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HIV Protective *KIR3DL1* and *HLA-B* Genotypes Influence NK Cell Function Following Stimulation with HLA-Devoid Cells

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Epidemiological studies in humans have implicated carriage of combinations of genes encoding certain *KIR3DL1* (killer Ig-like receptor 3DL1) alleles and their *HLA-Bw4* ligands in slower progression to AIDS, lower viral load and protection from infection. Given that the *KIR3DL1**h/*y/*HLA-B**57 genetic combination is strongly associated with favorable HIV outcomes, we measured responses from NK cells isolated from these individuals by multiparametric flow cytometry for cytokine secretion and degranulation in response to stimulation with HLA-devoid cells to assess whether the KIR/HLA compound genotypes linked to better HIV outcome favor increased NK cell functional potential. Our results indicate that NK cells from these individuals had increased functional potential, particularly in the *KIR3DL1*⁺ NK cell subset. These results support a link between KIR/HLA genotypes and NK cell function and could provide an explanation for the observation that some KIR/HLA combinations are associated protective phenotypes in the context of host-HIV interactions. *The Journal of Immunology*, 2010, 184: 2057–2064.

As cells of the innate arm of the immune system, NK cells are important for tumor and viral control. However, given their potent cytolytic and cytokine secretion potential, their activity needs to be tightly regulated. The activation state of an NK cell is determined partly by the integration of stimulatory and inhibitory signals after interaction of surface NK cell receptors (NCRs), including the highly diverse killer Ig-like receptor (KIR) family, with ligands found on target cells (1). The polygenic and polymorphic *KIR* genetic region encodes for receptors with varying protein structures (2), ligand affinities (3–5), cell surface expression levels (6, 7), and types (activating or inhibitory) of transmitted intracellular signals (2). These subtle variations could explain why NK cells from individuals with certain KIR-ligand combinations seem better able to activate NK cell responses in the presence of specific pathogens (8–11). This has been highlighted by a number of epidemiologic studies linking the generic *KIR3DS/L1* haplotypes with HIV disease progression or resistance to infection (8, 9, 12–15).

The *KIR3DS/L1* locus has the particularity of encoding for a highly polymorphic group of inhibitory alleles (*KIR3DL1*;

hereafter, 3DL1) and a smaller set of activating alleles (*KIR3DS1*; hereafter, 3DS1), of which *3DS1**013 is the most common across several populations (16). The polymorphisms of *3DL1* alleles translate into molecules with cell surface expression levels that can be high (*h; 3DL1*001, *002, *008, *009, *015, and *020), intermediate/low (*l; *005, *006, *007, *028, and *053), or null (not expressed at the surface; *004) (4, 7, 9).

In recent studies, various *3DL1* allotypes in combination with their known, or putative ligands, *HLA* alleles belonging to the *Bw4* group, were shown to be associated with differential HIV disease progression, protection from opportunistic infections and viral load (VL) set point (8–10). The genotype conferring the highest protection compared with Bw6 homozygotes (hmz), who lack any *HLA-B* ligand for *KIR3DL1*, with respect to these outcomes, was a combination referred to as **h/*y+B*57*. This genotype is composed of a *3DL1* genotype termed *3DL1**h/*y (i.e., lacking *3DL1**l alleles) with *HLA-B**57, an *HLA-Bw4* allele with an isoleucine at position 80 (i.e., *Bw4-80I*). In line with these findings, our group recently reported an association between carrying the **h/*y+B*57* combination with resistance to infection in a population of HIV exposed uninfected (EU) individuals (12). However, although these data support a role for NK cells in control of HIV that varies depending on carriage of **h/*y+B*57*, there are few studies linking this KIR/HLA combined genotype to improved antiviral NK cell function.

NK cells can be divided into two populations based on the intensity of the CD56 marker at the cell surface. CD56^{dim} NK cells are the more cytotoxic subset, whereas CD56^{bright} cells are poorly cytotoxic and preferentially secrete cytokines when activated (17, 18). However, the cytokine secretion and cytolytic functions are not exclusive to either cell subset (19–21). IFN- γ and TNF- α secretion as well as cytolysis have documented antiviral activity and are functions attributable to both NK and CD8⁺ T cells (18, 22–27). In HIV infection, where there is evidence that CD8⁺ T cells play a role in controlling virus, it is not so much the frequency or breadth of epitope recognition by these T cells, but rather the functional quality of their anti-HIV responses that correlates best with VL control (28, 29). This provided the

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Abbreviations used in this paper: *Bw4-80I*, *HLA-Bw4* allele with an isoleucine at position 80; EU, exposed uninfected; HCV, hepatitis C virus; hmz, homozygotes; IDU, injection drug user; KIR, killer Ig-like receptors; NCR, NK cell receptor; VL, viral load.

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rationale for characterizing the quality of NK cell functional potential by measuring secretion of cytokines (IFN- γ and TNF- α) and CD107a expression, a marker for degranulation (30, 31). To determine whether NK cells from individuals bearing the **h/*y+ B*57* combination associated with distinct outcomes with respect to HIV infection could be distinguished based on functional potential, we stimulated PBMCs with HLA-devoid K562 cells and measured the frequency of NK cells displaying all possible functional patterns. We found that individuals carrying this KIR/HLA genotype combination associated with control of HIV had increased frequencies of polyfunctional NK cells compared with study participants carrying other *Bw4* alleles and to *Bw6* hmz individuals.

