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

Gianfranco Pittari,* Xiao-Rong Liu,* Annamalai Selvakumar,* Zeguo Zhao,* Ernesto Merino,* Morgan Huse,* Joseph H. Chewning,*† Katharine C. Hsu,*†‡ and Bo Dupont*††

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 tolerated 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-C2 were 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: 000–000.
We report that 2DS1pos NK clones with anti–HLA-C2 reactivity can be obtained from individuals with any HLA-C genotype. The frequency of 2DS1pos clones with anti–HLA-C2 reactivity is equally high for donors with the HLA-C genotypes C1:C1 and C1:C2. In contrast, 2DS1pos 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 2DS1pos clones. In 2DL1pos NK clones, 2DS1 expression is verifiable by real-time RT-qPCR.

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 Biotech) (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-15Ra/IL-15 transfectants

The pSFG retroviral vectors containing full-length cDNA of human IL-15Ra 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-15Ra/IL-15 double-transfected pre-B-lymphocyte BaF/3 cells (BaF/3 IL-15Ra/IL-15) were obtained by limiting dilution, and stable expression of IL-15 and IL-15Ra 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 (CH1; Miltenyi Biotech), 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 (FACS Aria 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: 105 allogeneic EBV-B lymphoblastoid cell line (BLCL) (JY), 4 × 104 PBMCs obtained from three different donors, and 3 × 104 BaF/3 IL-15Ro/IL-15 cells. Feeders were γ irradiated (EBV-BLCL and PBMCs: 5.2 Gy; BaF/3 IL-15Ro/IL-15: 13.9 Gy). After sorting, plates were centrifuged at 500 rpm for 1 min and incubated in a 37°C, 5% CO2 humidified atmosphere. On day 5, 100 μl fresh medium and irradiated EBV-BLCL (105), PBMCs (4 × 104), and BaF/3 IL-15Ro/IL-15 (3 × 104) 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-15Ro/IL-15 (5 × 103). On day 22, 300 μl supernatant was removed from all wells and replaced with 500 μl medium and irradiated BaF/3 IL-15Ro/IL-15 (5 × 103). Between day 28 and 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-
pression (i.e., DX0536). 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-C2 reactivity by 2DS1+ clones. In addition, it was used in the analysis of a possible effect of 3DS1 on anti–HLA-C2 cytoactivity 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 9Cr 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 1:3 (×10^3 target cells per well). Where indicated, effectors were tested in the presence of 10 μg/ml anti-human NKGA2A, 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: GA, A*02:01/03:01, B*40:01/15:01, Cw*03:04/03:04; GB: HLA–killer cell Ig-like receptor ligand group Bw4 (Bw6); C: C1; C2; C3 30; 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 were ligated 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. RNA from Escherichia coli (New England Biolabs) was added in for-in-column digestion. For 10 pmRNA-bound cDNA (2 μN, 30°C, 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 7500 Real-Time 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 NKGA2A: 3 × 10^3–30; GAPDH: 3 × 10^3–30) 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 for each KIR and NKGA2A 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 NKGA2A was calculated as follows: [KIR or NKGA2A copy number/GAPDH copy number] × 10^7.

**Statistical analysis**

Frequencies of cytotoxic 2DS1+ 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 NKGA2A gene expression and NKGA2A 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).

**Quantitative PCR**

Primers and probes. Sequences for 2DL1 primers, as well as 2DS1, 2DL2-3, 3DS1, and NKGA2A 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 (qRT-PCR) experiments (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′-TCTCTACATGTCGCCATG-3′ (500), Reverse (Rev): 5′-AGGGCCCAGGAAGAAGTTT-3′ (500). Probe: 5′-6FAM-CAGCTTATGAGACCT-MGB-3′ (150); 2DL1: Fwd: 5′-GAGCCACCATGTCGCCCTT-3′ (300), Rev: 5′-GTCACTGGGAGCTG-ACAC-3′ (100), Probe: 5′-6FAM-CACATGGAGGTCACC-MGB-3′ (150); 2DS2: Fwd: 5′-TGCCAGAGAAGGAGAAG-3′ (300), Rev: 5′-CACGGCTCTCCGCA-3′ (300), Probe: 5′-6FAM-CTTGCGTCTCTGCGCTTG-3′ (500). GAPDH primers were 5′-CCACTGAAACGAAGTCCG-3′ (500), 5′-CCAGGACACTTGGATCA-3′ (500), 5′-6FAM-CTCGTGTGCTGCAAAAAC-MGB-3′ (150); 3DS1: Fwd: 5′-GACCCAGAGCACAACCCCA-3′ (300), Rev: 5′-TAGTGCTTCGGACGAC-3′ (300). Probe: 5′-6FAM-AATTTCCCTACG-3′ (300). Fwd: 5′-CTCCAGAGAAGCTCCATTGGT-3′ (500), Probe: 5′-6FAM-CTGATTTGTTATTTCTCCTACA-MGB-3′ (150); GAPDH: Fwd: 5′-TTCGCTCTCTGCTGCTCTCGT-3′, Rev: 5′-CTTCCGCTGCTCAGGCTTGA-3′.

