions approved all performed experiments.

Cell lines

The $CD4^+$ CEM.NKr-CCR5 T-cell line was obtained from the NIH AIDS Reagent Program, Divison of AIDS, NIAID, NIH. The HLA-I-devoid 721.221 cell line was kindly provided by Dr. Andrew Brooks (Department of Microbiology and Immunology, University of Melbourne).

HLA-C typing and KIR2DL1 expression

The Victorian Transplantation and Immunogenetics Service at The Australian Red Cross Blood Service performed sequence-based typing of HLA-C alleles to four-digit resolution for all 13 donors. Expression of KIR2DL1 by NK cells from all 13 donors was demonstrated by staining with FITC-conjugated anti-KIR2DL1 antibody (clone: 143221; R&D Systems) and detection by flow cytometry using a BD LSR Fortessa. This antibody clone specifically detects KIR2DL1 and exhibits no cross-reactivity with other KIR2D or KIR3D gene products. Flow Jo Version 9.2 (Tree Star) was utilized for analysis of flow cytometry data.

NK cell activation assays

To study HIV antibody specific NK cell activation, we used a previously described flow cytometric assay to detect NK cell IFNγ expression [17]. Briefly, CEM.NKr-CCR5 target cells were prepared by coating with HIV-1 $gp140_{AD8}$ (3µg/1.0X10^6 cells in 1ml of solution) for 90 minutes at 4°C. The HIV-1 $gp140_{AD8}$ was prepared as previously described [27]. Next, PBMC effector cells were combined with gp140-pulsed CEM.NKr-CCR5 target cells at a 10:1 effector to target ratio in the presence or absence of a 1:2000 final dilution of plasma from an HIV-1-infected donor, Brefeldin A (5µg/ml) (Sigma) and monensin (6µg/ml) (BD). Co-cultures were incubated for five hours at 37°C. To demonstrate that the assay specifically detects anti-HIV-1 antibody-dependent responses, three donors were assessed for NK cell activation in the presence of 1:2000 dilutions of both HIV-1-uninfected and HIV-1-infected plasma. The utilized plasma dilution was implemented due to previously reported prozone effects in assays measuring anti-HIV-1 ADCC [1, 17]. Following incubation, cells were surface stained with Per CP-conjugated anti-CD3 (clone: SK7; BD), PE-Cy7-conjugated anti-CD56 (clone: NCAM16.2; BD), FITC-conjugated anti-KIR2DL1 (clone: 143221; R&D Systems) and Pacific Blue-conjugated anti-CD57 (clone: HCD57; Biolegend) antibodies. Next, cells were fixed in formaldehyde, permeabilized with 1X Perm solution (BD) and stained with Alexa Fluor 700-conjugated anti-IFNγ antibody (clone: b27; BD). Lastly, cells were fixed in formaldehyde and acquired using a BD LSR Fortessa (BD). Data was analysed with FlowJo Version 9.2. Antibody-independent NK cell activation was also assessed in an identical manner as anti-HIV-1 antibody-dependent NK cell activation above, except PBMCs were cultured with the HLA-I-devoid 721.221 cell line in the absence of any antibody sources.

Autologous whole blood antibody-dependent NK cell activation assay

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To assess anti-HIV-1 antibody-dependent NK cell activation in an autologous setting, we utilized a previously described assay measuring antibody-dependent NK cell activation by autologous

$CD4^+$ cells that have bound HIV-1 envelope and antibodies derived from HIV-1-infected plasma. Briefly, 150µl of HIV-1-uninfected whole blood was combined with 50µl of HIV-1-infected plasma, 1µg/ml HIV-1 $gp140_{AD8}$, Brefeldin A (5µg/ml and monensin (6µg/ml). Control conditions containing whole blood alone or whole blood plus HIV-1-infected plasma were conducted simultaneously. All conditions were incubated for five hours at 37°C. Following incubation, blood was incubated with Per-CP-conjugated anti-CD3 (clone: SK7; BD), PE-Cy7-conjugated anti-CD56 (clone: NCAM16.2; BD) and FITC-conjugated anti-KIR2DL1 (clone: 143221; R&D Systems) antibodies. Next, red blood cells were lysed with 1X lysis buffer (BD), permeabilized with 1X Perm solution (BD) and stained with Alexa Fluor 700-conjugated anti-IFNγ antibody (clone: b27; BD). Samples were fixed in formaldehyde and acquired using a BD LSR Fortesa (BD). Data analysis was performed with FlowJo Version 9.2.

Assessment of CD16 expression on $KIR2DL1^+$ and $KIR2DL1^-$ NK cells

Whole blood from nine donors was incubated with Per CP-conjugated CD3 (clone: SK7; BD), PE-Cy7-conjugated CD56 (clone: NCAM16.2; BD), APC-conjugated anti-KIR2DL1 (clone: 143221; R&D Systems) and FITC-conjugated anti-CD16 (clone: 3G8; BD). Next, red blood cells were lysed with 1X lysis buffer, washed and fixed in formaldehyde. Samples were acquired using a BD LSR Fortessa (BD). Data was analysed with FlowJo Version 9.2.

Statistics

GraphPad Prism version 4.0 was used for statistical analyses. Within group differences were compared using the Wilcoxon matched pairs test. Data throughout the manuscript is presented in the [median (range) vs. median (range)] format.

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Results

Direct and anti-HIV-1 antibody-dependent activation of NK cells educated through KIR2DL1

The functional advantage of educated KIR2DL1$^+$ NK cells over the KIR2DL1$^-$ population, which contains both uneducated NK cells and cells educated through other HLA/KIR combinations, has been observed upon direct stimulation, for both HIV-1-infected and uninfected donors, and non-HIV-1 antibody-dependent stimulation, for HIV-1-uninfected donors [12, 22, 28]. The role of education through KIR2DL1 on anti-HIV antibody-dependent activation potential, however, has not yet been investigated. To address this issue we stimulated NK cell effectors within PBMCs, obtained from eight HLA-C2 carrying donors and five donors homozygous for HLA-C1 alleles (Table 1), with HIV-1_{AD8} gp140-coated CEM.NKr-CCR5 T-cells in the presence of plasma from an HIV-1-infected donor. This assay specifically detects anti-HIV-1 antibody-dependent NK cell activation, as activation is observed in the presence of HIV-1-infected plasma but not in the presence of HIV-1-uninfected plasma (Supplementary Figure 1) Simultaneously, in order to demonstrate that the utilized HLA-C2 carrying donors, but not the HLA-C1 homozygous donors, exhibit the previously reported functional advantage within the educated KIR2DL1$^+$ population upon direct stimulation, we stimulated NK cells within PBMC with the HLA-I-devoid 721.221 cell line. Following stimulation, samples were assessed by flow cytometry. The gating procedure used to identify KIR2DL1$^+$ and KIR2DL1$^-$ NK cells, as well as the percentage of NK cells within each population that became activated to produce IFNγ is depicted in Figure 1A. As expected, upon stimulation with 721.221 targets the percentage of NK cells activated to produce IFNγ was higher in the KIR2DL1$^+$ population than in the KIR2DL1$^-$ population for HLA-C2 carrying donors [16.2% (3.6-28.9%) vs. 10.4% (3.4-12.9%), p=0.0078] (Figure 1B). No differences in IFNγ production was observed between these NK cell populations in donors homozygous for HLA-C1 alleles [10.7% (5.2-16.9%) vs. 8.5% (7.3-14%), p=1.00] (Figure 1B). Similarly, when NK cells were stimulated in an anti-HIV-1 antibody-dependent manner, HLA-C2 carrying donors exhibited higher percentages of IFNγ producing NK cells in the KIR2DL1$^+$ population than the

KIR2DL1$^-$ population [6.6% (1.9-16.2%) vs. 3.5% (0.9-5.7%), p=0.0078] (Figure 1C). No differences were observed between these NK cell populations upon anti-HIV-1 antibody-dependent stimulation in donors homozygous for HLA-C1 alleles [6.2% (3.0-9.0%) vs. 6.2% (2.8-6.9%), p=0.6250] (Figure1 C). As antibody-dependent NK cell activation is triggered through CD16, we next questioned if these differences in antibody-dependent NK cell activation could simply be attributed to preferentially higher expression levels of CD16 on KIR2DL1$^+$ NK cells in HLA-C2 carrying donors. Although we noted higher CD16 ~~expression~~median fluorescence intensity (MFI) on KIR2DL1$^+$ than KIR2DL1$^-$ NK cells, this expression pattern was seen in both HLA-C2 carriers [3227 (1118-5549) vs. 2814 (877-4405); n=5] and HLA-C1 homozygotes [2994 (1134-8652) vs. 2534 (993-7355); n=4]. As such, CD16 expression levels do not account for the preferential activation of KIR2DL1$^+$ NK cells in HLA-C2 carrying donors upon antibody-dependent stimulation. –These data reaffirm a role for NK cell education through KIR2DL1 in determining the ability of NK cells to exhibit activation upon direct stimulation. Further, the data suggest that the role of education in determining NK cell functional potential extends to the ability of NK cells to exhibit anti-HIV-1 antibody-dependent NK cell activation.

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Impact of NK cell differentiation on differences in activation between KIR2DL1$^+$ and KIR2DL1$^-$ NK cells

The ability of NK cells to exhibit activation upon stimulation through CD16 is higher in NK cells expressing the CD57 differentiation marker [10, 16]. As higher percentages of CD57$^+$ NK cells express KIRs, the relative contributions of education and differentiation to antibody-dependent NK cell activation can become unclear upon assessments of the total NK cell population [10, 16]. We therefore assessed the influence of KIR2DL1 expression on the function of both CD57$^+$ and CD57$^-$ NK cells within our donors. Coinciding with previously published data, we observed higher anti-HIV-1 antibody-dependent NK cell activation in the CD57$^+$ NK cells than the CD57$^-$ NK cells in all 13 donors [6.5% (1.4-13%) vs. 2.4% (0.6-4.4%), p=0.0002] (Figure 2A) [10]. Additional assessments of CD57$^+$ and CD57$^-$ NK cells revealed a higher frequency of KIR2DL1 expression in the CD57$^+$ population across all

13 donors [25.3% (8.4-50.4%] vs. 12.7% (3.5-32.1%), *p*=0.0002] (Figure 2B). Lastly, we assessed if the differences in anti-HIV-1 antibody-dependent activation observed between KIR2DL1$^+$ and KIR2DL1$^-$ NK cells in HLA-C2 carrying donors were due to education, and not a bystander effect of NK cell differentiation. We compared the anti-HIV-1 antibody-dependent activation of CD57$^-$KIR2DL1$^+$ and CD57$^-$KIR2DL1$^-$ NK cells within HLA-C2 carrying donors and HLA-C1 homozygous donors. As depicted in Figure 2C, within HLA-C2 carrying donors KIR2DL1$^+$ NK cells within the CD57$^-$ population exhibited higher levels of IFNγ production upon anti-HIV-1 antibody-dependent stimulation than the KIR2DL1$^-$ population [4.6% (0.8-9.4%) vs. 2.2% (0.4-3.0%), *p*=0.0156]. No differences were observed between the CD57$^-$KIR2DL1$^+$ and CD57$^-$KIR2DL1$^-$ populations in donors homozygous for HLA-C1 [3.1% (0.1-3.5%) vs. 2.4% (1.3-4.5%), *p*=0.3125] (Figure 2C). These data further confirm a role for NK cell education through KIR2DL1 in determining the ability of NK cells to exhibit anti-HIV-1 antibody-dependent activation.