Materials and Methods

Study population

A total of 61 HIV seronegative individuals were included in this study. These were recruited from amongst the participants of the St. Luc injection drug user (IDU) cohort or were the seronegative partner in a serodiscordant couple (13, 32, 33). Twenty-nine met the criteria for inclusion in an HIV EU population because they had at least five documented parenteral or mucosal exposures to HIV. The remaining 32 individuals were enrolled in the St. Luc IDU cohort but did not meet the criteria for classification as EUs either because of insufficient documented exposure to HIV or because their needle sharing was with partners of unknown serostatus. All 61 individuals included in this study are KIR3DL1 homozygous to eliminate any possible confounding effect on NK function of carrying the activating allele at this locus, *KIR3DS1*. Informed consent was obtained from all study subjects, and the research conformed to ethical guidelines of all the authors' institutions.

MHC and KIR typing

Genomic DNA was extracted from PBMC or EBV-transformed cells using a QIAamp DNA blood kit (Qiagen, Mississauga, Ontario, Canada). All subjects were typed for MHC class I alleles by sequence-based typing using kits from Atria Genetics (South San Francisco, CA). Assign software was used to interpret sequence information for allele typing (Conexio Genetics, Perth, Australia). *KIR3DL1* generic genotyping was performed by PCR using two pairs of specific primers for amplification of either *KIR3DL1* or *KIR3DS1* as previously described (13). *KIR3DL1* allotyping was performed by sequencing *KIR3DL1* exons as previously described (12). Single nucleotide polymorphisms corresponding to the *3DL1* alleles were identified by aligning the sequenced DNA to a reference consensus sequence consisting of a contig of *3DL1* cDNA sequences. In this study, *KIR3DL1** \lt high expression alleles included *3DL1**001, *3DL1**002, *3DL1**008, *3DL1**009, *3DL1**015, and *3DL1**020, and *KIR3DL1**low expression alleles included *3DL1**005, *3DL1**007, and *3DL1**021. In addition, HLA-Bw6 hmz individuals were defined by the lack of any HLA-Bw4 epitope at the *HLA-A* and *-B* locus.

Cells

PBMCs were isolated by density gradient centrifugation (Ficoll-Paque, Pharmacia Upsala, Sweden) from whole blood obtained by venipuncture into tubes containing EDTA anticoagulant or by leukapheresis as previously reported (34). The cells were then cryopreserved in 10% DMSO (Sigma-Aldrich, St. Louis, MO) with 90% FBS (Wisent, St. Bruno, Quebec, Canada).

NK cell activation and staining for phenotype and function

Cryopreserved PBMCs were thawed and resuspended at 10^6 cells/ml in RPMI 1640 (Wisent) containing 10% FBS (Wisent), 2 mM L-glutamine (Wisent), 50 IU/ml penicillin (Wisent), and 50 μ g/ml streptomycin (Wisent). Brefeldin A (at 5 μ g/ml, Sigma-Aldrich), monensin (at 6 μ g/ml, Golgi Stop; BD Biosciences, Mississauga, Ontario, Canada) and anti-CD107a-FITC Ab (BD Biosciences) were added to the cells. One million PBMCs were stimulated with 1) MHC class I-devoid K562 cells (American Type Culture Collection, Manassas, VA) at a PBMC-to-target cell ratio of 5:1, 2) medium alone (negative control), or 3) PMA (1.25 μ g/ml); ionomycin (0.25 μ g/ml, Sigma-Aldrich) (positive control to ensure the cells being tested were able to respond; all stimulation data shown in the manuscript were from cells that generated positive stimulation results with PMA and ionomycin). Stimulated PBMCs were incubated 6 h at 37°C in a humidified 5% CO₂ incubator.

Cells were then stained for viability using the Aqua Live/Dead fixable dead cell stain kit (Invitrogen, Burlington, Ontario, Canada) according to the manufacturer's instructions. PBMCs were then stained for cell surface markers with anti-CD56-APC, anti-CD16-Pacific blue (BD Biosciences), and CD3-ECD, CD158e-PE (also known as, Z27-PE, Beckman Coulter, Mississauga, Ontario, Canada) for 30 min. Samples were washed with PBS containing 1% FBS (Wisent) and 0.1% sodium azide (Sigma-Aldrich), fixed and permeabilized using the Fix and Perm kit (Invitrogen), and stained for intracellular cytokines using Alexa 700 conjugated anti-IFN- γ and PE-Cy7 conjugated anti-TNF- α (BD Biosciences) for 30 min. After washing, samples were fixed with a solution of 1% paraformaldehyde (Fisher Scientific, Ottawa, Ontario, Canada) and kept in the dark at 4°C until acquisition.

Flow cytometry analysis

The gating strategy used to analyze the functional profile of NK cells stimulated with K562 cells is available in Supplemental Fig. 1. For all analyses, NK cells were defined as CD3 $^-$ and CD56 $^+$ CD16 $^+$, CD56 $^+$ CD16 $^-$, or CD56 $^-$ CD16 $^+$. Boolean gating was used to determine the trifunctional (CD107a, IFN- γ , and TNF- α), bifunctional (any combination of two of the measured functions), or monofunctional (a single measurable function) profile of 3DL1 $^+$ (i.e., Z27 hi), 3DL1 $^-$ (i.e., Z27 dim and Z27 $^-$), or all NK cells (3DL1 $^+$ and 3DL1 $^-$ NK cells). Between 400,000 and 600,000 total events were acquired for each sample on a LSRII flow cytometer (BD Biosciences). Flow cytometry analysis for NK cell activation after K562 stimulation or for NK cell phenotyping was performed using FlowJo software version 8.7.1 (TreeStar, Ashland, OR). The data obtained were corrected for background using unstimulated cells before statistical analysis.

