HHS Public Access

Author manuscript

J Immunol. Author manuscript; available in PMC 2017 April 15.

Published in final edited form as:

J Immunol. 2016 April 15; 196(8): 3398–3410. doi:10.4049/jimmunol.1502469.

KIR3DL1 and HLA-B density and binding calibrate NK education and response to HIV

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Abstract

Natural killer (NK) cells recognize “self” HLA via killer Ig-like receptors (KIR). Homeostatic HLA expression signals for inhibition via KIR, and downregulation of HLA, a common consequence of viral infection, allows NK activation. Like *HLA, KIR* are highly polymorphic, and allele combinations of the most diverse receptor-ligand pair, *KIR3DL1* and *HLA-B*, correspond to hierarchical HIV control. We used primary cells from healthy human donors to demonstrate how subtype combinations of *KIR3DL1* and *HLA-B* calibrate NK education and their consequent capacity to eliminate HIV-infected cells. High-density KIR3DL1 and *Bw4-80I* partnerships endow NK cells with the greatest reactivity against HLA-negative targets; NK cells exhibiting the remaining KIR3DL1/*HLA-Bw4* combinations demonstrate intermediate responsiveness; and *Bw4*-negative KIR3DL1^{+} NK cells are poorly responsive. Cytotoxicity against HIV-infected autologous CD4^{+} T cells strikingly correlated with reactivity to HLA-negative targets. These findings suggest that the programming of NK effector function results from defined features of receptor and ligand subtypes. KIR3DL1 and HLA-B subtypes exhibit an array of binding strengths. Like KIR3DL1, subtypes of HLA-Bw4 are expressed at distinct, predictable membrane densities. Combinatorial permutations of common receptor and ligand subtypes reveal binding strength, receptor density, and ligand density to be functionally important. These findings have immediate implications for prognosis in patients with HIV infection. Furthermore, they demonstrate how features of KIR and HLA modified by allelic variation calibrate NK cell reactive potential.

Keywords

Natural Killer cells; AIDS; Human; Viral; Cytotoxicity

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Introduction

NK cells weigh inhibitory input against activating signals to distinguish healthy from diseased cells (2). To simultaneously achieve self-tolerance and sensitivity to unhealthy cells, NK cells rely on interaction between cell surface killer Ig-like receptors (KIR) and their HLA class I ligands. KIR receptors convey inhibitory signaling upon binding to their HLA ligands, thereby preventing auto-aggression. Interestingly, KIR and HLA interaction is also critical for NK education, a poorly understood process that confers NK cell sensitivity to loss of normal HLA expression on diseased cells. Independent segregation of KIR and HLA genes frequently leads to the same individual harboring NK populations exhibiting KIR for self-HLA as well as NK populations exhibiting KIR for which the cognate ligand is lacking (3, 4). Educated cells expressing KIR molecules specific for self-HLA class I molecules readily respond to activation signals on target cells when HLA is absent or downregulated (3, 5–7). In contrast, uneducated NK cells lacking inhibitory receptors for "self" class I molecules require more potent stimulation, including inflammation or opsonizing antibodies, to be activated for effector function, but their insensitivity to inhibition by class I molecules is beneficial in conditions where HLA expression persists (8–10).

Among individuals with the same KIR and HLA genes, NK populations frequently exhibit dramatic differences in inhibitory sensitivities and reactive potentials, possibly attributable to significant allelic variation for both receptor and ligand (11–14). *KIR3DL1* and *HLA-B* form the most ancient and polymorphic receptor-ligand pair (15). Wide variation in *KIR3DL1* and *HLA-B* alleles occurs throughout global populations, but both genes are maintained at nearly 100% frequency, underscoring a co-evolution that has selected for substantial subtype diversity (16).

The 77 unique alleles of *KIR3DL1* are classified into four subtypes based on their surface expression density and sequence homology: *KIR3DS1*, null (*KIR3DL1-n*), low (*KIR3DL1-l*) and high (*KIR3DL1-h*) (17–19). Considerably more diverse, the HLA-B alleles can nevertheless be clustered based on polymorphisms at positions 77–83 that define either the Bw6 epitope, which does not interact with any KIR, or the Bw4 epitope, a ligand for KIR3DL1 (20). Based on a dimorphism (isoleucine vs threonine) at position 80 that affects interaction with KIR3DL1, the *HLA-Bw4* alleles can be segregated further into *Bw4-80I* or *Bw4-80T* subtypes.

In experiments using transfectant systems and tetramer binding, specific combinations of KIR3DL1 and HLA-Bw4 subtypes exhibit different receptor-ligand binding affinities and inhibitory strengths (13, 14, 21). KIR3DS1 and KIR3DL1-n subtypes are not known to engage Bw4 molecules on neighboring cells; however, specific peptides including those from HIV may facilitate engagement of KIR3DS1 by Bw4-80I (22). KIR3DL1-l and –h subtypes, in contrast, bind both Bw4 subtypes, with varying strengths. KIR3DL1*005, a common KIR3DL1-l isoform, binds Bw4-80I and -80T tetramers with similar affinity (21). KIR3DL1-h, notably the common KIR3DL1*001 and *015 isoforms, preferentially engage Bw4-80I over -80T tetramers (13, 21, 23). The functional relevance of such preferential

binding remains to be determined in primary NK cells, where additional factors, including receptor and ligand densities, might influence cell-cell interactions and NK education.

Combinations of *KIR3DL1* and *HLA-B* subtypes are associated with distinct rates of disease progression in persons infected with HIV (24). Notably, pairings of *Bw4-80I* with *KIR3DL1-h, -n,* or *KIR3DS1* are associated with the slowest HIV progression. The remaining combinations of *KIR3DL1* and *HLA-Bw4,* while less protective, are still superior to those lacking *HLA-Bw4* (24). HIV infection leads to downregulation of HLA-B (25, 26). Therefore, to the KIR3DL1$^+$ NK cell, the autologous HIV-infected cell may appear as a target cell lacking self-HLA, and NK cells educated for high sensitivity to "missing self" would be expected to mount a robust response. Challenged with HLA class I-negative targets, NK cells from individuals with *KIR3DL1-h* and *HLA-B*51* or *B*52*, both *Bw4-80I* subtypes, exhibit enhanced IFN-γ production compared with other *KIR3DL1/HLA-Bw4* subtype combinations (27). Furthermore, when *HLA-B*57,* a *Bw4-80I* subtype, is combined with *KIR3DL1-h,* a "trifunctional" NK population capable of cytotoxicity, cytokine and chemokine production is identifiable (28–30). Limited to only a few pairs, however, published analyses could only speculate about the molecular characteristics of receptor-ligand relationships responsible for governing NK cell education and HIV control.

To understand how epistatic interactions between KIR3DL1 and HLA-Bw4 define hierarchical control of HIV, we investigated 7 KIR3DL1 and 20 HLA-B allotypes, whose pairings were informative for receptor density, ligand density, and receptor-ligand binding strength. We now report that HLA-Bw4 subtypes exhibit significant differences in cell surface expression, and we demonstrate wide differences in strengths of binding between KIR3DL1 and HLA-B subtypes. We find that high cell surface expression of both receptor and ligand, as well as strong binding between KIR3DL1 and HLA-Bw4, cooperatively generate the most potent reactivity of primary NK cells against HLA-negative target cells and autologous CD4$^+$ cells infected with HIV. These new insights reveal how NK immunogenetics vary receptor and ligand interactions to control NK education and innate immunity against HIV.

Materials and Methods

Healthy Donor PBMCs and cell lines

Buffy coats were collected from volunteer blood donors at the New York Blood Center (http://nybloodcenter.org/). These samples were obtained anonymously; therefore, the MSKCC IRB waived the need for additional research consent. Peripheral blood was additionally collected from healthy donors at MSKCC following approval by the MSKCC IRB, and donors provided informed written consent. PBMC were isolated by ficoll purification, aliquoted and stored in liquid nitrogen prior to experimentation. DNA was isolated from PBMCs using DNeasy Blood and Tissue mini kits (Qiagen, Valencia, CA).

Expi293F cells were maintained in Expi293 expression medium according to the manufacturer's instructions (Life Technologies, Grand Island, NY). Phoenix A cells were obtained from ATCC and maintained in DMEM containing 10% FBS. 721.221 and Jurkat cells, kind gifts from Dr. Richard O'Reilly (Memorial Sloan Kettering Cancer Center) and

Dr. Steven Nimer (University of Miami, Miami FL), respectively, were maintained in RPMI containing 10% FBS.