Anti-HIV-1 antibody-dependent activation of educated KIR2DL1$^+$ NK cells against autologous targets

Although the preferential activation of educated KIR2DL1$^+$ NK cells against gp140-coated CEM.NKr-CCR5 target cells demonstrates a role for NK cell education in determining NK cell activation potential, this experimental system does not determine if target cells expressing the HLA-C2 ligand can activate KIR2DL1$^+$ NK cells. Indeed, CEM.NKr-CCR5 target cells are HLA-C1 homozygous (Dr Nicole F Bernard - personal communication). To address this issue we performed an autologous whole blood anti-HIV-1 antibody-dependent NK cell activation assay. This assay combines HIV-1-uninfected whole blood with HIV-1 gp140$_{AD8}$ and HIV-1-infected plasma. This allows CD4$^+$ cells to bind viral envelope, which binds anti-HIV-1 antibodies, and activate NK cells. Stimulation of NK cells within whole blood from five HLA-C2 carriers resulted in activation of both the KIR2DL1$^+$ and KIR2DL1$^-$ NK cell subsets (Figure 3). Intriguingly, four of the five donors screened exhibited higher activation in the KIR2DL1$^+$ [2.1% (1.8-18%) vs. 1.8% (1.3-10.7%)] than KIR2DL1$^-$ NK cell subset, while the fifth donor exhibited equal activation in both NK cell subsets. These data demonstrating that

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educated KIR2DL1^{+} NK cells can become activated in an anti-HIV-1 antibody-dependent manner against target cells expressing the HLA-C2 ligand, even maintaining a functional advantage over KIR2DL1^{-} NK cells in a majority of donors, highlight that anti-HIV-1 antibody-dependent stimulation at least partially overcomes inhibitory signals through KIR2DL1/HLA-C2 receptor ligand combinations.

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Discussion

The data presented in this manuscript provide the first demonstration that education of NK cells through KIR2DL1/HLA-C2 combinations enhances the ability of NK cells to respond upon anti-HIV-1 antibody-dependent activation. Additionally, and in concordance with previous studies, we provide data demonstrating an activation advantage of the KIR2DL1$^+$ NK cell population upon antibody-independent stimulation with HLA-I-devoid target cells [12, 22]. These observations are interesting in the context of recent studies implicating KIR2DL1/HLA-C2 combinations in providing protection from HIV-1 infection, or contributing to inhibiting viral replication during primary HIV-1 infection [21, 22]. Indeed, the data presented in the current manuscript could be of importance for understanding mechanisms contributing to protective outcomes upon HIV-1 exposure or infection.

Jennes et al recently suggested that KIR2DL1/HLA-C2 combinations could contribute to protection from HIV-1 infection [21]. In a cohort of Senegalese couples concordant and discordant for HIV-1 infection the authors observed cognate ligand matches between inhibitory KIR in HIV-1 recipients of concordant couples and HLA-I in the HIV-1 donors. In the discordant couples, cognate ligand mismatches were observed between the inhibitory KIR of the uninfected partner and the HLA-I of the infected partner. The HIV-1 uninfected partners were observed to carry the education-competent KIR2DL1/HLA-C2 combination, while their HIV-1 infected partners tended to be HLA-C1 homozygous. The authors further demonstrated that NK cells carrying KIR mismatched to HLA-I expressed on CD4$^+$ T-cells were capable of killing allogeneic CD4$^+$ T-cells, suggesting that lack of ligands for inhibitory KIRs can result in enhanced direct recognition of HIV-1-infected allogeneic target cells and offer protection from HIV-1 acquisition. The data presented in the current manuscript, showing that educated KIR2DL1$^+$ NK cells have an activation advantage for anti-HIV-1 antibody-dependent activation, might further explain the protection observed in Senegelese

serodiscordant couples. Although HIV-1 exposed uninfected individuals do not carry anti-HIV-1 IgG within their sera, it has recently been shown that antibodies passively provided by HIV-1-infected mothers to their children via breast milk can protect against virus transmission [29]. Others and we have recently observed anti-HIV-1 antibodies capable of activating NK cells and/or triggering ADCC in seminal plasma and vaginal fluids [30, 31]. As these antibodies are exchanged between HIV-1-infected donors and their uninfected partners upon exposure to HIV-1, it is possible that the NK cells within the exposed individual could utilize these antibodies to eliminate infected allogeneic lymphocytes or autologous lymphocytes infected early after exposure.

Although the prospect of eliminating HIV-1-infected allogeneic lymphocytes via ADCC is supported by the observation that KIR/HLA-I mismatched NK cells can directly kill allogeneic $CD4^+$ T-cells, the notion that autologous infected $CD4^+$ T-cells can be targeted is complicated by the presence of cognate HLA-I ligands on $CD4^+$ T-cells for the inhibitory KIRs expressed by educated NK cells. Indeed, Ward et al. demonstrated that the presence of HLA-C and HLA-E on HIV-1-infected $CD4^+$ T-cells inhibits NK cell-mediated ADCC via triggering inhibitory signals through KIR2DL1/2/3 and NKG2A [32]. Despite this observation, several additional studies assessing ADCC or antibody-dependent NK cell activation, triggered by polyclonal anti-HIV-1 antibodies or therapeutic anti-tumor monoclonal antibodies, have demonstrated that antibody-dependent NK cell stimulation can at least partially overcome inhibitory signals through HLA-I/KIR combinations [11, 17, 33, 34]. Indeed, we have recently demonstrated that educated $KIR3DL1^+$ NK cells from HLA-Bw4 carrying donors exhibit higher anti-HIV-1 antibody-dependent activation than $KIR3DL1^-$ NK cells upon stimulation with allogeneic $CD4^+$ T-cells expressing HLA-Bw4 [17]. ~~Unfortunately, the data in the current manuscript does not address the question of whether anti-HIV antibody-dependent activation of KIR2DL1$^+$ NK cells overcomes the inhibitory signals that the presence of HLA-C2 would initiate, as the CEM.NKr-CCR5 target cells used have been shown to be HLA-C1 homozygous (Dr. Nicole Bernard, McGill University, Personal communication)~~Additionally, we now show that $KIR2DL1^+$ NK cells can at least partially overcome inhibitory signals conferred through KIR2DL1/HLA-C2 combinations to become

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activated in an anti-HIV-1 antibody-dependent manner against autologous targets (Figure 3). Further research is required to resolve why some previous research has indicated robust inhibition of anti-HIV-1 antibody-dependent NK cell functions occurs through HLA-C and HLA-E interactions with KIR2DL1/2/3 and NKG2A, while we have reported anti-HIV-1 antibody-dependent stimulation at least partially overcomes inhibition through HLA-Bw4 ~~and~~ /KIR3DL1 and HLA-C2/KIR2DL1 combinations. ~~While these differences could reflect intrinsic signalling intensity differences between KIR3DL1 and KIR2DL1/2/3 or NKG2A receptors, it is perhaps most likely~~It is perhaps most likely that methodological differences account for the discrepancy. To assess anti-HIV-1 antibody-dependent responses Ward et al. utilized a pool of four monoclonal antibodies, while our studies implemented polyclonal anti-HIV-1 serum [11, 17, 32]. Smalls-Mantey et al. demonstrated that pooled monoclonal anti-HIV-1 antibodies mediate poor anti-HIV-1 ADCC compared to the polyclonal mixtures found in sera [35]. We hypothesize that the strength of the signal through CD16 determines the susceptibility of antibody-dependent NK cell responses to inhibition via inhibitory NK cell receptors. The differential ability of monoclonal and polyclonal antibodies to activate NK cells in an antibody-dependent manner is likely to determine if the preferential activation of educated NK cells reported in the current manuscript extends to antibody-dependent responses triggered against different antigenic targets. Future studies should assess the relative activation of educated and uneducated NK cells upon antibody-dependent stimulation against additional infectious disease targets~~s~~, such as influenza, or by monoclonal antibodies utilized for therapy of malignancies.

In addition to potentially modulating susceptibility to HIV-1 acquisition, the enhanced function of educated KIR2DL1^{+} NK cells have been suggested to contribute to control of HIV-1 replication during primary HIV-1 infection [22]. Indeed, educated KIR2DL1^{+} NK cells are expanded during primary HIV-1 infection, and these cells exhibit potent activation, compared to KIR2DL1^{-} NK cells, upon direct stimulation with HLA-I-devoid 721.221 target cells. Future research should assess the ability of educated KIR2DL1^{+} NK cells expanded during primary HIV-1 infection to mediate anti-HIV-1 antibody-dependent activation. As anti-HIV-1 antibodies capable of activating NK cells are

detected during primary HIV-1 infection, the anti-HIV-1 antibody-dependent activation potential of educated KIR2DL1^{+} NK cells might play a role in controlling early viral replication and establishing a lower viral set point [36].

The data presented in this manuscript adds to a growing body of literature on the importance of NK cell education and the interplay of activating and inhibitory receptors in determining the potential of NK cells to mediate anti-viral functions. Although highly~~likely~~ important for assisting with understanding the regulation of NK cell activation potential, the data presented in the current manuscript~~HIV-1 susceptibility and pathogenesis, the role of NK cell education in tuning anti-HIV-1 antibody-dependent activation potential~~ might also be useful ~~for designing anti- HIV-1 vaccines. Indeed, understanding the regulation of effector cells mediating anti-HIV-1 antibody-dependent responses will assist with optimizing antibody based vaccination protocols~~designing future research to enhance our understanding of HIV-1 susceptibility and pathogenesis.

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Author Contributions

The study was designed by MSP and SJK. Experimental work was performed by SLG and RJC. Data analysis was completed by SLG and MSP.

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Figure Legends

Figure 1. Direct and anti-HIV-1 antibody-dependent activation of KIR2DL1$^+$ and KIR2DL1$^-$ NK cells from HLA-C2$^+$ and HLA-C1 homozygous donors. (A) Direct and anti-HIV-1 antibody-dependent activation of NK cell effectors within PBMC was accomplished by stimulation with the HLA-I-devoid 721.221 cell line or HIV-1_{AD8} gp140-pulsed CEM.NKr-CCR5 in the presence of anti-HIV-1 antibodies, respectively. Following stimulation PBMCs were stained with fluorochrome-conjugated antibodies and assessed by flow cytometry. The FACs plots depict progressive gating upon CD3$^-$CD56$^+$ NK cells (Top Left), KIR2DL1$^+$ and KIR2DL1$^-$ NK cell subsets (Top Right), and the assessment of gated cells for IFNγ production in the non-stimulated (Bottom Left), 721.221 stimulated (Bottom Middle) and anti-HIV-1 antibody dependent stimulated conditions (Bottom Right). (B) Graphs depict the relative activation of the KIR2DL1$^+$ and KIR2DL1$^-$ NK cell subsets upon activation by the 721.221 cell line in eight donors carrying HLA-C2 alleles (Left) and five HLA-C1 homozygotes (Right). (C) Graphs depict the relative activation of the KIR2DL1$^+$ and KIR2DL1$^-$ NK cell subsets upon anti-HIV-1 antibody-dependent activation in eight donors carrying HLA-C2 alleles (Left) and five HLA-C1 homozygotes (Right).

Figure 2. The anti-HIV-1 antibody-dependent activation advantage of differentiated NK cells and the role of differentiation in the activation advantage of educated KIR2DL1^{+} NK cells. (A) The FACs plot depicts the gating utilized to identify the differentiated CD57^{+} and less differentiated CD57^{-} CD56^{dim} NK cell populations. The graph highlights the relative ability of CD57^{+}CD56^{dim} and CD57^{-}CD56^{dim} NK cells from all 13 donors to exhibit anti-HIV-1 antibody-dependent activation. (B) FACs plots depict the gating implemented to identify KIR2DL1^{+} and KIR2DL1^{-} NK cells within the CD57^{+} (Left) and CD57^{-} (Right) NK cell subsets. The graph depicts the relative percentages of CD57^{+}CD56^{dim} and CD57^{-} CD56^{dim} NK cells expressing the KIR2DL1 receptor in all 13 donors. (C) The graphs depict the relative anti-HIV-1 antibody-dependent activation of KIR2DL1^{+} and KIR2DL1^{-} NK cells within the CD57^{-}CD56^{dim} NK cell population in eight HLA-C2^{+} donors (Left) and five HLA-C1 homozygotes (Right).