Statistical analysis

Statistical analysis and graphical presentation were performed using GraphPad InStat 3.05 and GraphPad Prism 4.01. Mann-Whitney *U* tests were used for between-group comparisons. Wilcoxon signed-rank test was used to compare the percent of 3DL1 $^+$ to 3DL1 $^-$ polyfunctional NK cells within individuals. Spearman's correlation analysis was used to study the relationship between percent contribution of a response and its NK cell frequency. A *p* value of \lt 0.05 was considered significant. For between-group comparisons of the percent contribution of NK cells with different functional profiles to the total NK cell response, a Bonferroni correction for multiple comparisons was applied by multiplying the *p* value by the number of functional profiles evaluated ($n = 7$) to give a corrected *p* value, which was considered significant if \lt 0.05.

Results

The mAb Z27 (also known as CD158e) binds both KIR3DL1 and KIR3DS1. However, the fluorescence intensity of Ab binding to the inhibitory versus activating forms of these receptors differs, such that 3DL1 $^+$ NK cell (Z27 hi) and 3DL1 $^-$ NK cell (Z27 dim or Z27 $^-$) are readily distinguishable (35, 36). All 61 individuals included in this study are *3DL1* hmz. Forty-five are carriers of both the KIR3DL1 receptor and its *HLA-Bw4* ligand (termed *3DL1-Bw4* individuals) and 16 are *Bw6* hmz individuals who carry no *HLA* alleles able to act as a ligand for any *3DL1* receptor. Using the Z27 Ab, we were able to assess the function of 3DL1 $^+$ and 3DL1 $^-$ NK cells. It has been previously reported that expressing an NCR for self-*HLA* ligand leads to an increased NK cell response after stimulation with MHC-devoid K562 cells (37). As shown in Fig. 1, we observed a similar phenomenon for CD107a surface translocation, IFN- γ and TNF- α secretion. In *3DL1-Bw4* individuals, there was an increase in the percentage of 3DL1 $^+$ NK cells that were CD107a $^+$, IFN- γ $^+$, or TNF- α $^+$ after K562 stimulation when compared with the percentage of 3DL1 $^-$ NK cells ($p < 0.0001$ for all functions, Wilcoxon signed-rank test) (Fig. 1A–C, left panels). In *Bw6* hmz (Fig. 1A–C, right panels), only TNF- α $^+$ secretion differed significantly between 3DL1 $^+$ and 3DL1 $^-$ NK cells ($p = 0.0029$, Wilcoxon signed-rank test). When *3DL1-Bw4* individuals were compared with *Bw6* hmz, the percentage (median [range]) of 3DL1 $^+$ that were CD107a $^+$, IFN- γ $^+$, and TNF- α $^+$ was greater in *3DL1-Bw4* individuals than in *Bw6* hmz (11.4% [0.3–34.8] versus 6.4% [1.3–23.0], $p = 0.0242$), (15.0% [0.4–43] versus 6.1% [1.3–17.9], $p = 0.0143$), and (1.8% [0–12.1] versus 1.0% [0–5.8], $p = 0.1891$). There was no significant

