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Hierarchical System of the Human Natural Killer Cell Response Is Determined by Class and Quantity of Inhibitory Receptors for Self-HLA-B and HLA-C Ligands

Junli Yu,* Glenn Heller,† Joseph Chewning,‡ Sungjin Kim,¶ Wayne M. Yokoyama,‖ and Katharine C. Hsu*‡§

The interaction of NK inhibitory killer Ig-like receptors (KIRs) with self-MHC class I molecules mediates NK tolerance to self while conferring functional competence. Through single-cell analysis of intracellular IFN-γ production and NK clone cytotoxicity we evaluated the resting NK repertoire, analyzing the responsiveness of NK subgroups expressing discrete combinations of non-KIR and KIR class I-specific receptors. CD94:NKG2A and Ig-like transcript 2 (ILT2)-expressing cells have a modest response to class I-negative target cells, but NK cells expressing inhibitory KIRs to self-MHC class I ligands, both HLA-B and HLA-C ligands, achieve significantly higher effector capacity. There is a dose effect of KIR for self-MHC on effector capacity, but even in the most highly responsive NK cells expressing more than one inhibitory KIR for self-MHC the presentation of only one cognate MHC ligand is sufficient to abolish response. Among KIR+ cells there is preferential expression for inhibitory KIR for self-MHC. The likelihood of KIR expression is influenced by whether other KIRs are already expressed on the same cell, supporting a model of serial acquisition of KIR expression. These findings define how inhibitory receptor and autologous HLA interactions impact single-cell function and demonstrate that the resting human NK repertoire is highly attuned but variegated in response. These findings have important implications for the resting NK response to viral pathogens and malignancy, for donor selection in allogeneic hematopoietic cell transplantation, and for models of NK tolerance. The Journal of Immunology, 2007, 179: 5977–5989.

Natural killer cells are lymphocyte members of the innate immune system capable of recognizing and eliminating infected and transformed cells (1, 2). Regulated by activating and inhibitory signals, NK cells fulfill their role of effector function against pathogens and damaged cells while simultaneously scrutinizing and sparing cells expressing self-MHC class I proteins. Recognition of MHC class I proteins by cell surface inhibitory receptors prevents autoreactive behavior but also permits cytotoxicity against target cells that have down-regulated MHC class I Ag expression, where the lack of class I engagement by inhibitory receptors allows NK activation (3–6). In humans, inhibitory NK receptors with specificity for the human MHC (HLA) proteins include the lectin-like CD94:NKG2A heterodimer, which recognizes complexes of HLA-E bound to peptides from the leader sequences of other class I molecules; the Ig-like transcript 2 (ILT2,3 also referred to as LILRB1 and LIR1), whose recognition of a relatively conserved region in the class I molecule provides broad class I specificity; and the polymorphic killer Ig-like receptors (KIRs), whose members’ recognition of the HLA-C allotypes with asparagine 80 (HLA-Cw3 and related alleles or group C1 alleles), HLA-C allotypes with lysine 80 (HLA-Cw4 and related alleles or group C2 alleles), and HLA-A and B allotypes with the Bw4 epitope confer narrower specificity (6).

Although their role in NK effector function against class I-deficient target cells has been well-described, how these same inhibitory receptors simultaneously mediate NK self-tolerance and functional competence, particularly in individuals lacking the MHC ligands for their inhibitory receptors, has been the subject of renewed investigation. Recent findings indicate that not all NK cells behave alike and that an NK cell’s effector capacity depends not only on its inhibitory receptor phenotype but also the receptor’s specific recognition of self-MHC class I molecules. In the mouse, NK cells lacking all known self-MHC class I-specific inhibitory receptors exist and respond poorly to class I-deficient lymphoblasts (7). Studies using MHC congenic and transgenic strains of mice have shown that NK cells expressing a given self-MHC class I-specific inhibitory receptor are more competent than NK cells lacking such a receptor, suggesting that the engagement of such receptors may be required for full functional competence of NK cells (8). Consistent with this model, NK cells from β2-microglobulin-deficient or class I-deficient mice exhibit no functional competence; however, introduction of a transgene encoding an MHC class I molecule that interacts only with one inhibitory Ly49 receptor into the MHC-deficient background exclusively restores the functional competence of NK cells expressing the corresponding receptor (8). Additional gene transfer experiments have demonstrated that signaling via the ITIM within the cytoplasmic tail of Ig-like receptor; NS-KIR, inhibitory KIR expressed by NK cells for nonself-MHC class I; S-KIR, inhibitory KIR expressed by NK cells for self-MHC class I.

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3 Abbreviations used in this paper: ILT2, Ig-like transcript 2; AHSCT, allogeneic hematopoietic stem cell transplantation; BLCL, B lymphoblastoid cell line; KIR, killer
the receptor is required for functional competence. Taken together, the results from these studies indicate that direct interaction between inhibitory receptors and MHC class I molecules and signal transduction of the engaged receptor are necessary to endow the NK cell with functional competence.

In the human, the inhibitory KIR receptors KIR2DL2/2DL3, KIR2DL1, and KIR3DL1 are specific for the HLA-C1, HLA-C2, and HLA-Bw4 ligands, respectively. Extending the murine findings to the human, a recent study by Anfossi et al. (9) has shown that human NK cells expressing the inhibitory KIR2DL1 or KIR2DL2/2DL3, when derived from HLA-C2/C2 or HLA-C1/C1 donors, respectively, are more responsive by CD16 cross-linking, cytokine production, and CD107 mobilization to class I-negative target cells when compared with other NK cell subsets. Based on this observation, the authors demonstrate that autologous combinations of inhibitory KIRs with their HLA ligands is correlated with the acquisition of NK cell competence similar to that seen in murine NK cells. The response among NK cells negative for KIR expression but positive for other class I receptors such as CD94: NKG2A or ILT2 was not reported, and the role of the KIR3DL1 receptor specific for HLA-Bw4 in NK licensing was not explored.

Furthermore, it has not been reported whether the magnitude of NK response is influenced by the number of different types of class I-specific inhibitory receptors expressed.