KIR typing, *KIR3DL1* allele analysis and HLA genotyping

Medium resolution typing for *HLA* alleles was completed by Histogenetics, Inc. (Ossining, NY, USA). *HLA*-A, -*B* and -*C* epitopes were assigned to *HLA-Bw4, -Bw6, -C1,* and -*C2* subtypes using the HLA Immunopolymorphism database version 3.14.0. KIR genotyping and *KIR3DL1* subtyping were performed as previously described (19, 31, 32). Individuals with *KIR3DL1*002*-group high alleles were examined using a Luminex-based KIR SSO platform according to the manufacturer's instructions. The probe recognition site for bead region 64 spans the codons 236-239 and specifically targets the alleles *KIR3DL1*002* and *KIR3DL1*054. KIR3DL1*054* is a rare allele, lacking entirely from at least two patient cohorts (33, 34). Therefore, individuals positive for bead region 64 by Luminex and *KIR3DL1*002*-group typing by PCR-SSP were assumed to exhibit *KIR3DL1*002. KIR3DL1*002* and **015* represent a total of 70.2% of the alleles identified in the *KIR3DL1*002* group by PCR-SSP. Therefore, alleles not identified as *KIR3DL1*002* in this group were assigned the characteristics of *KIR3DL1*015.*

NK stimulation with HLA-negative 721.221 target cells

PBMCs were co-incubated with target cells at a 3:1 ratio for 4h in the presence of anti-CD107α antibody (LAMP-1, H4A3, BD Biosciences, San Jose, CA) to detect degranulation. The HLA-negative, NK-sensitive 721.221 BLCL were used as targets for NK activation assays. PBMCs were cultured overnight in RPMI-1640 media containing 10% FBS and 1000 IU/mL IL-2 prior to functional analysis. KIR3DL1^{+} NK cell responsiveness was normalized between donors by subtracting the background activation in the KIR^{-}NKG2A^{-} population from the specific activation observed in NK cells solely expressing KIR3DL1. Donors exhibiting *HLA-A* alleles that encode Bw4 epitopes were excluded from all analyses, although pilot experiments determined that they do not contribute to NK education (data not shown).

FACS analysis

Dead cells were excluded based on staining with live/dead viability dye (Life Technologies) and NK cells were identified as CD56^{+} (N901, Beckman Coulter, Jersey City, NJ) and CD3^{-} (OKT3, Biolegend, San Diego, CA, USA). NK cells expressing high and low alleles of KIR3DL1 were identified by co-staining with anti-KIR3DL1 DX9 (BD Biosciences), anti-KIR3DL1/S1 Z27 (Miltenyi Biotec, San Diego, CA) and anti-KIR3DL1 177407 (R&D systems, Minneapolis, MN). NK cells expressing additional inhibitory receptors were identified using the following antibodies: anti-KIR2DL2/L3/S2 (GL183, Beckman Coulter), anti-KIR2DL1/S1 (EB6, Beckman Coulter) and anti-NKG2A (Z199, Beckman Coulter). Data was collected using an LSR Fortessa and analyzed using FlowJo 9.7 software (Treestar, Ashland, OR, USA). NK cell education was assessed on NKG2A^{-} cells expressing KIR3DL1 as the only KIR molecule.

Two anti-HLA-Bw4 antibodies, clone 0007 and REA274, were used to establish expression of the Bw4-80I and Bw4-80T subtypes and to monitor loss of HLA-B expression following

HIV infection (FH0007 and BIH0007, One Lambda, Canoga Park, CA; and REA274, Miltenyi Biotec, Auburn, CA). HLA-C expression was monitored using supernatant from the DT9 hybridoma, kindly provided by Dr. Mary Carrington (NIH, Fredrick, MD) with secondary staining using anti-mouse IgG (eBiosciences, San Diego, CA). Data reported are median fluorescence intensities obtained using FITC conjugated clone 0007 (FH0007), which bound similarly to all HLA-Bw4 isoforms tested. To correct for slight variances in antibody affinity to HLA-Bw4 isoforms, binding values were calculated using median fluorescence intensities in the following formula: (Antibody binding to cells x antibody binding to bead-bound specific isoform)/antibody binding to bead-bound HLA-B*57:01 (35).

DHIV3 virus production and titration

The envelope-deleted DHIV3 infection model allows for non-productive infection of $CD4^+$ T cells and subsequent downregulation of HLA-B (26). DHIV3 plasmid and VSV-G were cotransfected into Phoenix A cells in the presence of 25uM chloroquine. Virus-containing supernatants were harvested 48 and 72h post-transfection and titrated using Jurkat cells, as described (36).

$CD4^+$ T cell expansion and DHIV3 infection

Non-tissue culture-treated plates were coated with 1μg/mL anti-CD2 (Santa Cruz Biotechnology, Dallas, TX), anti-CD3, and anti-CD28 (BD Biosciences), washed and blocked with PBS containing 5% FBS for 2h at 37°C. Total donor PBMC were seeded at 5×10^6 cells/mL in RPMI containing 10% human serum for 1 week. $CD4^+$ T cells were positively selected using Dynabeads (Life Technologies) and incubated on freshly-coated plates for 7–14 days in the presence of 350 IU/mL IL-2.

$CD4^+$ T cells were infected with DHIV3 virus by spinoculation for 1.5h in the presence of 10mg/mL polybrene, at 300 x *g* (37). Cells were returned to culture in freshly-coated plates in the presence of 350 IU/mL IL-2 for 48h prior to FACS analysis for intracellular HIV p24 protein (NIH reagents program) (38–40). Phenotyping was performed for CD4 (RPA-T4, BD Biosciences), CD8 (RPA-T8, BD Biosciences), CD3, HLA-ABC, HLA-Bw4 and HLA-C (41). Dead cells were excluded by live/dead staining.

NK challenge with autologous DHIV3-infected $CD4^+$ T cells

NK cells were purified from previously frozen PBMC by negative enrichment according to the manufacturer's instructions (StemCell Technologies, Vancouver, Canada). NK and DHIV3-infected autologous $CD4^+$ T cells were seeded at a 1:1 ratio in the presence of anti-CD107α for 6h prior to FACS analysis for phenotype, function and total infected cell killing. To quantitate infected cell killing, cell death of infected ($CD4^{low}$) and for uninfected ($CD4^{high}$) were compared for each sample.

Production of soluble KIR-Fc recombinant proteins

KIR-Fc constructs were created to produce soluble recombinant proteins for the *KIR3DL1-h* alleles **001*, **002*, *020 and for *KIR3DL1-l *005*. An additional KIR-Fc construct was created to represent *KIR3DL1*007* and **015*, alleles which are expressed at low and high

surface densities, respectively, but share a common ectodomain (42). Complementary DNA for the IL-2 signal peptide, exons encoding the extracellular domains of KIR3DL1, and the Fc region of human IgG1 were cloned into the mammalian expression vector pcDNA3.4. All constructs were prepared as per manufacturer's instructions using the HiSpeed Plasmid Maxi Kit (Qiagen, Valencia, CA, USA).

Expi293F cells (7.5×10^7) were transfected with 30 μg of the appropriate KIR-Fc plasmid using the Expifectamine 293F transfection reagent per manufacturer's instructions (Life Technologies). Supernatants containing the secreted recombinant proteins were harvested seven days post-transfection and the concentration of soluble KIR-Fc in the supernatants was determined using the Easy-Titer human IgG assay (Pierce Biotechnologies, Rockford, IL, USA). To assess the folding of each recombinant protein, Protein A-coated microspheres (Bangs Laboratories, Fishers, IN) were incubated with 1.0 μg of the KIR-Fc proteins for 1h at 4°C. The correctly folded KIR-Fc molecules were detected by flow cytometry using the conformation-specific KIR3DL1 antibodies, DX9 and Z27 (BD Biosciences and Beckman Coulter, respectively).

Detection of soluble KIR-Fc binding to HLA

KIR-Fc proteins were tested for binding to a panel of single antigen HLA molecules conjugated to uniquely labeled microspheres (One Lambda). The microspheres were incubated with KIR-Fc proteins (4.0 μg/ml) for 1h at room temperature with 300 rpm rotation. After washing, samples were probed with a PE-conjugated goat antibody specific for human IgG (One Lambda) and analyzed on a LABScan 100 flow analyzer using the Xponent software (Luminex, Austin, TX). The MFI values obtained for each interaction between KIR-Fc and HLA were first background-subtracted for binding to the negative control bead and compared to understand the relative binding affinities of each allelic partnership (35). HLA-B*27:05 was not bound efficiently by any KIR-Fc construct and was consequently not included in the binding heat map array. Total HLA loading onto beads was measured by staining with anti-β2m (clone bbm.1, BD Biosciences), and anti-HLA class I (clones G46-2.6 and W6/32 from BD Biosciences and eBiosciences, respectively). All antibodies exhibited the same relative MFIs for all HLA isoforms. KIR-Fc binding was determined using the following formula: (bead staining with KIR-Fc - background bead fluorescence)/(MFI for total HLA staining – background bead fluorescence) (12, 35). The specific peptides loaded onto the bead-bound HLA molecules are not disclosed by the manufacturer; however, as they are derived from EVB-transformed cell lines it is expected that they represent a heterogeneity of endogenous and EBV-derived peptides (43).

Statistical analysis

All statistical analyses were computed using GraphPad Prism 6.0e software (GraphPad Prism Software, Inc., La Jolla, CA). Differences between KIR3DL1 and HLA-B subtype combinations were compared using Student's t tests and 1-way ANOVA when more than two subtype groups were considered simultaneously. Tukey's post-hoc assessments were used to identify significance between specific subtype groups and to correct for multiple comparisons, and p values <0.05 were considered significant. Correlational analyses were performed by linear regression.