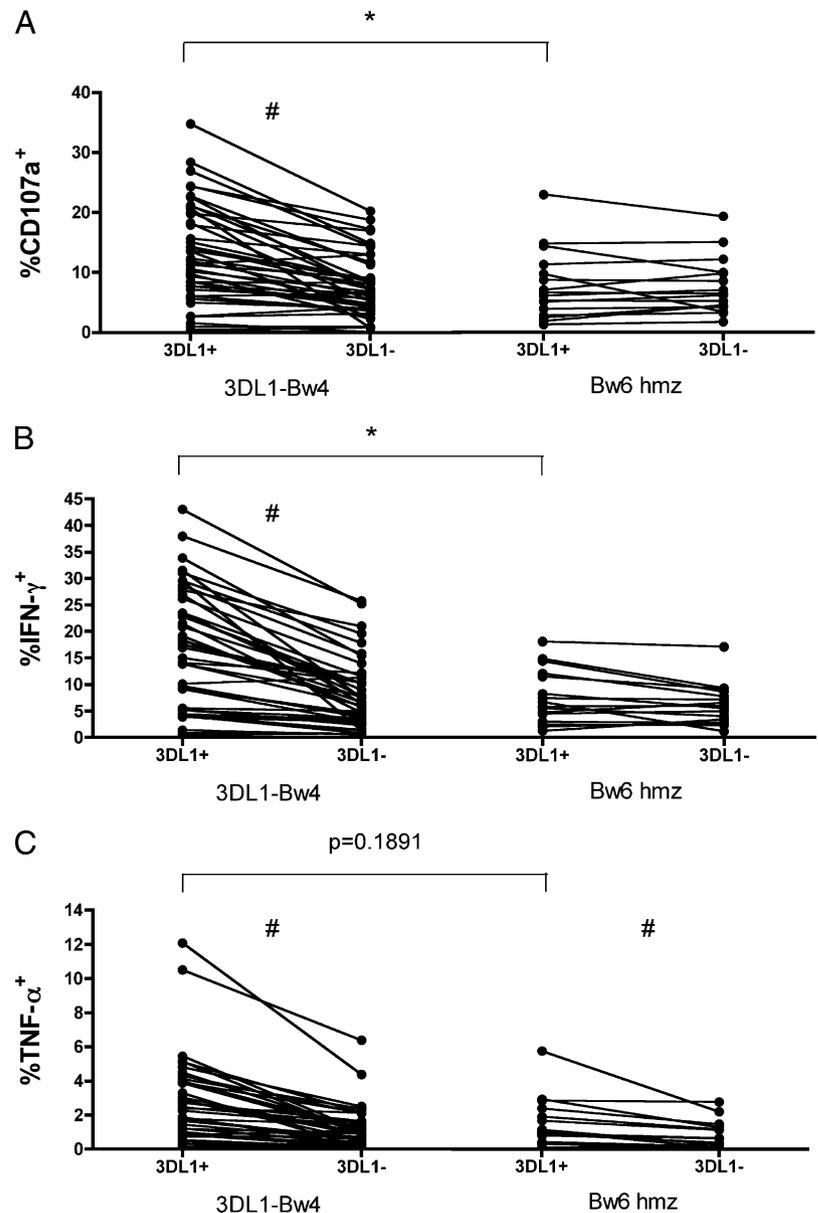


FIGURE 1. NK cells from individuals carrying KIR3DL1/HLA-Bw4 receptor-ligand pairs have a KIR3DL1⁺ subset with increased functional potential. The before and after plots show the percent of total 3DL1⁺ or 3DL1⁻ NK cells that are CD107a⁺ (A) IFN- γ ⁺ (B), or TNF- α ⁺ (C) in individuals who carry 3DL1-Bw4 receptor ligand pair (left-hand plots) or are Bw6 hmz (right-hand plots). A Mann-Whitney *U* test was used to assess the significance of between-group comparisons (3DL1-Bw4 versus Bw6 hmz) for each NK subset. $p < 0.05$ was considered significant and shown with an asterisk (*) over the line linking the two groups being compared. The number (#) symbol over the line linking two groups being compared represents a significant Wilcoxon signed-rank test comparing within-subject percent of 3DL1⁺ and 3DL1⁻ NK cells that are CD107a⁺, IFN- γ ⁺, or TNF- α ⁺.

difference between 3DL1-Bw4 and the Bw6 hmz in the percentage of 3DL1⁻ CD107a⁺, IFN- γ ⁺, or TNF- α ⁺ NK cells. Together, these results indicate that carriage of KIR3DL1 with its HLA-Bw4 ligand increases the functional capacity of 3DL1⁺ NK cells in response to stimulation with HLA-devoid cells.

Certain KIR3DL1 and HLA-Bw4 genotype combinations have been shown to be associated with time to AIDS and VL set point (9) or have been reported to be associated with a reduced risk of HIV infection in EUs (12). The 3DL1/Bw4 genetic combination that has been most strongly associated with these HIV outcomes is **h/*y+B*57* and this may be because this combined genotype confers a unique NK cell functional profile. To address whether NK cells from individuals carrying the **h/*y+B*57* combination have differential functional potential, we first measured the expression of CD107a and secretion of IFN- γ and TNF- α by NK cells in response to stimulation with K562 cells by eight-color flow cytometry in 11 subjects carrying **h/*y+B*57* and 16 Bw6 hmz. Of note, the median number of documented exposures to HIV was five (range: 0–92) in **h/*y+B*57* individuals and five (range: 0–134) in Bw6 hmz individuals ($p = 0.5869$, Mann-Whitney *U* test).

Given that the NK cell response is characterized using three functional markers, eight different functional permutations are possible and define eight different NK cell subsets. However, only seven of these subsets define responsive NK cells, the eighth permutation being negative for all three functions. For each Bw6 hmz and **h/*y+B*57* individual, the frequency for each response pattern was assessed and the contribution of each combination to the total NK cell response determined. Fig. 2A shows that the contribution of trifunctional NK cells to the total K562 stimulated response was significantly greater in NK cells from subjects with the **h/*y+B*57* combination (corrected $p = 0.0084$) compared with NK cells from Bw6 hmz (median, [range]) (4.8% [0.7–11.0] and 2.8% [0.0–4.2] for NK cells from individuals typing as **h/*y+B*57* and Bw6 hmz, respectively, Mann-Whitney *U* test). Therefore, NK cells from individuals carrying the **h/*y+B*57* genotype combination associated with slower HIV disease progression, lower VL, and/or protection from HIV infection have a greater multifunctional potential on stimulation with HLA-devoid cells when compared with NK cells from individuals who are Bw6 hmz. No significant differences were detected between these two study groups for the percent contribution of any of the