The finding that the interaction of inhibitory NK receptors with self-MHC class I ligands plays a role in endowing the NK cell with functional competence redefines our understanding of what comprises the functional NK repertoire. Because the human NK repertoire is comprised of phenotypically diverse subpopulations, differentiating how inhibitory NK receptor combinations with HLA genotype impact the magnitude of NK response is essential in understanding the NK repertoire, a critical interface between innate and adaptive immunity. We hypothesized that the NK repertoire is biased to maximize the number of functional NK cells while maintaining tolerance to self. Using multicolor staining and flow cytometric analysis to measure IFN-γ production, CD16 activation, and cytotoxicity, we extensively analyzed 10 KIR haplotype-A homozygous individuals who do not express any activating KIR that might interfere with the assessment of inhibitory KIR-HLA interaction. By analyzing the response at the single-cell level among NK populations with differential expression of class I-specific non-KIR and KIR receptors, we confirm that KIR3DL1 for HLA-Bw4 can confer responsiveness equivalent to the HLA-C-specific inhibitory KIR, and we demonstrate differences in response capacities between NK cells expressing KIRs vs non-KIR receptors. Furthermore, by evaluating the quantitative differences in response based on the qualitative differences in KIR number (as defined by the number of different KIR receptor types expressed on the cell surface), we show that there is a clear hierarchy of responses among NK subset populations and that the response is proportionate to the number of different inhibitory KIRs for self-MHC class I expressed on the cell surface. From these findings, we establish that the functional human NK repertoire is clearly influenced by self-HLA, that there is a wide distribution of NK responsiveness in the NK repertoire based on cell surface expression of inhibitory receptors for self-HLA, and that the distribution is biased toward NK competence through preferential expression of inhibitory KIR for self-HLA. Biostatistical modeling of the steady-state NK repertoires from these individuals confirms that inhibitory KIR expression occurs in a nonuniform fashion, supportive of serial acquisition of KIR expression. These studies support a model of NK licensing where graduated acquisition of inhibitory KIR expression endows the NK cell with higher effector capacity and represent a comprehensive analysis of how inhibitory receptor-MHC class I interaction dictates the diverse response of the resting human NK repertoire to nonself targets.

Materials and Methods

**Human subjects and blood samples**

Blood samples were obtained from healthy, unrelated volunteer donors after obtaining informed consent for sample procurement as approved by the Memorial Sloan-Kettering Cancer Center Institutional Review Board (New York, NY). PBMCs were isolated by Ficoll/Hypaque density gradient centrifugation, and genomic DNA was prepared from 5 × 10^6 PBMCs using the Puregene DNA Isolation Kit (Genta Systems) or the QIAamp DNA blood mini kit (Qiagen) according to the manufacturer’s instructions.

**KIR and HLA genotyping**

Previously published primers were used for the detection of the KIR genes 2DS1, 2DS2, 2DS3, 2DS4, 2DL1, 2DL2, 2DL3, 2DL4, 2DL5, 3DS1, 3DL1, 3DL2, and 3DL3 and the pseudogenes 2DP1 and 2DP1 (10). HLA typing was performed using low resolution sequence-specific oligonucleotide typing for HLA-A, HLA-B, HLA-C, HLA-D, and HLA-DQ loci (Dyna-System), followed by high resolution typing by sequence-specific oligo probe analysis (Orchid Diagnostics). Specific HLA allele identification was performed by a sequence-specific probe method (Pel-Freeze).

**Target cells and culture conditions**

The 721.221 parent cell line, 721.221 transfectants expressing the HLA-Bw4 ligand (HLA-B2705) and the HLA-Bw6 epitope (HLA-B3502), and B lymphoblastoid cell lines (BLCL) were provided by Dr. B. Dupont (Sloan-Kettering Institute, New York, NY). The 721.221 transfectant expressing the HLA-Cw4 ligand (HLA-Cw0304) and the 721.221 transfectant expressing the HLA-Cw4 ligand (HLA-Cw0401) were generated in Dr. P. Parham’s laboratory (Stanford University, Stanford, CA). All cell lines were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 100 µg/ml l-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin and incubated at 37°C with 5% CO2. Class I molecule expression was monitored weekly by using G46-2.6-FITC (anti-human HLA-A, HLA-B, and HLA-C Ab).

**Single-cell analysis by flow cytometry for KIR and intracellular IFN-γ**

The percentage of CD3+CD56+ cells was determined for peripheral blood samples by using anti-CD3-PerCP (clone SK7), anti-CD56-FITC (clone NCAM16.2), and anti-CD45-PE (clone HI30) (BD Biosciences). PBMCs containing 50,000 NK cells were mixed with target cells (721.221 and class-I transfectants) or without target cells (control) at an NK/target cell ratio of 1:1 in sterile 96-well round-bottom plates with RPMI 1640 plus 10% FBS, centrifuged at 1200 rpm for 1 min, and coincubated at 37°C with 5% CO2 for 14–17 h. To inhibit cytokine secretion, brefeldin-A (Sigma-Aldrich) was added for a final concentration of 10 µg/ml 1 h after mixing effectors and targets. For single-cell analysis of NK cell inhibitory receptor expression and intracellular IFN-γ, cells were first stained in the dark at room temperature for 20 min or at 4°C for 30 min with the selected mAbs anti-KIR2DL1/2DS1-allophycocyanin (clone 143211) (R&D Systems), anti-KIR2DL2/2DS1-FITC (clone HP-3E4), anti-KIR2DL2/2DL3/2DS2-FITC (clone CH-L), anti-KIR2DL2/2DL3/ 2DS2-PE (clone CH-L); anti-KIR3DL1-FITC (clone DX9), anti-KIR3DL1/ 3DS1-PE (clone DX9), anti-CD3-PerCP (clone SK7), and anti-CD56- PE-Cy7 (clone B159) (BD Biosciences), anti-NKG2A/CD94-PE (clone Z199), and anti-ILT2-PE (clone HP-F1) (Beckman Coulter) before fixation and permeabilization (Fix and Perm kit; Invitrogen Life Technologies) and addition of Alexa Fluor 700-conjugated anti-IFN-γ (clone G46-2.6) (BD Biosciences). Biostatistical modeling of the steady-state NK repertoires from these individuals confirms that inhibitory KIR expression occurs in a nonuniform fashion, supportive of serial acquisition of KIR expression. These studies support a model of NK licensing where graduated acquisition of inhibitory KIR expression endows the NK cell with higher effector capacity and represent a comprehensive analysis of how inhibitory receptor-MHC class I interaction dictates the diverse response of the resting human NK repertoire to nonself targets.
of interest. Each analysis was performed in triplicate on each donor on two separate occasions.

**NK cell stimulation**

CD16 mouse mAb (clone 3G8) (Beckman Coulter) was coated to the wells of enzyme immunosassay/radioimmunosassay high binding plate (Fisher Scientific) for 6–8 h in PBS at 4°C at 5 μg/well. After washing, 1 × 10⁵ PBMCs were plated in each well. Brefeldin-A was added for a final concentration of 10 μg/ml to each well 1 h after plating the cells. Cells were then incubated at 37°C with 5% CO₂ for 17 h and then transferred to 96-well round-bottom plates where they were stained and analyzed by flow cytometry as described above.

