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Role of education and differentiation in determining the potential of natural killer cells to respond to antibody-dependent stimulation

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Antibody-dependent activation of natural killer (NK) cells might facilitate protective outcomes in the context of HIV exposure or infection. Antibody-dependent activation is heightened in NK cells educated by interactions between killer immunoglobulin-like receptors (KIRs) and their major histocompatibility complex class I ligands during ontogeny. Differentiated NK cells, defined as CD57⁺, also exhibit enhanced antibody-dependent responsiveness. Although KIRs are more frequently expressed on CD57⁺ NK cells, the presented data suggest education and differentiation make independent contributions to NK cell anti-HIV envelope antibody-dependent activation.

A prophylactic vaccine is required to curb the HIV epidemic. Antibody-dependent functions of natural killer (NK) cells are attractive to utilize via vaccination. NK cells express CD16, which binds the Fc portion of antiviral immunoglobulin G bound to HIV envelope on infected cells. Antibody-dependent activation of NK cells facilitates cytolysis of infected cells and cytokine production [1,2]. Antibody-dependent NK cell responses associate with protective outcomes during HIV infection and may confer vaccine-mediated protection [3,4]. Understanding regulation of NK cell antibody-dependent activation may improve the efficacy of antibody-eliciting vaccines.

Regulation of NK cell potential to exhibit antibody-dependent activation occurs during the ontological process of education [5–8]. During education, NK cells expressing inhibitory receptors, in individuals expressing particular major histocompatibility complex class I [MHC-I or human leukocyte antigen (HLA) I] ligands, are conferred with improved potential to respond upon stimulation [9]. NK cells educated through killer immunoglobulin-like receptor (KIR)/HLA-I ligand combinations of KIR3DL1/HLA-Bw4 or KIR2DL/HLA-C demonstrate higher cytolysis and/or cytokine production upon stimulation with antibody-coated allogeneic or xenogeneic targets [5,7,8]. Higher activation of educated KIR3DL1⁺ NK cells, as compared with KIR3DL1⁻ NK cells, occurs against autologous targets coated with HIV antigens and antiviral antibodies [6].

Antibody-dependent NK cell activation is also influenced by their differentiation stage, as exemplified by CD56 and CD57 expression [10]. Early in development, NK cells exhibit a CD56^{bright}CD57⁻ phenotype and produce high levels of cytokines upon stimulation. CD56^{bright}CD57⁻ NK cells develop into CD56^{dim}CD57⁻ cells, which are more efficient at mediating cytolysis. Upon further development, CD56^{dim} NK cells express the CD57 differentiation marker [11]. CD57⁺ NK cells mediate more robust CD16-dependent activation than CD56^{dim}CD57⁻ NK cells [11]. The reason for increased function through CD16 is unclear. Although CD57⁺ NK cells express higher levels of CD16, there are also changes in the expression of other NK cell receptors throughout development. In particular, CD57⁺ NK cells more frequently express KIRs than CD57⁻ NK cells [11]. As KIR expression is increased on CD57⁺ NK cells, enhanced antibody-dependent activation of these cells may reflect higher levels of education. Alternatively, heightened antibody-dependent activation of KIR-expressing NK cells might reflect increased differentiation, and thus, higher CD16 expression.

To dissect the roles of education and differentiation in the antibody-dependent activation potential of NK cells, we utilized a previously described assay to measure anti-HIV antibody-dependent NK cell activation [1,12]. Briefly, 150 μ l of whole blood from six HIV-uninfected individuals, who expressed KIR3DL1 and carried HLA-A or HLA-B alleles containing the HLA-Bw4 epitope, was mixed with 50 μ l of HIV-infected plasma, from a donor carrying NK cell-activating antibodies [12], and incubated with 1 μ g/ml of HIV-1_{AD8} gp140 for 5 hours at 37°C. The envelope glycoprotein was produced as previously described [13]. Control conditions included incubation of blood alone, blood with gp140 only or blood with HIV⁺ plasma only. Fluorochrome-conjugated antibodies (CD3, CD56, CD57, CD16, and KIR3DL1) were utilized to detect subpopulations of NK cells expressing degranulation marker CD107a and interferon (IFN) γ by flow cytometry. Activation was defined as coexpression of IFN γ and CD107a by NK cells. Data are presented in the following format: [median (range) vs. median (range), *P* value].