Results

KIR3DL1 subtypes exhibit distinct expression patterns

We used a multiplex PCR typing assay to identify subtypes of *KIR3DL1/S1* alleles in 135 healthy donors (19). Using this typing method, the 77 known alleles of KIR3DL1/S1 are grouped into six subtypes and four phenotypically-distinct groups (17–20) (Figure 1A). Among the inhibitory KIR3DL1 subtypes detected on the cell surface by both antibodies, two phenotypes are established: low expression (KIR3DL1-l, the product of KIR3DL1*005 and *007 subtypes) and high expression (KIR3DL1-h, the product of KIR3DL1*001, *002, and *015 subtypes) (Figure 1A and B). Members of the "null" group (KIR3DL1-n), most commonly *KIR3DL1*004* and *019*, are not expressed on the cell surface under homeostatic conditions (44) and therefore cannot be detected by staining with either of the anti-KIR3DL1 antibodies, DX9 or Z27. KIR3DL1-n can, however, be stained intracellularly with clone 177407 or on the cell surface after overnight culture at 25°C (45, 46). The activating KIR3DS1 subtypes are weakly detected on NK surfaces by Z27 and are not bound at all by DX9 (18).

KIR3DL1$^+$ NK phenotypes also consistently define fixed proportions of KIR3DL1$^+$ NK cells in the repertoire. While the proportion of lymphocytes that are NK cells does not differ appreciably between donors with specific *KIR3DL1* subtypes (Figure 1C), KIR3DL1-l$^+$ and -h$^+$ NK cells are present in the NK repertoire at low and high frequencies, respectively (Figure 1D). In individuals expressing both *KIR3DL1* surface subtypes (*KIR3DL1-l+h*), two phenotypically distinct populations are observed (Figure 1A), and the total proportion of KIR3DL1$^+$ NK cells is intermediate when compared to the proportion in KIR3DL1-l+l individuals and KIR3DL1-h+h individuals (Figure 1D).

The distinct phenotypes of KIR3DL1$^+$ NK cells reflect differences in expression, rather than variable affinities for antibody binding and/or competition between antibodies for overlapping or proximal epitopes. Three different antibodies that recognize KIR3DL1 create the same pattern of low and high expression phenotypes. Further, competition between antibodies for shared epitopes is unlikely because simultaneous or serial co-staining with the same antibodies produces the same phenotypes (data not shown).

Subtype combinations of KIR3DL1 and HLA-B titrate NK cell reactivity

We investigated how subtype combinations of KIR3DL1 and HLA-B impact NK education. NK cells from healthy donors selected to represent the permutations of HLA-B subtypes (*Bw6*, *Bw4-80T*, or *Bw4-80I*) and *KIR3DL1* subtypes (*KIR3DL1-l*, *–h, -n,* or *KIR3DS1*) were analyzed for degranulation in response to the NK-sensitive, HLA-negative target cell line 721.221 (Figure 2A). As expected, NK cells exclusively expressing KIR3DL1 from *Bw4*-negative (*Bw6*+) donors were poorly responsive, reflecting a lack of NK education due to non-engagement of KIR3DL1 by Bw6. In contrast, KIR3DL1 monopositive cells from *HLA-Bw4*$^+$ donors demonstrated higher responsiveness, consistent with NK education. The magnitude of reactivity varied between educated NK cells, but followed a specific pattern predicted by *KIR3DL1* and *HLA-B* subtype combinations. KIR3DL1-h$^+$ NK cells from *Bw4-80I* individuals exhibited the highest reactivity among all groups. KIR3DL1-h$^+$ NK

cells from Bw4-80T individuals and KIR3DL1-l$^+$ NK cells from Bw4-80I$^+$ or Bw4-80T$^+$ individuals had similar, intermediate degrees of reactivity (Figure 2A, p=0.7609). KIR3DS1$^+$ NK cells were non-responsive to 721.221 target cells, irrespective of donor HLA-B background, confirming that this population is not educated by HLA-B (Supplementary Figure 1A). Finally, NK cells exhibiting KIR3DL1-n were educated by both Bw4-80I and -80T for an intermediate level of responsiveness (Supplementary Figure 1B), consistent with previous conclusions that the receptors' intracellular retention does not preclude education (45, 46) (Submitted for publication).

Our observations indicate that interactions between receptor and ligand subtypes predictably program NK education. To further distinguish the roles of allele subtype interactions from inter-donor variation in NK education, we compared the high and low density KIR3DL1$^+$ populations from *KIR3DL1-l+h* individuals, where the HLA environment is constant but the KIR3DL1 receptors and their consequent interaction with "self" HLA might differ. In response to stimulation with 721.221 target cells, KIR3DL1-l$^+$ and –h$^+$ NK cells derived from the same donors exhibit degranulation that is consistent with the level of education defined by their KIR3DL1 subtype. In a representative *Bw4-80I$^+$* donor, KIR3DL1-h$^+$ NK cells exhibit greater degranulation than KIR3DL1-l$^+$ NK cells (Figure 2B). In a representative *Bw4-80T$^+$ KIR3DL1-l+h* donor, the KIR3DL1-l$^+$ and –h$^+$ NK populations demonstrate similar reactivity, consistent with their comparable education by Bw4-80T. Together, these observations demonstrate that NK cell education results from the interactions of specific receptor-ligand pairs. The reproducible reactivity of these combinations educated in the same environment therefore indicates that NK education is defined by constant characteristics unique to receptor and ligand subtypes, and unaffected by inter-individual variation.

NK cell education is predicted by the strength of KIR3DL1 and HLA-B interactions

We hypothesized that strong binding between KIR3DL1 and HLA-B subtypes corresponds to high NK reactivity. In addition to the *80I* versus *80T* distinction in the Bw4 epitope, several polymorphic sites on KIR3DL1 titrate its affinity for HLA-Bw4 ligands (21, 31, 47). Therefore, binary categorization of KIR3DL1 and HLA-B molecules as "high" versus "low", or Bw4-80I versus -80T, respectively, likely underestimates the variation in binding of KIR3DL1 to HLA-Bw4 generated by these highly polymorphic receptor and ligand families. Previous studies have demonstrated different strengths of binding between specific KIR3DL1 and HLA-B subtypes (13, 14, 21, 23); however, a comprehensive analysis to codify the relative binding of all of the most common isoforms of HLA-B and KIR3DL1 subtypes has not been described.

To directly compare ligand specificity of the most common receptor allotypes, we generated soluble recombinant proteins exhibiting the ectodomains of two common KIR3DL1-l allotypes (KIR3DL1*005 and *007) and four common KIR3DL1-h allotypes (KIR3DL1*001, *002, *015, *020). Binding of these recombinant KIR3DL1-Fc proteins to 20 different HLA-Bw4 allotypes individually conjugated to microspheres was then evaluated, as described (11, 12). The relative binding of KIR3DL1 isoforms to different HLA-B subtypes was determined by comparing the mean fluorescent intensities generated

after secondary staining with a PE-labeled anti-Fc antibody to create a heat map array (Figure 3A).

As expected, KIR3DL1-Fc proteins did not bind the HLA-Bw6 allotypes (Figure 3). In general, Bw4-80T allotypes interacted with all KIR3DL1 subtypes weakly, with the exceptions of HLA-B*44 and B*47, which demonstrated notable binding to the *005 and *007 group low isoforms and the *001 and *015 group high KIR3DL1 isoforms. Bw4-80I allotypes exhibited a spectrum of binding to KIR3DL1 ranging from low to high. Most striking, HLA-B*57:01 exhibited the highest binding of all pairings when combined with KIR3DL1*001 or KIR3DL1*005. On the other extreme, HLA-B*52:01 and HLA-B*59:01 were bound very weakly by all subtypes of KIR3DL1. All of the Bw4-80I allotypes exhibited relatively poor binding to KIR3DL1*002 and *020, and established similar and hierarchical binding to each of *005, *007, *001 and *015 group alleles. These findings represent the most comprehensive assessment of KIR3DL1 and HLA-B binding completed in a single array, enabling consideration of relative binding interactions of receptor-ligand subtypes in studies of NK education.

Interestingly, B*27:05 did not bind to any of the recombinant Fc receptors, despite a known interaction between both high and low isoforms of KIR3DL1 and HLA-B*27 (13, 21). HLA-B*27:05 is unique among the HLA molecules because it forms homodimers on the cell surface (48); which might not be accurately recapitulated on the synthetic microspheres. To confirm poor binding strength between KIR3DL1 and HLA-B*27:05, hereafter referred to as B*27, we used 721.221 cells transfected with *HLA-B*27* or *HLA-B*44:02* to measure the relative affinities of KIR3DL1 subtypes. All KIR3DL1-Fc proteins bound poorly to 721.221 cells transfected with *HLA-B*27*, compared to 721.221 cells transfected with *HLA-B*44:02* (Figure 3B–C), confirming that the binding between KIR3DL1 and HLA-B is weak.