**NK cloning**

PBMCs obtained from healthy donors were sorted using MoFlo flow cytometry to isolate CD³⁺CD56⁺2DL¹⁺⁺⁺, CD³⁺CD56⁺2DL³⁻, and CD³⁺CD56⁺3DL¹⁻ NK cells. Cells were diluted and cultured at a target density of 5 cells/well or 10 cells/well in 96-well U-bottom plates containing 500 IU/ml IL-2 and irradiated (30 gray) allogeneic PBMCs as feeder cells. Cells were grown in serum-free Cellgro stem cell growth medium supplemented with 5% heat-inactivated, human AB serum. On day 4, half the medium of the well was removed and replaced with fresh medium containing 500 IU/ml IL-2. Cells were cultured for up to 5 wk and restimulated with irradiated PBMCs and IL-2 every week. Clonal NK populations were confirmed by flow cytometric assessment of the uniform expression of CD3, CD56, and inhibitory KIRs. Clones were then assayed for cytotoxicity 10 days after restimulation with irradiated PBMCs when all feeder cells were no longer viable.

**NK cytotoxicity**

Cytotoxicity assays were performed according to published methods (11). Briefly, 10⁵ target cells were incubated with the dye molecule PKH26 (Sigma-Aldrich), which inserts into plasma membranes. Target cells were allowed by two washes with PBS. Lysis was evaluated by flow cytometry and maximum TO-PRO-3 staining was obtained by incubating target cells from lysed cells by their ability to exclude TO-PRO-3. Spontaneous back-ground cell death was determined in PKH-26-labeled targets alone, and maximum TKO-PRO-3 staining was obtained by incubating target cells with 2 ml of PermeaFix for 20 min in the dark at room temperature, followed by two washes with PBS. Lysis was evaluated by flow cytometry and quantitated as the percentage of TO-PRO-3⁺PKH-26⁺/PKH26⁻. The percentage of target cell death was corrected for spontaneous background death by subtracting the percentage of dead cells in control samples (PKH-26-labeled target alone) from the percentage of dead cells within test samples.

**Statistical analysis**

To compute the p value for comparisons in response between NK cells expressing "self" KIR (S-KIR) vs "nonself" KIR (NS-KIR), a hierarchical permutation test was performed. The resamples were generated by the individual first and then within the individual by the S-KIR or NS-KIR group. A two-tailed paired Student’s t test was used within each individual to compare response between NK cells expressing different numbers of S-KIR. The paired Student’s t test was also used within individuals to compare percentages of KIR-expressing NK subgroups.

A test was performed to determine whether the proportion of NK cells differed as a function of the number of inhibitory KIR in the population. The observed proportion of NK cells with 1 KIR (j = 0, 1, 2, or 3) was recorded for each of 10 subjects. Due to the small number of subjects, a bootstrap resampling procedure was used to test the hypothesis of equality across the four KIR groups. The test statistic, the weighted average proportion of NK cells, was based on the contrast (3, 1, −1, and −3) and was used because of its power against the alternative hypothesis that the expected proportion of NK cells across the four KIR groups was monotonically decreasing. For the bootstrap test, 2000 bootstrap resamples were generated (using the subject as the resampling unit) and the p value was based on the achieved significance level.

KIR expression data from 10 KIR haplotype-A homozygous subjects with ~20,000 NK cells per subject was used to fit models of inhibitory KIR expression. Within each NK cell it was assumed that there are positions for as many as three KIRs. For the binomial Independent Bernoulli model, the estimated probability that a KIR filled a position was 0.25. Under the assumption that the number of KIRs per NK cell followed a binomial distribution, the expected proportions of cells with 0, 1, 2, and 3 KIRs are 0.422, 0.422, 0.141, and 0.016, respectively. A χ² goodness of fit test was computed to determine whether observed proportions of cells with j KIRs (j = 0, 1, 2, or 3) approximated the expected proportions under the binomial assumption and produced ρ < 0.001, indicating that the binomial model is not correct. For the correlated Bernoulli model, the number of KIRs within each cell was modeled as the sum of three correlated Bernoulli random variables with a constant probability (τ = 0.25) that a KIR fills a position and a constant pairwise correlation (ρ = 0.33) between the three Bernoulli random variables. The expected proportion of NK cells with each KIR number was then computed under the correlated Bernoulli model and compared with observed proportions.

Results

To allow evaluation of inhibitory KIRs and to minimize interference from potential class-I recognizing activating KIRs, we analyzed NK cells from 10 unrelated normal individuals, all of whom were homozygous for KIR haplotype-A. KIR haplotype-A contains the genes for the inhibitory KIR receptors KIR2DL3, KIR2DL1, and KIR3DL1, which are specific for the HLA-C1, HLA-C2, and HLA-Bw4 ligands, respectively, and contains at most one gene for an activating KIR, KIR2DS4, a receptor whose ligand is unknown and which displays no cross-reactivity with mAbs used to detect inhibitory KIR. Six individuals exhibited homozygosity for the KIR2DS4 allele KIR2DS4*003, a deletion variant presumed to encode a nonfunctional receptor (10, 12). All individuals exhibited unique HLA genotypes comprised of various combinations of KIR ligands as indicated in Table I.
NK cells expressing inhibitory KIR for self HLA-B or HLA-C ligands are functionally competent and silenced by target expression of ligand

To avoid contributions from more than one inhibitory KIR to the NK response, we studied the response of NK subgroups characterized by the expression of one inhibitory KIR only or cells with “exclusive KIR expression.” The use of six-color flow cytometry allowed segregation and simultaneous analysis of NK subsets with different inhibitory receptor phenotypes. Simultaneous staining demonstrated that all KIR-expressing cells belonged to the mature CD56dim population (data not shown). As illustrated by Fig. 1, A and B, NK response was measured by the production of intracellular IFN-γ in NK populations exclusively expressing a specific inhibitory KIR upon exposure to the class I-negative 721.221 target cell and to 721.221 transfectants expressing the HLA-Cw3, HLA-Cw4, HLA-Bw4, and HLA-Bw6 epitopes (Fig. 1, B–D) and in cell-free stimulation assays by CD16 cross-linking (Fig. 1E). NK cells expressing inhibitory KIR for self-MHC class I (“self” or S-KIR) exhibited higher functional capacity and responsiveness as demonstrated by increased IFN-γ production to 721.221 and to 721.221 transfectants lacking the cognate HLA ligand. In contrast, NK cells expressing inhibitory KIR for which the individual lacked the cognate HLA ligand (“nonself” or NS-KIR) demonstrated little or no response to target cells lacking the cognate ligand. Thus, in an HLA-C2 homozygous individual, NK cells exclusively expressing KIR2DL1 (KIR2DL1*2DL3*3DL1*) NK demonstrated significant IFN-γ response upon CD16 cross-linking or when stimulated with the class I-deficient 721.221 cell line or target cells lacking the HLA-C2 ligand. Ligand presentation by 721.221.HLA-Cw4 transfectants resulted in receptor engagement and subsequent inhibition with an appropriate decline in IFN-γ response (Fig. 1B). This was in sharp contrast to the response of NK cells exclusively expressing the NS-KIR KIR2DL3 from the same HLA-C2 homozygous individual. The 2DL3*2DL1*3DL1* NK cells were hyporesponsive not only to 721.221 but to all targets regardless of the absence of the HLA-C1 ligand, and there was no significant change even when the ligand was restored in 721.221.HLA-Cw3 transfectants (Fig. 1C). The same individual displayed an HLA-Bw4/Bw6 genotype and NK cells exclusively expressing inhibitory KIR3DL1 demonstrated high functional capacity (Fig. 1D), indicating that inhibitory KIR for HLA-Bw4 as well as inhibitory KIR for HLA-C ligands can confer NK responsiveness when the ligand is a self-MHC class I molecule. Furthermore, heterozygosity for HLA-Bw4 was sufficient to allow functional competence to NK cells expressing the cognate KIR3DL1 receptor to a comparable degree as NK cells expressing the KIR2DL1 receptor in the same HLA-Cw4 homozygous individual. We performed additional studies using as target cells BLCL derived from individuals with various HLA genotypes. Using these target cells, which could coexpress both self-MHC and nonself MHC KIR ligands depending on the KIR/HLA genotypes of the NK donor, the studies yielded consistent results (data not shown).