Recombinant plasmids. KIR, NKGA2A, 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, 60°C, 30 s, 72°C]; and stage 3: 7 min, 72°C. PCR cycling conditions 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-C2 reactivity by 2DS1+ clones.
Results

IL-15 trans-presentation supports generation of 2DS1pos NK clones

2DS1pos 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 2DS1pos 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 FACSC-sorted NK cells with murine Ba/F3 cells transfected with human IL-15Rs and human IL-15. This procedure supported clone development from all donors, irrespective of their HLA-C genotype. Each clone reached 0.25–4 × 10⁸ 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 2DS1SP phenotype and other 2DS1pos phenotypes (Table II). For example, 2DS1SP clones were obtained from donors with each HLA-C genotype (Table II, Columns A and B). Similarly, twenty-four 2DS1SP, which also expressed the inhibitory receptor CD94/NKG2A, were obtained from donors with any HLA-C genotype, when IL-15 trans-presentation is the NK growth factor.

Frequency of 2DS1SP NK clones with anti–HLA-C2 cytotoxicity is decreased only in donors homozygous for HLA-C2

A total of 91 2DS1SP 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 2DS1SP, 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 2DS1pos clones from donors heterozygous for HLA-C2. The 22 clones from C1:C2 heterozygous donors were tested on HLA-C2 heterozygous and homozgyous 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, 2DS1SP 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 2DS1SP.

We finally determined the effect of inhibitory KIRs with ligand specificity for nonself–HLA class I on the function of 2DS1pos clones. Thirteen 2DS1pos, 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 2DS1SP, C2:C2 clones (Fig. 2A, right, Fig. 2C; Table IIIC). Therefore, 2DS1pos, HLA-C2 homozygous clones with nonself inhibitory KIR display anti–HLA-C2 reactivity comparable to that of 2DS1SP clones from the same donor.

2DS1SP NK clones with anti–HLA-C2 reactivity are present in recipients of 2DS1pos allogeneic hematopoietic stem cell transplantation

Allogeneic, myeloablative hematopoietic stem cell transplantation (HCT) provides a possibility for evaluating de novo development of donor-derived 2DS1pos NK cells in the presence of cognate HLA-C2 ligand. We investigated two cases in which the graft was obtained from 2DS1pos C1:C2 donors. 2DS1SP NK cells were identified post-HCT in both recipients (Fig. 2D). In the first case, ten 2DS1SP clones were obtained 100 d post-HCT from an HLA-C2 homozygous recipient. Four of the 2DS1SP clones displayed anti–HLA-C2 cytotoxicity (Fig. 2D, Case 1). In the second case, four 2DS1SP 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 2DS1SP NK cells with ability to mediate anti–HLA-C2 cytotoxicity can be identified in an allogeneic host that expresses the cognate HLA-C2 ligand.

2DS1pos 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

<table>
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<tr>
<th>Sample ID</th>
<th>HLA-KIR Ligand Group</th>
<th>KIR Genes</th>
<th>KIR Haplotype*</th>
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<td></td>
<td>C</td>
<td>B</td>
<td></td>
</tr>
<tr>
<td>Healthy volunteers</td>
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<tr>
<td>UDN* 001</td>
<td>C1:C1</td>
<td>Bw4</td>
<td>2DS1/2DS2/3DS1</td>
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<td>Bw4</td>
<td>2DS1/2DS2/3DS1</td>
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*KIR haplotype numbers from Khakoo and Carrington (18).
*Unique donor number.
*Absence of KIR gene.
Anti–HLA-C2 cytotoxicity of 2DS1SP clones expressing the inhibitory receptor 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 2DS1SP 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:C2 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 2DS1SP NK cells

The reduced frequency of anti–HLA-C2 reactivity observed in HLA-C2 homozygous, 2DS1SP NK cells 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 2DS1SP, HLA-C2 homozygous clones expressing CD94/NKG2A. EB6 mAb cross-linking of the 2DS1 receptor in the presence of ICAM-I induces Ca2+ 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 2DS1SP/NKG2Apos clones. NKG2A mRNA transcript copy numbers correlated well with NKG2A receptor MFI in 2DS1 SP/NKG2Apos clones from C1:C1 and C2:C2 donors (p = 0.002).