To determine if NK education is driven by the strength of KIR-HLA interaction, we used the relative binding of KIR3DL1 and HLA-B subtype pairs in a linear regression analysis with degranulation to 721.221 as the dependent variable. Among $Bw4^+$ NK cells singly expressing KIR3DL1, increasing binding strength was positively associated with greater NK cell reactivity (Figure 4A, r^2=0.3591, p=0<0.0001). Separately, each of the KIR3DL1-h and –l populations exhibited a correlation between relative binding affinity and NK reactivity (KIR3DL1-l: r^2=0.3268, p=0.0054, Figure 4B; KIR3DL1-h: r^2=0.4112, p=0.0013, Figure 4C).

We next tested the responsiveness of NK cells exhibiting KIR3DL1*002 or *015-group high-density alleles in donors exhibiting Bw4-80I to evaluate whether strong binding is necessary to generate potent effector function. Despite its high surface expression, KIR3DL1*002 binds weakest to the Bw4 allotypes tested compared to the other KIR3DL1-h subtypes (Figures 3A and 5A). Although the responsiveness of KIR3DL1*002-expressing NK cells was lower than that of the other KIR3DL1*015-group isoforms, the difference was not statistically significant (Figure 5B). Likewise, HLA-B*51 binds strongly to low-expressing KIR3DL1*005 and weaker to the high-expressing KIR3DL1*002-group alleles. Nevertheless, KIR3DL1-high NK cells from HLA-$B*51^+$ donors exhibit greater reactivity

compared with KIR3DL1-low NK cells from HLA-B*51^{+} donors (Figure 5C). These data support an incomplete role of binding strength in predicting the responsiveness of KIR3DL1-h+ NK cells.

High density KIR3DL1 expression is necessary for strong reactivity

Our data suggest that low KIR3DL1 density limits the reactive potential of educated NK cells. We examined NK cells exhibiting KIR3DL1*007, a common allele that is expressed at a low surface density but shares identical ectodomain homology with KIR3DL1*015, a KIR3DL1-h isoform. Like KIR3DL1-h subtypes, KIR3DL1*007 binds Bw4-80I tetramers and HLA-coated microspheres preferentially over Bw4-80T (13, 14, 21, 42) (Figure 3A). If high KIR3DL1 surface density is necessary for potent responsiveness, the reactivity of KIR3DL1*007^{+} cells would be limited when compared with the KIR3DL1-h^{+} NK cells. We examined a *Bw4-80I*$^{+}$ donor encoding both *KIR3DL1*007* and a *KIR3DL1*015*-group high allele to understand if limited receptor density impacts NK cell education. In response to 721.221 target cell stimulation, the low density KIR3DL1 population indeed degranulated less than high density KIR3DL1^{+} NK cells from the same individual (Figure 6A).

To confirm that low expression of KIR is sufficient to limit NK cell responsiveness, we compared the low-expressing KIR3DL1*005 and KIR3DL1*007^{+} NK cells to the high-expressing KIR3DL1*015-group^{+} NK cells, all from donors exhibiting *Bw4-80I*. Binding strength between KIR3DL1 and Bw4-80I is similar for all combinations tested (Figure 3A), permitting examination of the impact of KIR3DL1 surface density on NK reactivity. NK cells from donors encoding KIR3DL1*007 phenocopied the KIR3LD1*005 low subtypes and exhibited equivalently low responses to 721.221 target cells when compared to NK cells with KIR3DL1*015 (Figure 6B–C). Therefore, despite high binding to the same ligand, KIR3DL1*007^{+} NK cells do not achieve the same responsiveness as NK cells expressing KIR3DL1*015. Together, these findings indicate that low receptor expression limits NK reactive potential and reveal that discrete cell surface phenotypes encoded by KIR3DL1 subtypes have functional consequences on NK education. Because KIR3DL1 expression density is categorical rather than continuous, it was not possible to perform linear regressions analysis using KIR3DL1 expression density as the dependent variable.

Ligand density predicts the magnitude of NK responsiveness

Collectively, our observations establish that the densities of KIR3DL1 allotypes and their binding to HLA-Bw4 calibrate NK education. We next measured surface expression of HLA-Bw4 to determine whether the availability of ligand may also factor in NK education. We confirmed that the anti-Bw4 antibody clone 0007 selectively stains Bw4 epitopes by testing its binding to HLA-coated microspheres. The antibody did not bind Bw6 allotypes, but bound all tested Bw4 allotypes similarly (Supplementary Figure 2). HLA-Bw4 is expressed at an array of densities ranging from low to high. This is most evident when staining NK cells from Bw4/Bw6 individuals, where Bw4 expression is contributed by one allele only. We evaluated 77 individuals with specific but common Bw4 allotypes (Figure 7A) and compared staining median fluorescence intensities. We found that, in general, Bw4-80T alleles are expressed with a low surface density that can increase when two Bw4-80T alleles are encoded. A single *Bw4-80I* allele is expressed with higher surface

density compared with a *Bw4-80T* allele. Co-expression of a second *HLA-Bw4* allele, either *Bw4-80I* or *–80T*, further enhances surface Bw4-I80 expression. Most strikingly, the allotypes HLA-B*27 and HLA-B*57 are expressed at markedly higher densities compared with all other allotypes (Figure 7A and B).

When Bw4 staining data from 65 Bw4/Bw6 individuals was combined, four discrete groups of Bw4 surface expression are evident. NK cells from *Bw4-80I/Bw6* individuals displayed consistently high median fluorescence, despite the heterogeneous composition of Bw4-I80 alleles represented. NK cells from *Bw4-80T/Bw6* individuals similarly revealed a consistent median fluorescence intensity that was markedly lower than that of *Bw4-80I/Bw6* donors. Finally, NK cells from individuals with *HLA-B*57* (Bw4-80I) or *HLA-B*27* (Bw4-80T), consistently displayed higher HLA-Bw4 surface densities than other members of their subtype groups (Figure 7B). Staining using a second anti-Bw4 antibody, clone REA274, revealed the same pattern, confirming that these distinctions result from different levels of protein expression on primary cells (Supplementary Figure 3).

Discrimination between Bw4 expression patterns allowed us to test the hypothesis that density of HLA-Bw4 impacts NK education. We compared the responsiveness of KIR3DL1-h+ NK cells educated by Bw4 alleles categorized by their expression pattern: Bw4-*80T, -80I, B*27* or *B*57:01*. Co-incubated with 721.221 target cells, KIR3DL1-h$^+$ NK cells from *Bw4-80T*$^+$ individuals displayed modest effector function (Figure 7C). KIR3DL1-h$^+$ NK cells from individuals with any *Bw4-80I* allele, including *HLA-B*57,* were strongly reactive toward 721.221 cells.

Strikingly, KIR3DL1-h$^+$ NK cells educated by HLA-B*27 exhibited enhanced responsiveness compared to KIR3DL1-h$^+$ NK cells educated by all other Bw4-80T subtypes, in spite of its poor binding (Figures 3C and 7C). Therefore, high-density ligand expression may be sufficient to enhance the overall avidity of KIR3DL1-HLA-Bw4 interactions. Collectively, these findings indicate that for maximum reactivity, strong receptor-ligand binding may be less important when both binding partners are abundant.

For individuals with two *Bw4* alleles, contributions from both alleles create a spectrum of Bw4 expression densities. Using linear regression analyses, we tested whether Bw4 expression density can predict the responsiveness of KIR3DL1+ NK cells. HLA-Bw4 expression predicted the responsiveness of the KIR3DL1-l$^+$ NK population (r^2=0.5077, p=0.0063, Figure 7D) and the KIR3DL1-h$^+$ NK population (r^2=0.5203, p<0.0001, Figure 7E) separately and combined (r^2=0.4466, p<0.0001, Figure 7F).

KIR3DL1 and *HLA-B* subtype combinations predict NK cytotoxicity against autologous HIV-infected CD4$^+$ cells

The hierarchy of response to 721.221 dictated by KIR3DL1 and HLA-B subtype combinations strikingly matches previously reported associations of specific subtype combinations with response to HIV *in vitro* and *in vivo* (24, 28, 30, 49, 50). In HIV+ patients, *KIR3DL1-h* and *Bw4-80I* subtype combinations are associated with the most delayed progression to AIDS, and combinations of *KIR3DL1-h* and *Bw4-80T* or *KIR3DL1-l* with either *Bw4* subtype are associated with lower protection (24). HIV infection mediates

downregulation of surface HLA-B expression (25, 26), creating a putative "HLA-disparate" target for educated KIR3DL1$^+$ NK cells. We therefore hypothesized that the magnitude of NK education would directly correspond to cytolysis of HIV-infected autologous CD4$^+$ T cells.