The different KIR/HLA combinations among the 10 KIR haplotype-A homozygous individuals represented all of the possible combinations for S-KIR and NS-KIR for HLA-C and HLA-B ligands, and the IFN-γ response pattern to the 721.221 target cell panel for each inhibitory KIR-expressing NK subset could be predicted based on the individual’s KIR/HLA genotypes (Table II). In all 10 individuals, NK cells exclusively expressing the inhibitory KIR for which the individual expressed the KIR ligand (S-KIR) demonstrated robust response to target cells lacking the cognate ligand, whereas NK cells exclusively expressing inhibitory KIR for which the individual lacked the ligand (NS-KIR) exhibited hyporesponsiveness. Effector response of all S-KIR-expressing NK subsets could be abrogated by challenge with a target cell expressing the cognate ligand (Table II).

Because the absolute measurement of IFN-γ response varies from individual to individual, we calculated the functional response relative to background IFN-γ expression, thereby allowing direct comparison of the responsiveness of NK expressing S-KIR to the responsiveness of NK expressing NS-KIR (Fig. 2). In this way multiple NK subsets from multiple individuals could be directly compared, confirming that NK cells expressing S-KIR for both HLA-C and HLA-B ligands are significantly more responsive than NK cells expressing NS-KIR (p < 0.001).

NK cells expressing multiple inhibitory KIRs achieve uniformity of NK response

To demonstrate how coexpression of multiple inhibitory KIRs alters the functional capacity of an individual NK cell, we compared the response pattern of NK populations exclusively expressing one inhibitory KIR (Fig. 3A) with the response pattern of populations nonexclusively expressing the same inhibitory KIR (e.g., inclusive of those cells that coexpress more than one inhibitory KIR) (Fig. 3B). In other words, instead of restricting the analysis to NK cells only expressing KIR2DL1+, we examined the entire KIR2DL1+ population inclusive of those cells that may coexpress 2DL3 or 3DL1. In an HLA-C2/C2, Bw4/Bw6 individual, NK cells exclusively expressing the S-KIR KIR2DL1 or KIR3DL1 were more responsive to target cells lacking their respective ligands than NK cells exclusively expressing the NS-KIR KIR2DL3 (Fig. 3A). The KIR− subset also exhibited poor response to all target cells. However, when “inclusive” populations were examined (Fig. 3B), not only were KIR2DL1+ and KIR3DL1+ cells functionally competent but KIR2DL3+-expressing cells became responsive to activation by 721.221, an observation most parsimoniously explained by preferred coexpression of the NS-KIR KIR2DL3 with the S-KIRs KIR2DL1 or KIR3DL1. This was confirmed by analysis of the KIR2DL3+ NK subset, which demonstrated that 73% of KIR2DL3+ cells were coexpressed with an S-KIR (28% coexpressed with 2DL1, 23% coexpressed with 3DL1, and 22% coexpressed with both 2DL1 and 3DL1). Interestingly, the degree of NK inhibition by the HLA-C ligands when their receptors were NS-KIR was consistently lower (53.6 ± 3.2%) than NK inhibition by the same class I ligands when their receptors were S-KIR (78.8 ± 5.1%), as demonstrated by the individual in Fig. 3, whose cells which coexpressed the NS-KIR2DL3 with an S-KIR, could not be efficiently inhibited by the nonself class I ligand HLA-Cw3 (Fig. 3B). KIR3DL1-expressing NK cells, however, achieved efficient inhibition (88.7 ± 6.1%) regardless of whether the KIR3DL1 receptor was S-KIR or NS-KIR (data not shown).

Expression of class I nonspecific inhibitory receptors CD94/NKG2A or ILT2 confers modest response

