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NK Cell Tolerance of Self-Specific Activating Receptor KIR2DS1 in Individuals with Cognate HLA-C2 Ligand

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NK cells are regulated by inhibiting and activating cell surface receptors. Most inhibitory receptors recognize MHC class I Ags and protect healthy cells from NK cell–mediated autoaggression. However, certain activating receptors, including the human activating killer cell Ig-like receptor (KIR) 2DS1, also recognize MHC class I. This fact raises the question of how NK cells expressing such activating receptors are tolerized to host tissues. We investigated whether the presence of HLA-C2, the cognate ligand for 2DS1, induces tolerance in 2DS1-expressing NK cells. Anti–HLA-C2 activity could be detected in vitro in some 2DS1 positive NK clones irrespective of the presence or absence of HLA-C2 ligand in the donor. The frequency of anti–HLA-C2 reactivity was high in donors homozygous for *HLA-C1*. Surprisingly, no significant difference was seen in the frequency of anti–HLA-C2 cytotoxicity in donors heterozygous for *HLA-C2* and donors without *HLA-C2* ligand. However, donors homozygous for *HLA-C2*, compared with all other donors, had significantly reduced frequency of anti–HLA-C2 reactive clones. The 2DS1 positive clones that express inhibitory KIR for self–HLA class I were commonly noncytotoxic, and anti–HLA-C2 cytotoxicity was nearly exclusively restricted to 2DS1 single positive clones lacking inhibitory KIR. 2DS1 single positive NK clones with anti–HLA-C2 reactivity were also present posttransplantation in *HLA-C2* positive recipients of hematopoietic stem cell transplants from *2DS1* positive donors. These results demonstrate that many NK cells with anti–HLA-C2 reactivity are present in *HLA-C1* homozygous and heterozygous donors with *2DS1*. In contrast, 2DS1 positive clones from *HLA-C2* homozygous donors are frequently tolerant to HLA-C2. *The Journal of Immunology*, 2013, 190: 4650–4660.

Protection of normal self tissues from immune aggression is tightly controlled. Autoaggressive T and B lymphocytes are mostly controlled through clonal deletion or anergy (1, 2). In contrast to T and B cells, NK cells develop tolerance to normal self-tissues largely by the "missing self–MHC class I" mechanism (3, 4). In this situation, inhibitory receptors with ligand specificity for self–MHC class I generate inhibitory signals upon interaction with cognate MHC ligand (5, 6). The NK repertoire, however, also contains activating receptors with ligand specificity for self-antigens. In mice, generalized expression of activating ligands results in reduced effector function and/or deletion of NK cells expressing cognate activating receptors, suggesting that NK cells receiving continuous activating receptor stimulation are either hyporesponsive or deleted (7–11). NK tolerance has also been reported in mixed allogeneic bone marrow chimeras (12). The human activating killer cell Ig-like receptor (KIR) 2DS1 (or, simply, 2DS1) recognizes HLA-C2 (C2, HLA–killer cell Ig-like receptor ligand group C2) Ags (i.e., Asn^{77}-Lys^{80} in the HLA-C H chain). *2DS1* is common among Caucasian populations, in which it ranges from 23 to 55%. The frequency of the natural ligand HLA-C2 is also high in the same populations, 54–66% (13). Because *KIR* and *HLA* segregate independently, *2DS1* is present in both *HLA-C2* positive [*HLA-C* genotypes *C2:C2* and *C1:C2* (C1, HLA–killer cell Ig-like receptor ligand group C1)] and *HLA-C2* negative individuals (*HLA-C* genotype *C1:C1*). 2DS1 positive ($2\mathrm{DS1}^{\mathrm{pos}}$) NK cells have been detected in individuals with *HLA-C2* (14–16). The frequency of peripheral blood NK cells expressing 2DS1 may exceed 20% (14). Recently, 2DS1 expression has been assessed on NK cells in peripheral blood from individuals with different *HLA-C* genotypes. $2\mathrm{DS1}^{\mathrm{pos}}$ NK cells lacking inhibitory KIR receptors [2DS1 single positive ($2\mathrm{DS1}^{\mathrm{SP}}$)] were identified in *HLA-C2* homozygous donors. $2\mathrm{DS1}^{\mathrm{SP}}$ NK cells from such individuals were not reduced in number but were found to be hyporesponsive when compared with $2\mathrm{DS1}^{\mathrm{SP}}$ NK cells from *HLA-C1* homozygous donors (16).

In this study, $2\mathrm{DS1}^{\mathrm{pos}}$ NK clones were developed from donors with all three *HLA-C* genotypes—*C1:C1*, *C1:C2*, and *C2:C2*—for the purpose of determining the effect of the natural ligand, HLA-C2, on their frequency, phenotype, and tolerance to the self-ligand.

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Abbreviations used in this article: AML, acute myeloid leukemia; BLCL, B lymphoblastoid cell line; Bw4, HLA–killer cell Ig-like receptor ligand group Bw4; Bw6, HLA–killer cell Ig-like receptor ligand group Bw6; C1, HLA–killer cell Ig-like receptor ligand group C1; C2, HLA–killer cell Ig-like receptor ligand group C2; 2DL1, inhibitory killer cell Ig-like receptor 2DL1; 2DL2, inhibitory killer cell Ig-like receptor 2DL2; 2DL3, inhibitory killer cell Ig-like receptor 2DL3; 2DS1, activating killer cell Ig-like receptor 2DS1; 2DS1pos, 2DS1 positive; 2DS1SP, 2DS1 single positive; 2DS2, activating killer cell Ig-like receptor 2DS2; 3DL1, inhibitory killer cell Ig-like receptor 3DL1; 3DS1, activating killer cell Ig-like receptor 3DS1; Fwd, forward; HCT, hematopoietic stem cell transplantation; IHWG, International Histocompatibility Working Group; KIR, killer cell Ig-like receptor; LILRB1, leukocyte Ig-like receptor, subfamily B, member 1; MFI, mean fluorescence intensity; Rev, reverse; RT-qPCR, quantitative RT-PCR.

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We report that 2DS1$^{\text{pos}}$ NK clones with anti–HLA-C2 reactivity can be obtained from individuals with any *HLA-C* genotype. The frequency of 2DS1$^{\text{SP}}$ clones with anti–HLA-C2 reactivity is equally high for donors with the *HLA-C* genotypes *C1:C1* and *C1:C2*. In contrast, 2DS1$^{\text{pos}}$ clones from donors homozygous for *HLA-C2* have significantly decreased frequency of anti–HLA-C2 reactivity, consistent with tolerance of 2DS1 to HLA-C2. We also find that the inhibiting receptor CD94/NKG2A is not a critical regulator of tolerance to HLA-C2 in *HLA-C2* homozygous NK cells. Finally, we observe that 2DS1-mediated anti–HLA-C2 cytotoxicity in all donors almost exclusively is restricted to 2DS1$^{\text{SP}}$ clones.

Materials and Methods

NK cell donors

NK cells were obtained from seven individuals (five healthy donors and two transplant recipients). HLA class I genotyping was performed on genomic DNA by a combination of PCR amplification with sequence-specific primers and with sequence-specific oligonucleotide probes (17). KIR genotyping was performed by KIR sequence-specific primers (KIR Genotyping SSP Kit; Invitrogen) and KIR haplotypes, and genotypes were assigned (18) (Table I). NK cells from healthy donors were negatively selected from freshly isolated PBMCs obtained from 30 ml peripheral blood, using a mixture of magnetically labeled mAbs specific for non-NK lineage Ags (Miltenyi Biotec) (19). For all experiments, postisolation NK cell purity was >90%. NK cells from transplant recipients were directly FACS sorted from bulk PBMCs (see *NK cloning*).

BaF/3 IL-15Rα/IL-15 transfectants

The pSFG retroviral vectors containing full-length cDNA of human IL-15Rα or IL-15 (kindly provided by Dr. Thomas A. Waldmann, Metabolism Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD) were transfected into Phoenix E packaging cell line, to produce retroviral supernatants. BaF/3 cells were incubated with retroviral supernatants, for 6–8 h, in fibronectin-coated plates (Takara Biomedicals). Clones of IL-15Rα/IL-15 double-transfected pre–B-lymphocyte BaF/3 cells (BaF/3 IL-15Rα/IL-15) were obtained by limiting dilution, and stable expression of IL-15 and IL-15Rα was confirmed by monthly mAb staining. The cell line was maintained in RPMI 1640 supplemented with 10% FCS, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 2 mM L-glutamine (all provided by the Core Media Preparation Facility, Memorial Sloan-Kettering Cancer Center, New York, NY).

NK cloning

NK clones were developed following single-cell deposition (Fig. 1) and propagated by IL-15 *trans*-presentation. NK cell subpopulations displaying specific combinations of KIR/NKG2A expression were identified by the following mAbs: CD3-PE/Texas Red (S4.1; Invitrogen), CD56-PE/Cyanine7 (MEM-188; BioLegend), inhibitory killer cell Ig-like receptor 2DL1 (2DL1)/S1-PerCP/Cyanine5.5 (HP-MA4; BioLegend), 2DL1-allophycocyanin (143211; R&D Systems), inhibitory killer cell Ig-like receptor 2DL2 (2DL2)–inhibitory killer cell Ig-like receptor 2DL3 (2DL3)/killer cell Ig-like receptor 2DS2 (2DS2)-FITC (CH-L; Miltenyi Biotec), inhibitory killer cell Ig-like receptor 3DL1 (3DL1)–Alexa Fluor 700 (DX9; BioLegend), 3DL1/killer cell Ig-like receptor 3DS1 (3DS1)-PE (Z27; Beckman Coulter), NKG2A-PE (131411; R&D Systems), and CD85j/ILT2 [leukocyte Ig-like receptor, subfamily B, member 1 (LILRB1)]–PE (HP-F1; Beckman Coulter). Single NK cells from selected subpopulations were FACS sorted (FACSAria III; BD Biosciences) and deposited into U-shaped polystyrene 96-well plates (one cell per well) containing 100 μl CellGro SCGM Medium (CellGenix) supplemented with 10% heat-inactivated AB human serum (Gemini Bioproducts), 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 2 mM L-glutamine. The following feeders were added to the medium: 10^4 allogeneic EBV-B lymphoblastoid cell line (BLCL) (JY), 4×10^4 PBMCs obtained from three different donors, and 3×10^3 BaF/3 IL-15Rα/IL-15 cells. Feeders were γ irradiated (EBV-BLCL and PBMCs: 5.2 Gy; BaF/3 IL-15Rα/IL-15: 13.9 Gy). After sorting, plates were centrifuged at 500 rpm for 1 min and incubated in a 37°C, 5% CO_2 humidified atmosphere. On day 5, 100 μl fresh medium and irradiated EBV-BLCL (10^4), PBMCs (4×10^4), and BaF/3 IL-15Rα/IL-15 (3×10^4) were added to each well. On day 10, 80 μl supernatant was removed and substituted with medium and irradiated feeders, as on day 5. On day 15, NK cell growth could be detected by a colorimetric change (purple to yellow) of the microculture supernatant. Proliferating NK clones were collected, transferred in 48-well plates, and supplemented with 800 μl medium and irradiated BaF/3 IL-15Rα/IL-15 (5×10^5). On day 22, 300 μl supernatant was removed from all wells and replaced with 500 μl medium and irradiated BaF/3 IL-15Rα/IL-15 (5×10^5). Between day 28 and day 32, NK cells were screened by flow cytometry to determine viability, clonality, and receptor expression. NK clones were harvested, functionally characterized, and cryopreserved for subsequent molecular studies.