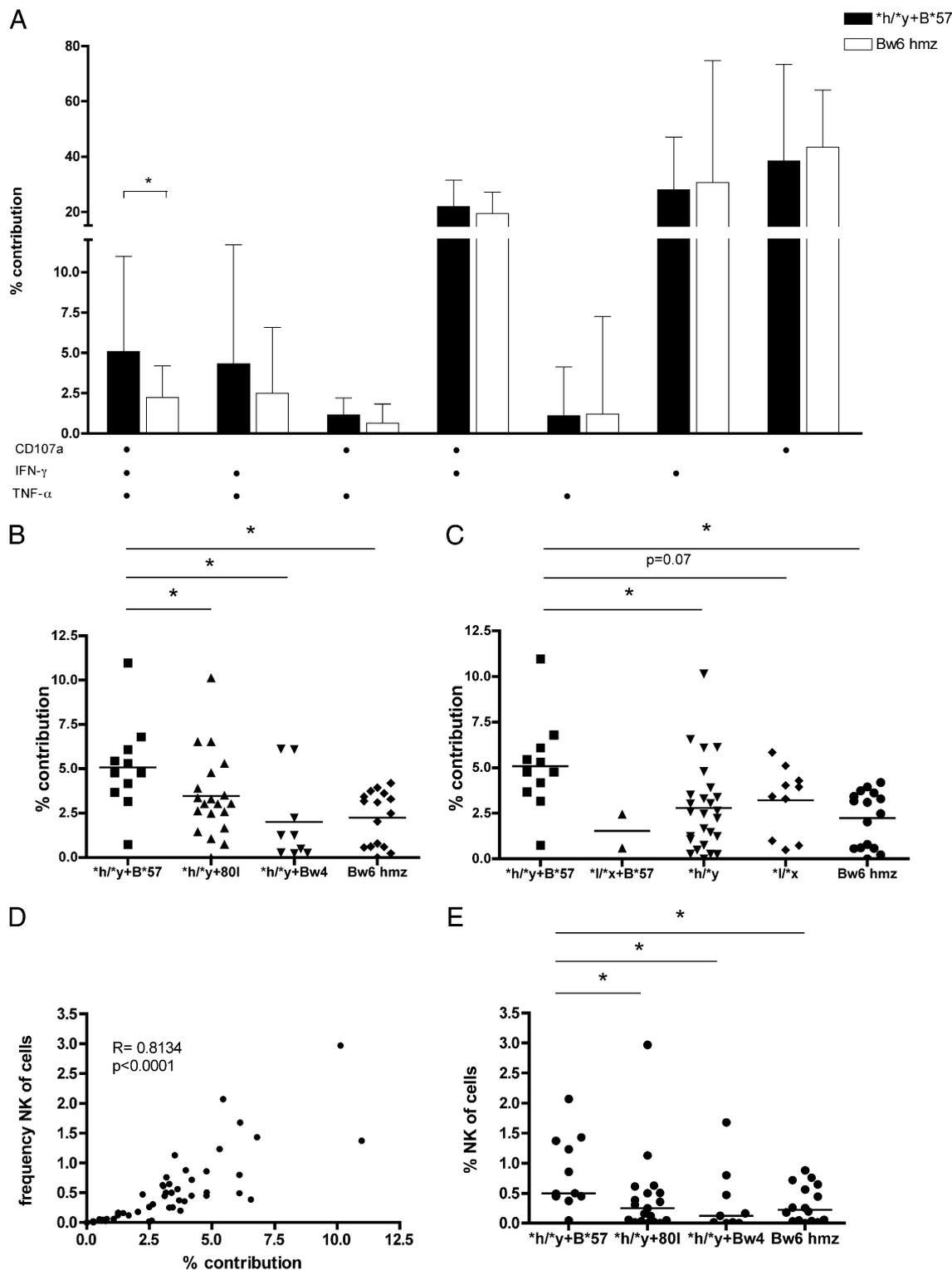


FIGURE 2. NK cells from *KIR3DL1* *h/*y+*HLA-B**57 positive individuals have a distinct functional profile. **A**, The percent contribution of seven different functional profiles to the total NK cell response to K562 *HLA*-devoid cells from individuals with carrying *KIR3DL1* *h/*y+*HLA-B**57 (*h/*y+B*57, $n = 11$) and *HLA-Bw6* hmz (Bw6 hmz, $n = 16$). Below the x -axis, dots refer to the presence of each functional marker (CD107a, IFN- γ , and TNF- α) in that profile. The height of each bar represents the median for the group and the height of the error bar the upper range for that group. An asterisk (*) over the line linking two bars indicates that the contribution of that functional subset to the NK cell response was significantly different in the two study populations. Trifunctional CD107a⁺IFN- γ ⁺TNF- α ⁺ NK cell contribution to the total response was compared between *h/*y+B*57 individuals and non-B*57 individuals who were *h/*y+80I, *h/*y+Bw4 or Bw6 hmz (**B**) and to *I/*x+B*57 individuals and non-B*57 individuals who carried *h/*y, *I/*x, or Bw6 hmz (**C**). A Mann-Whitney U test was used to assess the significance of between-group comparisons and $p < 0.05$ was considered significant. **D**, The correlation between a trifunctional response's contribution to the total NK cell response and the frequency of NK cells expressing this function. **E**, The same analyses as in **B** but expressed as the percentage of NK cells. *h/*y+B*57 = cocarriage of an *KIR3DL1* *h/*y genotype with an *HLA-B**57 allele; *h/*y+80I = cocarriage of an *KIR3DL1* *h/*y genotype with an *HLA-Bw4* allele with an 80I of the HLA H chain that is not *HLA-B**57; *h/*y+Bw4 = cocarriage of an *KIR3DL1* *h/*y genotype with an *HLA-Bw4* allele that does not have an 80I of the HLA H chain; *I/*x+B*57 = cocarriage of an *KIR3DL1* *I/*x genotype with an *HLA-B**57 allele.

other NK cell functional profiles to the total NK cell response. Therefore, for subsequent analyses reported in this study, we have focused on results relating to NK cells having a trifunctional profile, although data on all functional profiles were collected and compared.