To clarify the contribution of inhibitory receptors other than KIR to the functional NK repertoire, we simultaneously stained cells for CD94/NKG2A and ILT2 in addition to KIR and specifically examined the responsiveness of the KIR+ populations compared with the KIR− populations. We found that NK cells lacking all inhibitory NK receptors (KIR− CD94/NKG2A− ILT2−) represented up to 9.68% of the NK cell population and were essentially nonresponsive (Fig. 3C, blue bars). NK cells negative for KIR expression but positive for CD94/NKG2A or ILT2 expression represented a much higher percentage of NK cells (up to 32.1%). At best modestly responsive to the class I-negative 721.221 target
FIGURE 1. Exclusion gating of single-cell flow cytometric analysis demonstrates higher functional capacity in NK cells expressing inhibitory KIR for self-MHC class I and inhibition by the cognate ligand. A and B, PBMC preparations from a C2/C2, Bw4/Bw6 donor (no. 9) were incubated with no target, 721.221, 721.221 transfected with the HLA-Cw3, HLA-Cw4, HLA-Bw4, or HLA-Bw6 epitopes, or stimulated with CD16 mAb and stained for KIR and intracellular IFN-γ. Directed analysis of NK cells exclusively expressing specific inhibitory KIR was performed as demonstrated for KIR2DL1-expressing cells in A and B. Gating for CD3 CD56+ NK cells was followed by the exclusion of KIR3DL1+ and 2DL3+ cells (A). KIR2DL1-expressing cells were detected from the KIR3DL1-2DL3+ cell population and analyzed for individual cell production of IFN-γ after coincubation with the indicated target cells (B). KIR2DL1-expressing cells recognize the self-MHC class I ligand HLA-C2 and are depicted as S-KIR. Upper quadrant values in B represent the percentage of IFN-γ+ KIR2DL1 among the KIR2DL3-3DL1- NK population, and lower quadrant values represent the percentage of IFN-γ+ KIR2DL1 among the KIR2DL3+3DL1+ NK population. Values in parentheses indicate the proportion within the KIR2DL1 subset (upper quadrant) and within the KIR− subset (lower quadrant). Exclusion gating for NK cells exclusively expressing KIR3DL1 and 2DL3 was performed as for 2DL1 (not depicted). C, KIR2DL3 is specific for the MHC class I ligand HLA-C1, which is absent in this individual. Percentages of IFN-γ+ KIR2DL3 among the KIR2DL1-3DL1- NK population are shown and demonstrate poor response. D, KIR3DL1-expressing cells recognize the self-MHC class I ligand HLA-Bw4 and are depicted as S-KIR. Percentages of IFN-γ+ KIR3DL1 among the KIR2DL1+3DL1+ NK population are shown and demonstrate robust response to target cells lacking the cognate ligand. E, PBMC preparations were stimulated by CD16 isotype control or CD16 mAb. CD16 cross-linking of NK cell subsets exclusively expressing the S-KIR 2DL1 and 3DL1 have highest response, whereas the NK cell subset expressing KIR2DL3 is hyporesponsive. The KIR− populations in the lower quadrants are negative for all inhibitory KIRs. Dot blots depict one representative experiment for NK activation by target cell coincubation (n = 10) and CD16 activation (n = 4).
cell, CD94/NKG2A–ILT2+/KIR− cells still displayed appropriate inhibition of response upon coinubcation with target cells expressing any class I ligand, consistent with the broad class I specificity of the receptors (Fig. 3C, red, yellow, and green bars), a population corresponding to 22.7–30% of all NK cells. These cells exhibited the same response patterns for S-KIR and NS-KIR as seen in cells coexpressing KIR and CD94/NKG2A or ILT2, and the magnitude of response in NK cells exclusively expressing S-KIR was still significantly greater than that of the NK cells expressing only CD94/NKG2A or ILT2. Thus, based on inhibitory receptor phenotype alone, NK cells display a clear hierarchy of response: cells exclusively expressing S-KIR are significantly more responsive than KIR CD94/NKG2A+ and KIR ILT2+ cells (p = 0.0002), which, in turn, are more responsive than cells exclusively expressing NS-KIR (p = 0.003). Cells expressing both S-KIR and CD94/NKG2A or ILT2 are slightly more responsive overall, and cells lacking all inhibitory receptors are essentially nonresponsive to class I-negative target cells. These results were confirmed using single-cell analysis of IFN-γ production to CD16 cross-linking in a cell-free system (Fig. 3D).

**NK cells with more than one inhibitory KIR for self-MHC exhibit progressively higher effector response while preserving a binary “on/off” inhibitory response**

Previous studies have focused on phenotypic descriptions of NK subsets; however, it is now clear that an inhibitory receptor phenotype in the context of self-MHC class I defines a diverse functional NK repertoire. Whereas the expression of one S-KIR leads to functional competence, we sought to determine whether the expression of more than one S-KIR increases functional capacity further. Of the 10 KIR haplotype-A homozygous individuals, six individuals had more than one inhibitory S-KIR: four individuals had two S-KIRs and one NS-KIR and two individuals had three S-KIRs. NK cell subsets were gated in a hierarchical manner and segregated into groups: NK cells with one S-KIR, NK cells with two S-KIRs, and NK cells with one S-KIR and one NS-KIR whose intracellular IFN-γ responses to a class I-negative target cell were then compared. Thus, for an HLA-Bw4/Bw6, C2/C2 individual whose KIR2DL1 and KIR3DL1 are both S-KIR and whose KIR2DL3 is an NS-KIR, 2DL1+2DL3+3DL1− cells (one S-KIR) were compared with 2DL1+2DL3+2DL1− cells (two S-KIRs) and 2DL1+2DL3−3DL1− cells (one S-KIR and one NS-KIR). For the same individual, a separate comparison could be made between cells with 3DL1+2DL1+2DL3− (one S-KIR), 3DL1−2DL1−2DL3+ (two S-KIRs), and NK cells with 3DL1−2DL3+3DL1− (one S-KIR and one NS-KIR). Similar comparisons were then made in individuals with various HLA genotype backgrounds after segregating NK cells according to S-KIR or NS-KIR expression. In all comparisons, NK cells with two S-KIRs were significantly more responsive than those with one S-KIR (p < 0.001), and coexpression of a NS-KIR with an S-KIR did not diminish the degree of response of the NK cell subset (Fig. 4A). The same findings were recapitulated in cell-free stimulation experiments by

**FIGURE 2.** NK cells expressing inhibitory KIRs for self-MHC class I (S-KIRs) are significantly more responsive than NK cells expressing inhibitory KIR for nonself MHC class I (NS-KIRs). Relative responsiveness to the class I-negative 721.221 target cell was calculated for NK subsets with exclusive inhibitory KIR expression by the formula $(\text{KIR} \cdot \text{IFN-} \gamma) / \text{KIR} \cdot \text{IFN-} \gamma/KIR^{-}$. Within each of 10 individuals, inhibitory KIRs were segregated into S-KIR and NS-KIR groups, and the responsiveness of NK expressing S-KIR (All) was compared with the responsiveness of NK expressing NS-KIR (All). A hierarchical permutation test was performed to compute the p value for this comparison. NK cells expressing S-KIR were significantly more responsive than NK cells expressing NS-KIR (p < 0.001). Segregation of S-KIR populations by the specific inhibitory KIRs KIR2DL1, KIR2DL3, and KIR3DL1 demonstrates comparable levels of functional competence.
CD16 cross-linking and analyzed by flow cytometric measurement of intracellular IFN-γ production (Fig. 4B). Furthermore, because cytokine secretion and cytotoxicity do not always correlate precisely, we examined cytotoxicity among NK clones with discrete combinations of S-KIRs and NS-KIRs. By isolating NK clones exclusively expressing combinations of inhibitory KIRs and testing for cytotoxicity by flow cytometric quantitation of 721.221 TO-PRO-3 uptake, we could demonstrate that the NK clone cytotoxic response to 721.221 is proportionate to number of expressed S-KIRs (Fig. 4C). The cytotoxicity results from 78 individual NK clones agreed with the IFN-γ results, confirming that the capacity for NK effector function, as evaluated by cytokine secretion and cytotoxicity, is directly related to the number of inhibitory KIRs for self-HLA expressed on the NK cell surface (Fig. 4D). Two individuals with HLA-Bw4/C1/C2 genotyping had three S-KIRs, the maximum number of S-KIRs possible in a KIR haplotype-A homozygous individual. Again, there was a clear hierarchy in IFN-γ response where NK cells expressing three S-KIRs were more responsive than those with two S-KIRs (p < 0.01), which, in turn, were more responsive than NK cells expressing one S-KIR (p < 0.001) (data not shown).