Characterization of NK clones

KIR/NKG2A receptor expression. KIR and NKG2A expression was tested by flow cytometry. The mRNA copy numbers for individual KIR (see *Quantitative PCR*) were used for estimation of KIR surface expression when KIR receptors could not be individually recognized by monospecific mAbs. Normalized mRNA copy numbers for 2DL1, 2DS1, 2DL2–3, and 3DS1 were used to determine the lowest number associated with surface expression. Cell surface expression of 2DL1, 2DS1, 2DL2–3, and 3DS1 was assigned to one of three groups: KIR expression present; KIR expression absent; and KIR surface expression not tested. Because the z27 mAb used to detect 3DS1 receptor also recognizes 3DL1 (20), the analysis for determining the relationship between 3DS1 cell surface expression and mRNA copy numbers was exclusively based on clones lacking 3DL1 ex-

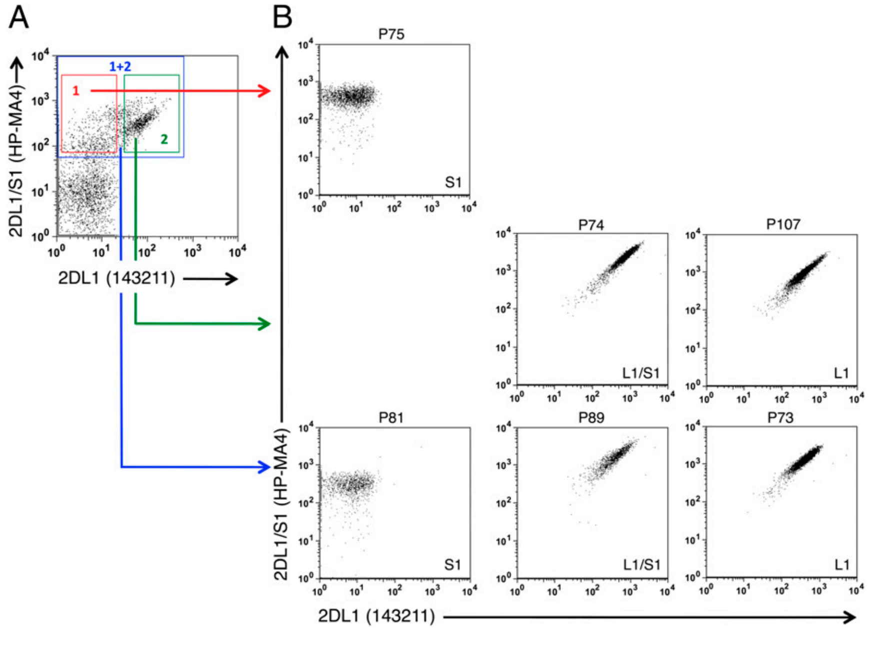

FIGURE 1. Generation of NK clones from single NK cells with specific receptor repertoires. (**A**) Flow cytometric representation of NK subsets identified by HP-MA4 and 143211 mAbs. HP-MA4 recognizes NK cells expressing 2DS1, 2DL1, or both (subset 1+2, *blue*). Combined use of HP-MA4 and 143211 mAbs allows discrimination between 2DS1$^{\text{pos}}$/2DL1$^{\text{neg}}$ (subset 1, *red*) and 2DS1$^{\text{pos}}$/2DL1$^{\text{pos}}$ or 2DS1$^{\text{neg}}$/2DL1$^{\text{pos}}$ (subset 2, *green*) NK cells. Resting NK cells obtained from a healthy donor are depicted. (**B**) P75 and P81: 2DS1$^{\text{pos}}$/2DL1$^{\text{neg}}$ (HP-MA4$^{\text{pos}}$/143211$^{\text{neg}}$) NK clones. P74, P107, P89, and P73: 2DL1$^{\text{pos}}$ (HP-MA4$^{\text{pos}}$/143211$^{\text{pos}}$) NK clones. In 2DL1$^{\text{pos}}$ NK clones, 2DS1 expression is verified by real-time RT-qPCR.

pression (i.e., DX9$^{\text{neg}}$). The lowest KIR transcript number associated with detectable receptor surface expression was 40 copies for 2DL1, 13 copies for 2DS1, 23 copies for 2DL2-3, and 98 copies for 3DS1. These values were set as the minimal copy number of transcripts necessary for surface expression of each KIR. This procedure for identification of NK clones with KIR surface expression was applied to evaluate the effects of inhibitory KIR for self–HLA class I on anti–HLA-C2 reactivity by 2DS1$^{\text{pos}}$ clones. In addition, it was used in the analysis of a possible effect of 3DS1 on anti–HLA-C2 cytotoxity mediated by 2DS1.

Mean fluorescence intensity (MFI) values were used to determine expression levels of 2DS1 receptor.

Cytotoxicity. Cytotoxicity against EBV-BLCL was measured in standard ^{51}Cr release assays performed in triplicate (or in duplicate for clones with limited cell number) for 4 h, at 37°C, at E:T ratio of 10:1 (3×10^3 target cells per well). Where indicated, effectors were tested in the presence of 10 μg/ml anti-human NKG2A, 4E (anti–HLA-B/C), or control anti-mouse F(ab′)$_2$ fragment. EBV-BLCL target cells were obtained from the International Histocompatibility Working Group (IHWG; www.ihwg.org/reference/index.html Consanguineous_Reference_Panel) or generated in our laboratory. EBV-BLCL possessed the following *HLA* class I genotypes: GK, *A*02:01/*03:01*, *B*40:01/*15:01*, *Cw*03:04/*03:04* [*B* group: HLA–killer cell Ig-like receptor ligand group Bw6 (*Bw6*); *C* group: *C1*]; KA, *A*03:01/*68:01*, *B*15:01/*51:01*, *Cw*04:01/*07:04* [*B* group: HLA–killer cell Ig-like receptor ligand group Bw4 (*Bw4*); *C* group: *C1:C2*]; 9036, *A*02:01/*02:01*; *B*44:02/*44:02*, *Cw*05:01/*05:01* (*B* group: *Bw4*; *C* group: *C2*); DD, *A*02:01/03:01*, *B*35:02/41:01*, *Cw*04:01/17:01* (*B* group: *Bw6*; *C* group: *C2*). Targets were maintained in RPMI 1640 supplemented with 10% FCS, 100 U/ml penicillin, and 0.1 mg/ml streptomycin up to 3 mo before being discarded. All EBV-BLCL were tested for expression of HLA-E, using PE-conjugated anti–HLA-E mAb (3D12; BioLegend).

We determined the values for nonspecific ^{51}Cr release in 2DS1$^{\text{pos}}$ clones by performing 101 cytotoxicity assays in which 2DS1-mediated activation could not be involved in target lysis. In 60 assays, 2DL1$^{\text{pos}}$/2DS1$^{\text{neg}}$ clones were tested against an *HLA-C2* group positive EBV-BLCL, and in 41 assays, 2DS1$^{\text{SP}}$ clones were tested against an *HLA-C2* group negative EBV-BLCL. No cytotoxicity from such combinations could be accounted for by "missing self–HLA class I recognition." The uppermost percentage of lysis observed in these assays was 13.1%. A threshold at 13.1% lysis was therefore set to distinguish between nonspecific ^{51}Cr release and specific 2DS1-mediated NK response.

Supported lipid bilayers and live imaging. Bilayers were generated as described from small unilamellar vesicles containing a 10:1 mixture of 1,2-dioleoyl-sn-glycero-3-phosphocholine and biotinyl cap phosphoethanolamine (Avanti Polar Lipids) (21). After formation, bilayers were incubated with streptavidin, washed with PBS, and then incubated with 2 mg/ml biotinylated EB6 mAb (anti-2DL1/S1), 1 mg/ml biotinylated ICAM-I, and 1 mg/ml biotinylated HLA-E. For comparative studies in which one or more constituents were left out of the bilayer, the nonstimulatory biotinylated mouse MHC molecule H2-D$^{\text{b}}$ was added to keep the total protein concentration constant for that experiment. After protein loading, bilayers were stored at room temperature for up to 4 h prior to use.

Prior to imaging, NK cell clones were loaded with 5 mg/ml Fura 2-AM and then transferred into RPMI 1640 supplemented with 5% FCS and lacking phenol red. Next, 2×10^5 NK cells were added to a chambered coverglass bearing stimulatory supported lipid bilayers and then imaged using an inverted fluorescence video microscope (IX-81; Olympus) fitted with a 20× objective lens (0.75 NA; Olympus) and attached to an electron-multiplying charge-coupled device camera (Hamamatsu Photonics). Ratiometric Fura 2 images were collected using a DG-4 Xenon lamp (Sutter Instrument Company) with 340- and 380-nm bandpass filters in place. Differential interference contrast and Fura 2 fluorescence images were acquired every 30 s for 20–30 min after addition of cells to the stimulatory bilayer. All experiments were performed at 37°C. Data were collected using Slidebook Software (Intelligent Imaging Innovations) and analyzed using Slidebook and Excel by computing the average Fura 2 ratio for all cells in the imaging field for each time point.