Robust NK cell activation was observed in all donors upon incubation with combinations of gp140 and HIV⁺ plasma, but not after incubation with gp140 or HIV⁺ plasma alone. Fig. 1a depicts representative gating used to identify activated NK cells. Comparisons of the activation of KIR3DL1⁺ educated NK cells and the KIR3DL1⁻ subset, as well as comparisons of the

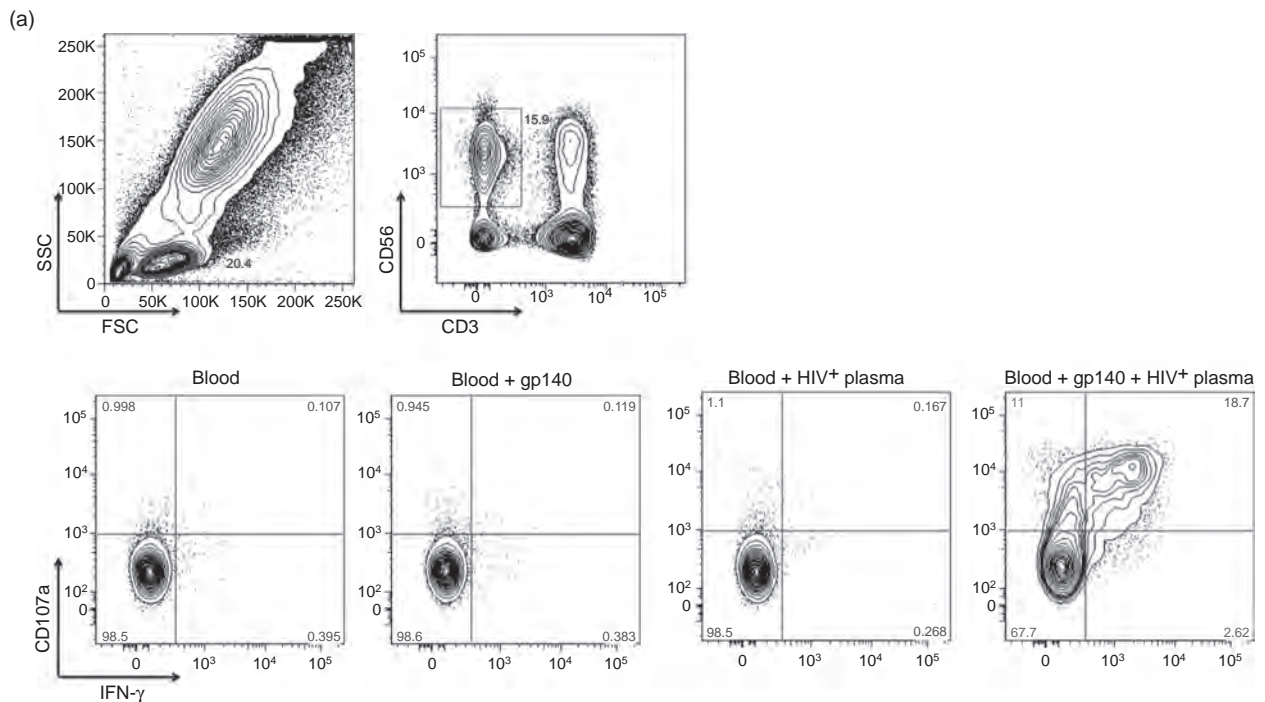


Fig. 1. Detection of antibody-dependent natural killer cell activation in educated and differentiated subsets. (a) Contour plots depict the general gating strategy utilized to assess natural killer (NK) cell activation upon antibody-dependent activation. After utilizing forward and side scatter to gate upon lymphocytes, NK cells were defined as $CD3^{-}CD56^{dim}$ and were assessed for expression of the CD107a degranulation marker and production of interferon (IFN) γ under nonstimulating (i.e. blood alone, blood and gp140, and blood and HIV⁺ plasma) and stimulating conditions (i.e. blood and gp140 and HIV⁺ plasma). (b) Contour plots were used to gate upon $CD57^{+}$ differentiated and $CD57^{-}$ nondifferentiated cells (top left) or $KIR3DL1^{+}$ educated and $KIR3DL1^{-}$ cells (top right) within the $CD3^{-}CD56^{dim}$ NK cell gate. Graphs on the bottom depict the relative antibody-dependent activation exhibited by differentiated and nondifferentiated (left) or $KIR3DL1^{+}$ and $KIR3DL1^{-}$ (right) NK cells. (c) Contour plots were used to gate differentiated $CD57^{+}$ and nondifferentiated $CD57^{-}$ NK cell subsets (top left), which were analyzed for $KIR3DL1$ expression (bottom left). The graph on the right depicts the percentage of differentiated and nondifferentiated NK cells expressing $KIR3DL1$. (d) To determine the relative contribution of NK cell education and differentiation on NK cell antibody-dependent functional potential, NK cells exhibiting different phenotypic permutations of $CD57$ and $KIR3DL1$ were assessed for the $CD107a^{+}IFN\gamma^{+}$ phenotype under nonstimulating (i.e. blood alone) and stimulating conditions (i.e. blood and gp140 and HIV⁺ plasma). Background NK cell activation in the nonstimulating condition, the total NK cell activation upon stimulation for anti-HIV antibody-dependent NK