DHIV3 is a replication-defective HIV mutant strain that differs only from wild type virus by deletion of the gene encoding the envelope protein (51). We pseudotyped DHIV3 using the glycoprotein from VSV to enable infection of primary CD4$^+$ T cells (51). Virus-containing supernatants infected 15.5 ± 9.3% of CD4$^+$ T cells, measured by intracellular staining for the HIV p24 protein. Compared to virus-exposed but uninfected T cells in the same well, DHIV3-infected T cells demonstrated diminished cell-surface expression of CD4 and HLA-Bw4, but HLA-C expression remained unchanged (Figure 8A–B and Supplementary Figure 4A). Despite their distinct cell surface expression densities, expression of Bw4-80T and Bw4-80I allotypes was equivalently reduced, by approximately 60% (Figure 8B–C).

NK cells from *HLA-Bw4*$^+$ donors exclusively expressing KIR3DL1 degranulate upon co-culture with autologous, infected CD4$^+$ T cells, while those harvested from individuals lacking *Bw4* do not mount a significant response (Figure 8D). In contrast, neither educated nor uneducated NK cells expressing the HLA-C specific KIR2DL1/L2/L3 receptors respond to DHIV3-infected autologous CD4$^+$ T cells. Similarly, neither of the KIR3DL1, KIR2DL1 or KIR2DL2/3$^+$ NK cells degranulated in response to autologous uninfected CD4$^+$ T cells after expansion (Supplementary Figure 4B). These findings reflect the selective downregulation of HLA-Bw4 by HIV infection and implicate educated KIR3DL1$^+$ NK cells as the major NK population responding to HIV infection.

To directly measure NK-mediated killing of DHIV3-infected cells, we assessed the viability of infected cells after co-culture with autologous NK cells. The proportion of CD4$^+$ T cells infected with DHIV3 was measured by p24 staining prior to addition of NK cells at a 1:1 ratio with infected cells. Identification of the intracellular p24 protein requires cell fixation and permeabilization, precluding its utility to sort infected cells for coculture with NK cells. CD4 dim vs bright expression on CD3$^+$ cells was therefore used as a marker of DHIV3-infection, which permitted same well comparison of the viabilities of infected and uninfected populations following exposure to autologous NK cells (Supplementary Figure 4A) (37).

The hierarchy of NK-mediated cytotoxocity against DHIV3-infected cells (Figure 8E) strikingly recapitulated the pattern seen in NK cell responses to the 721.221 HLA-negative targets (Figure 2A). Reflecting the reactivity of educated NK cells to HLA downregulation on infected cells, NK cells from *KIR3DL1-h* or *–l* and *HLA-Bw4*$^+$ donors mediated significantly greater killing of autologous DHIV3-infected cells compared to NK cells derived from *Bw6* donors. NK cells from donors with *KIR3DL1-h* and *Bw4-80I* exhibited the highest cytotoxicity of any subtype combination, illustrating that strong NK education is consistent with high cytolytic function against autologous cells exhibiting diminished class I. Together, these findings explain how KIR3DL1 and HLA-Bw4 isoforms combine to define NK education and impact the control of HIV.

Consistent with their known protective association against HIV infection and progression to AIDS (52–55), NK cells from KIR3DS1 homozygous donors lysed autologous HIV-infected $CD4^+$ T cells. This outcome was notably pronounced in NK cells from a *Bw4-80I*$^+$ donor (Supplementary Figure 4C). Similarly, NK from *HLA-Bw4* donors homozygous for *KIR3DL1-n* exhibited higher cytolysis of HIV-infected autologous cells compared with NK cells from *Bw6* donors homozygous for *KIR3DL1-n* (Supplementary Figure 4D). These findings reflect NK education and explain the observed benefit of the *KIR3DL1-n* + *Bw4* subtype combination against HIV progression (24, 45). Collectively, these findings demonstrate that NK cell education, titrated by *KIR3DL1* and *HLA-B* subtype combinations, predicts their reactivity against diseased cells exhibiting diminished levels of HLA-B. These observations provide functional insight for known genetic influences of *KIR3DL1* and *HLA-B* on HIV control, and illustrate how immunogenetic combinations prepare NK cells for direct recognition and cytolysis of infected cells.

Discussion

NK cells play a prominent role in controlling HIV infection, and subtype combinations of *KIR3DL1* and *HLA-B* are associated with protection from primary infection and delayed progression to AIDS (24, 49, 54, 56, 57). We now demonstrate that defined characteristics of *KIR3DL1* and *HLA-B* subtypes define their interactions and determine NK responsiveness against HLA-negative targets and autologous HIV-infected $CD4^+$ T cells. Using unmanipulated NK cells from healthy donors, we demonstrate that both the binding affinity and cell surface density of receptor-ligand pairs correlate with the magnitude of NK reactivity. Analysis of naturally occurring, common subtypes reveals that high densities of both receptor and ligand can compensate for low binding affinity to endow strong NK reactivity. The clinical importance of variable NK education is most aptly illustrated in HIV infection, in which highly educated NK cells are the effector population most responsible for viral clearance. The data presented here demonstrate a clear biological explanation for the prior observation that epistatic interactions between *KIR3DL1-h* and *Bw4-80I* are associated with the greatest protection from HIV progression to AIDS (24). More broadly, they demonstrate that allelic variation for the ubiquitous *KIR3DL1* and *HLA-B* genes generates substantial effector diversity, relevant for other viral and malignant pathologies resulting in downregulation of HLA class I (8, 9, 58, 59).

The exact mechanisms by which NK education occurs remain incompletely understood, and universal consensus is lacking. The “disarming” model asserts that NK cells whose inhibitory receptors enable sensitivity to “self” HLA will be rescued from activation-induced anergy and consequently maintain higher responsiveness (60). The “licensing” model ascribes a more active mechanism for education, wherein NK cells capable of “self” HLA recognition are endowed with greater potential for reactivity (61). Regardless of their differences, both models support a program of NK education in which KIR and HLA calibrate NK effector function to ensure that reactive potential is counterbalanced by inhibitory capacity.

Graded NK education is noted in murine NK cells, where functional studies demonstrate that the quantity and strength of binding between inhibitory Ly49, the murine ortholog to

human KIR, and “self” MHC class I correspond with the magnitude of their responsiveness. MHC-deficient, hemizygous and homozygous mice display increasing densities of MHC; consequently, NK cells expressing cognate Ly49 inhibitory receptors demonstrate increasing reactive potential against class I-negative target cells (62–64). Further, $Ly49C^+I^+NKG2A^+$ NK cells, which carry three inhibitory receptors sensitive to the $H\text{-}2k^b$ MHC I carried by B6 mice, exhibit the greatest cytotoxic response to cells lacking self-MHC, while NK cells carrying 2, 1, and 0 inhibitory receptors demonstrate graded decreases in effector capacity (65). Finally, $Ly49A^+$ NK cells, which engage an array of MHC I molecules, demonstrate the highest responsiveness when they develop in the presence of a high-affinity ligand (66). Altogether, these results implicate that both the number of interactions and their binding strength — the avidity of receptor-ligand engagement — calibrates NK education in mice.

In humans, inter-donor allelic variation creates extensive complexity by driving diversity in HLA and KIR expression and affinity, with consequences on NK function and education. Increasing copy number of HLA-Bw4 alleles is associated with enhanced reactivity of $KIR3DL1^+$ NK cells (67, 68). We now demonstrate that HLA-Bw4 subtypes are similarly expressed at discrete and subtype-specific levels, where higher Bw4 density is associated with enhanced effector potential. Like in mice, co-expression of multiple types of self-sensitive inhibitory KIR corresponds to enhanced NK effector potential (7). We now further show that NK education can vary based on differential expression of a single receptor subtype. We find that because high density KIR3DL1 expression is necessary for maximum effector potential, education is dependent on the number of receptors expressed. Paired with a highly expressed ligand, high-density receptors can achieve the maximum effector response. Low expression of either receptor or ligand limits the number of interactions, establishing a lower effector capacity.

It should be noted that the Bw4-80I allotypes exhibit both qualities important for NK education: high density and strong binding to most KIR3DL1 isoforms. For this reason, we cannot attribute the high NK education in KIR3DL1-h + Bw4-80I individuals to one quality in isolation of the other. We can, however, conclude that poor binding can be overcome by a high-density of both receptor and ligand, but strong binding is inadequate for maximal NK education when the availability of receptor and/or ligand is limited.

Two naturally-occurring allotypes support our conclusion that high-density ligands can overcome the requirement for high affinity, HLA-B*51 and B*27:05. Uniquely, HLA-B*27:05 forms surface homodimers that present peptides and exclude β2m. Both the canonical single-chain HLA-B*27:05-β2m and homodimer HLA-B*27:05 complexes interact with KIR3DL1 (69). While it is possible that homodimerization influences NK education in an additional and undetermined manner, our data imply that the high-density expression of Bw4 molecules is sufficient to potentiate NK cells. In agreement, HLA-B*51, which is expressed at the high-density characteristic of the Bw4-80I group, which does not homodimerize and binds KIR3DL1-h alleles with only moderate affinity, can similarly potentiate NK cells for high effector function. Taken together, B*51 and B*27:05 demonstrate that the high-density expression of Bw4 is associated with strong NK education.