Quantitative PCR

Primers and probes. Sequences for 2DL1 primers, as well as 2DS1, 2DL2-3/S2, 3DS1, and NKG2A primers and probes, were previously reported (22, 23). A 2DL1 probe and GAPDH primers were designed in our laboratory. Nanomolar oligonucleotide concentrations for real-time quantitative RT-PCR (RT-qPCR) reactions (indicated in parentheses) were established by optimization matrix for both primers (range: 100–900 nM) and probes (range: 50–250 nM). Shorter probes with conjugated minor groove binder groups were preferred over standard DNA probes to increase reaction specificity. 2DS1: Forward (Fwd): 5′-TCTCCATCAGTCGCATGAR-3′ (500), Reverse (Rev): 5′-AGGGCCCAGAGGAAAGTT-3′ (500), Probe: 5′-6FAM-AGGTCTATATGAGAAACCT-MGB-3′ (150); 2DL1: Fwd: 5′-GCAGCACCATGTCGCTCT-3′ (300), Rev: 5′-GTCACTGGGAGCTGACAC-3 (100), Probe: 5′-6FAM-CACATGAGGGAGTCCAC-MGB-3′ (100); 2DS2: Fwd: 5′-TGCACAGAGAGGGGAAGTA-3′ (300), Rev: 5′-CACGCTCTCTCCTGCCAA-3′ (300), Probe: 5′-6FAM-GTCATCACAGGTCTATATGA-MGB-3′ (100); 2DL2: Fwd: 5′-GGAGGGGGAGGCCCATGAAT-3′ (300), Rev: 5′-GTCGGGGGTTACCGGTTTTA-3′ (500), Probe: 5′-6FAM-CCAAGGTCAACGGAACA-MGB-3′ (150); 2DL3: Fwd: 5′-CCACTGAACCAAGCTCCG-3′ (500), Rev: 5′-CAGGAGACAACTTTGGATCA-3′ (500), Probe: 5′-6FAM-CTGGTGCTGCAACAA-MGB-3′ (150); 3DS1: Fwd: 5′-GCACCCAGCAACCCCA-3′ (300), Rev: 5′-TAGGTCCCTGCAAGGGCAC-3′ (500), Probe: 5′-6FAM-AATTTCTCCATCGGTTCCATGA-MGB-3′ (150); NKG2A, Fwd: 5′-CTCCAGAGAAGCTCATTGTTGG-3′ (500), Rev: 5′-CACCAATCCATGAGGATGGTG-3′ (500), Probe: 5′-6FAM-CGATAGTTGTTATTCCCTCTACA-MGB-3′ (150); GAPDH, Fwd: 5′-TTCGCTCTCTGCTCCTCCTG-3′, Rev: 5′-CTTCCCGTTCTCAGCCTTGA-3′.

Recombinant plasmids. KIR, NKG2A, and GAPDH cDNA was PCR amplified from two donors, using the above-listed primers (500 nM). Thermal cycling conditions were as follows: stage 1: 2 min, 94°C; stage 2: 30 cycles of [30 s, 94°C; 30 s, 60°C; 30 s, 72°C]; and stage 3: 7 min, 72°C. PCR products were ligated (pGEM-T Easy Vector; Promega) and transformed into MAX Efficiency DH5α Competent Cells (Invitrogen). Recombinant plasmid DNA was extracted, the insert sequenced, and the concentration determined at 260 nm (NanoDrop 1000; Thermo Scientific).

cDNA synthesis from NK clones. cDNA for real-time RT-qPCR was extracted from cryopreserved NK clones using the MACS One-Step cDNA technology (Miltenyi Biotec). Briefly, poly(A)$^+$ tails of mRNA in cell lysates were hybridized with oligo(dT) microbeads. Magnetically labeled mRNA retained in micro columns was used as template for cDNA synthesis (1 h, 42°C). Prior to reverse transcription, RNase-free DNase I (Applied Biosystems) was added to mRNA (10 U, 2 min, room temperature), to completely remove traces of genomic DNA. RNase H from *Escherichia coli* (New England Biolabs) was added for in-column digestion of mRNA-bound cDNA (2 U, 30 min, 37°C). cDNA was stored at −20°C.

PCR amplification of cDNA. PCR amplifications used 2 μl NK clone cDNA in buffer solution in a 50-μl reaction mix containing FastStart Universal Probe Master (Roche Applied Science) and the primer/probe oligonucleotides described above. Quantification of housekeeping GAPDH was performed by TaqMan Gene Expression Assay for GAPDH (HS99999905_m1; Applied Biosystems). Reactions were performed in duplicate using an ABI 7300 PCR System (Applied Biosystems), under the following thermal cycling conditions: stage 1: 2 min, 50°C; stage 2: 10 min, 95°C; stage 3: 40 cycles of [15 s, 95°C]; stage 4: 1 min, 60°C. Nontemplate controls were set up in triplicate for each reaction.

Absolute quantification of NK clone transcripts. Samples containing 10-fold serial dilutions (KIR and NKG2A: 3×10^5–30; GAPDH: 3×10^6–3×10^2) of known gene copy numbers in recombinant plasmids were amplified in triplicate, along with each real-time RT-qPCR run. Five-point standard curves were generated to quantify each KIR and NKG2A transcript. Standard curves with a linearity (r^2) ≥ 0.985 and an efficiency ranging from 85 to 110% were considered acceptable. Threshold cycle values > 36 were considered nonspecific and discarded. Normalization of copy numbers for KIR and NKG2A was calculated as follows: [KIR or NKG2A copy number/GAPDH copy number] $\times 10^3$.

Statistical analysis

Frequencies of cytotoxic 2DS1$^{\text{pos}}$ clones possessing different receptors or different *HLA-C* genotypes were compared using the two-sided χ^2 test. Specific cytotoxicity values or receptor expression levels observed in different clone groups were compared by nonparametric two-tailed Mann–Whitney or Wilcoxon signed rank tests for independent or paired observations, respectively. Correlation between NKG2A gene expression and NKG2A MFI or percentage of cytotoxicity was determined by Spearman's rank correlation coefficient. All statistical tests were performed using Prism 5 for Mac OS X (GraphPad). A p value ≤ 0.05 was considered significant.

Institutional review board approval

Informed written consent was obtained from all donors according to Memorial Sloan-Kettering Cancer Center Institutional Review Board Protocol (IRB# 95-054A for healthy donors and IRB# 09-141 for transplant recipients).

Results

IL-15 trans*-presentation supports generation of 2DS1^{pos} NK clones*

2DS1^{pos} clones have previously been obtained from donors lacking cognate HLA-C2 ligand (i.e., donors homozygous for the HLA-C1 ligand). In contrast, very few 2DS1^{pos} clones were obtained from donors expressing HLA-C2 (19). Because IL-15 *trans*-presentation is the major growth and survival signal for NK cells (24–27), we investigated whether human NK cloning efficiency and clone survival could be enhanced by IL-15 *trans*-presentation in vitro (Fig. 1). *Trans*-presentation was achieved by coculture of FACS-sorted NK cells with murine BaF/3 cells transfected with human IL-15Rα and human IL-15. This procedure supported clone development from all donors, irrespective of their *HLA-C* genotype. Each clone reached $0.25–4 \times 10^6$ cells, and the overall cloning efficiency was 35–40%. Clones were developed from seven donors, representing the three *HLA-C* genotypes: *C1:C1*, *C1:C2*, and *C2:C2*. *HLA-KIR* ligand groups and *KIR* genes for each NK donor are listed in Table I. We analyzed 386 clones, which included the 2DS1^{SP} phenotype and other 2DS1^{pos} phenotypes (Table II). For example, 2DS1^{SP} clones were obtained from donors with each *HLA-C* genotype (Table II, Columns A and B). Similarly, twenty-four 2DS1^{SP}, which also expressed the inhibitory receptor CD94/NKG2A, were obtained (Table II, Column F). Accordingly, clones with a broad KIR repertoire, including 2DS1, can be obtained from donors with any *HLA-C* genotype, when IL-15 *trans*-presentation is the NK growth factor.

Frequency of 2DS1^{SP} NK clones with anti–HLA-C2 cytotoxicity is decreased only in donors homozygous for HLA-C2

A total of 91 2DS1^{SP} clones were isolated, of which 56 had anti–HLA-C2 cytotoxicity (Table III). Clones with anti–HLA-C2 cytotoxicity were obtained from any donor, regardless of the *HLA-C* genotype. Anti–HLA-C2 cytotoxicity was detected in 29 of 42 clones with the *C1:C1* genotype (69%) and in 19 of 22 clones with the *C1:C2* genotype (86%). In contrast, anti–HLA-C2 cytotoxicity was observed in only 8 of 27 clones with the *C2:C2* genotype (30%) (*C1:C1* versus *C2:C2*, $p = 0.001$, and *C1:C2* versus *C2:C2*, $p < 0.0001$) (Fig. 2A, Table IIIA).

The frequency of anti–HLA-C2 cytotoxicity among 2DS1^{SP}, *C1:C2* clones was similar to that observed among *C1:C1* clones (Fig. 2A, *left* and *center*). This finding demonstrates that clonal deletion or clonal anergy is not characteristic of 2DS1^{SP} clones from donors heterozygous for *HLA-C2*. The 22 clones from *C1:C2* heterozygous donors were tested on *HLA-C2* heterozygous and homozygous target cells. The *C1:C2* clones were significantly less frequently cytotoxic against target cells with the autologous *C1:C2* genotype ($p < 0.0001$) (Fig. 2A, *center*, Fig. 2B; Table IIIB). Thus, 2DS1^{SP} clones with anti–HLA-C2 reactivity derived from donors with the *C1:C2* genotype are rarely cytotoxic to autologous targets. This decrease in frequency of anti–HLA-C2 cytotoxicity cannot be ascribed to the effect of inhibitory KIR expressed by the clones, because they all are 2DS1^{SP}.

We finally determined the effect of inhibitory KIRs with ligand specificity for nonself–HLA class I on the function of 2DS1^{pos} clones. Thirteen 2DS1^{pos}, *C2:C2* clones, which also expressed the inhibitory receptor 2DL3 with ligand specificity for HLA-C1, were obtained. Six of 13 (46%) clones had anti–HLA-C2 reactivity, which is not significantly different from the results obtained with 2DS1^{SP}, *C2:C2* clones (Fig. 2A, *right,* Fig. 2C; Table IIIC). Therefore, 2DS1^{pos}, *HLA-C2* homozygous clones with nonself inhibitory KIR display anti–HLA-C2 reactivity comparable to that of 2DS1^{SP} clones from the same donor.

2DS1^{SP} NK clones with anti–HLA-C2 reactivity are present in recipients of 2DS1^{pos} allogeneic hematopoietic stem cell transplantation

Allogeneic, myeloablative hematopoietic stem cell transplantation (HCT) provides a possibility for evaluating de novo development of donor-derived 2DS1^{pos} NK cells in the presence of cognate HLA-C2 ligand. We investigated two cases in which the graft was obtained from 2DS1^{pos} *C1:C2* donors. 2DS1^{SP} NK cells were identified post-HCT in both recipients (Fig. 2D). In the first case, ten 2DS1^{SP} clones were obtained 100 d post-HCT from an *HLA-C2* homozygous recipient. Four of the 2DS1^{SP} clones displayed anti–HLA-C2 cytotoxicity (Fig. 2D, Case 1). In the second case, four 2DS1^{SP} clones were obtained from an *HLA-C1:C2* heterozygous recipient, 200 d post-HCT. Two clones displayed anti–HLA-C2 cytotoxicity (Fig. 2D, Case 2). Therefore, donor-derived 2DS1^{SP} NK cells with ability to mediate anti–HLA-C2 cytotoxicity can be identified in an allogeneic host that expresses the cognate HLA-C2 ligand.