cell activation and net NK cell activation after subtraction of background were assessed to detect activation differences between NK cells expressing different phenotypic permutations of $CD57$ and $KIR3DL1$. (e) Whole blood was incubated alone or in the presence of the 3G8 anti-CD16 monoclonal antibody for 5 hours and assessed by flow cytometry for expression of the CD107a degranulation marker and IFN γ production. Fluorescence-activated cell sorting plots on the left depict gating upon lymphocytes, $CD3^{-}CD56^{+}$ NK cells and assessment for CD107a and IFN γ in stimulated (i.e. blood and 3G8) and nonstimulated conditions (i.e. blood alone). The graph on the right depicts the percentages of $CD107a^{+}IFN\gamma^{+}$ NK cells within NK cell subsets expressing different phenotypic permutations of $KIR3DL1$ and $CD57$ under nonstimulating and stimulating conditions. (f) Graphs depict the relative median fluorescence intensities of CD16 on $CD57^{+}$ vs. $CD57^{-}$ (left), $KIR3DL1^{+}$ vs. $KIR3DL1^{-}$ (middle), and $CD57^{+}KIR3DL1^{+}$ vs. $CD57^{-}KIR3DL1^{+}$ (right) NK cells. The P value above each graph was obtained using Wilcoxon Match Pairs tests. Differences were considered statistical significant when $P < 0.05$.

activation of the $CD57^{+}$ and $CD57^{-}$ NK cells were conducted. $KIR3DL1^{+}$ and $CD57^{+}$ NK cells, respectively, exhibited more activation upon stimulation than $KIR3DL1^{-}$ [11.05% (4.7–26.1%) vs. 4.8% (1.8–10.7%), $P < 0.05$] or $CD57^{-}$ NK cells [9.05% (4.2–17.7%) vs. 3.5% (1–7.8%), $P < 0.05$] (Fig. 1b).

As higher percentages of $CD57^{+}$ NK cells express $KIR3DL1$ [30.8% (13.1–46.8%) vs. 19.8% (6.2–30.5%), $P < 0.05$] [11] (Fig. 1c), we questioned if the heightened

potential of $CD57^{+}$ and $KIR3DL1^{+}$ NK cells to exhibit antibody-dependent activation reflected the same phenomenon. Despite observing no pre-existing ex-vivo differences between NK cell subsets expressing different phenotypic permutations of $KIR3DL1$ and $CD57$ (Fig. 1d), anti-HIV antibody-dependent activation data suggest that education and differentiation make independent contributions to antibody-dependent NK cell activation. Upon subtraction of the low nonstimulated background (<4%) activation from anti-HIV