Figure 3. Anti-HIV-1 antibody-dependent activation of KIR2DL1^{+} and KIR2DL1^{-} NK cells from HLA-C2 carrying donors by autologous target cells. NK cells within whole blood from five HLA-C2 carrying donors were stimulated using the autologous whole blood anti-HIV-1 antibody-dependent activation assay. Data was collected by flow cytometry and CD3^{-}CD56^{+}KIR2DL1^{+} and CD3^{-}CD56^{+}KIR2DL1^{-} NK cells were assessed for IFNγ production. The graph depicts the relative production of IFNγ by KIR2DL1^{+} and KIR2DL1^{-} NK cells from each donor.

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Supplementary Figure Legend

Supplementary Figure 1. Anti-HIV-1-specific antibody-dependent activation of NK cells against gp140-coated CEM.NKr-CCR5 target cells. NK cell effectors within PBMC were stimulated with gp140-coated CEM.NKr-CCR5 target cells in the presence 1:2000 dilutions of HIV-1^+ or HIV-1^- plasma. Antibody-dependent NK cell activation was assessed by flow cytometry. The FACS plot at the top depicts gating to identify $CD3^-CD56^+$ NK cells. The FACS plots on the bottom depict the relative amounts of NK cell activation, measured as IFNγ production, observed when NK cells and gp140-coated CEM.NKr-CCR5 cells were co-cultured in the presence of HIV-1^- (Right plot) or HIV-1^+ (Left plot) plasma. The depicted data from this single PBMC donor is representative of three independent PBMC donors tested.

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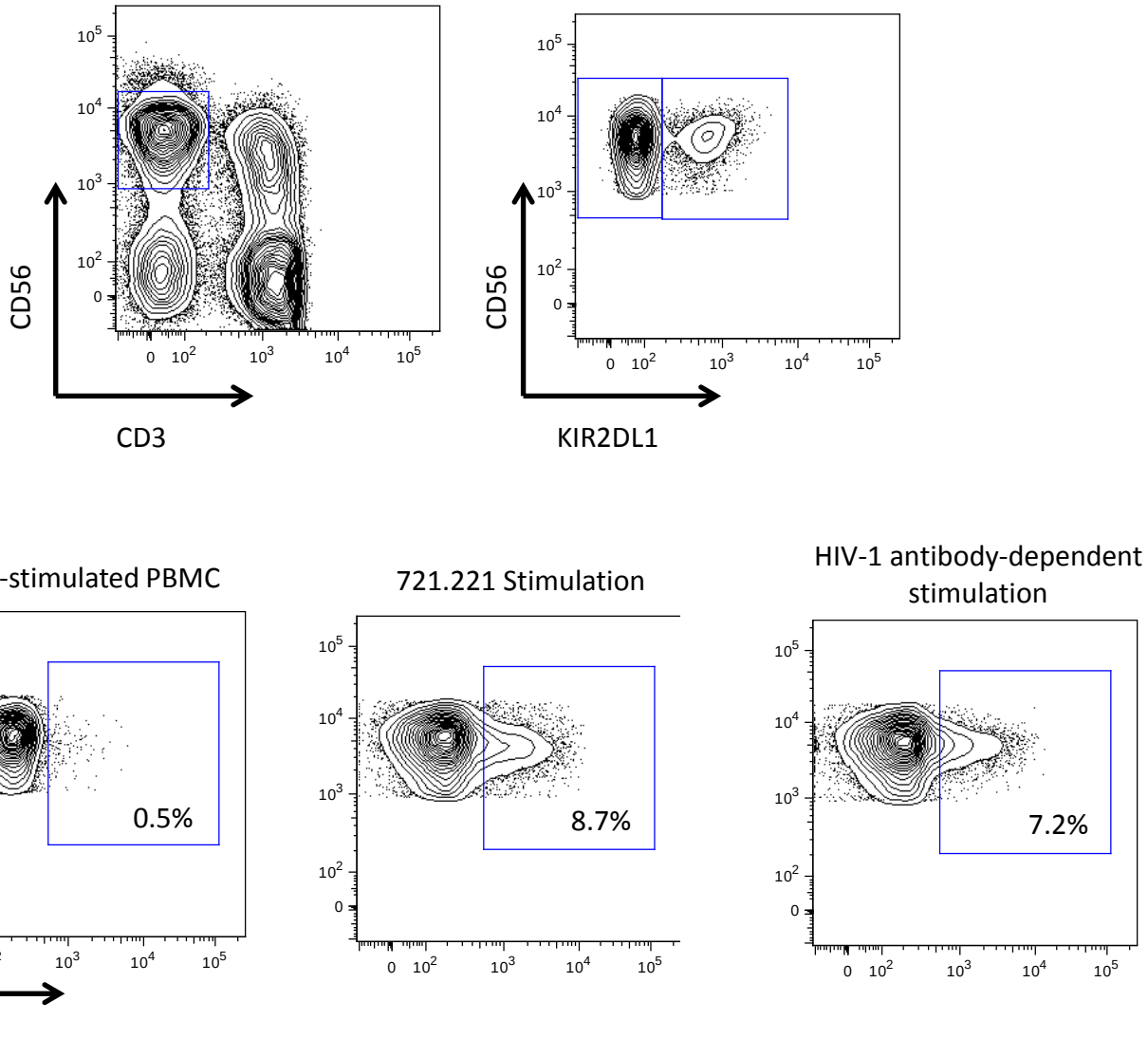
Table 1. HLA-C typing of KIR2DL1 carrying effector cell donors.

Donor ID	HLA-C alleles	HLA-C1/C2 typing
1	04:01, 14:02	C1/C2
2	07:02, 17:01	C1/C2
3	02:02, 03:04	C1/C2
4	07:02, 07:02	C1/C1
5	05:01, 12:03	C1/C2
6	07:04, 12:03	C1/C1
7	04:03, 07:02	C1/C2
8	04:01, 07:01	C1/C2
9	03:03, 07:02	C1/C1
10	03:04, 07:01	C1/C1
11	07:01, 16:02	C1/C2
12	01:02, 01:02	C1/C1
13	04:01, 12:02	C1/C2

Figure 1A



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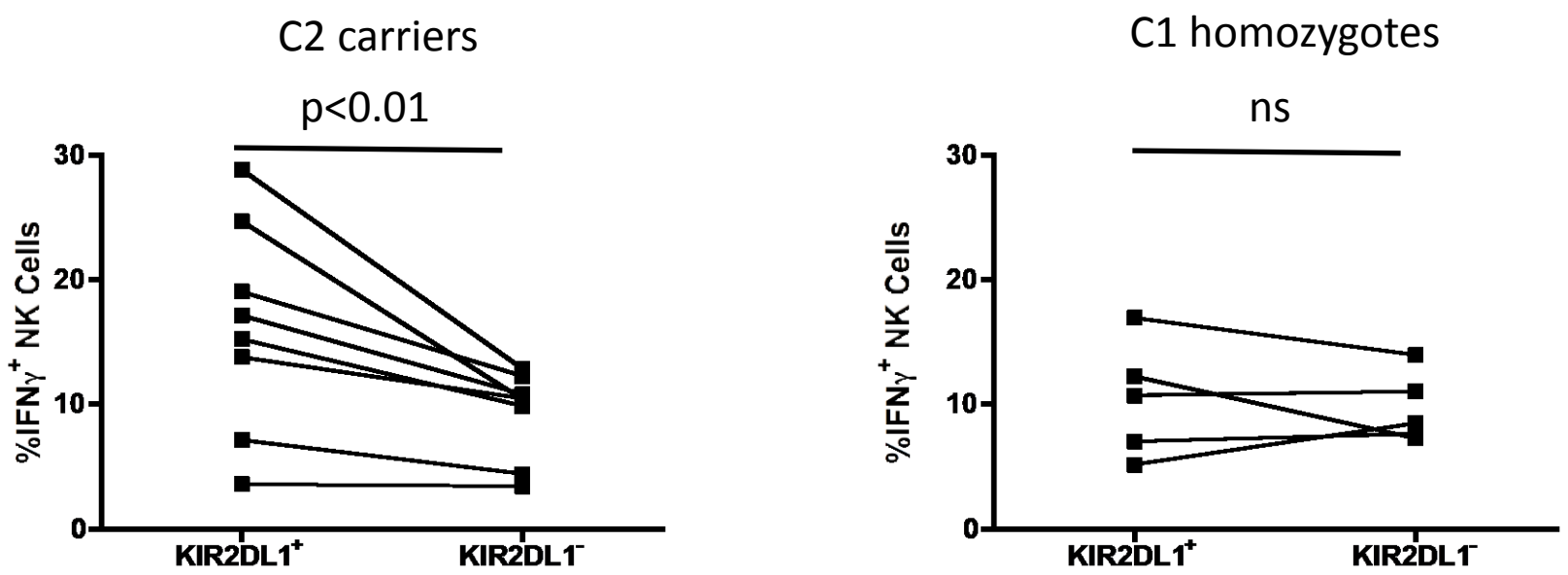


C.

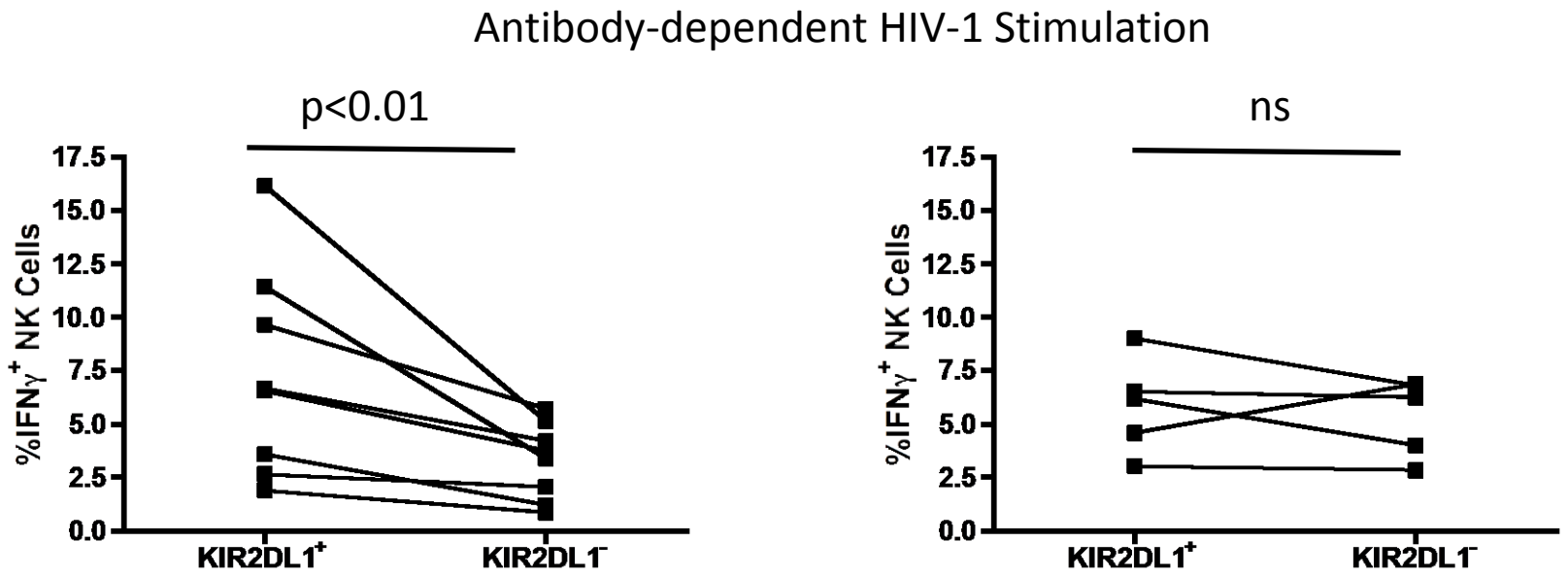
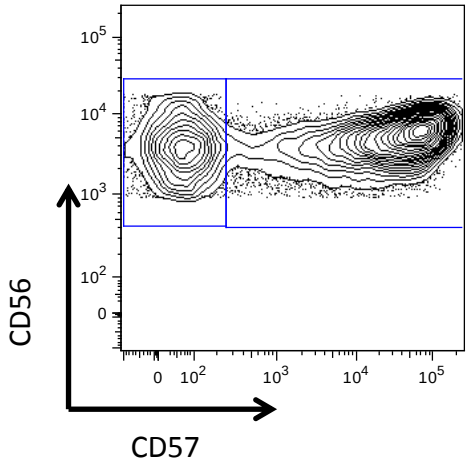
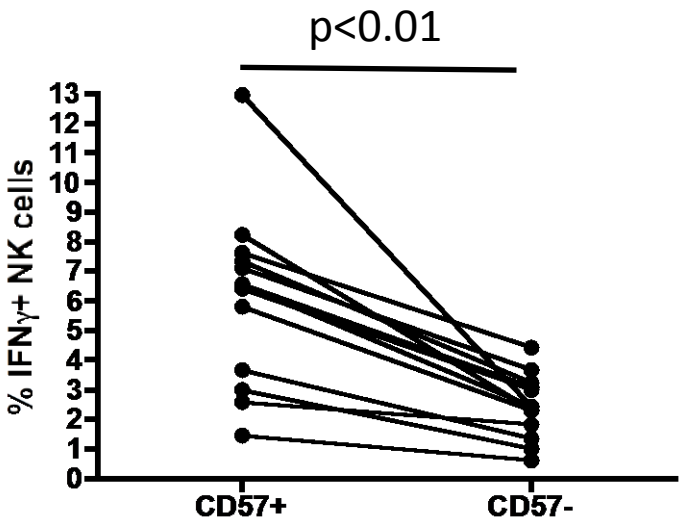