2DS1^{pos} NK clones expressing at least one inhibitory KIR for self–HLA class I are tolerant

Among the inhibitory KIR with HLA class I ligand specificity, only 2DL1 and 3DL1 can be individually recognized by mAbs. The remaining inhibitory KIR 2DL2, 2DL3, and the activating receptor 2DS2 cannot be distinguished by monospecific Abs. Similarly, 2DS1 is not distinguishable from 2DL1, when both receptors are present on the same cell. KIR phenotyping was therefore supplemented with determination of mRNA copy numbers for each of the KIRs with ambiguous phenotypes. Absolute RT-qPCR quan-

Table I. Donor HLA class I and KIR

	HLA-KIR Ligand Group		*KIR* Genes		
Sample ID	C	B	Activating	Inhibitory	KIR Haplotype[a]
Healthy volunteers					
UDN[b] 001	C1:C1	Bw4	*2DS1/—[c]/3DS1*	*2DL1/2DL3/3DL1*	A, B [A1, B3]
UDN 002	C1:C1	Bw4	*2DS1/2DS2/3DS1*	*2DL1/2DL2–3/3DL1*	A, B [A1, B15]
UDN 003	C2:C2	Bw4	*2DS1/—/3DS1*	*2DL1/2DL3/3DL1*	A, B [A1, B3]
UDN 004	C1:C2	Bw4	*2DS1/2DS2/3DS1*	*2DL1/2DL3/3DL1*	B, B [B4, B17]
UDN 005	C1:C2	Bw4	*2DS1/2DS2/3DS1*	*2DL1/2DL2–3/3DL1*	B, B [B7, B24]
HCT donors					
UDN 006	C1:C2	Bw6	*2DS1/2DS2/—*	*2DL1/2DL2–3/3DL1*	B, B [B31, B34]
UDN 007	C1:C2	Bw4	*2DS1/—/3DS1*	*2DL1/2DL3/3DL1*	A, B [A1, B3]

[a] KIR haplotype numbers from Khakoo and Carrington (18).

[b] Unique donor number.

[c] Absence of *KIR* gene.

Table II. NK cloning strategy

	Donor *HLA-C* Genotype	Number of NK Clones per FACS Sorting Gate[a]						Total NK Clones by *HLA-C* Genotype
		A	B	C	D	E	F	
	C1:C1	55	33	18	37	3	0	146
	C1:C2	43	23	36	19	12	0	133
	C2:C2	30	25	15	9	4	24	107
Total NK clones by sorting gate		128	81	69	65	19	24	386
Possible KIR phenotypic combinations		2DS1[b] or 2DL1[b] or 2DS1/2DL1[b]	2DS1	2DL1[b] or 2DS1/2DL1[b]	2DS1 and 2DL2/2DL3/2DS2[c]	2DS1/3DL1	2DS1/NKG2A	

[a] Sorting of single NK cells using the following mAb: anti-2DL1/2DS1 (HP-MA4), anti-2DL1 (143211), anti-2DL2/2DL3/2DS2 (CH-L), anti-3DL1 (DX9), anti-NKG2A (131411). FACS sorting gates: A, HP-MA4^{pos}; B, HP-MA4^{pos}, 143211^{neg}, CH-L^{neg}, DX9^{neg}; C, HP-MA4^{pos}, 143211^{pos}; D, HP-MA4^{pos}, 143211^{neg}, CH-L^{pos}, DX9^{neg}; E, HP-MA4^{pos}, 143211^{neg}, CH-L^{neg}, DX9^{pos}; and F, HP-MA4^{pos}, 143211^{neg}, CH-L^{neg}, DX9^{neg}, 131411^{pos}.

[b] Possible expression of additional KIR with ligand specificity for HLA class I.

[c] Expression of at least one inhibitory (2DL2/2DL3) or activating (2DS2) receptor.

tification assays were performed for 2DL1 (166 clones), 2DS1 (285 clones), and 2DL2–3 (229 clones) KIR transcripts. The mRNA copy numbers for such KIRs were determined, and the minimal copy number associated with KIR surface expression was identified, as described in *Materials and Methods* and in Fig. 3. These minimal copy number values were used as reference to assign a KIR receptor phenotype to a total of 29 clones, whose surface expression of one or more KIR could not be unambiguously determined by flow cytometric analysis alone. This method allowed distinction of 2DL3 expression from 2DS2 expression in 16 clones and detection of 2DS1 expression in 12 2DL1^{pos} clones. Moreover, we could identify 2DL1 and 2DS1 expression in three clones that were characterized by a mAb with specificity for both 2DL1 and 2DS1.

Forty-six 2DS1^{pos} clones expressing at least one inhibitory KIR for self–HLA class I were obtained. Thirty-two clones were heterozygous for *HLA-C2*, and 14 clones were homozygous for *HLA-C2*. Only one clone displayed anti–HLA-C2 cytotoxicity (2%) (Table IV). Therefore, 2DS1^{pos} NK cells expressing at least one inhibitory KIR for self–HLA class I very rarely mediate anti–HLA-C2 cytotoxicity, when the donor has *HLA-C2*. In contrast, 2DS1^{pos}, *HLA-C1* homozygous clones coexpressing inhibitory KIR for self–HLA class I frequently display anti–HLA-C2 cytotoxicity (Table V and Ref. 19).

Anti–HLA-C2 cytotoxicity of 2DS1^{SP} clones expressing the inhibitory receptor LILRB1

LILRB1 is expressed on NK cell subsets and other cells belonging to the myeloid and lymphoid lineage (28, 29) and delivers inhibitory signals upon interaction with a wide range of HLA class I Ags (30, 31). Twenty-two 2DS1^{SP} clones expressed LILRB1. Eight clones were obtained from *HLA-C1* homozygous donors and 14 clones from *HLA-C2* homozygous donors. All clones from *C1* donors displayed anti–HLA-C2 cytotoxicity. In contrast, anti–HLA-C2 cytotoxicity was observed in only 5 of 14 clones from *C2* donors (*C1:C1* versus *C2:C2*, $p = 0.003$). Therefore, LILRB1 may in some instances contribute to inhibition and to maintaining tolerance of 2DS1 signals in *HLA-C2* homozygous donors.

Effect of inhibitory receptor CD94/NKG2A on tolerance development of HLA-C2 homozygous 2DS1^{SP} NK cells

The reduced frequency of anti–HLA-C2 reactivity observed in *HLA-C2* homozygous, 2DS1^{SP} clones is consistent with NK tolerance observed in mice transgenic for activating receptor ligands (7–11). In humans, the inhibitory receptor CD94/NKG2A could potentially counteract 2DS1 activation by the HLA-C2 ligand. We first determined if the 2DS1 receptor is signaling competent in 2DS1^{SP}, *HLA-C2* homozygous clones expressing CD94/NKG2A. EB6 mAb cross-linking of the 2DS1 receptor in the presence of ICAM-I induces Ca^{2+} flux (*Materials and Methods*). This activation signal is inhibited when HLA-E, the ligand for CD94/NKG2A (32), is added (Fig. 4A). Similar results were obtained with three additional clones, demonstrating that the 2DS1 receptor is signaling competent. Next, we determined the correlation between NKG2A expression levels and anti–HLA-C2 reactivity of 2DS1^{SP}/NKG2A^{pos} clones. NKG2A mRNA transcript copy numbers correlated well with NKG2A receptor MFI in 2DS1^{SP}/NKG2A^{pos} clones from *C1:C1* and *C2:C2* donors ($p = 0.002$)

Table III. Cytotoxicity of 2DS1^{SP} NK clones

Donor *HLA-C* Genotype	KIR Phenotype	Target *HLA-C* Genotype	*n*	Cytolytic		% Lysis Median (Range)	*p*[a]	*p*[b]
				Yes, *n* (%)	No, *n* (%)			
A								
C1:C1	2DS1^{SP}	*C2:C2*	42	29 (69)	13 (31)	34.3 (13.3–67.5)	1	1
C1:C2	2DS1^{SP}	*C2:C2*	22	19 (86)	3 (14)	39.6 (13.3–62.6)	NS	NS
C2:C2	2DS1^{SP}	*C2:C2*	27	8 (30)	19 (70)	24.9 (13.5–43.4)	0.001	NS
B								
C1:C2	2DS1^{SP}	*C2:C2*	22	19 (86)	3 (14)	39.6 (13.3–62.6)	1	1
C1:C2	2DS1^{SP}	*C1:C2*	22	3 (14)	19 (86)	24.3 (22.9–29.7)	<0.0001	NS
C								
C2:C2	2DS1^{SP}	*C2:C2*	27	8 (30)	19 (70)	24.9 (13.5–43.4)	1	1
C2:C2	2DS1^{pos}/L3^{pos}	*C2:C2*	13	6 (46)	7 (54)	17.7 (13.2–27.7)	NS	NS

[a] Frequency of anti–HLA-C2 cytolytic clones in each group is compared.

[b] Magnitude of anti–HLA-C2 cytotoxic responses for each clone group is compared.