Educated NK cells are characterized by low activation thresholds when challenged with target cells exhibiting stimulatory ligands in the absence of normal class I expression. Whether inhibitory sensitivity of individual cells is similarly tuned remains an outstanding question in the larger field of NK cell biology. We find that NK cells from *Bw4-80I*$^{+}$ donors that express KIR3DL1*007 and KIR3DL1*015 exhibit medium and high responsiveness, respectively, despite identical binding affinity. However, challenged with HLA-B*58:01 (a Bw4-80I epitope), KIR3DL1*015 conveys a more profound inhibition compared with KIR3DL1*007 (14). It is possible that in both instances, the density of KIR3DL1 on the cell surface may be a determining factor: for education, high-density expression is associated with greater effector potential. In the context of inhibition, a low cell surface density of KIR3DL1*007 may diminish the overall avidity of the NK cells' interaction with HLA-B, limiting the signal for inhibition.

In mice, educated NK cells exhibit altered organization of activating receptors in the plasma membrane, priming them for responsiveness (70). Further, educated NK cells adopt an "open" conformation of LFA-1, an adhesion molecule that facilitates NK-target cell binding (71). In human cells, combinations of *KIR3DL1-h* and *HLA-B*57* (80I) highly educate NK cells, which are simultaneously capable of cytotoxicity, chemokine, and cytokine production (28, 30, 50). Therefore, in addition to enhancing degranulation and direct cytotoxicity, higher NK education may also facilitate pro-inflammatory signaling, promote chemotaxis, or block chemokine receptors. Complementing a known capacity of educated NK cells to limit the spread of HIV (30, 52, 72), our use of a replication-defective mutant of HIV conclusively demonstrates a role for NK cells in the direct recognition and elimination of infected cells.

Because the availability of activating or inhibitory signals varies by pathology, and KIR and HLA are highly polymorphic, the designation of immunogenetic combinations as "beneficial" depends on the disease and its manipulation of HLA expression. In instances where HLA expression is diminished, including HIV and HSV (25, 26, 73, 74), highly educated NK cells would be expected to mount the most efficient cytotoxic response. In pathologies where HLA expression persists, uneducated NK cells, lacking receptors to self-HLA and therefore refractory to inhibition, mediate superior clinical outcomes (8, 9, 31, 46, 58). In both pathologic circumstances, benefit may occur with an intracellularly retained receptor, which in the presence of its ligand, permits effector capacity while remaining immune to inhibition (Submitted for publication). By altering receptor and ligand density and binding, KIR3DL1 and HLA-B subtypes diversify the function of NK cells to establish complementary functions. KIR3DL1 and HLA-B therefore represent an immunogenetic axis that can be screened for prognosis and manipulated for therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This project was supported by NIH R01 HL088134, NIH U01 AI069197, NIH P01 CA23766, NIH P30 CA008748-46, NIH P03 CA008748 (MSKCC Core Grant), and NIH R01 AI065361 (EB).

The authors thank Dr. Mary Carrington for the DT9 hybridoma used to produce the anti-HLA-C monoclonal antibody. The anti-p24 antibody was obtained from Dr. Susan Zolla-Pazner through the AIDS Reagent Program, Division of AIDS, NIAID. The authors thank Dr. Daniel McVicar for kindly providing cDNA for KIR3DL1*001, *005 and *015 from which KIR-Fc constructs were created.

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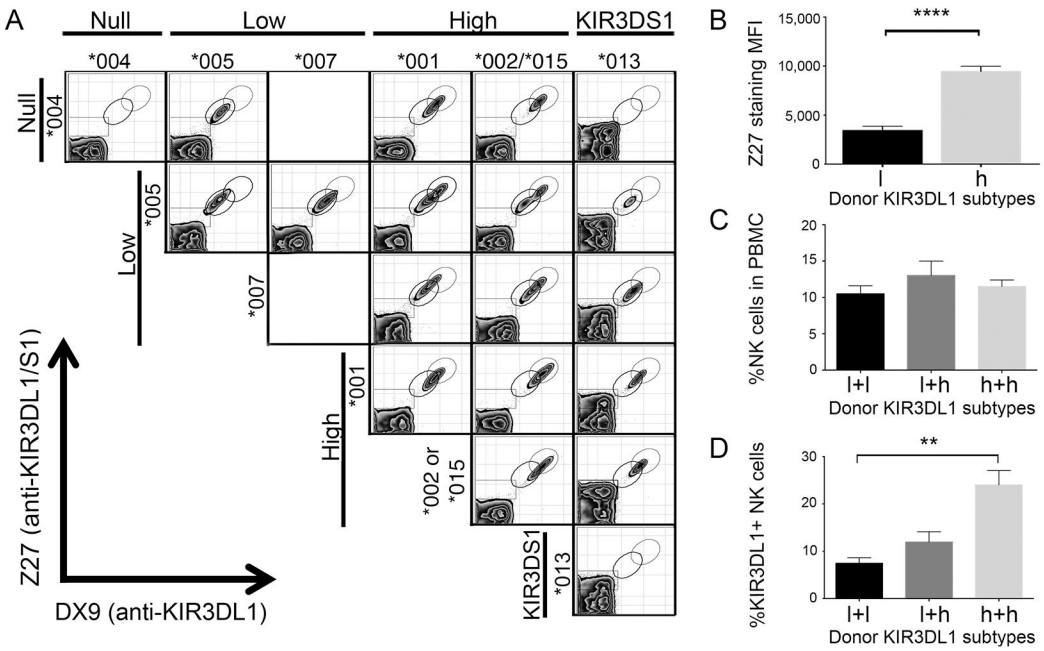

Figure 1. KIR3DL1 allele subtyping predicts NK cell phenotype and representation in the repertoire

Viable $CD3^{-}CD56^{+}$ NK cells from healthy human donors were assessed by KIR3DL1 PCR-SSP and stained for flow cytometry using DX9 (anti-KIR3DL1) and Z27 (anti-KIR3DL1/S1) monoclonal antibodies. (A) Phenotyping panel of healthy donor PBMC, gated on NK cells to represent all available major allele subtype combinations. Labels indicate the most common allele in each KIR3DL1 subtype. A representative donor from each subtype combination is displayed. (B) Z27 staining MFI from individuals expressing KIR3DL1-l or –h. (C) Percentage of PBMC that are NK cells, segregated based on donor compound *KIR3DL1* subtypes. (D) Percentage of peripheral blood NK cells that express KIR3DL1, organized based on donor KIR3DL1 subtypes. Bar graphs represent means ± SEM and a minimum of 2 independent experiments with 3–5 donors per group. **, $p<0.01$; ****, $p<0.0001$. Means are compared by Student's t test (1B) or one-way ANOVA (1C–D).

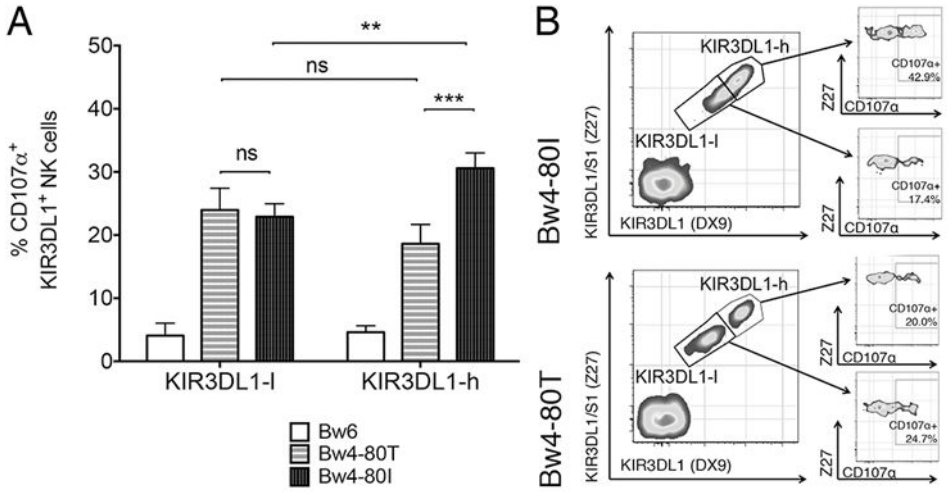

Figure 2. Allelic combinations of KIR3DL1 and HLA-B subtypes confer variable NK education and reactivity toward HLA-negative 721.221

(A) Total PBMCs from 54 healthy donors were stimulated with the HLA-negative, NK-sensitive target cell line 721.221. $CD3^{-}CD56^{+}$ NK cells were gated to include either KIR3DL1-high or KIR3DL1-low NK cells that lacked NKG2A and other KIR. Individuals encoding both high and low populations were included after dividing the populations by FACS gating. Each bar represents mean ± SEM for a minimum of 5 independent donors. **, $p<0.01$; ***, $p<0.001$, calculated by one-way ANOVA using Tukey's post-hoc test. (B) Representative donors co-expressing high and low alleles demonstrate variable education by *Bw4-80I* and *Bw4-80T* donors.