Given the higher response in NK cells expressing more than one S-KIR, we sought to determine whether the NK repertoire is biased toward a higher frequency of NK cells expressing multiple inhibitory S-KIRs. Comparison of percentages of NK cells expressing different numbers of inhibitory KIR from 10 individuals revealed that the size of each NK cell subset was inversely related to the number of expressed KIRs regardless of whether the KIR was S-KIR or NS-KIR (Fig. 4E). Statistical testing through a bootstrap resampling procedure revealed that

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**FIGURE 3.** A response hierarchy exists among NK subsets with different class I-specific inhibitory receptors and disappears with coexpression. The percentage of IFN-γ-producing cells among NK subsets from an HLA-C2/C2, Bw4/Bw6 individual (no. 8) upon coincubation with indicated target cells or CD16 activation is shown. A, NK cell subsets with exclusive expression of the indicated inhibitory KIR demonstrate higher response among NK cells expressing the S-KIR 2DL1 and 3DL1 as compared with the NS-KIR 2DL3 or KIR group, and the response is nearly completely inhibited upon challenge with the cognate class I ligand. B, Response to the class I-negative 721.221 equalizes among NK cell subsets expressing the indicated inhibitory KIR where the subset is inclusive of NK cells coexpressing other KIRs, indicating the retention of functional capacity through S-KIR coexpression. Challenge with nonself MHC class I (HLA-Cw3) does not inhibit the NS-KIR/2DL3-expressing subset (51%) to a comparable extent as self-MHC class I ligands inhibit the S-KIR-expressing subsets (79%). Data are consistent among analyses from eight individuals. C and D, KIR-negative NK cells expressing CD94/NKG2A and/or ILT2 do not achieve the same level of response as NK cells expressing S-KIR, and NK cells lacking all class I-specific inhibitory receptors (KIR/CD94/NKG2A/ILT2) are markedly hyporesponsive as assessed by intracellular IFN-γ production to the 721.221 target cell panel (C) or by CD16 activation (D) (data representative of analysis from four individuals).
FIGURE 4. The magnitude of NK response is directly related to the number of expressed S-KIRs, while the frequency of NK cells is indirectly related to the number of expressed KIRs. 

A, Intracellular IFN-γ response to 721.221 was compared between NK cells with differing numbers of S-KIRs or NS-KIRs from four individuals (nos. 3, 4, 8, and 9).

B, Intracellular IFN-γ response to CD16 stimulation was compared between NK cells with differing numbers of S-KIRs or NS-KIRs from three individuals (nos. 4, 8, and 9).

C, In an HLA-C2/C2, Bw4/Bw6 individual (no. 8), NK clones (n = 6) expressing both S-KIR 2DL1 and 3DL1 displayed higher cytotoxicity than cells exclusively expressing either 2DL1 (n = 16) or 3DL1 (n = 14) alone. NK clones exclusively expressing NS-KIR2DL3 (n = 5) were hyporesponsive and displayed the lowest cytotoxicity.

D, Cytotoxicity to 721.221 at an E:T ratio of 10:1 was compared among 78 NK clones with differing numbers of S-KIRs or NS-KIRs from two individuals (nos. 3 and 8). Each dot and line represents one comparison group within an individual. The paired Student’s t test was performed to compute the p values for these comparisons. NK cells expressing two S-KIRs have higher response than NK cells expressing one S-KIR (p = 0.001), which have higher response than NK cells expressing one NS-KIR (p < 0.001).

E, Among all individuals the KIR− NK subset represents the highest percentage of cells within the NK repertoire. Frequencies of NK populations are progressively smaller with increased numbers of expressed S-KIRs as seen in four individuals (nos. 3, 4, 8, and 9) with two S-KIRs. Similar results can be seen in individuals with three S-KIRs (data not shown).
the proportions of NK cells with zero, one, two, or three inhibitory KIRs were not equal, instead decreasing with each additional expressed KIR \((p < 0.001)\). When the analysis was restricted to examination of S-KIR only, the inverse relationship persisted, indicating progressively smaller proportions of NK cells as the number of S-KIRs increased.

To determine whether NK cells expressing more than one S-KIR are less likely to be inhibited by the presentation of a single ligand, we evaluated the degree of inhibition by each of the relevant cognate ligands. The heightened response in cells with more than one S-KIR could still be abolished upon interaction with a target cell expressing any one of the cognate class I ligands (Fig. 5) to a comparable degree as NK cells expressing only one S-KIR (77–89% inhibition). These results indicate that although the NK effector repertoire is variegated based on number of S-KIRs expressed, the inhibitory response adheres to a binary algorithm similar to an “on/off” switch.

Is KIR expression equiprobable?

The frequency of NK cells is inversely related to the number of expressed inhibitory KIRs, implying that the distribution of KIR expression is not uniform. Using the KIR phenotyping and frequency data of 20,000 NK cells per subject from 10 haplotype-A homozygous individuals, we sought to model the likelihood of KIR expression in the NK repertoire. We assumed that for each NK cell in KIR haplotype-A homozygous individuals there are “positions” for as many as three different inhibitory KIRs. When we fit the data to a binomial model hypothesizing that within a cell the probability that a KIR fills each position is constant and does not depend on the outcome of KIRs in the other positions on the cell (independent Bernoulli model), the data did not fit (Fig. 6).

However, when we examined the hypothesis that additional KIR expression is dependent upon whether another KIR is already expressed in a correlated Bernoulli model, the data showed agreement with the model-based expected proportions, demonstrating that the correlated Bernoulli model is appropriate. Thus, for cells that achieve expression of KIR, expression of a second inhibitory KIR on the cell surface is influenced by whether another one is already present, a schema most consistent with a developmental model of the serial acquisition of inhibitory KIR expression. These findings also indicate that the expression of a KIR within a position...
is not favored (only 0.25 probability), leading to the observed high proportion of KIR− NK cells (0.50) within all studied individuals.

**KIR+ NK cells exhibit preferential expression of S-KIR**

Because the proportion of an NK population is influenced by its number of KIRs, we sought to determine whether there is a general bias toward the expression of S-KIR, favoring a more functional and responsive repertoire. We performed pairwise comparisons among different KIR-expressing subgroups within all 10 individuals. Among NK cells expressing a single inhibitory KIR there was a higher probability that the receptor would be an S-KIR rather than an NS-KIR (p < 0.001) (Fig. 7). Among NK cells expressing two inhibitory KIRs, there was preferential coexpression of one S-KIR with one NS-KIR, superseding not only the coexpression of two S-KIRs (p < 0.001) but also the coexpression of two NS-KIRs (p = 0.004) but also the coexpression of two NS-KIRs (p = 0.006) (Fig. 7).