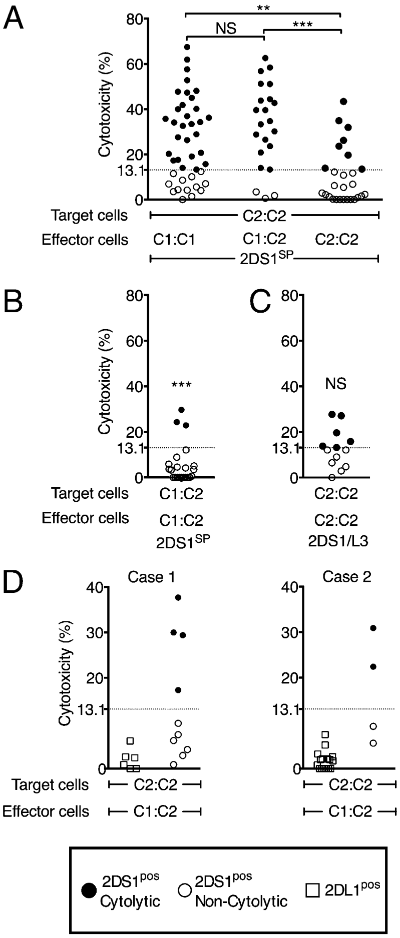

FIGURE 2. 2DS1^{SP} NK clones with anti–HLA-C2 cytotoxicity. (**A**–**C**) Clones from healthy donors. (**D**) Donor-derived clones obtained from HCT recipients. Collected data are from 14 independent experiments. Assays were performed in duplicate or triplicate, with E:T ratio 10:1. Dotted line: 13.1% cutoff between clone cytotoxicity and noncytotoxicity. ●, 2DS1^{pos} clones with anti–HLA-C2 cytotoxicity; ○, 2DS1^{pos} clones without anti–HLA-C2 cytotoxicity; □, 2DL1^{pos} clones. 2DS1^{SP} denotes clones expressing the activating 2DS1 receptor, but no inhibitory KIR with ligand specificity for HLA class I (2DL1, 2DL2, 2DL3, and 3DL1). (A) *C1:C1* (n = 42), *C1:C2* (n = 22), and *C2:C2* (n = 27) 2DS1^{SP} clones were tested for cytotoxicity against *C2:C2* EBV-BLCL (IHWG 9036) target cells. (B) *C1:C2* 2DS1^{SP} clones (n = 22) were tested for cytotoxicity against *C1:C2* EBV-BLCL (KA) target cells. The same *C1:C2* clones were tested for cytotoxicity against *C2:C2* target cells, shown in (A). Statistical analysis compares the frequency of anti–HLA-C2 cytotoxic *C1:C2* clones detected using *C2:C2* or *C1:C2* target cells. (C) *C2:C2* 2DS1^{pos}/2DL3^{pos} clones (n = 13) were tested for cytotoxicity against *C2:C2* EBV-BLCL (IHW 9036) target cells. Statistical analysis compares the frequency of anti–HLA-C2 cytotoxic 2DS1^{pos}/2DL3^{pos} *C2:C2* clones, shown here, and of 2DS1^{SP} *C2:C2* clones, shown in (A), to the same *C2:C2* target cells. (D) Donor-derived clones obtained from HCT recipients were tested against *C2:C2* EBV-BLCL (IHW 9036) target cells. Case 1, HCT 1. Cytotoxicity of 2DL1^{pos} (n = 6) and 2DS1^{SP} (n = 10) *C1:C2* clones, obtained from a *C2:C2* HCT recipient 100 d posttransplantation. Case 2, HCT 2. Cytotoxicity of 2DL1^{pos} (n = 16) and 2DS1^{SP} (n = 4) *C1:C2* clones, obtained from a *C1:C2* HCT recipient 200 d posttransplantation. $^{**}p$ = 0.001, $^{***}p < 0.0001$.

(Fig. 4B). These data validated the inclusion of NKG2A mRNA copies for our correlation studies. We expected that high cytotoxicity would be observed in clones with low NKG2A mRNA copies. However, NKG2A mRNA copy numbers were not found to correlate with anti–HLA-C2 cytotoxicity in 2DS1^{SP}, *C2:C2* clones (Fig. 4C). Therefore, expression of CD94/NKG2A inhibitory receptor does not predict whether 2DS1^{SP}, *HLA-C2* homozygous clones mediate anti–HLA-C2 reactivity or whether they are tolerant to HLA-C2.

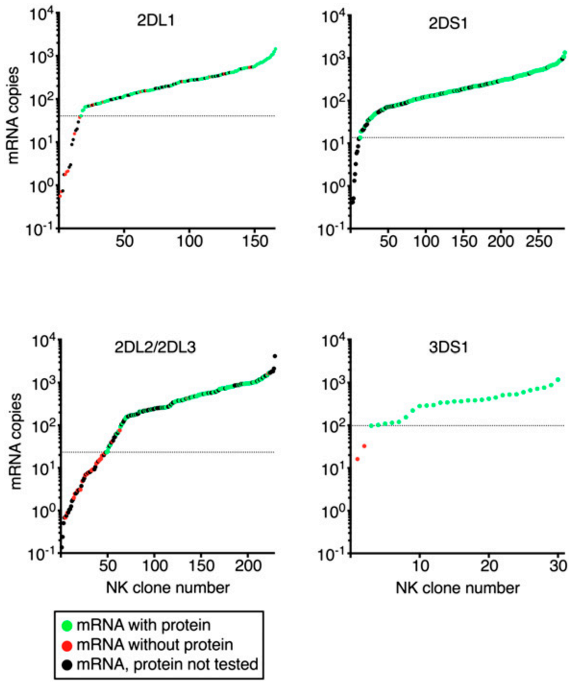

FIGURE 3. mRNA copy numbers for KIR receptors with ligand specificity for HLA-C Ags. Quantitative determination of mRNA expression for 2DL1, 2DS1, 2DL2–3, and 3DS1 performed by real-time RT-qPCR. Dots represent individual NK clones: mRNA expression with protein surface expression (green), mRNA expression without surface expression (red), and mRNA expression with untested surface expression (black). Dotted lines in each plot identify the minimal mRNA copy number values associated with KIR surface expression. 2DL1: 166 clones, minimal value: 40 copies. 2DS1: 285 clones, minimal value: 13 copies. 2DL2–3: 229 clones, minimal value: 23 copies. 3DS1: 30 clones, minimal value: 98 copies. For each sample, mRNA copy number determination is performed in duplicate.

To directly test the inhibitory function of CD94/NKG2A on 2DS1^{SP}/NKG2A^{pos}, *HLA-C2* homozygous clones, we determined the effect of anti-NKG2A F(ab′)$_2$ fragment on the cytotoxicity of 10 noncytolytic (Fig. 4D) and 14 cytolytic (Fig. 4E) clones. Anti–HLA-C2 cytotoxicity was determined using HLA-A**02:01* homozygous target (BLCL 9036), which expresses several HLA class I alleles with HLA-E binding leader peptides (33). HLA-E expression on this target was confirmed by mAb staining. Only one noncytolytic clone changed from noncytolytic to cytolytic when the CD94/NKG2A receptor was blocked with anti-NKG2A F(ab′)$_2$ (Fig. 4D). Furthermore, all 14 2DS1^{SP} clones with anti–HLA-C2 reactivity displayed enhanced cytotoxicity following blocking with anti-NKG2A F(ab′)$_2$ (Fig. 4E). Therefore, CD94/NKG2A provides only a modulatory, attenuating effect on 2DS1-mediated anti–HLA-C2 cytotoxicity, and is not a major factor controlling 2DS1 tolerance to HLA-C2 in *HLA-C2* homozygous donors. These results also agree with a recent report of a single 2DS1^{pos}/NKG2A^{pos} clone in which 2DS1-mediated cytotoxicity was unaffected by the presence of CD94/NKG2A (34).

2DS1 activation, missing self–HLA class I recognition, and KIR inhibition

We investigated the relationship between 2DS1 activation by HLA-C2 ligand, NK activation by missing self–HLA class I, and inhibition of NK cell activation by inhibitory KIR to self–HLA class I.

Table IV. 2DS1^{pos} NK clones with one or more inhibitory KIR for self–HLA class I

Donor *HLA-C* Genotype	Effector Cell KIR Phenotype	Target Cell *HLA-KIR* Ligands	*n*	Cytolytic	
				Yes, *n* (%)	No, *n* (%)
C1:C2		*C1:C2;Bw4*	32	1 (3)	31 (97)
C1:C2	2DS1/2DL1	*C1:C2;Bw4*	5	0	5
C1:C2	2DS1/2DL1/2DL3	*C1:C2;Bw4*	1	0	1
C1:C2	2DS1/2DL1/2DL3/3DL1	*C1:C2;Bw4*	1	0	1
C1:C2	2DS1/2DL3	*C1:C2;Bw4*	14	0	14
C1:C2	2DS1/3DL1	*C1:C2;Bw4*	11	1	10
C2:C2		*C2:C2;Bw4*	14	0 (0)	14 (100)
C2:C2	2DS1/2DL1	*C2:C2;Bw4*	5	0	5
C2:C2	2DS1/2DL1/3DL1	*C2:C2;Bw4*	3	0	3
C2:C2	2DS1/3DL1	*C2:C2;Bw4*	6	0	6
Total			46	1 (2)	45 (98)

Eight 2DS1^{pos} clones that also expressed the inhibitory receptor 3DL1 were obtained from a donor heterozygous for *C1:C2* and homozygous for *HLA-Bw4*. The clones were tested against a panel of target cells homozygous for the 2DS1 activating ligand (i.e., *C2:C2* homozygous) or lacking the activating ligand (i.e., *C1:C1* homozygous). The presence of inhibitory ligand for 3DL1 (i.e., *Bw4* homozygous) or absence of 3DL1 ligand (i.e., *Bw6* homozygous) was similarly tested in different combinations of target cells (Fig. 5). Six of eight clones were inhibited by a target homozygous for *HLA-C2;Bw4* (Fig. 5, *left*), consistent with the finding that clones with inhibitory KIR for self–HLA class I in most instances do not respond to HLA-C2 activation (Table IV). The same clones were tested on a target homozygous for *HLA-C2* but lacking the HLA-Bw4 ligand for 3DL1 (i.e., HLA genotype *C2:C2;Bw6:Bw6*). All eight clones displayed anti–HLA-C2 cytotoxicity, demonstrating the combined activating effect of 2DS1 signaling and recognition of "missing self–HLA class I" (Fig. 5, *center*). Finally, the effect of lack of HLA-C2 ligand and absence of HLA-Bw4 on target cells (i.e., no stimulation of 2DS1 but recognition of "missing self–HLA class I") is shown. Three clones were cytolytic, whereas five were inhibited (Fig. 5, *right*). Therefore, "missing self–HLA class I" recognition in the absence of 2DS1 activation provides a variable activation signal.

Tolerance of 2DS1^{SP} clones to cognate ligand in HLA-C2 homozygous donors is not dependent upon ligand-mediated downregulation of the 2DS1 receptor

Studies in transgenic mice have demonstrated ligand-mediated downregulation of the activating NK receptor (10, 11). A similar mechanism could be involved in 2DS1 tolerance to *HLA-C2*. MFI of 2DS1 expression was determined on the 8 *C2:C2* 2DS1^{SP} clones displaying anti–HLA-C2 cytotoxicity and on the 19 *C2:C2* 2DS1^{SP} clones lacking anti–HLA-C2 cytotoxicity to the *HLA-C2* homozygous target. 2DS1 expression levels were similar in these *HLA-C2* homozygous 2DS1^{SP} clones, irrespective of their anti–HLA-C2 responsiveness (Fig. 6).