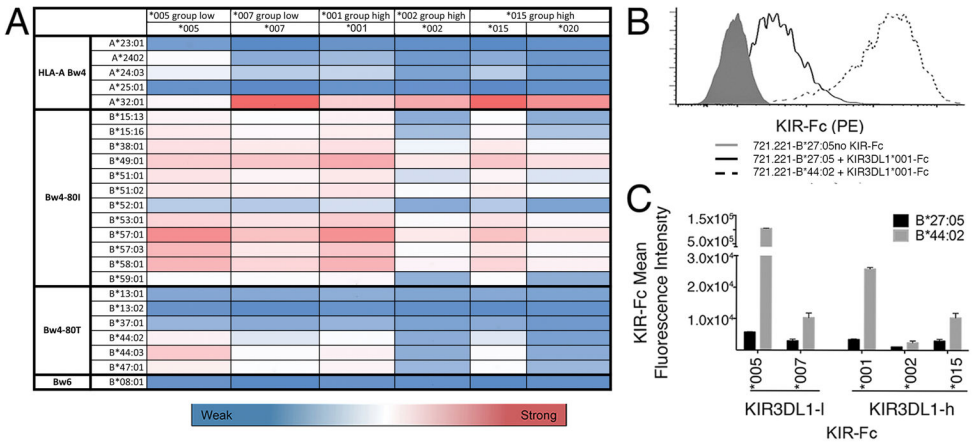

Figure 3. Binding strengths between KIR3DL1 and HLA-B

(A) Heat map array demonstrating relative binding strengths between KIR3DL1 subtype-Fc recombinant proteins and HLA-B allotypes tethered to microspheres, after correction for total bead-bound HLA. The blue-red scale indicates weakest to strongest relative binding. HLA-B*08:01 is shown as a representative *Bw6* allotype. The binding assay was performed in triplicate and replicated in two separate experiments. (B) Representative KIR3DL1*001-Fc binding to 721.221 transfectant cells expressing HLA-B*44:02 or B*27:05. The shaded grey histogram represents 721.221-B*27:05 without KIR-Fc; the solid black histogram represents KIR3DL1*001-Fc binding to 721.221-B*27:05 and dashed histogram represents KIR3DL1*001-Fc binding to 721.221-B*44:02. (C) Quantification of binding of the KIR3DL1-Fc proteins to 721.221 transfectants expressing HLA-B*44:02 or B*27:05. Bars represent SEM and 2–3 donors each.

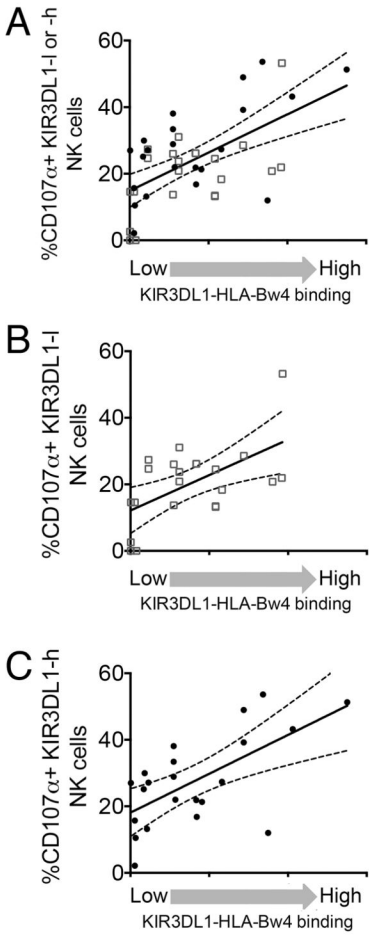

Figure 4. Strong interaction between KIR3DL1 and HLA-B is predictive but not necessary for NK cell education

Linear regression analysis for binding affinity and reactive potential among (A) combined KIR3DL1^{+} NK cells; (B) KIR3DL1-l or (C) KIR3DL1-h. Because no binding to HLA-B*27 was detected on HLA-coated microspheres, donors exhibiting this *Bw4* isoform were excluded from these linear regressions.

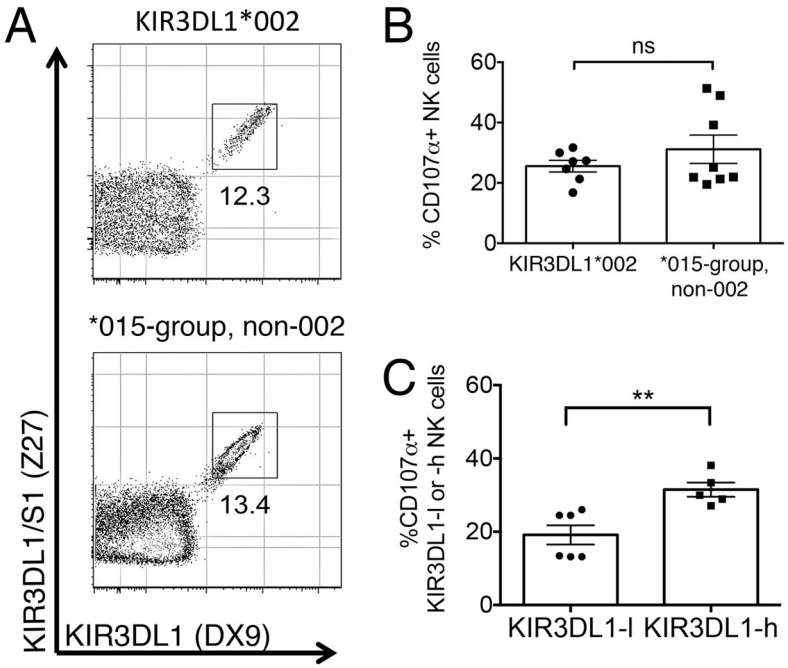

Figure 5. High-density receptor expression can compensate for poor binding affinity in NK education

(A) Phenotypes of KIR3DL1*002 or KIR3DL1*015. (B) Degranulation of NK cells expressing KIR3DL1*002 or KIR3DL1*015-group high alleles in response to 721.221 cells. (C) Degranulation of KIR3DL1-high or –low NK cells from Bw4-80I^{+} donors in response to stimulation with 721.221 targets. A minimum of five *Bw4-80I*$^{+}$ donors per group is shown, means are compared using two-tailed Student's t test and errors represent SEM.

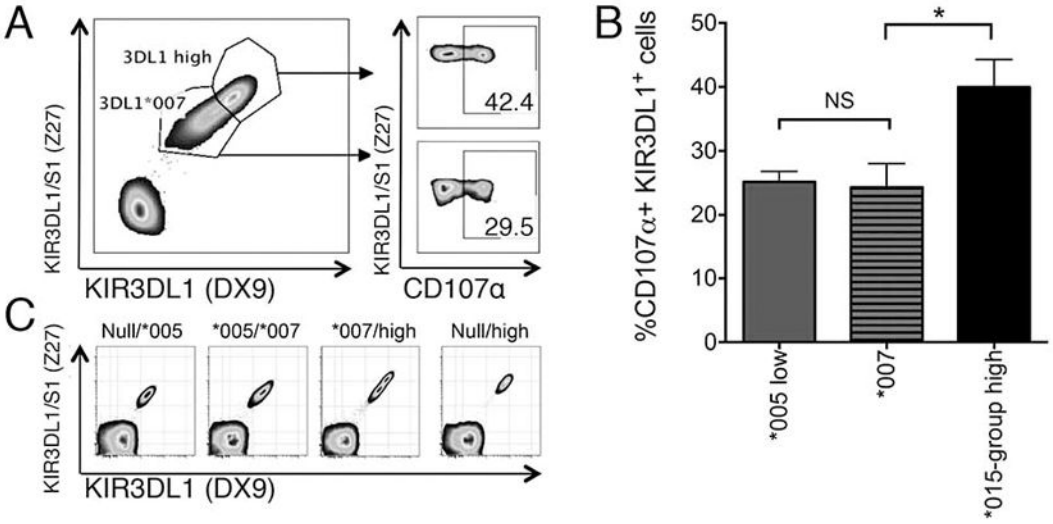

Figure 6. High KIR surface expression is critical for maximum NK cell reactivity

(A) NK cell phenotype and function of KIR3DL1-high or –low monopositive NK cells from a *Bw4-80I*$^{+}$ donor encoding *KIR3DL1*007* and *KIR3DL1*015.* (B) Surface staining of NK cells expressing *007 demonstrates a similar low-expression phenotype as NK cells expressing KIR3DL1*005. Staining of NK cells expressing high and null alleles is shown for comparison. (C) Degranulation of KIR3DL1^{+} monopositive NK cells from individuals with the indicated genotypes in response to 721.221 target cells. All samples shown are derived from *Bw4-80I*$^{+}$ donors and a minimum of three independent donors/group, assessed in two separate experiments, are included in the bar graph. *, $p<0.05$, computed using one-way ANOVA with Tukey's post-hoc test.