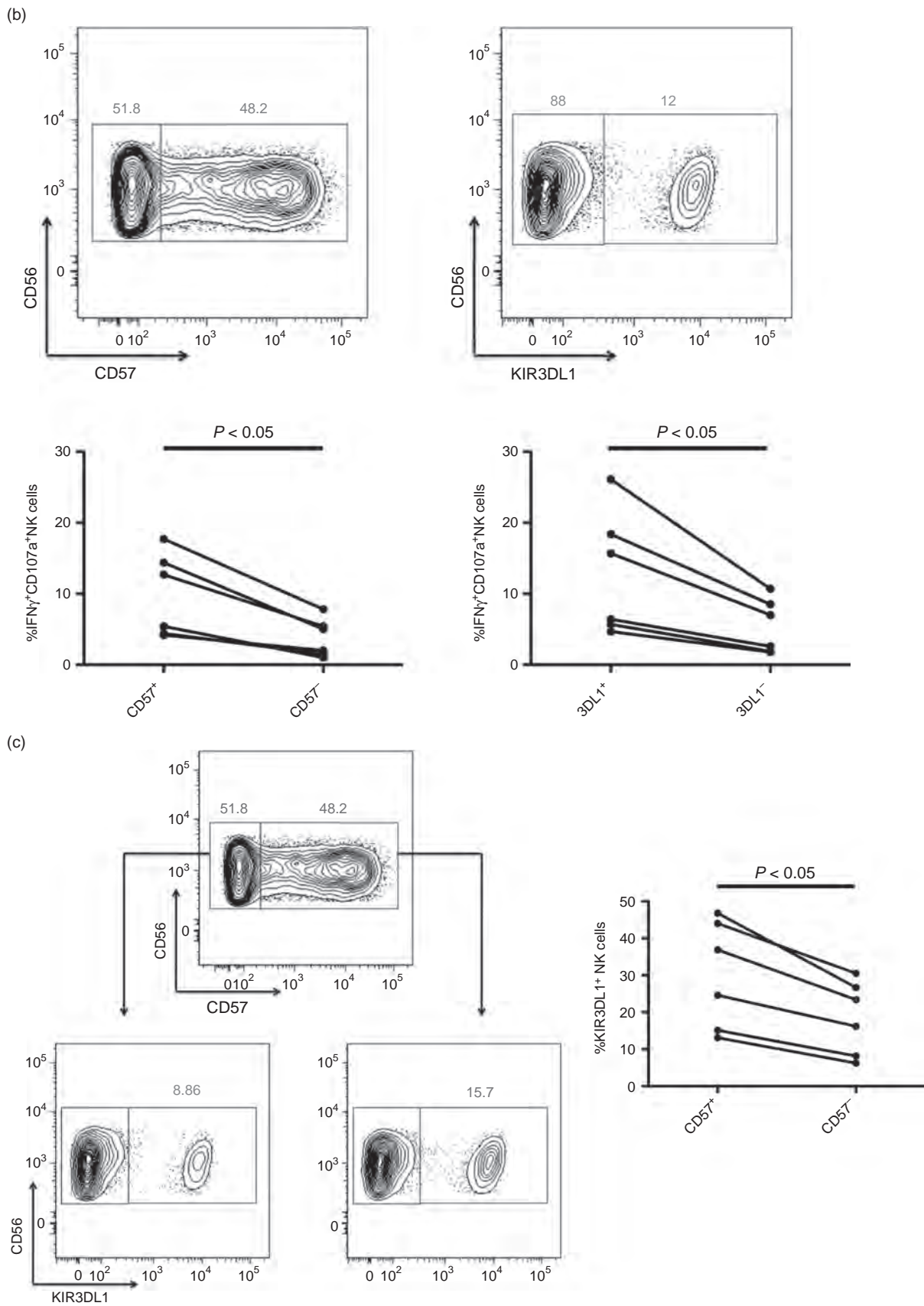


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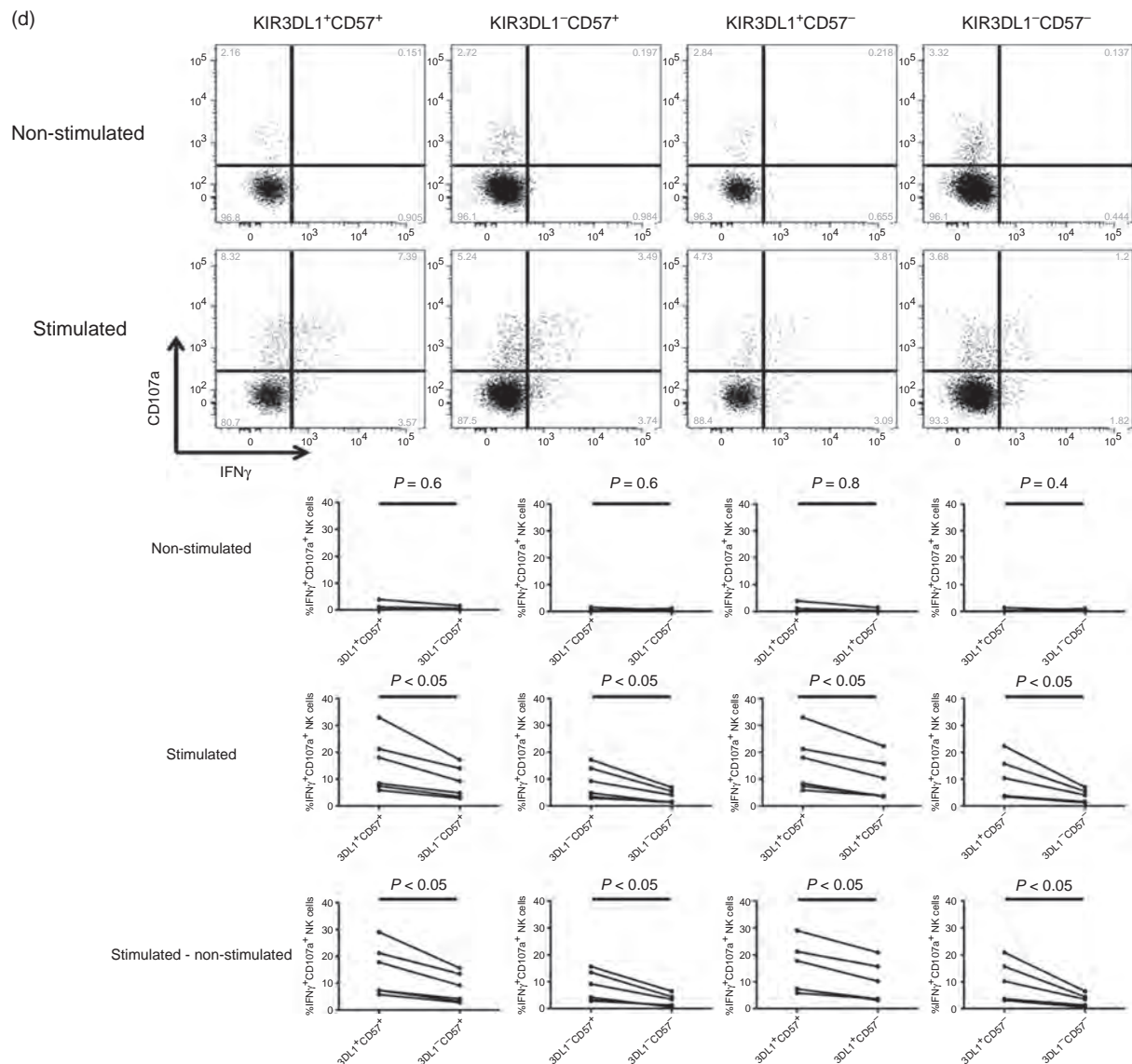


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antibody-dependent activation, KIR3DL1⁺CD57⁻ cells exhibited higher activation than KIR3DL1⁻CD57⁻ cells [6.9% (3.1–20.9%) vs. 2.4% (0.5–6.5%), $P < 0.05$] and KIR3DL1⁺CD57⁺ cells exhibited higher activation than KIR3DL1⁺CD57⁻ cells [12.6% (5.8–29%) vs. 6.9% (3.1–20.9%), $P < 0.05$] (Fig. 1d). Further, CD57⁺KIR3DL1⁻ cells exhibited higher activation than CD57⁻KIR3DL1⁻ cells [6.7% (2.8–15.7%) vs. 2.4% (0.5–6.5%), $P < 0.05$] and CD57⁺KIR3DL1⁺ cells exhibited higher activation than CD57⁻KIR3DL1⁻ cells [12.6% (5.8–29%) vs. 6.7% (2.8–15.7%), $P < 0.05$] (Fig. 1d). Furthermore, the differences in anti-HIV antibody-dependent NK cell activation between these subsets were still observed without background subtraction (Fig. 1d). Similarly, if NK cells were activated by the addition of the 3G8 anti-CD16 antibody to whole blood (as described previously [12]), the same patterns of activation were observed between NK cell

subsets expressing different phenotypic permutations of KIR3DL1 and CD57 (Fig. 1e).