3DS1 does not contribute to anti–HLA-C2 reactivity of 2DS1^{SP} clones

Because the genes encoding the two activating receptors 2DS1 and 3DS1 are in strong positive genetic linkage disequilibrium (35, 36), they frequently occur together. It is therefore potentially difficult to distinguish between a 3DS1- and a 2DS1-mediated effect. The minimal 3DS1 mRNA copy number needed for expression of 3DS1 receptor was 98 (Fig. 3; see *Materials and Methods)*. This value was used as reference for determination of 3DS1 receptor expression. Ninety 2DS1^{SP} clones from donors with different *HLA-C* genotypes were tested for 3DS1 expression. Sixty-five clones (72%) had the 2DS1^{SP}/3DS1^{pos} phenotype with 3DS1 mRNA copy numbers consistent with 3DS1 cell surface expression (median: 544; range: 102–2340). The remaining 25 clones had the 2DS1^{SP}/3DS1^{neg} phenotype. Their 3DS1 mRNA copies were either undetectable or present in numbers below the calculated minimal value needed for expression (median: 0; range: 0–79). The frequency of anti–HLA-C2 cytotoxicity was compared between 2DS1^{SP}/3DS1^{pos} and 2DS1^{SP}/3DS1^{neg} clones. The analysis was done separately for different *HLA-C* groups of donors because the *HLA-C* genotype affects the frequency of anti–HLA-C2 reactive 2DS1^{SP} clones (Fig. 2). It is demonstrated in Table VI that the presence or absence of 3DS1 does not affect anti–HLA-C2 reactivity in 2DS1^{SP}clones. This finding was observed in clones from *HLA-C1* positive (i.e., *C1:C1* and *C1:C2*) and clones from *HLA-C2* homozygous donors. Therefore, 3DS1 does not contribute to anti–HLA-C2 cytotoxicity by 2DS1^{SP} clones.

Discussion

We demonstrate that 2DS1^{pos} clones are readily obtained from normal donors, irrespective of their *HLA-C* genotype. The presence of both the activating 2DS1 receptor and its cognate ligand does not result in extensive deletion of such NK cells. Furthermore, 2DS1^{pos} clones from *HLA-C2* heterozygous donors display anti–HLA-C2 reactivity in vitro similar to that in *HLA-C1* homozygous donors, who do not carry the cognate ligand. Therefore,

Table V. 2DS1^{pos} C1:C1 NK clones coexpressing inhibitory KIR for self–HLA class I

Donor *HLA-C* Genotype	KIR Phenotype	Target *HLA-C* Genotype	*n*	Cytolytic		*p*
				Yes, *n* (%)	No, *n* (%)	
C1:C1	2DS1 and 2DL2/3	*C2:C2;Bw4*	46	42 (91)	4 (9)	$<0.0001^{a}$
		C1:C2;Bw4		1 (5)	18 (95)	
C1:C1	2DS1 and 3DL1	*C2:C2;Bw4*	2	0 (0)	2 (100)	

[a] Frequency of cytolytic clones to targets with different *HLA-C* genotype is compared.

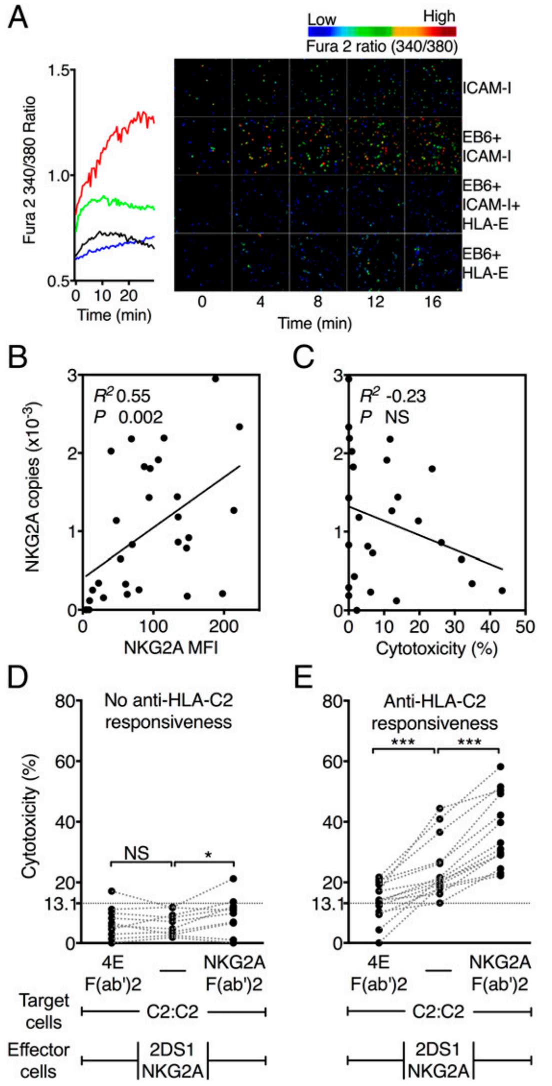

FIGURE 4. The inhibitory receptor CD94/NKG2A attenuates but does not block 2DS1-mediated activation signals. (**A**) Determination of intracellular Ca^{2+} concentration of a representative *C2:C2* $2DS1^{SP}/NKG2A^{pos}$ clone triggered by EB6 mAb cross-linking of the 2DS1 receptor in the presence of ICAM-I, with or without HLA-E ligand for NKG2A inhibitory receptor. Activation was measured during exposure to mAb- and/or ligand-coated lipid bilayers (0–29.5 min). Shifts of intracellular Ca^{2+} concentrations were determined by assessing changes of Fura 2-AM 340/380 fluorescence ratio. Results are representative of four independent experiments. Green line, ICAM-I; red line, EB6+ICAM-I; blue line, EB6+ICAM-I+HLA-E; black line, EB6+HLA-E. (**B**) Correlation between NKG2A mRNA copy numbers and NKG2A receptor MFI in $2DS1^{SP}/NKG2A^{pos}$ clones. This analysis was performed on *C1:C1* (*n* = 12) and *C2:C2* (*n* = 18) clones. (**C**) Correlation between NKG2A mRNA copy numbers and anti–HLA-C2 cytotoxicity in $2DS1^{SP}/NKG2A^{pos}$ clones. This analysis was performed on *C2:C2* (*n* = 26) clones. (**D** and **E**) Noncytolytic (*n* = 10) (D) and cytolytic (*n* = 14) (E) $2DS1^{SP}/NKG2A^{pos}$ *C2:C2* clones were tested against *C2:C2* EBV-BLCL (IHWG 9036), with or without anti-NKG2A $F(ab')_2$–mediated NKG2A inhibition. Data were generated in three independent experiments. Each test was performed in duplicate, with E:T ratio 10:1. $F(ab')_2$ concentration was 10 μg/ml. 4E $F(ab')_2$, anti–HLA-B/C $F(ab')_2$; NKG2A $F(ab')_2$, anti-hNKG2A $F(ab')_2$. The dotted line represents the 13.1% cutoff between cytotoxicity and noncytotoxicity. $*p < 0.05$, $***p \leq 0.0001$. EB6, Anti-KIR2DL1/S1; HLA-E, hNKG2A ligand.

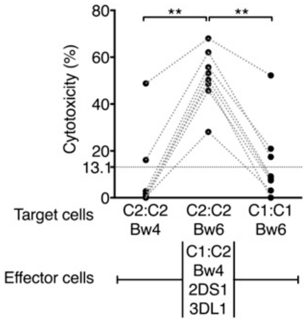

FIGURE 5. 3DL1 interaction with cognate HLA-Bw4 can override 2DS1 activation. $2DS1^{pos}/3DL1^{pos}$ clones (*n* = 8) were obtained from a *2DS1* healthy donor with the *HLA-C1:C2;Bw4* genotype and were tested for cytotoxicity against EBV-BLCL target cells with different *HLA-C* and *-B* genotype. *Left*, Cytotoxicity against *C2:C2;Bw4* EBV-BLCL (IHWG 9036) target cells. *Center*, Cytotoxicity against *C2:C2;Bw6* EBV-BLCL (DD) target cells. *Right*, Cytotoxicity against *C1:C1;Bw6* EBV-BLCL (GK) target cells. Data were generated in two independent experiments. Assays were performed in duplicate, with E:T ratio 10:1. The dotted line represents the 13.1% cutoff between clone cytotoxicity and non-cytotoxicity. The degree of cytotoxic response between different groups is compared. $**p < 0.01$.

donors heterozygous for *HLA-C2* do not express sufficient ligand to induce 2DS1 tolerance. In contrast, $2DS1^{pos}$ clones from *HLA-C2* homozygous donors have significantly reduced frequency of anti–HLA-C2 reactive clones. These results demonstrate that NK cells with an activating KIR specific for a self–major histocompatibility Ag are not all deleted from the repertoire, but are rendered tolerant when sufficient density of the ligand is expressed. NK cell tolerance has been reported in mouse models of NK cells that express activating receptors for self-antigens (7–11), but the differential effect of tolerance induction by homozygous versus heterozygous expression of the activating ligand has not previously been reported. NK tolerance was also observed in mice with mixed allogeneic bone marrow chimerism. NK cells in these mice expressed the activating Ly49D receptor, and one strain also expressed the putative MHC class I ligand $H2\text{-}D^d$ (12, 37, 38). These reports, as well as the current study, demonstrate self-tolerance of activating MHC class I specific receptors in the absence of known inhibitory receptors to self–MHC class I.

Nearly all $2DS1^{pos}$ clones with anti–HLA-C2 reactivity are $2DS1^{SP}$ or $2DS1^{pos}$ with irrelevant, nonself inhibitory KIR. These clones would be expected to display hyporesponsiveness owing to lack of NK licensing (39, 40). It is therefore surprising that $2DS1^{SP}$ clones from *HLA-C1* homozygous and heterozygous donors display potent anti–HLA-C2 responses, because they lack inhibitory receptors for self–MHC class I. Our findings are, however, consistent with the recently reported phenomenon termed “functional NK plasticity” described in mouse models. In these models, mature NK cells were transferred between wild -ype mice and MHC class I–deficient hosts. These mature NK cells displayed functional plasticity and adapted to the MHC environment of the host (41, 42). Our studies confirm and extend these findings by demonstrating NK plasticity of developing NK cells in both the syngeneic and the allogeneic *HLA-C2* positive host.

It is surprising that $2DS1^{pos}$ clones from donors heterozygous for *HLA-C2* do not display any evidence of reduced in vitro responses to HLA-C2. Determination of HLA-C2 Ag binding to 2DS1 in vitro demonstrates very weak binding affinity (43, 44). Because HLA Ags are codominantly expressed, the gene products from both *HLA-C* alleles will be displayed. *HLA-C2* homozygous individuals will therefore express twice the amount of HLA-C2 as will *HLA-C2* heterozygous donors. Our study indicates that the amount of HLA-C2 ligand expressed by *HLA-C2* homozygous host cells is sufficient to induce tolerance in $2DS1^{pos}$ NK cells, whereas the amount expressed by *HLA-C2* heterozygous donors is

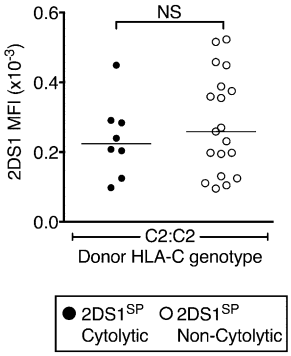

FIGURE 6. 2DS1 surface expression on *HLA-C2* homozygous, 2DS1^{SP} clones. *Left*, Anti–HLA-C2, cytolytic (n = 8). *Right*, Anti–HLA-C2, noncytolytic (n = 19). MFI was used to determine 2DS1 surface expression levels. Anti–HLA-C2 cytotoxicity is determined by reactivity against the *HLA-C2* homozygous EBV-BLCL (IHW 9036). Horizontal bars indicate medians. MFI levels of 2DS1 expression are compared between the two groups.

insufficient for activation of 2DS1. We also demonstrate lack of ligand-induced receptor downmodulation of 2DS1 on NK cells from *HLA-C2* homozygous donors. In contrast, the Ly49H receptor is downregulated in mice expressing the m157 viral ligand (10, 11). Collectively, these results support that 2DS1 interactions with HLA-C2 are weak, which agrees with results from binding affinity studies (43, 44).