Figure 2A





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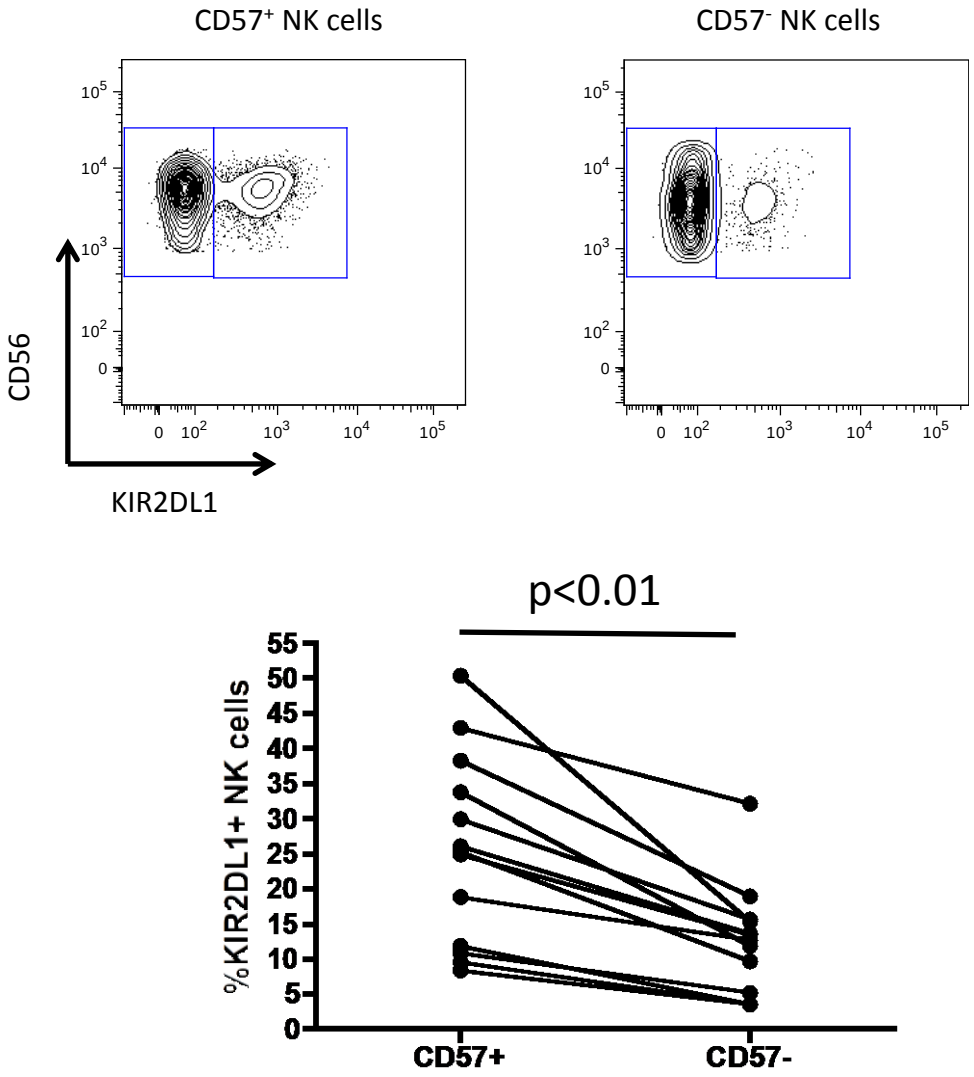

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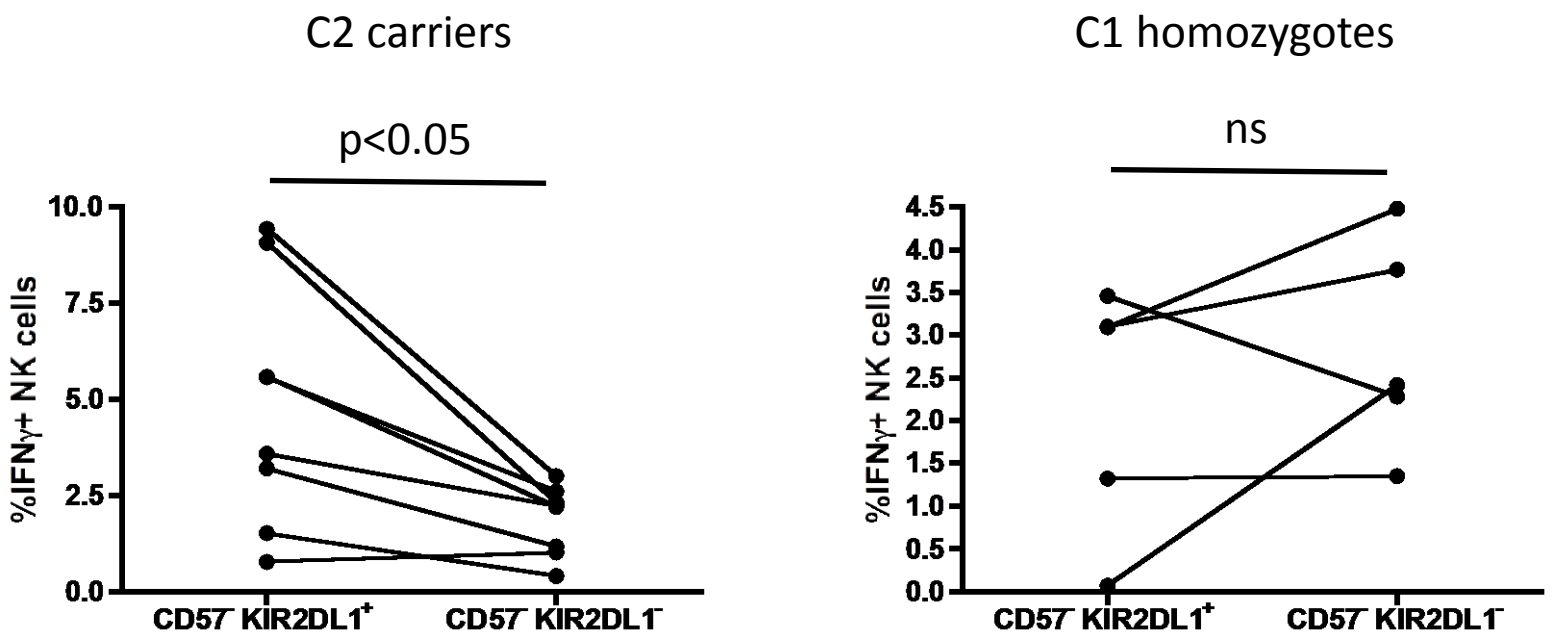
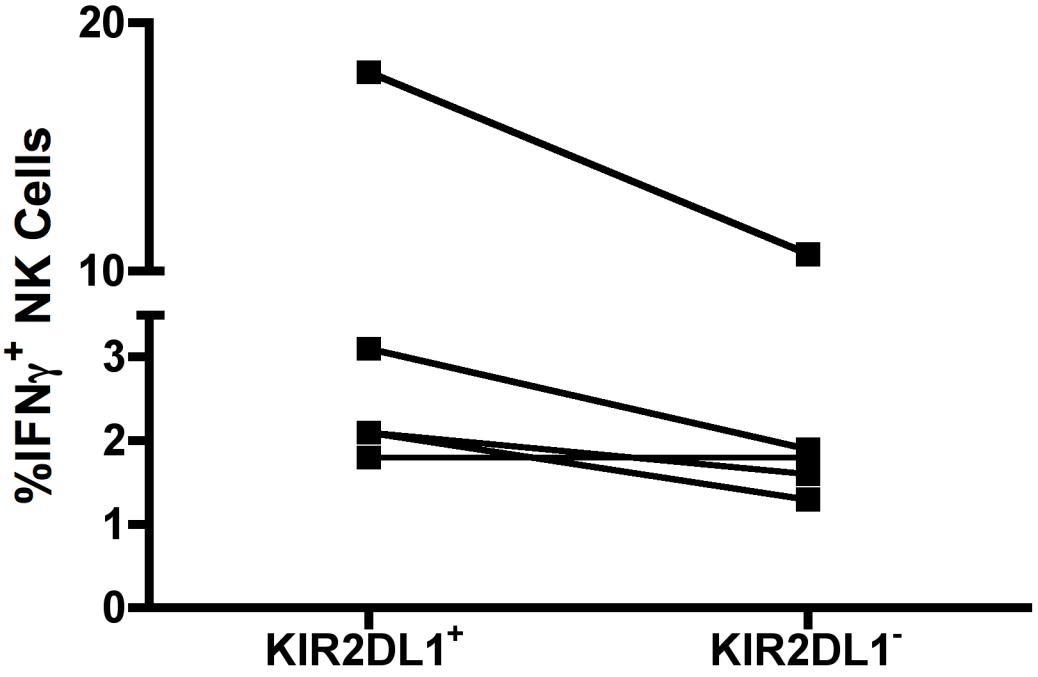
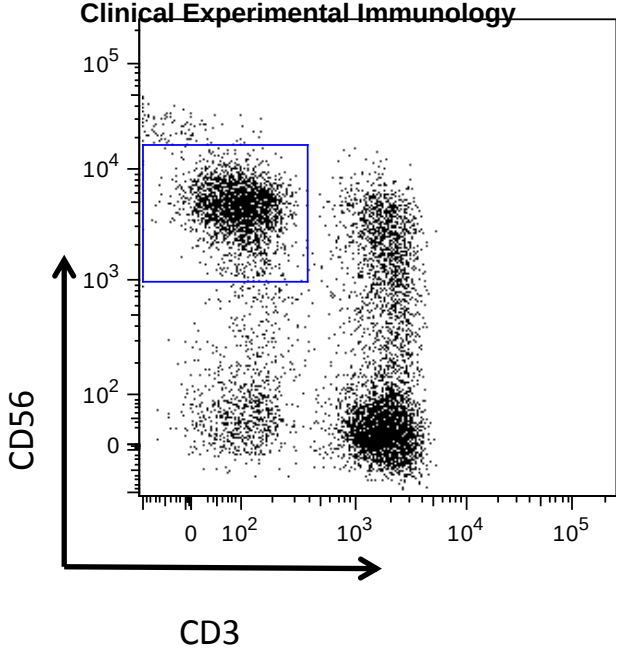
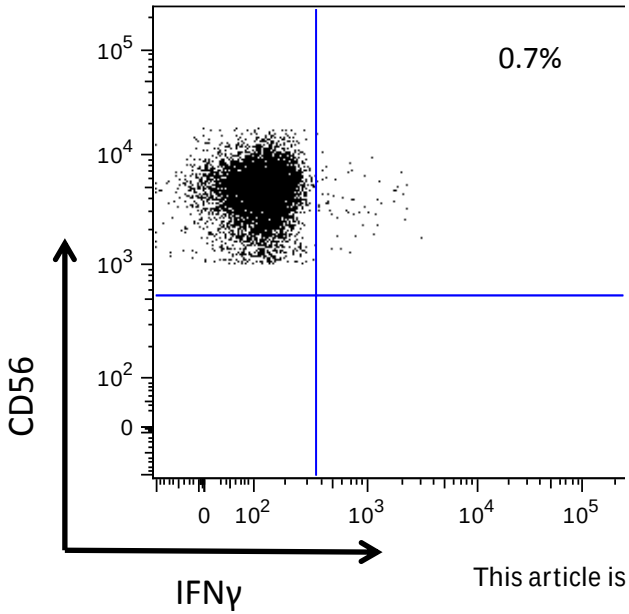