Finally, to determine if higher activation in differentiated and educated NK cells was because of differences in expression of CD16, we compared nonstimulated CD57⁺ with CD57⁻, KIR3DL1⁺ with KIR3DL1⁻, and CD57⁺KIR3DL1⁺ with CD57⁻KIR3DL1⁺ NK cells for CD16 expression. Median fluorescence intensity of CD16 did not differ between KIR3DL1⁺ and KIR3DL1⁻ NK cells [1866 (644–2541) vs. 1772 (617–2421), $P = 0.31$]. As previously reported [11], CD57⁺ cells trended towards higher CD16 levels than CD57⁻ cells [1851 (670–2480) vs. 1759 (568–2346), $P = 0.06$]; however, CD57⁺KIR3DL1⁺ and CD57⁻KIR3DL1⁺ NK cells did not differ in CD16 expression levels [1826 (626–2566) vs. 1967 (670–2529), $P = 0.84$] (Fig. 1f). As CD57⁺KIR3DL1⁺ NK cells exhibit higher

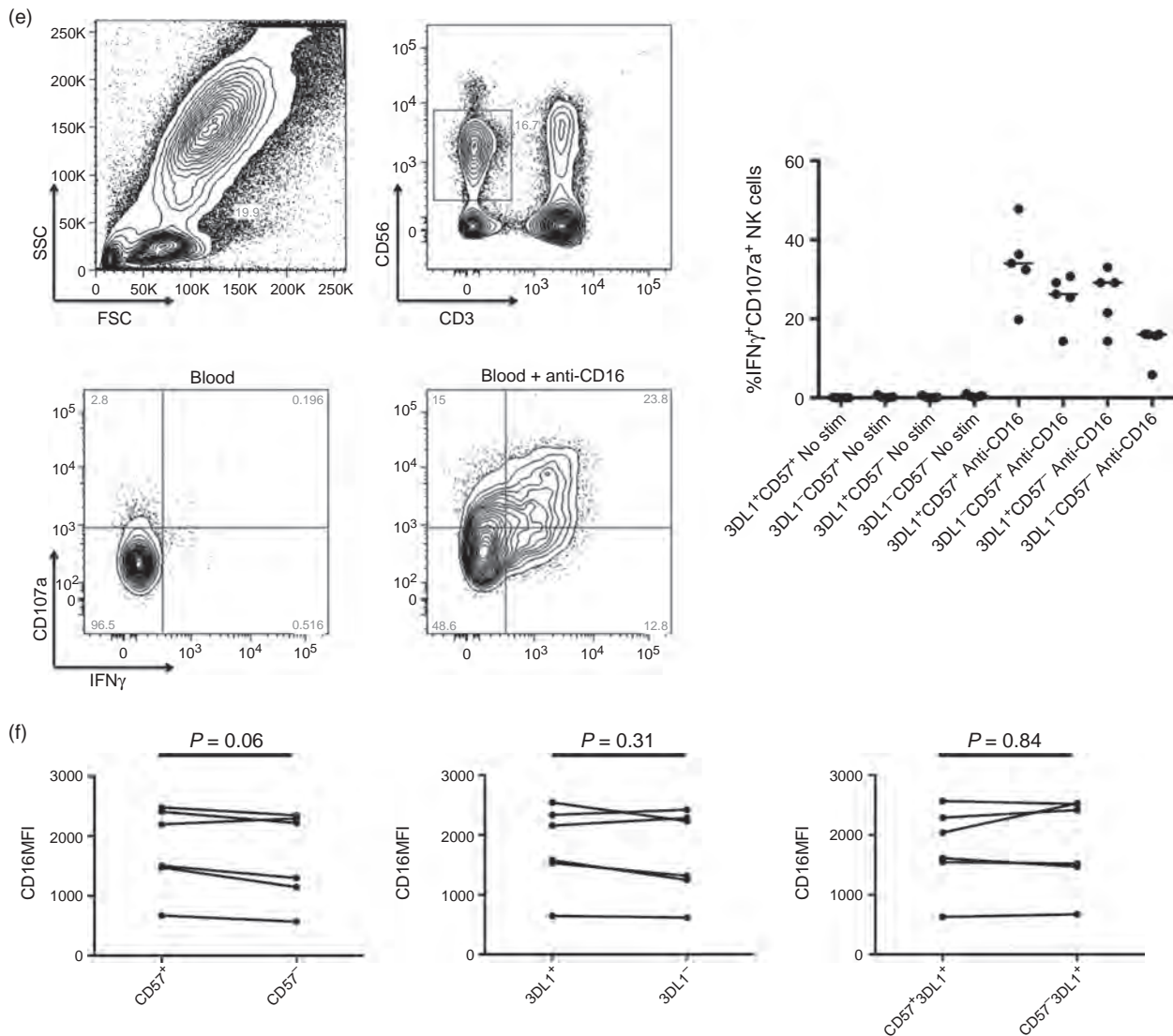


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antibody-dependent activation than CD57⁻KIR3DL1⁺ NK cells, these data collectively suggest, within these donors, that the antibody-dependent NK cell activation advantages of educated and differentiated NK cells are not because of CD16 expression.

Our results confirm previously published data demonstrating enhanced antibody-dependent activation in educated and differentiated NK cells [5–8,11]. We now show that education and differentiation make independent contributions to antibody-dependent NK cell activation potential. The antibody-dependent activation advantage in differentiated NK cells might be of importance for understanding HIV-related immunopathogenesis. Despite CD57^{bright} NK cells being over-represented within the CD56^{dim} NK cell subset during HIV infection [14], HIV-infected individuals with progressive infections exhibit reduced NK cell activation through CD16 [15]. However, CD57⁺ NK cells from

HIV-infected donors express higher levels of HLA-DR *ex vivo* [14], suggesting these NK cells are being activated *in vivo* and potentially reducing their responsiveness in NK cell activation assays.