**Discussion**

Through single-cell analysis of NK function we have examined within individuals the functional human NK repertoire, as defined by its inhibitory receptors, both KIR and non-KIR, and we have further analyzed how the repertoire is shaped by MHC class I and inhibitory KIR interaction. Although CD94:NKG2A, ILT2, and KIR are inhibitory receptors that can mediate tolerance through the recognition of self-MHC class I, the inhibitory KIRs specific for self-MHC class I molecules are the receptors most effectively involved in mediating functional competence. It is now clear that receptor expression defines functional capacity; how the human NK repertoire is comprised of various receptor expression groups provides insight to the functional diversity of the innate immune system.

Several models have been proposed to reconcile how inhibitory NK receptors can mediate effector function against class I-deficient target cells and yet do not mediate self-destruction when the individual lacks the class I ligands for the relevant encoded inhibitory receptor, a circumstance that occurs in >60% of the population (13). Contrary to the “at least one” model, which postulates that although an individual NK cell may express an inhibitory receptor that does not recognize self-MHC class I it coexpresses an inhibitory receptor that does (14, 15), we and others have demonstrated that a significant NK population in the human lacks all known inhibitory receptors specific for class I molecules, self or nonself (16). These cells fail to respond to class I-deficient target cells, rendering them self-tolerant by their hyporesponsive nature. Comparable in its poor response is the NK population expressing inhibitory KIRs specific for nonself class I, demonstrating that these cells with the potential for self-destructive behavior are tolerated to self through their inability to respond to a lack of cognate ligand. NK cells expressing receptors for self-MHC class I are inherently tolerant, as the engagement of an autologous class I ligand on the target cell leads to NK inhibition. While achieving tolerance to self-MHC class I in these diverse manners, these same populations, however, differ considerably in terms of effector function.

Simultaneous staining with multiple receptor and cytokine-specific mAbs permitted the analysis of individual NK cells characterized by specific receptor groupings. Because all individuals studied were homozygous for KIR haplotype-A, the only inhibitory KIRs that could mediate inhibition were KIR2DL3 and KIR3DL1, and no class-I specific activating KIRs were present to mediate activation or to confound cell surface phenotyping. This cohort of KIR haplotype-A homozygous individuals allowed for the testing of NK cells with similar KIR genetics but whose diverse HLA backgrounds influenced KIR-mediated NK function and phenotype in an ultimately predictable manner. Our data support data published in the mouse (8) and recently in the human (9) indicating that NK cells expressing inhibitory receptors for self-MHC class I are functionally competent. Moreover, we demonstrate that there is a clear hierarchy in responses such that NK cells expressing receptors with broad specificity for class I such as CD94:NKG2A and ILT2 (the latter not previously studied), while capable of responding to the lack of class I on the target cell as measured by intracellular IFN-γ production, only do so to a modest degree in comparison to NK cells expressing inhibitory KIR specific for self-MHC class I, and we show for the first time that the expression of progressively higher numbers of inhibitory KIRs for self-MHC class I is correlated with increased effector capacity. This includes the inhibitory KIR3DL1, which recognizes the HLA-Bw4 epitope and has not previously been reported to confer functional competence. By using a panel of 721.221 target cells expressing known specific HLA ligands, we could demonstrate clearly the KIR-HLA receptor-ligand relationships simultaneously mediate both effector and inhibitory response. BLCL targets with known HLA genotypes produced similar results, indicating that the responses to the 721.221 panel were not due to expression in the cell line of a non-HLA protein recognized by NK activating receptors.

Cell-free stimulation by CD16 cross-linking and IL-2-activated NK clone cytotoxicity assays confirmed the same differences in response based on KIR and non-KIR inhibitory receptor phenotypes. It has been reported that culturing murine NK cells in IL-2 for several days can lead to a partial reversal of the hyporesponsive phenotype of CI/NK2− NK cells, and cytokine stimulation in vitro can result in an enhanced capacity to stimulate otherwise unlicensed NK cells by anti-NK1.1, suggesting that the cytokine milieu of the NK cell may overcome strict MHC-dependent licensing requirements (7, 8). Our cytotoxicity results using human NK clones cocultured in IL-2 demonstrated that both NS-KIR- and
S-KIR-bearing NK clones were comparably up-regulated in response but that the degree of response disparity between unlicensed and licensed NK clones was preserved.

Although the acquisition of NK response by inhibitory KIRs for HLA-C has been described (9), we demonstrate for the first time that NK cells expressing inhibitory KIR3DL1 specific for the self-HLA-Bw4 ligand are equally functionally competent as NK cells expressing inhibitory KIRs specific for the HLA-C KIR ligands. These results may have ramifications in the clinical settings of allogeneic hematopoietic stem cell transplantation and adoptive NK cell therapy where HLA-mismatched donors may be selected on the basis of the likelihood of NK alloreactivity due to “missing self,” and they support broadening the desired HLA mismatch to include HLA-B in addition to HLA-C KIR ligands (17–19). It remains of interest to determine whether the inhibitory KIR allelic variation, previously shown to affect inhibitory capacity, frequency, and level of cell surface expression (20–22), may diversify NK effector capacity as well. KIR3DL1 allele typing of several of the individuals in this cohort could not demonstrate a correlation between specific alleles and effector function (data not shown); however, a significantly larger cohort comprised of specific HLA ligand combinations and 3DL1 allele combinations would be needed to specifically address this question.

In individual NK cells the preference for the expression of S-KIR over NS-KIR indicates that the class I repertoire of an individual can affect the NK repertoire. This is the first indication that HLA can affect receptor expression within an individual at the level of a single cell and is consistent with NK repertoire studies in the Japanese population (21) and in HLA-identical sibling studies in which bulk NK populations were phenotyped for KIR expression (23). From a functional viewpoint class I influence on KIR expression might be predicted, because the preference for the expression of an inhibitory KIR for self-MHC class I ensures a more robust NK effector response. A logical extension of this argument might then predict that the NK repertoire would be biased toward the expression of more than one inhibitory KIR for self-MHC class I, particularly as we have shown that these cells clearly have an even higher functional capacity and require ligand engagement of only one inhibitory receptor to silence the activation. We found, however, that a bias toward the expression of multiple S-KIRs did not exist and that instead there was an underlying bias against KIR expression leading to an indirect relationship between the size of an NK subgroup and the number of inhibitory KIR expressed by that subgroup, even if the KIRs were S-KIRs.