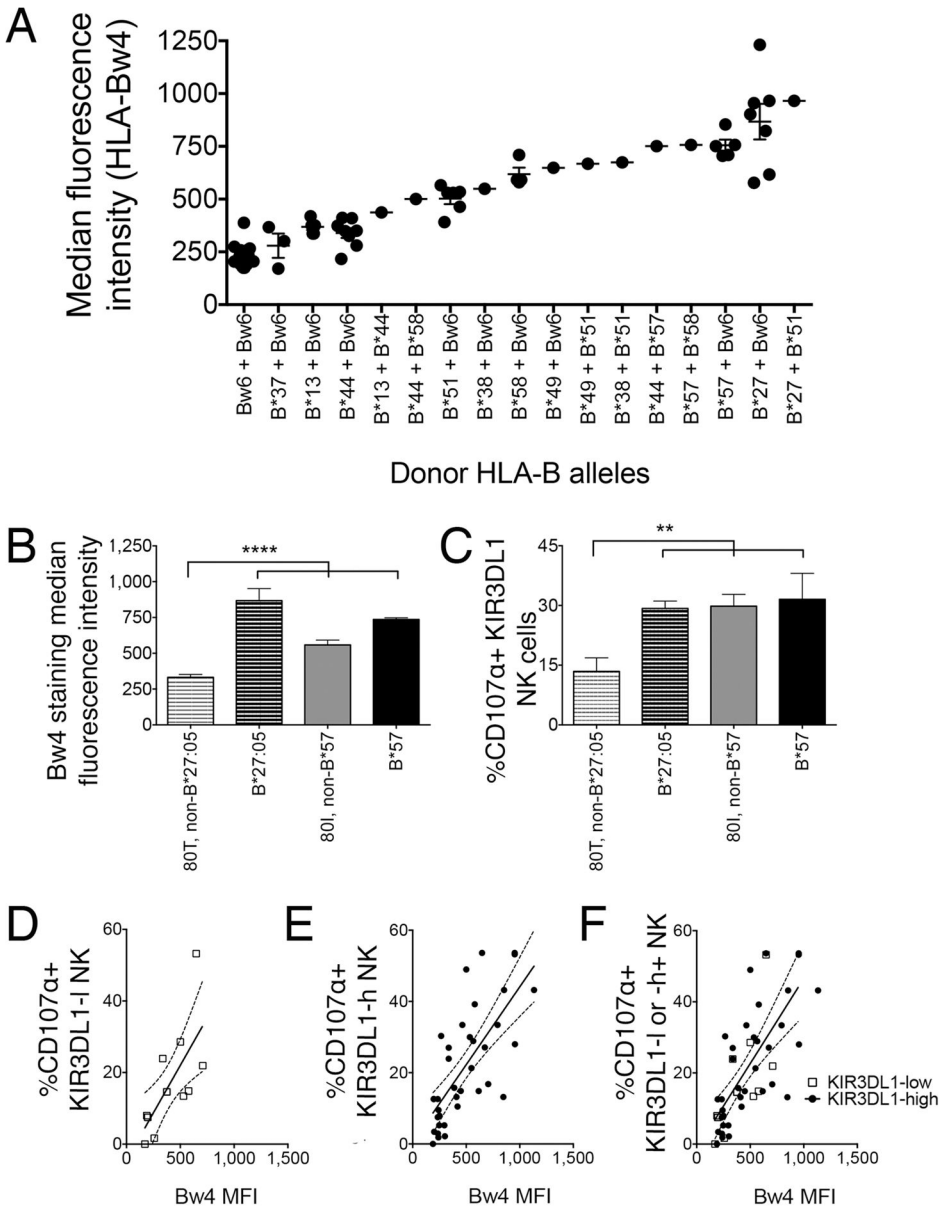

Figure 7. HLA-Bw4 subtypes are expressed at discrete densities and predict KIR3DL1+ NK education

(A) Median fluorescence intensity of HLA-Bw4 staining on 77 PBMC from donors with the indicated HLA-B allotypes. (B) Median fluorescence intensity of Bw4 alleles, categorized by subtype. Donors heterozygous for Bw4 and Bw6 were used. Bw4-80T (non-B*27:05) was used for comparison. Bars represent means ± SEM for a minimum of 5 donors each and three independent tests. ****, $p<0.0001$ computed using one-way ANOVA with Tukey's post-hoc test. (C) Responsiveness of KIR3DL1-h+ NK cells, educated by the indicated HLA-B subtypes, in response to 721.221 target cells. Each bar represents a minimum of four donors, and data were collected in two independent experiments. **, $p<0.01$, computed by one-way ANOVA using Tukey's post-hoc test, comparing to Bw4-80T, non-B*27:05. (D–F) Linear regression analysis comparing Bw4 surface density and reactivity of KIR3DL1-

monopositive NK cells toward 721.221 target cells: (D) KIR3DL1-l, (E) KIR3DL1-h and (F) KIR3DL1-l and KIR3DL1-h combined.

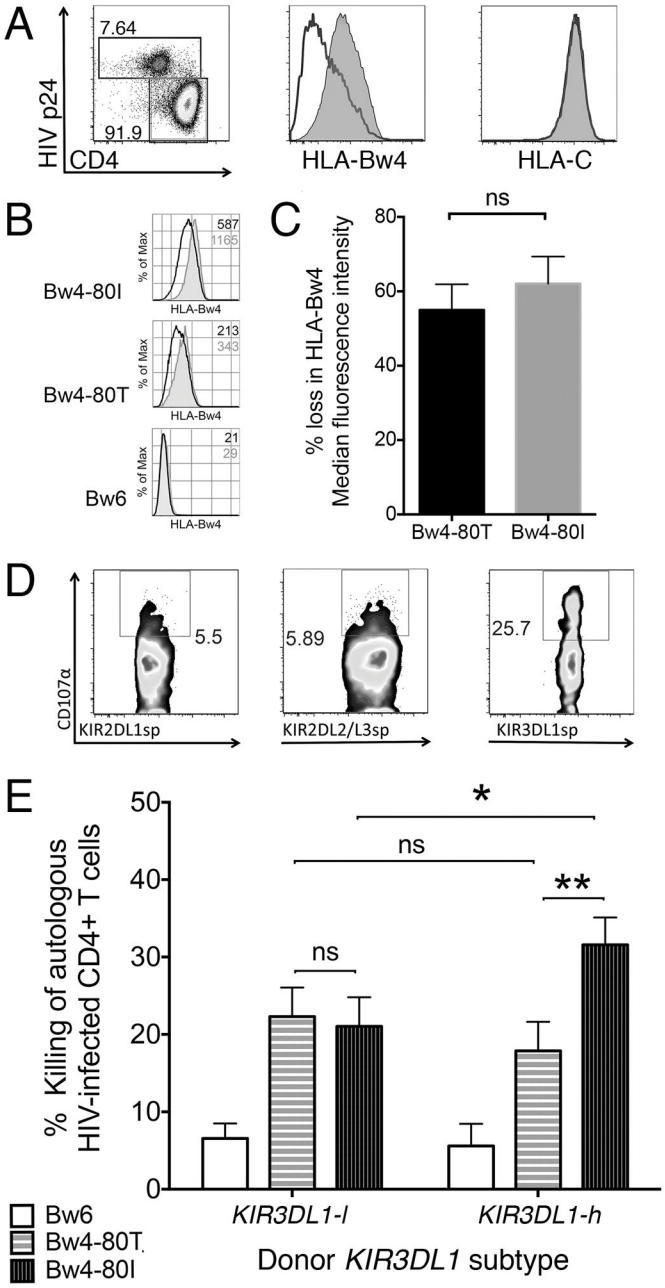

Figure 8. Autologous HIV-infected CD4$^+$ T cells behave as HLA-negative targets for educated NK cells

(A) Phenotype of CD4$^+$ T cells infected with HIV. Intracellular p24 staining confirms HIV infection; CD4 is diminished only in the HIV p24+ population. Histograms depict HLA-Bw4 and HLA-C expression among infected cells (black histograms) and uninfected cells (shaded histograms). (B) Representative staining of HLA-Bw4 on infected cells (black histograms) and uninfected cells (shaded histograms) from donors with the indicated HLA-B subtypes. Values indicate the median fluorescent intensity of HLA-Bw4 staining. (C) Percent change in HLA-Bw4 median fluorescence intensity 48h after HIV infection in Bw4-80I or Bw4-80T$^+$ donors. (D) Reactivity of NK cells monopositive for KIR2DL1,

KIR2DL2/3, or KIR3DL1 from a representative *Bw4-80I*$^{+}$*, C1C2*$^{+}$ donor. Data are representative of five independent experiments, each including a minimum of 6 independent donors. (E) *In vitro* cytotoxicity against autologous DHIV3-infected CD4^{+} T cells mediated by purified NK cells from individuals with the indicated KIR3DL1 and HLA-B subtypes. Each bar represents mean ± SEM a minimum of three donors, and data represents five independent trials. *, $p<0.05$; **, $p<0.01$. Data are analyzed by one-way ANOVA and Tukey's post-hoc test.