Within the presented data, heightened CD16 expression levels did not explain the increased function of educated and differentiated NK cells. It is possible that increased activation in differentiated NK cells could be because of education through other inhibitory NK cell receptors. However, we found CD57⁺ NK cells that lack expression of KIR3DL1 and other education competent receptors, such as NKG2A, KIR2DL1, KIR2DL2, and KIR2DL3 still exhibit higher activation than CD57⁻ NK cells lacking these receptors (not shown). Although education appears to contribute to the activation potential of differentiated and nondifferentiated NK cells (Fig. 1d), differentiated NK cells appear to have enhanced antibody-dependent activation for reasons not solely

dependent upon education. These observations imply NK cell antibody-dependent activation of differentiated NK cells might be obtainable outside the genetic constraints imposed by NK cell education. Vaccines utilizing antibody-dependent NK cell effector functions may be improved by selectively boosting differentiated NK cell populations.

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Conflicts of interest

There are no conflicts of interest.

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Successful treatment of aplastic anaemia associated with HIV infection with eltrombopag: implications for a possible immunomodulatory role

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We report the first successful treatment with the thrombopoietin receptor mimetic eltrombopag in a patient with severe aplastic anaemia (SAA) associated with HIV infection, thereby avoiding the use of standard immunosuppressive agents for treatment of SAA. Eltrombopag induced a trilineage haematological response. We also show that eltrombopag had an immunomodulatory role with a decrease in proinflammatory T helpers (Th1 and Th17 cells) with increased T-regulatory cell/T-helper ratio, thus contributing to recovery of haemopoiesis.

A 67-year-old man with HIV (CD4⁺ cell count 93 cells/ μ l, viral load <40 copies/ml) developed a purpuric rash and pancytopenia. Haemoglobin (Hb) was 105 g/l, white cell count (WBC) 2.8×10^9 /l, neutrophils 1.2×10^9 /l, platelets 3×10^9 /l, reticulocytes 5×10^9 /l). An initial diagnosis of immune