The bias against KIR expression may not be a functional one but rather a developmental one. Statistical modeling of the data suggests that KIR expression is impacted by whether other KIR are already pre-existing on the cell surface, consistent with a developmental model of sequential acquisition of inhibitory KIR expression that has been supported by computational modeling of Ly49 receptor expression in the mouse (24, 25). Modeling of NK development and KIR expression in the human has been less clear and has favored a selection model over a sequential model (26), although previous computations were based on only two individuals where phenotyping could not distinguish between activating and inhibitory receptor forms (14). Nevertheless, studies examining early NK development from umbilical cord stem cells and thymocytes in humans and from developing murine NK cells have described a coordinated acquisition of inhibitory NK receptor expression such that the acquisition of CD94/NKG2A precedes the acquisition of any KIR receptor (27–29), a finding that is recapitulated in NK reconstitution following hematopoietic stem cell transplantation (30, 31). It is likely then that KIR expression is a developmentally distal event, one that the majority of NK cells do not achieve, instead pausing, perhaps terminally, at inhibitory receptor phenotype stages along the way. In a cellular variation of ontogeny recapitulating phylology, the delay of KIR expression in NK development may be reflective of its more recent introduction in the genome compared with the older NKG2 genes (32, 33). Additional studies in NK development will be needed, however, to confirm the model of serial KIR receptor acquisition. It remains to be determined whether all KIR–NK cells are immature, although it has been recently described that they can acquire functional capacity upon coculture with IL-15 and a murine stromal cell line (16). Furthermore, a recent study has shown that the immature CD56(dim)CD16+ cells can acquire CD16 and KIR expression after cytokine activation (34). It is therefore quite possible that the steady-state NK cell repertoire, heavily weighted in moderately responsive KIR cells, behaves as a reservoir for the generation of more responsive KIR cells produced by cytokines elaborated in inflammatory states such as infection. Higher cytokine activation with overwhelming infection may then induce NK cells with multiple S-KIRs and even higher responsive potential.

Nevertheless, among KIR-expressing steady-state cells the NK repertoire demonstrates a tendency to maximize its effector potential by preferentially expressing inhibitory KIRs for self-MHC class I. Even in NK cells expressing an inhibitory KIR for nonself MHC class I, there was a clear predisposition to coexpress a KIR specific for self-MHC class I, thereby rescuing the NK cell from an otherwise hyporesponsive status. No matter how responsive the cell is, however, the ability to inhibit response is maintained such that, even in the most responsive NK cells with multiple KIRs for self-MHC class I, inhibitory signaling from the interaction of only one cognate class I ligand with its KIR receptor is sufficient to abrogate response. This finding supports a common signaling pathway between the multiple S-KIR such that inhibitory signaling arising from ligand engagement of one inhibitory KIR is adequate to silence the additional effector capacity associated with the non-engaged S-KIR. These data describe a diverse functional repertoire where the majority of NK cells exhibit a moderate effector response due to the lack of inhibitory KIR expression while maintaining CD94/NKG2A or ILT2 expression, but where NK cells that attain KIR expression secure their functional competence by preferentially expressing S-KIR. Fully tolerant to self, the NK repertoire is delineated on one side by the hyporesponsive subset devoid of all inhibitory class I receptors and on the other side by the hyperresponsive subset characterized by multiple S-KIR expression. This diversity of response may result in an early titrated response to pathogens or autologous cells with abnormal self-MHC expression while preserving a binary “on/off” capacity for inhibition by autologous cells expressing self-MHC class I.

That the engagement of one ligand is sufficient to inhibit a highly potent response in an NK cell expressing multiple S-KIRs may be more consistent with the model of NK “licensing” through the interaction of an inhibitory KIR for self-MHC class I in contrast to the “anergy” or “disarming” model, which proposes that hyporesponsive NK cells arise as a result of persistent in vivo stimulation unless counteracted by inhibitory signals from class I-specific receptors (35). Extending this reasoning to a teleological end, the anergy model might therefore predict that highly responsive NK cells require inhibitory signaling through multiple S-KIRs to adequately inhibit activation, a prediction contrary to our finding that the engagement of only one ligand is sufficient for inhibition. In conjunction with the statistical modeling of the steady-state repertoire data from our cohort, the existence of these highly potent multiple KIR-expressing cells supports a model in which the serial acquisition of multiple inhibitory KIRs for self-MHC confers increasing effector capacity to the NK cell. Additional studies are
hemopoietic stem cell transplantation (AHSCT) (13, 17, 18, 37–39) in certain leukemias, resulting in improved outcome in allogeneic transplantation. However, a study of a larger cohort of individuals could specify the role of KIR ligands for donor inhibitory KIR even if the donor is HLA matched, implying that donor NK cells with inhibitory KIR for nonself MHC class I are responsive when challenged with the MHC ligands (13, 38, 39). NK alloreactivity due to a “missing ligand” in an HLA-matched setting may be possible if CD34-derived NK or transferred NK cells express inhibitory KIR for nonself MHC class I. These findings are likely also influenced by variability in KIR expression following transplantation (17), implying a plasticity in NK tolerance development more dependent upon the HLA environment of encountered target cells that are NK phenotype of the NK cell itself (36). These findings are likely also influenced by variability in KIR expression following AHSCT, where reconstitution of donor NK phenotype can take well over a year to occur in some patients (30) and where KIR2DL1 and KIR3DL1 expression can lead to NK cell education by both HLA-identical and nonidentical donor HLA-A and -B alleles, as well as multiple subsets. Therefore, NK cell education by unrelated donors is likely to influence tolerance during NK cell development.

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Disclosures

The authors have no financial conflict of interest.

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30. Cooley, S., V. McCullar, R. Wangen, T. Bergemann, S. Spellman, D. Weisfurther, and J. Miller. 2005. KIR reconstitution is altered by T cells in the graft and
not IRGC. Furthermore, although the statement on page 7197 “Humans have only one intact p47 GTPase (IRGC) whose expression has been reported from testis but not THP-1 cells” is correct, it has little relevance to our work because we only measured the transcript of IRGM.


The authors apologize for any confusion caused and are grateful to colleagues in the field for bringing the errors to their attention. In particular, the authors wish to acknowledge Dr. Jonathan Howard of the University of Cologne Institute for Genetics (Cologne, Germany) for pointing out these errors and for helpful discussion and advice on preparing the erratum. Overall, the findings with IRGM were modest and do not form a major part of the conclusions, particularly those concerning Cathelicidin LL-37 and its role in resistance to tuberculosis.


In Footnotes, the country listed for grant support is incorrect. The footnote should read: 1 This work was supported by grant 2005-SGR00037 from the Generalitat de Catalunya, Spain.

In Results, under the heading Participation of gray cells in cytotoxic contact reactions, “archaeocytes” is misspelled in the fourth sentence of the first paragraph. The sentence should read: “However, it is likely that this migration of archaeocytes has as a final outcome their differentiation into gray cells (Fig. 2C) rather than a direct action of their own (7).”


In Materials and Methods, under the heading Statistical analysis on page 5979, the phrases “within each individual” and “within individuals” in the last two sentences of the first paragraph are incorrect. The sentences should read: “A two-tailed paired Student’s t test was used to compare response between NK cells expressing different numbers of S-KIR. The paired Student’s t test was also used to compare percentages of KIR-expressing NK subgroups.”


There is an error in the affiliation line for the seventh author due to an incorrect symbol. The correct affiliations for Byoung S. Kwon are: *Department of Biomedicine and ‡Immunomodulation Research Center, University of Ulsan, Ulsan, South Korea.