The *HLA-B*57* allele belongs to the Bw4 serotype and has an 80I. We questioned whether the increased trifunctional potential of NK cells from **h/*y+B*57* individuals was due to the presence of *B*57* or could also be observed by NK cells from individuals with other *HLA* alleles belonging to either the Bw4 or Bw4-80I allele subsets. To answer this question, the trifunctional response of NK cells from non-*B*57* individuals carrying *KIR3DL1 *h/*y* with other Bw4 or Bw4-80I alleles was compared with the response from the **h/*y+B*57* group. The contribution of trifunctional responses by stimulated NK cells from **h/*y+B*57* individuals was greater than that from 20 non-*B*57* individuals who were **h/*y+80I* (3.0% [0.0–10.1], $p = 0.039$) or from nine individuals who did not belong to either previous groups but were **h/*y+Bw4* (1.2% [0.2–6.1], $p = 0.0276$) (Mann-Whitney *U* test) (Fig. 2B). The percent contribution of stimulated trifunctional NK cells in these later two study populations was not significantly different from that seen in Bw6 hmz. These results suggest that *B*57* is unique among Bw4 and Bw4-80I *HLA* alleles in mediating increased NK trifunctional potential.

To determine the role of *3DL1 *h/*y* in increasing NK cell function in **h/*y+B*57* individuals, the trifunctional response of NK cells from non-*B*57* individuals carrying *3DL1 *h/*y* ($n = 26$) or *3DL1 *l/*x* ($n = 10$) was compared with the response from the **h/*y+B*57* group (Fig. 2C). Individuals carrying *3DL1 *h/*y* without *B*57* had a lower NK cell trifunctional response contribution ($p = 0.0083$) when compared with the **h/*y+B*57* group (4.8% [0.7–11.0] and 2.5% [0.0–10.1] for NK cells isolated from individuals typing as **h/*y+B*57* and **h/*y* non-*B*57*, respectively, Mann-Whitney *U* test). There was a nonsignificant trend toward an increased contribution of trifunctional NK cells to the total NK cell response in individuals carrying **h/*y+B*57* versus *3DL1 *l/*x* without *HLA-B*57* ($p = 0.0726$, 4.8% [0.7–11.0] and 3.7% [0.5–5.8] for NK cells isolated from individuals typing as **h/*y+B*57* and **l/*x* non-*B*57*, respectively). The percent of trifunctional NK cells from non-*B*57* individuals carrying either *3DL1 *h/*y* or *3DL1 *l/*x* was similar to that seen in Bw6 hmz. Finally, only two individuals in our population typed as having the **l/*x+B*57* genetic combination, which was insufficient for statistical analyses. It is noteworthy, however, that the contribution of trifunctional NK cells to the total response of 0.6% and 2.5% was more similar to NK cells from Bw6 hmz individuals than from **h/*y+B*57* individuals. These data suggest that carrying **h/*y* or *B*57* alone is not sufficient for an increased NK cell functional potential and that both the receptor and ligand may be required.

The data presented in Fig. 2A–C are reported as “percent contribution to the total response,” a measure often used to describe the quality of immune responses. The strength of a response is more accurately reported by the “frequency of total NK cells.” Both measures correlate with each other ($R = 0.8134$, $p < 0.0001$, Spearman correlation, Fig. 2D) and a similar conclusion is obtained when the frequency of NK cells is used to compare study populations as seen in Fig. 2E.

Based on Z27 staining, it was possible to attribute function to either the $3DL1^+$ or $3DL1^-$ NK cell subset for the populations carrying **h/*y+B*57* or Bw6 hmz. As shown in Fig. 3, the percent of $3DL1^+$ K562-stimulated NK cells that were trifunctional was greater in **h/*y+B*57* individuals than Bw6 hmz (1.4% [0.2–3.9] and 0.1% [0.0–1.5] for **h/*y+B*57* and Bw6 hmz, respectively,

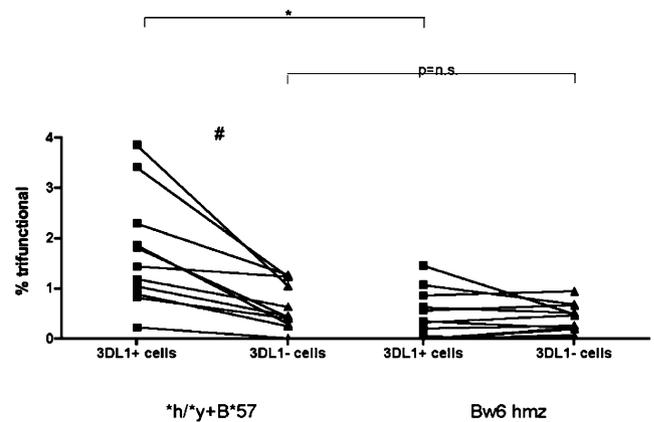


FIGURE 3. $KIR3DL1^+$ NK cells account for most of the trifunctional contribution of stimulated NK cells from *KIR3DL1 *h/*y+B*57* positive individuals. The before and after plots show the percent of the total $3DL1^+$ and $3DL1^-$ NK cells that are $CD107a^+IFN-\gamma^+TNF-\alpha^+$ in subjects who are *KIR3DL1 *h/*y+B*57* (left-hand plots) carriers and Bw6 hmz (right-hand plots). A Mann-Whitney *U* test was used to assess the significance of between group comparisons (**h/*y+B*57* versus Bw6 hmz); $p < 0.05$ was considered significant and shown with an asterisk (*) over the line linking the two groups being compared. The number (#) symbol over the line linking the two groups being compared represents a significant p value resulting from a Wilcoxon signed-rank test comparing within-subject percent of $3DL1^+$ cells and $3DL1^-$ NK cells that are trifunctional.