Figure 3





PBMC + gp140-coated CEM.NKr-CCR5 + HIV-1$^-$ Plasma



PBMC + gp140-coated CEM.NKr-CCR5 + HIV-1$^+$ Plasma

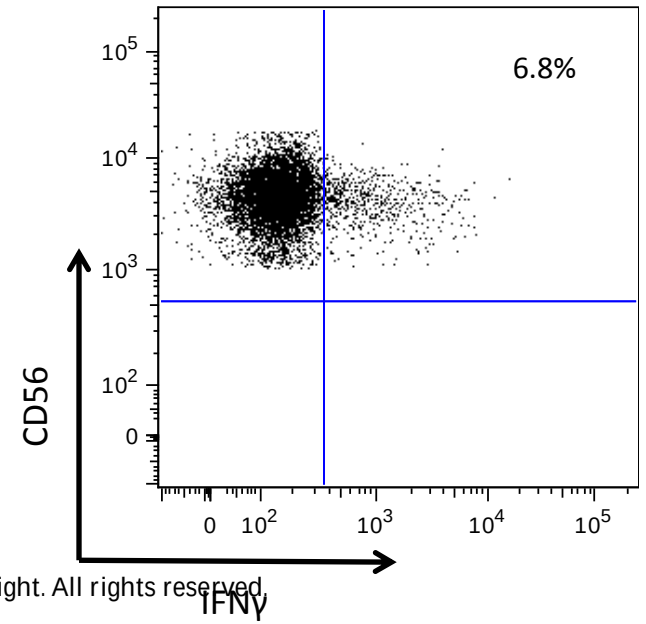


Figure 1A

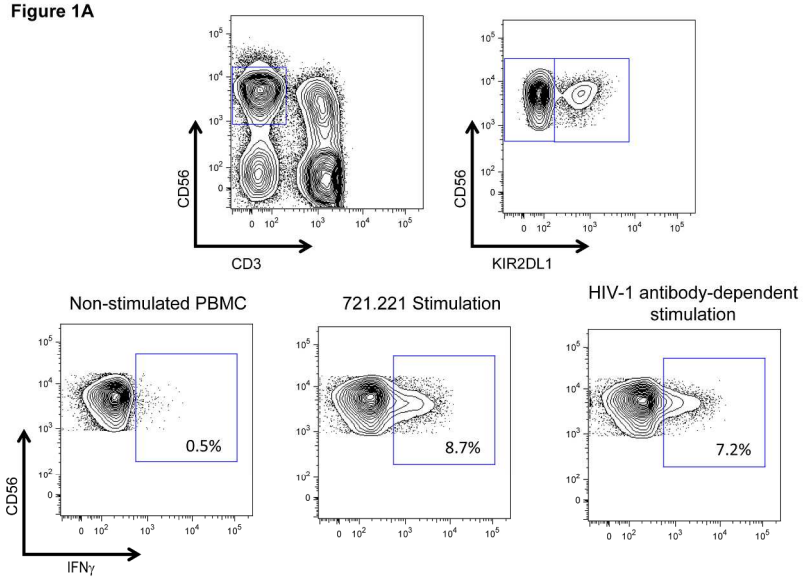


Figure 1B

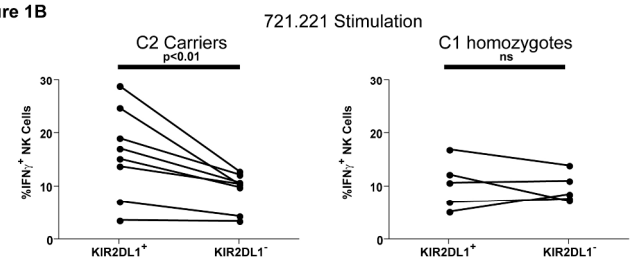


Figure 1C

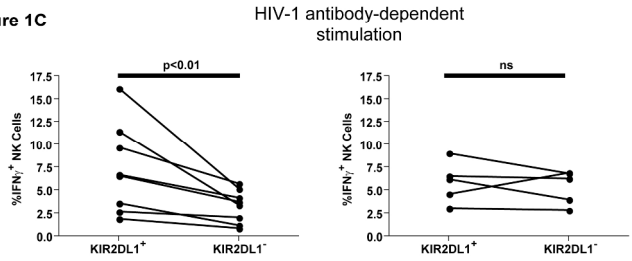


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Figure 2A

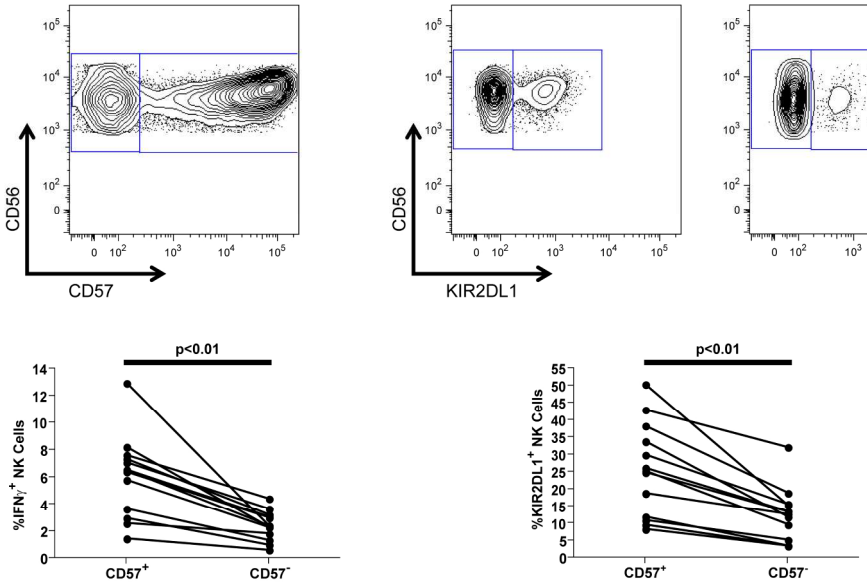

Figure 2B

Figure 2C

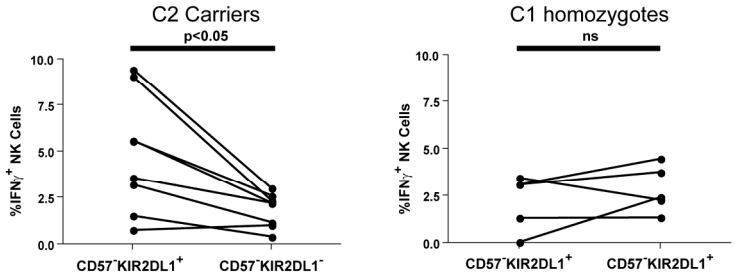

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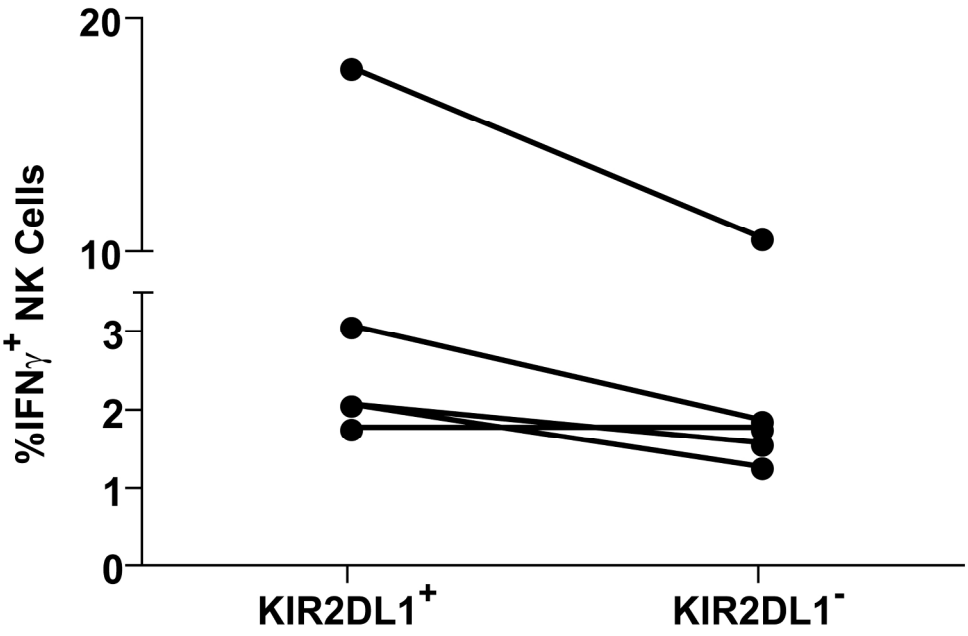
Figure 3



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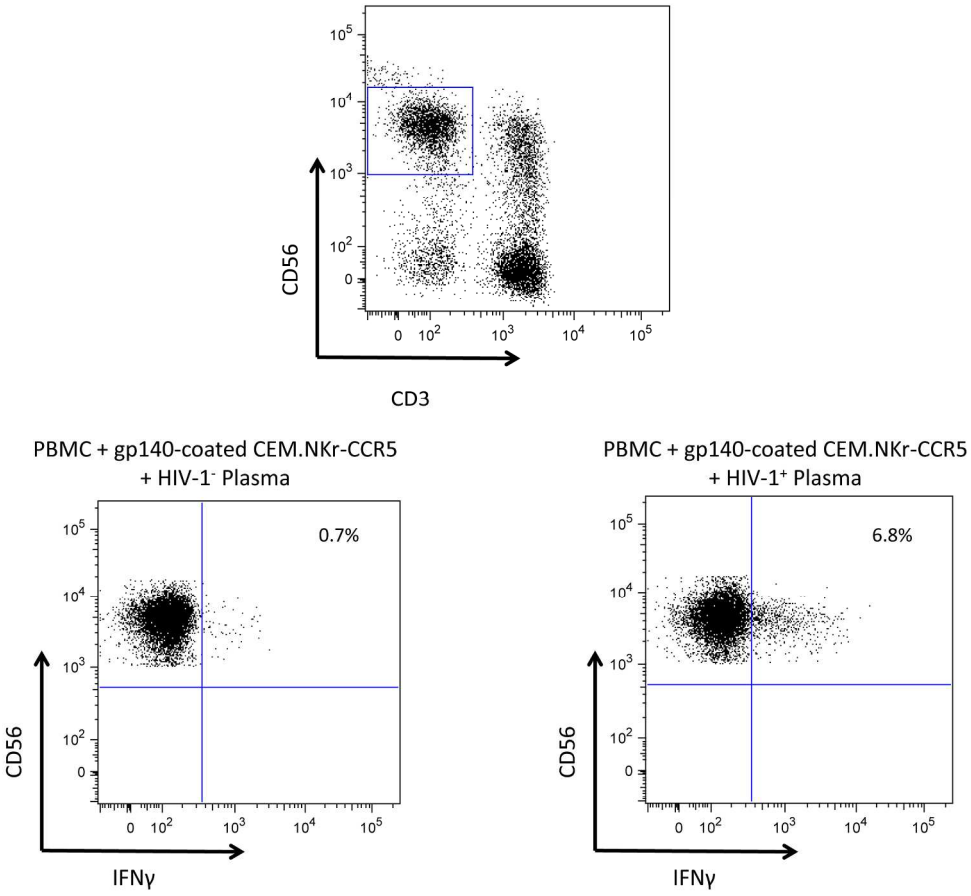

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Functional advantage of educated KIR2DL1^{+} natural killer cells for anti-HIV-1 antibody-dependent activation

Running head: NK cell education and ADCC

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* The authors declare no conflicts of interest.