The aim of the current study was to define the genetic basis for 2DS1 activation and ligand-induced tolerance. We did explore some possible mechanisms for tolerance to self in 2DS1^{pos} NK cells in donors homozygous for *HLA-C2*. Inhibition of 2DS1 activation by CD94/NKG2A only occurs very rarely. In contrast, inhibitory KIR with ligand specificity for self–HLA class I almost invariably suppressed 2DS1 activity. The effect of LILRB1 on *HLA-C2* homozygous 2DS1^{SP} clones was also tested. Such clones frequently did not display anti–HLA-C2 reactivity. However, we also demonstrate that the *HLA-C* genotype influences the frequency of anti–HLA-C2 reactivity, which is high in *HLA-C1* homozygous and low in *HLA-C2* homozygous donors. Therefore, our results suggest only a possible contribution of LILRB1 receptor to tolerance development and maintenance.

It has recently been proposed that NK activation is controlled by the localization of activating receptors in the NK plasma membrane. The presence or absence of inhibitory receptors with ligand specificity for self–HLA class I is, in these studies, suggested to regulate the activating receptor (45). It is possible that the 2DS1 receptor in IL-15–primed NK clones could obtain a similar localization in the plasma membrane, facilitating 2DS1 activation. Another possible mechanism for mediating self-tolerance to the HLA-C2 ligand in *HLA-C2* homozygous donors is *cis* interactions between the 2DS1 receptor and the HLA-C2 ligand on the individual NK cell (46). The present study does not address this issue. Ongoing studies with functional human NK cells in HLA class I transgenic mice may provide new insight on this issue (X.-R. Liu, Z. Zhao, L.D. Shultz, D.L. Greiner, and B. Dupont, unpublished observations).

NK alloreactivity is known to affect hematopoietic stem cell engraftment. Rejection of murine parental bone marrow grafts by F1 hybrid NK cells is regulated by missing self–MHC class I recognition, in combination with signals from activating receptor–ligand interactions. In some mouse strains, the activating NKG2D and its ligands are dominating, whereas in other strains the activating Ly49D receptor in the presence of H2-D^{d} mediates graft rejection (38, 47). Allogeneic NK cells also participate in protecting HCT recipients against leukemia relapse, and this effect is primarily observed in patients with acute myeloid leukemia (AML). The initial clinical studies involved HLA-haploidentical transplants for which the recipients lacked HLA class I ligands for inhibitory KIRs present in the donor (48). Donor-derived NK alloactivation was interpreted as being caused by "missing self–HLA class I ligand" in the recipient. Another mechanism for development of alloreactive NK cells is HLA-C2–mediated activation of 2DS1^{pos} NK cells from *HLA-C1* homozygous individuals (19, 49). We have recently demonstrated protection from relapse of AML following HCT from *2DS1* donors with the *HLA-C* genotypes *C1:C1* and *C1:C2*; this benefit is absent if the HCT donor has the *C2:C2* genotype. The present study of 2DS1^{pos} clones with anti–HLA-C2 reactivity derived from donors with different *HLA-C* genotypes provides a mechanistic interpretation of these clinical observations: *2DS1* donors with the *C1:C1* and *C1:C2* genotypes have similar ability to generate a large number of anti–HLA-C2 clones, but *HLA-C2* homozygous donors have significantly reduced frequency of such clones. These results support a model in which rejection of developing leukemic cells in many cases may be mediated by NKG2D activation of NK cells by NKG2D ligands (50, 51). Such NKG2D responses frequently require additional stimulatory signals. Amplifying, stimulatory signals might be provided by "missing self–HLA class I ligand," as observed in HLA-haploidentical HCT (48) or by donor-derived 2DS1^{pos} NK cells activated by HLA-C2 Ags in the recipient (36).

The present report addresses the functional effects of interactions between the activating receptor, 2DS1, and its ligand, HLA-C2. However, *2DS1* and the gene for another activating receptor, *3DS1,* frequently exist together owing to strong positive genetic linkage disequilibrium (35). Clinical genetic association studies of hematopoietic transplantation in AML have demonstrated different functional associations for the two genes in transplantation outcome. Specifically, *2DS1*, but not *3DS1*, was found to be associated with protection against posttransplantation leukemia relapse, whereas *3DS1* was associated with improved survival (36).

Table VI. Impact of 3DS1 expression on the anti–HLA-C2 cytotoxicity of 2DS1^{SP} clones

Donor *HLA-C* Genotype	KIR Phenotype	Target *HLA-C* Genotype	n[a] (%)	Cytolytic Yes, n (%)	 No, n (%)	p[b]
C1:C1, C1:C2	2DS1^{SP}/3DS1^{pos}	*C2:C2;Bw4*	45 (70)	33 (73)	12 (27)	NS
	2DS1^{SP}/3DS1^{neg}		19 (30)	15 (79)	4 (21)	
C2:C2	2DS1^{SP}/3DS1^{pos}	*C2:C2;Bw4*	20 (77)	7 (35)	13 (65)	NS
	2DS1^{SP}/3DS1^{neg}		6 (23)	1 (17)	5 (83)	

[a] Clones are identical to those described in Fig. 2 and Table III. One *C2:C2* clone was not included in this analysis owing to lack of cDNA for 3DS1 RT-qPCR amplification.

[b] Frequency of anti–HLA-C2 cytolytic clones in each group is compared.

In agreement with these clinical findings, we demonstrate in this article that the anti–HLA-C2 reactivity of 2DS1^{SP} clones is independent of the presence or absence of 3DS1 expression. It is currently not known how 3DS1 affects NK function, but studies of AIDS patients with HIV-1 indicate that the 3DS1 receptor might bind HLA-B (Bw4-80I) in HIV-1–infected cells and target NK cells toward infected cells (52). Collectively, these studies and ours suggest that important NK effector functions are mediated by activating receptors with ligand specificity for HLA class I.