$p = 0.0004$, Mann-Whitney *U* test). Although not as dramatic, this was also the case for $3DL1^-$ NK cells in that the percent of trifunctional $3DL1^-$ NK cells was 0.5% (0.0–1.3) and 0.2% (0.0–0.9) after stimulation of cells from **h/*y+B*57* subjects and Bw6 hmz, respectively ($p = 0.0513$; Mann-Whitney *U* test). Furthermore, within the **h/*y+B*57* group, trifunctional NK cells were more likely to be $3DL1^+$ (1.4% [0.2–3.9]) than $3DL1^-$ (0.5% [0.0–1.3]) ($p = 0.001$, Wilcoxon signed-rank test) (Fig. 3). In contrast, in Bw6 hmz, the contributions of the $3DL1^+$ and $3DL1^-$ subsets to the trifunctional NK response stimulated by K562 was similar (Fig. 3). In summary, the increased functional potential of NK cell from subjects carrying **h/*y+B*57* was most apparent in $KIR3DL1^+$ NK cells.

Discussion

In this report, we compared the functional profile of NK cells from individuals carrying different allele combinations of *KIR3DL1* and *HLA-B*. NK cells from individuals with **h/*y+B*57* exhibited an increased contribution of trifunctional NK cells to the total HLA-devoid K562-stimulated response. This genetic combination has been previously linked to slower HIV disease progression, decreased VL, and/or protection from HIV infection (9, 12). For cells from individuals with the **h/*y+B*57* combined genotype, the increased trifunctionality was more likely to be seen in NK cells expressing $3DL1$.

*HLA-B*57* is an *HLA* allele previously reported to be associated with slow HIV disease progression (9, 38–40). It has been reported that $CD8^+$ T cell responses restricted by this allele may have unique qualities that influence the efficiency with which they control HIV (41–43). For example, $CD8^+$ T cell responses restricted by *HLA-B*57* contribute more to the total HIV-specific response during early infection, recognize a greater fraction of potential HIV-restricted epitopes, and are associated with a reduction in the level of the response restricted by other alleles carried by the same individual (41–43). However, given the role of NK cells in controlling viral infections through cytokine secretion and cytotoxicity, the potency, and polyfunctionality of these innate immune cells in *HLA-B*57*

individuals may provide an additional mechanism contributing to slow disease progression that has been linked to carrying this allele. Potent NK cell-mediated antiviral responses induced early in HIV infection may directly suppress HIV replication as well as induce dendritic cell maturation via a number of mechanisms, including IFN- γ secretion, favoring a Th1 response that could be beneficial in controlling the infection (44, 45).

Although epidemiological data suggest a role for the $*h/*y+B*57$ combined genotype in controlling HIV infection, we could not exclude the possibility that carriage of $B*57$ was sufficient for increased NK cell trifunctionality. *HLA-B*57* has a frequency of ~6% in the predominantly white population from which the study subjects tested were drawn and the frequency of the $*h/*y$ *KIR3DL1* genotype is greater than that of the $*l/*x$ genotype. For this reason, we were only able to test two individuals who were $B*57$ positive without carrying $3DL1*h/*y$. The contribution of trifunctional NK cells in these two subjects appeared to be lower than that seen in the $*h/*y+B*57$ population. In $*h/*y+B*57$ individuals, NK cell trifunctionality occurred more frequently in $3DL1^+$ than $3DL1^-$ cells. Given that *HLA-B*57* is associated with HIV slow disease progression, within an HIV seropositive slow progressor population, we were able to identify six additional subjects who were $*h/*y+B*57$ and three who were $*l/*x+B*57$. The median percent contribution of NK cells with trifunctional potential after K562 stimulation was 3.2% and 1.24%, respectively ($p = 0.03$) (not shown). Because HIV infection has been reported to negatively affect NK cell function, we elected not to pool results from HIV-positive subjects with those shown for HIV-negative individuals shown in Fig. 2 (46). Taken together, these results suggest that $B*57$ is probably involved in the modulation of the NK response through its interaction with *KIR3DL1*. In contrast, because NK cells from subjects carrying *Bw4* alleles other than $B*57$ or $*h/*y$ *KIR3DL1* genotypes without the $B*57$ ligand did not exhibit increased trifunctionality after stimulation with K562 cells (Fig. 2B, 2C), it is clear that $B*57$ plays a central role in modulating the NK cell functional profile.

It is not known why this *HLA* allele is superior to other *Bw4* or *Bw4-80I* alleles in favoring the development of polyfunctional NK cells. Among *Bw4-80I* alleles, which are particularly good receptors for $3DL1$ (47–49), $B*5705$ and $B*5711$ (neither of which were present in our population) are the only members of the $B*57$ family that do not have a valine residue at position 97 (50). Of the other *Bw4-80I* alleles, only $B*5813$ has a valine at this position; the remaining *Bw4-80I* alleles have arginine, asparagines, threonine, tryptophan, or serine at this position (50). These residues differ markedly from valine in terms of either polarity or size. Although it is known that the amino acid at position 97 is involved in modulating the interaction between *HLA-B* and $3DL1$, its precise role is still unclear (51). It is possible that the presence of a valine at position 97 influences receptor-ligand interactions, which directly affect NK licensing. Licensing is an NK cell maturation process in which cells acquire inhibitory *KIRs* for self-*HLA* ligands until tolerance to self is achieved (9, 21, 52). Mature NK cell subsets that express potent inhibitory receptors for self-*HLA* may therefore have greater activation potential when the inhibitory signal is interrupted through interactions with *HLA*-deficient cells such as K562 or HIV-infected cells, in which *Nef* downmodulates *HLA-A* and *-B* cell surface expression (53, 54). The licensing concept provides an explanation for how an inhibitory *KIR*, such as *KIR3DL1*, may play a role in NK cells function that ultimately influences activation of antiviral functions.