Abstract

Evidence from the RV144 HIV-1 vaccine trial implicates anti-HIV-1 antibody-dependent cellular cytotoxicity (ADCC) in vaccine-conferred protection from infection. Amongst effector cells ~~capable of~~that mediate ADCC are natural killer (NK) cells. The ability of NK cells to be activated in an antibody-dependent manner is reliant upon several factors. In general, NK cell mediated antibody-dependent activation is most robust in terminally differentiated $CD57^+$ NK cells, as well as NK cells educated through ontological interactions between inhibitory killer immunoglobulin-like receptors (KIR) and their major histocompatibility complex class I (MHC-I or HLA-I) ligands. With regards to anti-HIV-1 antibody-dependent NK cell activation, previous research has demonstrated that the epidemiologically relevant KIR3DL1/HLA-Bw4 receptor/ligand combination confers enhanced activation potential. In the present study we assessed the ability of the KIR2DL1/HLA-C2 receptor/ligand combination to confer enhanced activation upon direct stimulation with HLA-I-devoid target cells or antibody-dependent stimulation with HIV-1 gp140-pulsed CEM.NKr-CCR5 target cells in the presence of an anti-HIV-1 antibody source. Amongst donors carrying the HLA-C2 ligand for KIR2DL1, higher IFNγ production was observed within $KIR2DL1^+$ NK cells than in $KIR2DL1^-$ NK cells upon both direct and antibody-dependent stimulation. No differences in $KIR2DL1^+$ and $KIR2DL1^-$ NK cell activation were observed in HLA-C1 homozygous donors. Additionally, higher activation in $KIR2DL1^+$ than $KIR2DL1^-$ NK cells from HLA-C2 carrying donors was observed within less differentiated $CD57^-$ NK cells, ~~implying~~ demonstrating observed differences were due to education and not an overabundance of $KIR2DL1^+$ NK cells within differentiated $CD57^+$ NK cells. These observations are relevant for ~~optimizing antibody-based HIV-1 vaccines~~understanding the regulation of anti-HIV-1 antibody-dependent NK cell responses.

Introduction

A prophylactic vaccine is desired to reduce the number of new HIV-1 infections. Anti-HIV-1 antibodies capable of triggering antibody-dependent cellular cytotoxicity (ADCC) might be important to elicit through vaccination. Following binding of viral epitopes on the surface of HIV-1-infected cells, the constant regions of ADCC antibodies engage the CD16 constant region receptor on innate immune cells, such as NK cells and monocytes. Engagement of CD16 can result in the lysis of the HIV-1-infected target cell [1-3]. Additionally, NK cells activated upon stimulation through CD16 release chemokines and cytokines [4, 5]. Chemokines produced by activated NK cells can directly inhibit HIV-1 replication [6]. In the context of vaccination, ADCC would allow for the elimination of autologous cells that become infected upon HIV-1 exposure, as well as HIV-1-infected allogeneic cells delivered within infected bodily fluids. The modestly successful RV144 vaccine trial has indicated a role for ADCC-competent antibodies in vaccine-conferred protection against HIV-1 infection [7-9]. Indeed, ADCC antibodies were associated with a lower likelihood of infection in vaccinees that carried low levels of anti-envelope IgA that could compete with IgG for antigen binding and block anti-HIV-1 ADCC [7, 9]. This observation highlights the need for further research into the factors regulating the ability of innate immune effector cells to mediate antibody-dependent functions, facilitating utilization of the full potential of ADCC antibodies in HIV-1 vaccine development.

The ability of NK cells to mediate anti-HIV antibody-dependent functions is dependent upon several NK cell factors, including the education status and state of differentiation of the cell [10, 11]. The education status of an NK cell is determined through interactions of activating and inhibitory NK cell receptors with self-major histocompatibility complex class I (MHC-I or HLA-I) ligands, which tunes the functional potential of the NK cell [12-14]. In general, NK cells that express inhibitory receptors capable of binding self-HLA-I are tuned for higher functional potential; whereas, NK cells lacking inhibitory receptors or expressing inhibitory receptors that do not recognize self HLA-I are tuned for reduced functional potential [12, 14]. Furthermore, NK cells expressing activating

receptors capable of binding self HLA-I are tuned for lower functional potential [13]. The education status of NK cells is linked to their ability to become activated by antibody-dependent and independent stimuli [12]. With regards to anti-HIV antibody-dependent NK cell activation, our group has previously demonstrated that educated NK cells expressing the inhibitory killer immunoglobulin-like receptor 3DL1 (KIR3DL1), derived from donors carrying the HLA-Bw4 ligand, exhibit a functional advantage over autologous KIR3DL1$^-$ NK cells and allogeneic KIR3DL1$^+$ NK cells, derived from donors lacking the HLA-Bw4 ligand [11]. In addition to education, NK cells independently undergo a differentiation process whereby they phenotypically progress from CD56^{bright}CD16$^-$CD57$^-$ to CD56^{dim}CD16$^+$CD57$^-$, and finally develop into CD56^{dim}CD16$^+$CD57$^+$NK cells [15]. Along with changes in phenotype, the functional profile of NK cells is also altered by differentiation. Indeed, NK cells expressing the CD57 differentiation marker exhibit more robust activation upon stimulation through CD16 [10, 16]. Differentiated CD57$^+$ NK cells are also more likely to express inhibitory KIRs [16]. Although differentiated NK cells expressing inhibitory KIR for self HLA-I would be educated for higher functional potential, the contributions of NK cell education and differentiation to NK cell antibody-dependent functional potential appear to be at least partially distinct [10].

Our previous research regarding the contribution of NK cell education to the antibody-dependent functional potential of NK cells focused upon the KIR3DL1/HLA-Bw4 receptor/ligand combination [10, 11, 17]. The results of those KIR3DL1/HLA-Bw4 studies corroborate epidemiological studies that have linked allelic combinations of this receptor/ligand pair to protection from HIV-1 infection and progression to AIDS [18, 19]. Several recent studies have now suggested that the inhibitory KIR2DL1 receptor might also be important for understanding susceptibility to HIV-1 infection and HIV-1 pathogenesis. The KIR2DL1 receptor binds a subset of HLA-C alleles, termed HLA-C2, which are characterized by the presence of lysine at amino acid position 80 [20]. Non-HLA-C2 alleles are termed HLA-C1 and are characterized by the presence of asparagine at amino acid position 80. ~~Evidence for a potential role of~~An association between the KIR2DL1/HLA-C2 receptor/ligand combination and ~~in~~ protection from HIV-1 infection has been provided by the

observation that exposed but uninfected Senegalese carry the education-competent KIR2DL1/HLA-C2 combination, while their infected partners lack HLA-C2 ligands that could inhibit the recognition and cytolysis of HIV-1-infected allogeneic leukocytes [21]. ~~Suggestive of a role for~~A potential link has also been noted between KIR2DL1 ~~in~~ and HIV-1 disease progression ~~is~~ through the demonstration by Korner et al. that KIR2DL1$^+$ NK cells are expanded in primary HIV-1 infection in individuals carrying the HLA-C2 ligand [22]. These expanded KIR2DL1$^+$ NK cells also exhibited a functional advantage over KIR2DL1$^-$ NK cells upon stimulation with HLA-I-devoid target cells. Interestingly, the frequency of KIR2DL1$^+$ NK cells appears to wane during chronic HIV-1 infection, further indicating a role in HIV-1 pathogenesis. Indeed, this phenomenon has been demonstrated in HIV-1 clade C-infected South Africans and clade A and D-infected Ugandans [23, 24]. Additional research has demonstrated that while the functional advantage of NK cells expressing the HLA-C binding KIR2DL1/2/3 receptors upon stimulation with HLA-I-devoid target cells is observed in HIV-1-uninfected donors, this advantage is not present in chronically HIV-1-infected donors [25].

Given that recent evidence has implied that educated KIR2DL1$^+$ NK cells might play a role in protection from HIV-1 infection and suppressing viral replication during primary HIV-1 infection, we sought to determine if education of NK cells through KIR2DL1/HLA-C2 interactions enhanced the ability of NK cells to become activated in an anti-HIV-1 antibody-dependent manner. We now present data demonstrating that education of NK cells through KIR2DL1/HLA-C2 interactions enhances NK cell activation upon exposure to antibody-dependent and antibody-independent stimuli. Furthermore, we demonstrate, through assessing CD57$^-$ NK cells, that the functional advantage of educated KIR2DL1$^+$ NK cells upon antibody-dependent stimulation is not a bystander effect of NK cell differentiation. These results enhance our understanding of the regulation of ~~ADCC effector cells and will be important for understanding how to optimize vaccines that induce ADCC-competent antibodies~~anti-HIV-1 antibody-dependent NK cell responses.

Materials and methods

Participants

Blood was collected from 13 HIV-1-uninfected donors by forearm venepuncture into vacuettes containing sodium heparin anti-coagulant. Ficoll Paque PLUS (GE Healthcare Life Sciences) density gradient centrifugation was employed to obtain PBMCs from whole blood. These PBMCs were utilized as effector cells in NK cell activation assays. As a source of anti-HIV-1 antibodies, plasma was obtained from an HIV-1-infected client of the Melbourne Sexual Health Centre. This HIV-1-infected donor's plasma has previously been shown to carry antibodies capable of activating NK cells in an anti-HIV-1-dependent manner [26]. All donors provided informed consent prior to collection of biological samples and the ethics committees of the participating institutions approved all performed experiments.

Cell lines

The $CD4^+$ CEM.NKr-CCR5 T-cell line was obtained from the NIH AIDS Reagent Program, Divison of AIDS, NIAID, NIH. The HLA-I-devoid 721.221 cell line was kindly provided by Dr. Andrew Brooks (Department of Microbiology and Immunology, University of Melbourne).

HLA-C typing and KIR2DL1 expression

The Victorian Transplantation and Immunogenetics Service at The Australian Red Cross Blood Service performed sequence-based typing of HLA-C alleles to four-digit resolution for all 13 donors. Expression of KIR2DL1 by NK cells from all 13 donors was demonstrated by staining with FITC-conjugated anti-KIR2DL1 antibody (clone: 143221; R&D Systems) and detection by flow cytometry using a BD LSR Fortessa. This antibody clone specifically detects KIR2DL1 and exhibits no cross-reactivity with other KIR2D or KIR3D gene products. Flow Jo Version 9.2 (Tree Star) was utilized for analysis of flow cytometry data.