Table III. Cytotoxicity of 2DS1SP NK clones

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<tr>
<th>Donor HLA-C Genotype</th>
<th>KIR Phenotype</th>
<th>Target HLA-C Genotype</th>
<th>n</th>
<th>Yes, n (%)</th>
<th>No, n (%)</th>
<th>% Lysis Median (Range)</th>
<th>p&lt;sup&gt;a&lt;/sup&gt;</th>
<th>p&lt;sup&gt;b&lt;/sup&gt;</th>
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<tbody>
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<tr>
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<td>29 (69)</td>
<td>13 (31)</td>
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<td>19 (86)</td>
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<td>8 (30)</td>
<td>19 (70)</td>
<td>24.9 (13.5–43.4)</td>
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<td>6 (46)</td>
<td>7 (54)</td>
<td>17.7 (13.2–27.7)</td>
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</tbody>
</table>

<sup>a</sup>Frequency of anti–HLA-C2 cytolytic clones in each group is compared.

<sup>b</sup>Magnitude of anti–HLA-C2 cytotoxic responses for each clone group is compared.
Using 2DS1SP clones (Fig. 4C). Therefore, expression of CD94/NKG2A inhibits cytotoxicity against C2:C2 target cells. Statistical analysis compares the frequency of anti–HLA-C2 cytotoxic clones obtained from healthy donors. (A) 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. Donor-derived clones were tested against EBV-BLCL (IHWG 9036) target cells. Case 1, HCT 1. Cytotoxicity of 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). (B) C1:C2 clones detected in the effect of anti-NKG2A F(ab\(^{-}\))\(_9\) 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 homologous 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\(^{-}\))\(_9\), showing that CD94/NKG2A blocks 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 2DS1SP/NKG2Apos clone in which 2DS1-mediated cytotoxicity was unaffected by the presence of CD94/NKG2A (34).

The effect of anti-NKG2A F(ab\(^{-}\))\(_9\) on the cytotoxicity of 2DS1SP clones was tested (Fig. 4D). Furthermore, all 14 2DS1 SP clones with anti–HLA-C2 reactivity displayed enhanced cytotoxicity following blocking with anti-NKG2A F(ab\(^{-}\))\(_9\) (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.

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.

![Image 90x383 to 239x732](image-url)
Eight 2DS1pos 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”) was 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 2DS1SP 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 2DS1SP clones displaying anti–HLA-C2 cytotoxicity and on the 19 C2:C2 2DS1SP clones lacking anti–HLA-C2 cytotoxicity to the HLA-C2 homozygous target. 2DS1 expression levels were similar in these HLA-C2 homozygous 2DS1SP clones, irrespective of their anti–HLA-C2 responsiveness (Fig. 6).

3DS1 does not contribute to anti–HLA-C2 reactivity of 2DS1SP 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 2DS1SP clones from donors with different HLA-C genotypes were tested for 3DS1 expression. Sixty-five clones (72%) had the 2DS1pos/3DS1pos phenotype with 3DS1 mRNA copy numbers consistent with 3DS1 cell surface expression (median: 544; range: 102–2340). The remaining 25 clones had the 2DS1pos/3DS1neg 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 2DS1SP/3DS1pos and 2DS1SP/3DS1neg 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 2DS1SP clones (Fig. 2). It is demonstrated in Table VI that the presence or absence of 3DS1 does not affect anti–HLA-C2 reactivity in 2DS1SP 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 2DS1SP clones.

**Discussion**

We demonstrate that 2DS1pos 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, 2DS1pos clones from HLA-C2 homozygous 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 IV. 2DS1pos NK clones with one or more inhibitory KIR for self–HLA class I

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

### Table V. 2DS1pos C1:C1 NK clones coexpressing inhibitory KIR for self–HLA class I

<table>
<thead>
<tr>
<th>Donor HLA-C Genotype</th>
<th>KIR Phenotype</th>
<th>Target HLA-C Genotype</th>
<th>n</th>
<th>Cytolytic</th>
<th>p</th>
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<tbody>
<tr>
<td>C1:C1</td>
<td>2DS1 and 2DL2/3</td>
<td>C2:C2;Bw4</td>
<td>46</td>
<td>42 (91)</td>
<td>4 (9)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C2:C2;Bw4</td>
<td>1</td>
<td>1 (5)</td>
<td>18 (95)</td>
</tr>
<tr>
<td>C1:C1</td>
<td>2DS1 and 3DL1</td>
<td>C2:C2;Bw4</td>
<td>2</td>
<td>0 (0)</td>
<td>2 (100)</td>
</tr>
</tbody>
</table>