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Disclosures

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References

1. Hogquist, K. A., T. A. Baldwin, and S. C. Jameson. 2005. Central tolerance: learning self-control in the thymus. *Nat. Rev. Immunol.* 5: 772–782.
2. Wardemann, H., S. Yurasov, A. Schaefer, J. W. Young, E. Meffre, and M. C. Nussenzweig. 2003. Predominant autoantibody production by early human B cell precursors. *Science* 301: 1374–1377.
3. Kärre, K., H. G. Ljunggren, G. Piontek, and R. Kiessling. 1986. Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy. *Nature* 319: 675–678.
4. Ljunggren, H. G., and K. Kärre. 1990. In search of the ‘missing self’: MHC molecules and NK cell recognition. *Immunol. Today* 11: 237–244.
5. Karlhofer, F. M., R. K. Ribaudo, and W. M. Yokoyama. 1992. MHC class I alloantigen specificity of Ly-49+ IL-2-activated natural killer cells. *Nature* 358: 66–70.
6. Daniels, B. F., F. M. Karlhofer, W. E. Seaman, and W. M. Yokoyama. 1994. A natural killer cell receptor specific for a major histocompatibility complex class I molecule. *J. Exp. Med.* 180: 687–692.
7. Ogasawara, K., J. Benjamin, R. Takaki, J. H. Phillips, and L. L. Lanier. 2005. Function of NKG2D in natural killer cell-mediated rejection of mouse bone marrow grafts. *Nat. Immunol.* 6: 938–945.
8. Oppenheim, D. E., S. J. Roberts, S. L. Clarke, R. Filler, J. M. Lewis, R. E. Tigelaar, M. Girardi, and A. C. Hayday. 2005. Sustained localized expression of ligand for the activating NKG2D receptor impairs natural cytotoxicity in vivo and reduces tumor immunosurveillance. *Nat. Immunol.* 6: 928–937.
9. Wiemann, K., H. W. Mittrücker, U. Feger, S. A. Welte, W. M. Yokoyama, T. Spies, H. G. Rammensee, and A. Steinle. 2005. Systemic NKG2D downregulation impairs NK and CD8 T cell responses in vivo. *J. Immunol.* 175: 720–729.
10. Sun, J. C., and L. L. Lanier. 2008. Tolerance of NK cells encountering their viral ligand during development. *J. Exp. Med.* 205: 1819–1828.
11. Tripathy, S. K., P. A. Keyel, L. Yang, J. T. Pingel, T. P. Cheng, A. Schneeberger, and W. M. Yokoyama. 2008. Continuous engagement of a self-specific activation receptor induces NK cell tolerance. *J. Exp. Med.* 205: 1829–1841.
12. Zhao, Y., H. Ohdan, J. O. Manilay, and M. Sykes. 2003. NK cell tolerance in mixed allogeneic chimeras. *J. Immunol.* 170: 5398–5405.
13. Single, R. M., M. P. Martin, X. Gao, D. Meyer, M. Yeager, J. R. Kidd, K. K. Kidd, and M. Carrington. 2007. Global diversity and evidence for coevolution of KIR and HLA. *Nat. Genet.* 39: 1114–1119.
14. Pende, D., S. Marcenaro, M. Falco, S. Martini, M. E. Bernardo, D. Montagna, E. Romeo, C. Cognet, M. Martinetti, R. Maccario, et al. 2009. Anti-leukemia activity of alloreactive NK cells in KIR ligand-mismatched haploidentical HSCT for pediatric patients: evaluation of the functional role of activating KIR and redefinition of inhibitory KIR specificity. *Blood* 113: 3119–3129.
15. Cognet, C., C. Farnarier, L. Gauthier, C. Frassati, P. André, A. Magérus-Chatinet, N. Anfossi, F. Rieux-Laucat, E. Vivier, and N. Schleinitz. 2010. Expression of the HLA-C2-specific activating killer-cell Ig-like receptor KIR2DS1 on NK and T cells. *Clin. Immunol.* 135: 26–32.
16. Fauriat, C., M. A. Ivarsson, H. G. Ljunggren, K. J. Malmberg, and J. Michaëlsson. 2010. Education of human natural killer cells by activating killer cell immunoglobulin-like receptors. *Blood* 115: 1166–1174.
17. Hsu, K. C., C. A. Keever-Taylor, A. Wilton, C. Pinto, G. Heller, K. Arkun, R. J. O’Reilly, M. M. Horowitz, and B. Dupont. 2005. Improved outcome in HLA-identical sibling hematopoietic stem-cell transplantation for acute myelogenous leukemia predicted by KIR and HLA genotypes. *Blood* 105: 4878–4884.
18. Khakoo, S. I., and M. Carrington. 2006. KIR and disease: a model system or system of models? *Immunol. Rev.* 214: 186–201.
19. Chewning, J. H., C. N. Gudme, K. C. Hsu, A. Selvakumar, and B. Dupont. 2007. KIR2DS1-positive NK cells mediate alloresponse against the C2 HLA-KIR ligand group in vitro. *J. Immunol.* 179: 854–868.
20. Trundley, A., H. Frebel, D. Jones, C. Chang, and J. Trowsdale. 2007. Allelic expression patterns of KIR3DS1 and 3DL1 using the Z27 and DX9 antibodies. *Eur. J. Immunol.* 37: 780–787.
21. Abeyweera, T. P., E. Merino, and M. Huse. 2011. Inhibitory signaling blocks activating receptor clustering and induces cytoskeletal retraction in natural killer cells. *J. Cell Biol.* 192: 675–690.
22. Uhrberg, M., N. M. Valiante, B. P. Shum, H. G. Shilling, K. Lienert-Weidenbach, B. Corliss, D. Tyan, L. L. Lanier, and P. Parham. 1997. Human diversity in killer cell inhibitory receptor genes. *Immunity* 7: 753–763.
23. Cooley, S., F. Xiao, M. Pitt, M. Gleason, V. McCullar, T. L. Bergemann, K. L. McQueen, L. A. Guethlein, P. Parham, and J. S. Miller. 2007. A subpopulation of human peripheral blood NK cells that lacks inhibitory receptors for self-MHC is developmentally immature. *Blood* 110: 578–586.
24. Dubois, S., J. Mariner, T. A. Waldmann, and Y. Tagaya. 2002. IL-15Ralpha recycles and presents IL-15 In trans to neighboring cells. *Immunity* 17: 537–547.
25. Burkett, P. R., R. Koka, M. Chien, S. Chai, D. L. Boone, and A. Ma. 2004. Coordinate expression and trans presentation of interleukin (IL)-15Ralpha and IL-15 supports natural killer cell and memory CD8+ T cell homeostasis. *J. Exp. Med.* 200: 825–834.
26. Koka, R., P. Burkett, M. Chien, S. Chai, D. L. Boone, and A. Ma. 2004. Cutting edge: murine dendritic cells require IL-15R alpha to prime NK cells. *J. Immunol.* 173: 3594–3598.
27. Huntington, N. D., H. Puthalakath, P. Gunn, E. Naik, E. M. Michalak, M. J. Smyth, H. Tabarias, M. A. Degli-Esposti, G. Dewson, S. N. Willis, et al. 2007. Interleukin 15-mediated survival of natural killer cells is determined by interactions among Bim, Noxa and Mcl-1. *Nat. Immunol.* 8: 856–863.
28. Colonna, M., F. Navarro, T. Bellón, M. Llano, P. García, J. Samaridis, L. Angman, M. Cella, and M. López-Botet. 1997. A common inhibitory receptor for major histocompatibility complex class I molecules on human lymphoid and myelomonocytic cells. *J. Exp. Med.* 186: 1809–1818.
29. Vitale, M., R. Castriconi, S. Parolini, D. Pende, M. L. Hsu, L. Moretta, D. Cosman, and A. Moretta. 1999. The leukocyte Ig-like receptor (LIR)-1 for the cytomegalovirus UL18 protein displays a broad specificity for different HLA class I alleles: analysis of LIR-1 + NK cell clones. *Int. Immunol.* 11: 29–35.
30. Borges, L., M. L. Hsu, N. Fanger, M. Kubin, and D. Cosman. 1997. A family of human lymphoid and myeloid Ig-like receptors, some of which bind to MHC class I molecules. *J. Immunol.* 159: 5192–5196.
31. Colonna, M., J. Samaridis, M. Cella, L. Angman, R. L. Allen, C. A. O’Callaghan, R. Dunbar, G. S. Ogg, V. Cerundolo, and A. Rolink. 1998. Human myelomonocytic cells express an inhibitory receptor for classical and nonclassical MHC class I molecules. *J. Immunol.* 160: 3096–3100.
32. Braud, V. M., D. S. Allan, C. A. O’Callaghan, K. Söderström, A. D’Andrea, G. S. Ogg, S. Lazetic, N. T. Young, J. I. Bell, J. H. Phillips, et al. 1998. HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C. *Nature* 391: 795–799.
33. Lee, N., M. Llano, M. Carretero, A. Ishitani, F. Navarro, M. López-Botet, and D. E. Geraghty. 1998. HLA-E is a major ligand for the natural killer inhibitory receptor CD94/NKG2A. *Proc. Natl. Acad. Sci. USA* 95: 5199–5204.
34. Foley, B., D. De Santis, L. Lathbury, F. Christiansen, and C. Witt. 2008. KIR2DS1-mediated activation overrides NKG2A-mediated inhibition in HLA-C C2-negative individuals. *Int. Immunol.* 20: 555–563.
35. Hsu, K. C., X. R. Liu, A. Selvakumar, E. Mickelson, R. J. O’Reilly, and B. Dupont. 2002. Killer Ig-like receptor haplotype analysis by gene content: evidence for genomic diversity with a minimum of six basic framework haplotypes, each with multiple subsets. *J. Immunol.* 169: 5118–5129.
36. Venstrom, J. M., G. Pittari, T. A. Gooley, J. H. Chewning, S. Spellman, M. Haagenson, M. M. Gallagher, M. Malkki, E. Petersdorf, B. Dupont, and K. C. Hsu. 2012. HLA-C-dependent prevention of leukemia relapse by donor activating KIR2DS1. *N. Engl. J. Med.* 367: 805–816.
37. Hanke, T., H. Takizawa, C. W. McMahon, D. H. Busch, E. G. Pamer, J. D. Miller, J. D. Altman, Y. Liu, D. Cado, F. A. Lemonnier, et al. 1999. Direct assessment of MHC class I binding by seven Ly49 inhibitory NK cell receptors. *Immunity* 11: 67–77.
38. George, T. C., J. R. Ortaldo, S. Lemieux, V. Kumar, and M. Bennett. 1999. Tolerance and alloreactivity of the Ly49D subset of murine NK cells. *J. Immunol.* 163: 1859–1867.
39. Kim, S., J. Poursine-Laurent, S. M. Truscott, L. Lybarger, Y. J. Song, L. Yang, A. R. French, J. B. Sunwoo, S. Lemieux, T. H. Hansen, and W. M. Yokoyama. 2005. Licensing of natural killer cells by host major histocompatibility complex class I molecules. *Nature* 436: 709–713.
40. Anfossi, N., P. André, S. Guia, C. S. Falk, S. Roetynck, C. A. Stewart, V. Breso, C. Frassati, D. Reviron, D. Middleton, et al. 2006. Human NK cell education by inhibitory receptors for MHC class I. *Immunity* 25: 331–342.
41. Elliott, J. M., J. A. Wahle, and W. M. Yokoyama. 2010. MHC class I-deficient natural killer cells acquire a licensed phenotype after transfer into an MHC class I-sufficient environment. *J. Exp. Med.* 207: 2073–2079.
42. Joncker, N. T., N. Shifrin, F. Delebecque, and D. H. Raulet. 2010. Mature natural killer cells reset their responsiveness when exposed to an altered MHC environment. *J. Exp. Med.* 207: 2065–2072.
43. Biassoni, R., A. Pessino, A. Malaspina, C. Cantoni, C. Bottino, S. Sivori, L. Moretta, and A. Moretta. 1997. Role of amino acid position 70 in the binding affinity of p50.1 and p58.1 receptors for HLA-Cw4 molecules. *Eur. J. Immunol.* 27: 3095–3099.
44. Stewart, C. A., F. Laugier-Anfossi, F. Vély, X. Saulquin, J. Riedmuller, A. Tisserant, L. Gauthier, F. Romagné, G. Ferracci, F. A. Arosa, et al. 2005.

Recognition of peptide-MHC class I complexes by activating killer immunoglobulin-like receptors. *Proc. Natl. Acad. Sci. USA* 102: 13224–13229.

45. Guia, S., B. N. Jaeger, S. Piatek, S. Mailfert, T. Trombik, A. Fenis, N. Chevrier, T. Walzer, Y. M. Kerdiles, D. Marguet, et al. 2011. Confinement of activating receptors at the plasma membrane controls natural killer cell tolerance. *Sci. Signal.* 4: ra21.
46. Doucey, M. A., L. Scarpellino, J. Zimmer, P. Guillaume, I. F. Luescher, C. Bron, and W. Held. 2004. Cis association of Ly49A with MHC class I restricts natural killer cell inhibition. *Nat. Immunol.* 5: 328–336.
47. Beilke, J. N., J. Benjamin, and L. L. Lanier. 2010. The requirement for NKG2D in NK cell-mediated rejection of parental bone marrow grafts is determined by MHC class I expressed by the graft recipient. *Blood* 116: 5208–5216.
48. Ruggeri, L., M. Capanni, E. Urbani, K. Perruccio, W. D. Shlomchik, A. Tosti, S. Posati, D. Rogaia, F. Frassoni, F. Aversa, et al. 2002. Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. *Science* 295: 2097–2100.
49. Sivori, S., S. Carlomagno, M. Falco, E. Romeo, L. Moretta, and A. Moretta. 2011. Natural killer cells expressing the KIR2DS1-activating receptor efficiently kill T-cell blasts and dendritic cells: implications in haploidentical HSCT. *Blood* 117: 4284–4292.
50. Druker, B. J., S. Tamura, E. Buchdunger, S. Ohno, G. M. Segal, S. Fanning, J. Zimmermann, and N. B. Lydon. 1996. Effects of a selective inhibitor of the Abl tyrosine kinase on the growth of Bcr-Abl positive cells. *Nat. Med.* 2: 561–566.
51. Boissel, N., D. Rea, V. Tieng, N. Dulphy, M. Brun, J. M. Cayuela, P. Rousselot, R. Tamouza, P. Le Bouteiller, F. X. Mahon, et al. 2006. BCR/ABL oncogene directly controls MHC class I chain-related molecule A expression in chronic myelogenous leukemia. *J. Immunol.* 176: 5108–5116.
52. Alter, G., M. P. Martin, N. Teigen, W. H. Carr, T. J. Suscovich, A. Schneidewind, H. Streeck, M. Waring, A. Meier, C. Brander, et al. 2007. Differential natural killer cell-mediated inhibition of HIV-1 replication based on distinct KIR/HLA subtypes. *J. Exp. Med.* 204: 3027–3036.