NK cell activation assays

To study HIV antibody specific NK cell activation, we used a previously described flow cytometric assay to detect NK cell IFNγ expression [17]. Briefly, CEM.NKr-CCR5 target cells were prepared by coating with HIV-1 $gp140_{AD8}$ (3µg/1.0X10^6 cells in 1ml of solution) for 90 minutes at 4°C. The HIV-1 $gp140_{AD8}$ was prepared as previously described [27]. Next, PBMC effector cells were combined with gp140-pulsed CEM.NKr-CCR5 target cells at a 10:1 effector to target ratio in the presence or absence of a 1:2000 final dilution of plasma from an HIV-1-infected donor, Brefeldin A (5µg/ml) (Sigma) and monensin (6µg/ml) (BD). Co-cultures were incubated for five hours at 37°C. To demonstrate that the assay specifically detects anti-HIV-1 antibody-dependent responses, three donors were assessed for NK cell activation in the presence of 1:2000 dilutions of both HIV-1-uninfected and HIV-1-infected plasma. The utilized plasma dilution was implemented due to previously reported prozone effects in assays measuring anti-HIV-1 ADCC [1, 17]. Following incubation, cells were surface stained with Per CP-conjugated anti-CD3 (clone: SK7; BD), PE-Cy7-conjugated anti-CD56 (clone: NCAM16.2; BD), FITC-conjugated anti-KIR2DL1 (clone: 143221; R&D Systems) and Pacific Blue-conjugated anti-CD57 (clone: HCD57; Biolegend) antibodies. Next, cells were fixed in formaldehyde, permeabilized with 1X Perm solution (BD) and stained with Alexa Fluor 700-conjugated anti-IFNγ antibody (clone: b27; BD). Lastly, cells were fixed in formaldehyde and acquired using a BD LSR Fortessa (BD). Data was analysed with FlowJo Version 9.2. Antibody-independent NK cell activation was also assessed in an identical manner as anti-HIV-1 antibody-dependent NK cell activation above, except PBMCs were cultured with the HLA-I-devoid 721.221 cell line in the absence of any antibody sources.

Autologous whole blood antibody-dependent NK cell activation assay

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To assess anti-HIV-1 antibody-dependent NK cell activation in an autologous setting, we utilized a previously described assay measuring antibody-dependent NK cell activation by autologous

$CD4^+$ cells that have bound HIV-1 envelope and antibodies derived from HIV-1-infected plasma. Briefly, 150µl of HIV-1-uninfected whole blood was combined with 50µl of HIV-1-infected plasma, 1µg/ml HIV-1 $gp140_{AD8}$, Brefeldin A (5µg/ml and monensin (6µg/ml). Control conditions containing whole blood alone or whole blood plus HIV-1-infected plasma were conducted simultaneously. All conditions were incubated for five hours at 37°C. Following incubation, blood was incubated with Per-CP-conjugated anti-CD3 (clone: SK7; BD), PE-Cy7-conjugated anti-CD56 (clone: NCAM16.2; BD) and FITC-conjugated anti-KIR2DL1 (clone: 143221; R&D Systems) antibodies. Next, red blood cells were lysed with 1X lysis buffer (BD), permeabilized with 1X Perm solution (BD) and stained with Alexa Fluor 700-conjugated anti-IFNγ antibody (clone: b27; BD). Samples were fixed in formaldehyde and acquired using a BD LSR Fortesa (BD). Data analysis was performed with FlowJo Version 9.2.

Assessment of CD16 expression on $KIR2DL1^+$ and $KIR2DL1^-$ NK cells

Whole blood from nine donors was incubated with Per CP-conjugated CD3 (clone: SK7; BD), PE-Cy7-conjugated CD56 (clone: NCAM16.2; BD), APC-conjugated anti-KIR2DL1 (clone: 143221; R&D Systems) and FITC-conjugated anti-CD16 (clone: 3G8; BD). Next, red blood cells were lysed with 1X lysis buffer, washed and fixed in formaldehyde. Samples were acquired using a BD LSR Fortessa (BD). Data was analysed with FlowJo Version 9.2.

Statistics

GraphPad Prism version 4.0 was used for statistical analyses. Within group differences were compared using the Wilcoxon matched pairs test. Data throughout the manuscript is presented in the [median (range) vs. median (range)] format.

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Results

Direct and anti-HIV-1 antibody-dependent activation of NK cells educated through KIR2DL1

The functional advantage of educated KIR2DL1$^+$ NK cells over the KIR2DL1$^-$ population, which contains both uneducated NK cells and cells educated through other HLA/KIR combinations, has been observed upon direct stimulation, for both HIV-1-infected and uninfected donors, and non-HIV-1 antibody-dependent stimulation, for HIV-1-uninfected donors [12, 22, 28]. The role of education through KIR2DL1 on anti-HIV antibody-dependent activation potential, however, has not yet been investigated. To address this issue we stimulated NK cell effectors within PBMCs, obtained from eight HLA-C2 carrying donors and five donors homozygous for HLA-C1 alleles (Table 1), with HIV-1_{AD8} gp140-coated CEM.NKr-CCR5 T-cells in the presence of plasma from an HIV-1-infected donor. This assay specifically detects anti-HIV-1 antibody-dependent NK cell activation, as activation is observed in the presence of HIV-1-infected plasma but not in the presence of HIV-1-uninfected plasma (Supplementary Figure 1) Simultaneously, in order to demonstrate that the utilized HLA-C2 carrying donors, but not the HLA-C1 homozygous donors, exhibit the previously reported functional advantage within the educated KIR2DL1$^+$ population upon direct stimulation, we stimulated NK cells within PBMC with the HLA-I-devoid 721.221 cell line. Following stimulation, samples were assessed by flow cytometry. The gating procedure used to identify KIR2DL1$^+$ and KIR2DL1$^-$ NK cells, as well as the percentage of NK cells within each population that became activated to produce IFNγ is depicted in Figure 1A. As expected, upon stimulation with 721.221 targets the percentage of NK cells activated to produce IFNγ was higher in the KIR2DL1$^+$ population than in the KIR2DL1$^-$ population for HLA-C2 carrying donors [16.2% (3.6-28.9%) vs. 10.4% (3.4-12.9%), p=0.0078] (Figure 1B). No differences in IFNγ production was observed between these NK cell populations in donors homozygous for HLA-C1 alleles [10.7% (5.2-16.9%) vs. 8.5% (7.3-14%), p=1.00] (Figure 1B). Similarly, when NK cells were stimulated in an anti-HIV-1 antibody-dependent manner, HLA-C2 carrying donors exhibited higher percentages of IFNγ producing NK cells in the KIR2DL1$^+$ population than the

KIR2DL1$^-$ population [6.6% (1.9-16.2%) vs. 3.5% (0.9-5.7%), p=0.0078] (Figure 1C). No differences were observed between these NK cell populations upon anti-HIV-1 antibody-dependent stimulation in donors homozygous for HLA-C1 alleles [6.2% (3.0-9.0%) vs. 6.2% (2.8-6.9%), p=0.6250] (Figure1 C). As antibody-dependent NK cell activation is triggered through CD16, we next questioned if these differences in antibody-dependent NK cell activation could simply be attributed to preferentially higher expression levels of CD16 on KIR2DL1$^+$ NK cells in HLA-C2 carrying donors. Although we noted higher CD16 ~~expression~~median fluorescence intensity (MFI) on KIR2DL1$^+$ than KIR2DL1$^-$ NK cells, this expression pattern was seen in both HLA-C2 carriers [3227 (1118-5549) vs. 2814 (877-4405); n=5] and HLA-C1 homozygotes [2994 (1134-8652) vs. 2534 (993-7355); n=4]. As such, CD16 expression levels do not account for the preferential activation of KIR2DL1$^+$ NK cells in HLA-C2 carrying donors upon antibody-dependent stimulation. –These data reaffirm a role for NK cell education through KIR2DL1 in determining the ability of NK cells to exhibit activation upon direct stimulation. Further, the data suggest that the role of education in determining NK cell functional potential extends to the ability of NK cells to exhibit anti-HIV-1 antibody-dependent NK cell activation.

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Impact of NK cell differentiation on differences in activation between KIR2DL1$^+$ and KIR2DL1$^-$ NK cells

The ability of NK cells to exhibit activation upon stimulation through CD16 is higher in NK cells expressing the CD57 differentiation marker [10, 16]. As higher percentages of CD57$^+$ NK cells express KIRs, the relative contributions of education and differentiation to antibody-dependent NK cell activation can become unclear upon assessments of the total NK cell population [10, 16]. We therefore assessed the influence of KIR2DL1 expression on the function of both CD57$^+$ and CD57$^-$ NK cells within our donors. Coinciding with previously published data, we observed higher anti-HIV-1 antibody-dependent NK cell activation in the CD57$^+$ NK cells than the CD57$^-$ NK cells in all 13 donors [6.5% (1.4-13%) vs. 2.4% (0.6-4.4%), p=0.0002] (Figure 2A) [10]. Additional assessments of CD57$^+$ and CD57$^-$ NK cells revealed a higher frequency of KIR2DL1 expression in the CD57$^+$ population across all

13 donors [25.3% (8.4-50.4%] vs. 12.7% (3.5-32.1%), *p*=0.0002] (Figure 2B). Lastly, we assessed if the differences in anti-HIV-1 antibody-dependent activation observed between KIR2DL1$^+$ and KIR2DL1$^-$ NK cells in HLA-C2 carrying donors were due to education, and not a bystander effect of NK cell differentiation. We compared the anti-HIV-1 antibody-dependent activation of CD57$^-$KIR2DL1$^+$ and CD57$^-$KIR2DL1$^-$ NK cells within HLA-C2 carrying donors and HLA-C1 homozygous donors. As depicted in Figure 2C, within HLA-C2 carrying donors KIR2DL1$^+$ NK cells within the CD57$^-$ population exhibited higher levels of IFNγ production upon anti-HIV-1 antibody-dependent stimulation than the KIR2DL1$^-$ population [4.6% (0.8-9.4%) vs. 2.2% (0.4-3.0%), *p*=0.0156]. No differences were observed between the CD57$^-$KIR2DL1$^+$ and CD57$^-$KIR2DL1$^-$ populations in donors homozygous for HLA-C1 [3.1% (0.1-3.5%) vs. 2.4% (1.3-4.5%), *p*=0.3125] (Figure 2C). These data further confirm a role for NK cell education through KIR2DL1 in determining the ability of NK cells to exhibit anti-HIV-1 antibody-dependent activation.

Anti-HIV-1 antibody-dependent activation of educated KIR2DL1$^+$ NK cells against autologous targets

Although the preferential activation of educated KIR2DL1$^+$ NK cells against gp140-coated CEM.NKr-CCR5 target cells demonstrates a role for NK cell education in determining NK cell activation potential, this experimental system does not determine if target cells expressing the HLA-C2 ligand can activate KIR2DL1$^+$ NK cells. Indeed, CEM.NKr-CCR5 target cells are HLA-C1 homozygous (Dr Nicole F Bernard - personal communication). To address this issue we performed an autologous whole blood anti-HIV-1 antibody-dependent NK cell activation assay. This assay combines HIV-1-uninfected whole blood with HIV-1 $gp140_{AD8}$ and HIV-1-infected plasma. This allows CD4$^+$ cells to bind viral envelope, which binds anti-HIV-1 antibodies, and activate NK cells. Stimulation of NK cells within whole blood from five HLA-C2 carriers resulted in activation of both the KIR2DL1$^+$ and KIR2DL1$^-$ NK cell subsets (Figure 3). Intriguingly, four of the five donors screened exhibited higher activation in the KIR2DL1$^+$ [2.1% (1.8-18%) vs. 1.8% (1.3-10.7%)] than KIR2DL1$^-$ NK cell subset, while the fifth donor exhibited equal activation in both NK cell subsets. These data demonstrating that

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educated KIR2DL1^{+} NK cells can become activated in an anti-HIV-1 antibody-dependent manner against target cells expressing the HLA-C2 ligand, even maintaining a functional advantage over KIR2DL1^{-} NK cells in a majority of donors, highlight that anti-HIV-1 antibody-dependent stimulation at least partially overcomes inhibitory signals through KIR2DL1/HLA-C2 receptor ligand combinations.

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Discussion

The data presented in this manuscript provide the first demonstration that education of NK cells through KIR2DL1/HLA-C2 combinations enhances the ability of NK cells to respond upon anti-HIV-1 antibody-dependent activation. Additionally, and in concordance with previous studies, we provide data demonstrating an activation advantage of the KIR2DL1$^+$ NK cell population upon antibody-independent stimulation with HLA-I-devoid target cells [12, 22]. These observations are interesting in the context of recent studies implicating KIR2DL1/HLA-C2 combinations in providing protection from HIV-1 infection, or contributing to inhibiting viral replication during primary HIV-1 infection [21, 22]. Indeed, the data presented in the current manuscript could be of importance for understanding mechanisms contributing to protective outcomes upon HIV-1 exposure or infection.