Given that the $*h/*y+80I$ genetic combination has also been linked to slower HIV disease progression, we expected that NK cells from individuals with this genotype would also have

a greater functional potential when compared with *Bw6* hnz. NK cells from 20 subjects who were $*h/*y+Bw4-80I$ and nine who were $*h/*y+Bw4$ (but not *Bw4-80I*) were similar to *Bw6* hnz in terms of their trifunctional potential after stimulation with K562. However, K562 stimulated a higher percentage of $3DL1^+$ NK cells from carriers of the $3DL1$ -*Bw4* receptor ligand pair, including $*h/*y+Bw4-80I$ individuals, than *Bw6* hnz for measures of $CD107a^+$, $IFN-\gamma^+$, and $TNF-\alpha^+$ (Fig. 1). Even if $*h/*y+B*57$ carriers are excluded from this analysis, the significance of the pairwise comparison (i.e., % $3DL1^+$ versus % $3DL1^-$) is maintained in the $3DL1$ -*Bw4* group ($p < 0.0001$ for all functions measured). The between-group (i.e., $3DL1$ -*Bw4* versus *Bw6* hnz) difference in the percentage of responding $3DL1^+$ NK cells falls below the level of statistical significance when $*h/*y+B*57$ individuals are excluded from the analysis. This may be due in part to the decreased sample size as suggested by the persistence of nonstatistically significant trends for these comparisons for $IFN-\gamma$ secretion ($p = 0.054$) and $CD107a$ expression ($p = 0.1071$). Therefore, although NK cells from $*h/*y+80I$ individuals do not differ from those from *Bw6* hnz in terms of trifunctionality, their $3DL1^+$ NK cells have a tendency to be more responsive for individual functions that play a role in antiviral immunity; this elevated responsiveness is most evident for $CD107a$ translocation and $IFN-\gamma$ secretion. It is also possible that the effects of $*h/*y+80I$ on NK cell function would be better assessed using functional markers not measured by our experiments, such as perforin or granzyme, recently proposed to be better correlates of immune protection in HIV-specific T cell responses (22). Finally, perhaps the epidemiological effect observed on HIV disease progression in individuals with this genetic combination is dependent on the presence of the virus, as it has been proposed in the case of *KIR3DS1* and *HLA-Bw4-80I*, another combined genotype linked to slower HIV disease progression (8, 55, 56). If this is the case, a different experimental system in which induction of the NK cells occurs in the context of HIV-infected cell stimulation may be required to observe the effect of the $*h/*y+80I$ genotype on NK cell function (55). In HIV-infected cells, the *Nef* gene product downregulates surface expression of *HLA-A* and *-B* but does not affect *HLA-C* and *-E* levels, which are ligands for some NCRs. In addition, the presence of HIV viral peptides bound to the *HLA* molecules could influence their binding to *KIR* molecules. Thus, although K562 stimulation of NK cells provides insights into their functional potential, there are limitations associated with this method and the next step in understanding the mechanisms underlying the association of certain *KIR/HLA* combination with favorable HIV outcome would be to investigate the influence of these combined genotypes on NK function using HIV-infected cells as stimuli.

As shown in Fig. 2B and 2C, we observed a high level of variability in the distribution of the contribution of the trifunctional responses in some genetic groups, such as the *Bw6* hnz or $*l/*x$ individuals. This variability may be the result of other *KIR/HLA* combinations or other NCRs that may be expressed at the NK cell surface. These could partly explain the absence of statistically significant differences in the contribution of the trifunctional NK cell response in $*l/*x$ individuals when compared with the $*h/*y+B*57$ group. The effect of other *HLA* alleles on the variability of a response within a group is demonstrated by the *Bw4* epitope at the *HLA-A* locus. Inclusion of individuals carrying the $A*23$, $A*24$, $A*25$, or $A*32$ alleles, which bear the *Bw4* epitope and may interact with *KIR3DL1*, but not carrying any *HLA-B* *Bw4* alleles in the *Bw6* hnz population, increased the variability and the median of the trifunctional response observed in this control group (data not shown) (4, 9, 57). Therefore, the functional profile of NK cells is not likely to be solely dependent on

KIR3DL1 and HLA-Bw4 interactions and a more complete understanding of NK cell functional profiles may depend on the consideration of the full genotypes of NCRs and their ligands.

One possible limitation of this report is that the study population is at high risk for HIV acquisition. The majority of the individuals included belong to an injection drug user cohort; many (51/61) are positive for anti-hepatitis C virus (HCV) Abs. Although injection drug use and HCV infection may affect NK cell function, the current study was not designed to address this issue. In our population, HIV-exposure, HCV-seropositivity and IDU are similar across *KIR/HLA* genetic groups, minimizing the possibility that any of these factors would bias our results. It will remain important to confirm our results in a low-risk population.

In conclusion, we provide a link between a *KIR3DL1/HLA-B* genotype that has been associated with slower HIV disease progression and resistance to infection and increased NK cell functional potential. Understanding the mechanisms underlying these effects will contribute to our appreciation of the role of the innate immune system in HIV infection and may lead to new HIV vaccine design strategies.

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Disclosures

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