*Frequency of cytolytic clones to targets with different HLA-C genotype is compared.*
HUMAN NK CELL TOLERANCE TO SELF–MHC LIGAND

FIGURE 4. The inhibitory receptor CD94/NKG2A attenuates but does not block 2DS1-mediated activation signals. (A) Determination of intracellular Ca2+ concentration of a representative C2:C2 2DS1(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 Ca2+ 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(pos) NKG2A clones. This analysis was performed on C1:C1 (n = 12) and 2:C2 (n = 18) clones. (C) Correlation between NKG2A mRNA copy numbers and anti-HLA-C2 cytotoxicity in 2DS1(pos) NKG2A clones. This analysis was performed on C2:C2 (n = 26) clones. (D and E) Noncytolytic (n = 10) (D) and cytolytic (n = 14) (E) 2DS1(pos) 2: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 ≤ 0.0001. EB6, Anti-KIR2DL1/S1; HLA-E, hNKG2A ligand.

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 homologous versus heterologous 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-D9 (41, 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(pos)/3DL1(pos) clones (n = 8) 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 9306) target cells. **Center**, Cytotoxicity against C2:C2;Bw6 EBV-BLCL (DD) target cells. **Right**, Cytotoxicity against C1:C1;Bw6 EBV-BLCL (GG) 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 noncytotoxicity. The degree of cytotoxic response between different groups is compared. **p < 0.01.

FIGURE 5. 3DL1 interaction with cognate HLA-Bw4 can override 2DS1 activation. 2DS1(pos)/3DL1(pos) clones (n = 8) 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 (GG) 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 noncytotoxicity. The degree of cytotoxic response between different groups is compared. **p < 0.01.

NKG2A F(ab′)2, anti–HLA-B/C F(ab′)2, anti–HLA-C2 F(ab′)2, anti–HLA-B/C F(ab′)2, anti–HLA-C2 F(ab′)2. The dotted line represents the 13.1% cutoff between cytotoxicity and noncytotoxicity. *p < 0.05, **p ≤ 0.0001. EB6, Anti-KIR2DL1/S1; HLA-E, hNKG2A ligand.
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 2DS1pos NK cells in donors homozygous for HLA-C2. Inhibition of 2DS1 activation by CD94/NKG2A only occurs very rarely. 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).

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-Dd 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 2DS1pos 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 2DS1pos clones with anti–HLA-C2 reactivity derived from donors with different HLA-C genotypes provides a mechanistic interpretation of some 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 2DS1pos 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 2DS1pos Clones

| Donor HLA-C Genotype | KIR Phenotype | Target HLA-C Genotype | n (%) | Yes, n (%) | No, n (%) | p
<table>
<thead>
<tr>
<th></th>
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<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>C1:C1, C1:C2</td>
<td>2DS1pos, 3DS1pos, 3DS1neg</td>
<td>C2:C2, Bw4</td>
<td>45 (70)</td>
<td>33 (73)</td>
<td>12 (27)</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>2DS1pos, 3DS1neg</td>
<td></td>
<td>19 (30)</td>
<td>15 (79)</td>
<td>4 (21)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2DS1pos, 3DS1neg</td>
<td></td>
<td>20 (77)</td>
<td>7 (35)</td>
<td>13 (65)</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>2DS1pos, 3DS1neg</td>
<td></td>
<td>6 (23)</td>
<td>1 (17)</td>
<td>5 (83)</td>
<td></td>
</tr>
</tbody>
</table>

*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.

*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 2DS1P 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 currently not known how 3DS1 affects NK function, but studies of dependent of the presence or absence of 3DS1 expression. It is HLA and KIR typing; and Meighan Gallagher for technical support.

References

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Disclosures
The authors have no financial conflicts of interest.

HUMAN NK CELL TOLERANCE TO SELF–MHc LIGAND

In 3DS1-mediated activation overrides NKG2A-mediated inhibition in HLA-C

activating receptors with ligand specificity for HLA class I. AIDS patients with HIV-1 indicate that the 3DS1 receptor might currently not known how 3DS1 affects NK function, but studies of dependent of the presence or absence of 3DS1 expression. It is

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