Jennes et al recently suggested that KIR2DL1/HLA-C2 combinations could contribute to protection from HIV-1 infection [21]. In a cohort of Senegalese couples concordant and discordant for HIV-1 infection the authors observed cognate ligand matches between inhibitory KIR in HIV-1 recipients of concordant couples and HLA-I in the HIV-1 donors. In the discordant couples, cognate ligand mismatches were observed between the inhibitory KIR of the uninfected partner and the HLA-I of the infected partner. The HIV-1 uninfected partners were observed to carry the education-competent KIR2DL1/HLA-C2 combination, while their HIV-1 infected partners tended to be HLA-C1 homozygous. The authors further demonstrated that NK cells carrying KIR mismatched to HLA-I expressed on CD4$^+$ T-cells were capable of killing allogeneic CD4$^+$ T-cells, suggesting that lack of ligands for inhibitory KIRs can result in enhanced direct recognition of HIV-1-infected allogeneic target cells and offer protection from HIV-1 acquisition. The data presented in the current manuscript, showing that educated KIR2DL1$^+$ NK cells have an activation advantage for anti-HIV-1 antibody-dependent activation, might further explain the protection observed in Senegelese

serodiscordant couples. Although HIV-1 exposed uninfected individuals do not carry anti-HIV-1 IgG within their sera, it has recently been shown that antibodies passively provided by HIV-1-infected mothers to their children via breast milk can protect against virus transmission [29]. Others and we have recently observed anti-HIV-1 antibodies capable of activating NK cells and/or triggering ADCC in seminal plasma and vaginal fluids [30, 31]. As these antibodies are exchanged between HIV-1-infected donors and their uninfected partners upon exposure to HIV-1, it is possible that the NK cells within the exposed individual could utilize these antibodies to eliminate infected allogeneic lymphocytes or autologous lymphocytes infected early after exposure.

Although the prospect of eliminating HIV-1-infected allogeneic lymphocytes via ADCC is supported by the observation that KIR/HLA-I mismatched NK cells can directly kill allogeneic $CD4^+$ T-cells, the notion that autologous infected $CD4^+$ T-cells can be targeted is complicated by the presence of cognate HLA-I ligands on $CD4^+$ T-cells for the inhibitory KIRs expressed by educated NK cells. Indeed, Ward et al. demonstrated that the presence of HLA-C and HLA-E on HIV-1-infected $CD4^+$ T-cells inhibits NK cell-mediated ADCC via triggering inhibitory signals through KIR2DL1/2/3 and NKG2A [32]. Despite this observation, several additional studies assessing ADCC or antibody-dependent NK cell activation, triggered by polyclonal anti-HIV-1 antibodies or therapeutic anti-tumor monoclonal antibodies, have demonstrated that antibody-dependent NK cell stimulation can at least partially overcome inhibitory signals through HLA-I/KIR combinations [11, 17, 33, 34]. Indeed, we have recently demonstrated that educated $KIR3DL1^+$ NK cells from HLA-Bw4 carrying donors exhibit higher anti-HIV-1 antibody-dependent activation than $KIR3DL1^-$ NK cells upon stimulation with allogeneic $CD4^+$ T-cells expressing HLA-Bw4 [17]. ~~Unfortunately, the data in the current manuscript does not address the question of whether anti-HIV antibody-dependent activation of $KIR2DL1^+$ NK cells overcomes the inhibitory signals that the presence of HLA-C2 would initiate, as the CEM.NKr-CCR5 target cells used have been shown to be HLA-C1 homozygous (Dr. Nicole Bernard, McGill University, Personal communication)~~Additionally, we now show that $KIR2DL1^+$ NK cells can at least partially overcome inhibitory signals conferred through KIR2DL1/HLA-C2 combinations to become

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activated in an anti-HIV-1 antibody-dependent manner against autologous targets (Figure 3). Further research is required to resolve why some previous research has indicated robust inhibition of anti-HIV-1 antibody-dependent NK cell functions occurs through HLA-C and HLA-E interactions with KIR2DL1/2/3 and NKG2A, while we have reported anti-HIV-1 antibody-dependent stimulation at least partially overcomes inhibition through HLA-Bw4 ~~and~~ /KIR3DL1 and HLA-C2/KIR2DL1 combinations. ~~While these differences could reflect intrinsic signalling intensity differences between KIR3DL1 and KIR2DL1/2/3 or NKG2A receptors, it is perhaps most likely~~It is perhaps most likely that methodological differences account for the discrepancy. To assess anti-HIV-1 antibody-dependent responses Ward et al. utilized a pool of four monoclonal antibodies, while our studies implemented polyclonal anti-HIV-1 serum [11, 17, 32]. Smalls-Mantey et al. demonstrated that pooled monoclonal anti-HIV-1 antibodies mediate poor anti-HIV-1 ADCC compared to the polyclonal mixtures found in sera [35]. We hypothesize that the strength of the signal through CD16 determines the susceptibility of antibody-dependent NK cell responses to inhibition via inhibitory NK cell receptors. The differential ability of monoclonal and polyclonal antibodies to activate NK cells in an antibody-dependent manner is likely to determine if the preferential activation of educated NK cells reported in the current manuscript extends to antibody-dependent responses triggered against different antigenic targets. Future studies should assess the relative activation of educated and uneducated NK cells upon antibody-dependent stimulation against additional infectious disease targets~~s~~, such as influenza, or by monoclonal antibodies utilized for therapy of malignancies.

In addition to potentially modulating susceptibility to HIV-1 acquisition, the enhanced function of educated KIR2DL1^{+} NK cells have been suggested to contribute to control of HIV-1 replication during primary HIV-1 infection [22]. Indeed, educated KIR2DL1^{+} NK cells are expanded during primary HIV-1 infection, and these cells exhibit potent activation, compared to KIR2DL1^{-} NK cells, upon direct stimulation with HLA-I-devoid 721.221 target cells. Future research should assess the ability of educated KIR2DL1^{+} NK cells expanded during primary HIV-1 infection to mediate anti-HIV-1 antibody-dependent activation. As anti-HIV-1 antibodies capable of activating NK cells are

detected during primary HIV-1 infection, the anti-HIV-1 antibody-dependent activation potential of educated KIR2DL1^{+} NK cells might play a role in controlling early viral replication and establishing a lower viral set point [36].

The data presented in this manuscript adds to a growing body of literature on the importance of NK cell education and the interplay of activating and inhibitory receptors in determining the potential of NK cells to mediate anti-viral functions. Although highly~~likely~~ important for assisting with understanding the regulation of NK cell activation potential, the data presented in the current manuscript~~HIV-1 susceptibility and pathogenesis, the role of NK cell education in tuning anti-HIV-1 antibody-dependent activation potential~~ might also be useful ~~for designing anti- HIV-1 vaccines. Indeed, understanding the regulation of effector cells mediating anti-HIV-1 antibody-dependent responses will assist with optimizing antibody based vaccination protocols~~designing future research to enhance our understanding of HIV-1 susceptibility and pathogenesis.

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Author Contributions

The study was designed by MSP and SJK. Experimental work was performed by SLG and RJC. Data analysis was completed by SLG and MSP.

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Figure Legends

Figure 1. Direct and anti-HIV-1 antibody-dependent activation of KIR2DL1^{+} and KIR2DL1^{-} NK cells from HLA-C2^{+} and HLA-C1 homozygous donors. (A) Direct and anti-HIV-1 antibody-dependent activation of NK cell effectors within PBMC was accomplished by stimulation with the HLA-I-devoid 721.221 cell line or HIV-1_{AD8} gp140-pulsed CEM.NKr-CCR5 in the presence of anti-HIV-1 antibodies, respectively. Following stimulation PBMCs were stained with fluorochrome-conjugated antibodies and assessed by flow cytometry. The FACs plots depict progressive gating upon CD3^{-}CD56^{+} NK cells (Top Left), KIR2DL1^{+} and KIR2DL1^{-} NK cell subsets (Top Right), and the assessment of gated cells for IFNγ production in the non-stimulated (Bottom Left), 721.221 stimulated (Bottom Middle) and anti-HIV-1 antibody dependent stimulated conditions (Bottom Right). (B) Graphs depict the relative activation of the KIR2DL1^{+} and KIR2DL1^{-} NK cell subsets upon activation by the 721.221 cell line in eight donors carrying HLA-C2 alleles (Left) and five HLA-C1 homozygotes (Right). (C) Graphs depict the relative activation of the KIR2DL1^{+} and KIR2DL1^{-} NK cell subsets upon anti-HIV-1 antibody-dependent activation in eight donors carrying HLA-C2 alleles (Left) and five HLA-C1 homozygotes (Right).

Figure 2. The anti-HIV-1 antibody-dependent activation advantage of differentiated NK cells and the role of differentiation in the activation advantage of educated KIR2DL1^{+} NK cells. (A) The FACs plot depicts the gating utilized to identify the differentiated CD57^{+} and less differentiated CD57^{-} CD56^{dim} NK cell populations. The graph highlights the relative ability of CD57^{+}CD56^{dim} and CD57^{-}CD56^{dim} NK cells from all 13 donors to exhibit anti-HIV-1 antibody-dependent activation. (B) FACs plots depict the gating implemented to identify KIR2DL1^{+} and KIR2DL1^{-} NK cells within the CD57^{+} (Left) and CD57^{-} (Right) NK cell subsets. The graph depicts the relative percentages of CD57^{+}CD56^{dim} and CD57^{-} CD56^{dim} NK cells expressing the KIR2DL1 receptor in all 13 donors. (C) The graphs depict the relative anti-HIV-1 antibody-dependent activation of KIR2DL1^{+} and KIR2DL1^{-} NK cells within the CD57^{-}CD56^{dim} NK cell population in eight HLA-C2^{+} donors (Left) and five HLA-C1 homozygotes (Right).

Figure 3. Anti-HIV-1 antibody-dependent activation of KIR2DL1^{+} and KIR2DL1^{-} NK cells from HLA-C2 carrying donors by autologous target cells. NK cells within whole blood from five HLA-C2 carrying donors were stimulated using the autologous whole blood anti-HIV-1 antibody-dependent activation assay. Data was collected by flow cytometry and CD3^{-}CD56^{+}KIR2DL1^{+} and CD3^{-}CD56^{+}KIR2DL1^{-} NK cells were assessed for IFNγ production. The graph depicts the relative production of IFNγ by KIR2DL1^{+} and KIR2DL1^{-} NK cells from each donor.

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Supplementary Figure Legend

Supplementary Figure 1. Anti-HIV-1-specific antibody-dependent activation of NK cells against gp140-coated CEM.NKr-CCR5 target cells. NK cell effectors within PBMC were stimulated with gp140-coated CEM.NKr-CCR5 target cells in the presence 1:2000 dilutions of HIV-1^+ or HIV-1^- plasma. Antibody-dependent NK cell activation was assessed by flow cytometry. The FACS plot at the top depicts gating to identify $CD3^-CD56^+$ NK cells. The FACS plots on the bottom depict the relative amounts of NK cell activation, measured as IFNγ production, observed when NK cells and gp140-coated CEM.NKr-CCR5 cells were co-cultured in the presence of HIV-1^- (Right plot) or HIV-1^+ (Left plot) plasma. The depicted data from this single PBMC donor is representative of three independent PBMC donors tested.

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Table 1. HLA-C typing of KIR2DL1 carrying effector cell donors.

Donor ID	HLA-C alleles	HLA-C1/C2 typing
1	04:01, 14:02	C1/C2
2	07:02, 17:01	C1/C2
3	02:02, 03:04	C1/C2
4	07:02, 07:02	C1/C1
5	05:01, 12:03	C1/C2
6	07:04, 12:03	C1/C1
7	04:03, 07:02	C1/C2
8	04:01, 07:01	C1/C2
9	03:03, 07:02	C1/C1
10	03:04, 07:01	C1/C1
11	07:01, 16:02	C1/C2
12	01:02, 01:02	C1/C1
13	04:01, 12:02	C1/C2