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KIR2DS1-Positive NK Cells Mediate Alloresponse against the C2 HLA-KIR Ligand Group In Vitro

Joseph H. Chewning,* Charlotte N. Gudme,† Katharine C. Hsu,‡ Annamalai Selvakumar,† and Bo Dupont‡†

The inhibitory 2DL1 and activating 2DS1 killer Ig-like receptors (KIR) both have shared ligand specificity for codon sequences in the C2 group HLA-Cw Ags. In this study, we have investigated NK cell activation by allogeneic target cells expressing different combinations of the HLA-KIR ligand groups C1, C2, and Bw4. We demonstrate that fresh NK cells as well as IL-2-propagated NK cells from 2DS1-positive donors that are homozygous for the C1 ligand group are activated in vitro by B lymphoblastoid cell lines expressing the C2 group. This response is, in part, due to the absence of C1 group recognition mediated by the inhibitory receptor 2DL2/3. This “missing self” alloresponse to C2, however, is rarely observed in NK cells from donors lacking 2DS1. Even in presence of 2DS1, the NK alloresponse is dramatically reduced in donors that have C2 group as “self.” Analysis of selected NK clones that express 2DS1 mRNA and lack mRNA for 2DL1 demonstrates that activation by the C2 ligand and mAb cross-linking of 2DS1 in these clones induces IFN-γ. Furthermore, this C2 group-induced activation is inhibited by Abs to both HLA class I and the receptor. Collectively, these studies demonstrate that NK cells from 2DS1-positive donors are activated by target cells that express the C2 group as an alloantigen. This leads to increased IFN-γ-positive fresh NK cells and induces NK alloreactivity in IL-2-propagated polyclonal NK cells and NK clones. This study also provides support for the concept that incompatibility for the HLA-KIR ligand groups C1, C2, and Bw4 dominates NK alloactivation in vitro. The Journal of Immunology, 2007, 179: 854–868.

Activation of NK cells is tightly regulated by multiple inhibiting and activating receptors (reviewed in Ref. 1). The inhibitory receptors with MHC class I ligand specificity provide the recognition structures responsible for most of the protection against NK autoreactivity as defined by the “missing self” paradigm (2). Additional inhibitory NK receptor with non-MHC class I ligand specificity have been reported, but their significance for the functional integration and regulation of the overall NK response has yet to be determined (3). Human NK receptors that recognize HLA class I molecules belong to the C-type lectin family, the leukocyte Ig-like receptor (LILR)3 family, and the killer Ig-like receptor (KIR) family. NKG2A/CD94 is a member of the C-type lectin family and recognizes the widely expressed non-classical HLA class I molecule HLA-E (reviewed in Refs. 1 and 4). The inhibitory receptor LILRB1 (ILT2/LIR1) of the LILR family is expressed on NK cells and has ligand specificity for a broad range of HLA class I molecules (5). There are, however, four inhibitory KIRs that have ligand specificity for codon sequences present in only some HLA class I alleles: KIR2DL1, 2DL2, 2DL3, and 3DL1. The 2DL2 and 2DL3 receptors, whose respective genes share an allelic relationship, recognize with rare exceptions the HLA-Cw molecules with Ser77Asn80 in the HLA H chain: HLA-Cw1, -Cw3, -Cw7, -Cw8, -Cw12, -Cw14, and -Cw16 (HLA-KIR ligand C1 group). 2DL1 receptors recognize the HLA-Cw molecules with Asn77Lys80 in the HLA H chain: HLA-Cw2, -Cw4, -Cw5, -Cw6, -Cw15, Cw*-1602, -Cw17, and -Cw18 (HLA-KIR ligand C2 group) (reviewed in Refs. 1 and 4). KIR3DL1 recognizes HLA-B molecules possessing the Bw4 serological epitope (HLA-KIR ligand Bw4 group). Profound differences in binding affinity for KIR3DL1 exist between Bw4 allospecificities, and these disparities in binding affinity translate into differences in KIR3DL1-mediated NK inhibition (6).

There are pairs of inhibiting and activating KIRs with highly homologous codon sequences in the extracellular domain, namely KIR2DL2/KIR2DS2, KIR2DL1/KIR2DS1, and KIR3DL1/KIR3DS1. In contrast to the inhibitory KIR, ligands for the activating KIR are largely unknown despite possessing similar extracellular regions. Possible HLA class I ligand specificities for these activating KIRs have been investigated in multiple studies. It was initially observed that some NK clones that had ligand specificity for HLA-Cw4 or other C2 group molecules were activated and not inhibited by these HLA-Cw ligands (7, 8). cDNA clones corresponding to activating KIRs with putative ligand specificity for HLA-Cw3 or other C1 group molecules were identified, and some NK clones with specificity for HLA-KIR ligand C1 group have been reported (8, 9). Subsequent studies on mapping the binding sites between inhibitory
KIRs and their cognate ligands were performed and compared with the codon sequences for the corresponding activating KIRs (10–13). Most recently, studies applying KIR tetramers in binding assays have established that 2DL1 and 2DS1 both have ligand specificity for C2 group molecules (13). In contrast, it could not be conclusively determined that 2DS2 had ligand specificity for C1 group molecules (10–13). It is also evident that the inhibitory 2DL1 has significantly higher affinity for C2 group molecules than the corresponding activating receptor 2DS1 (10, 12, 13). Therefore, the inhibitory pathway appears to dominate the activating pathway, thereby preventing NK-mediated autoreactivity. The activating KIR gene 3DS1 has recently been reported to encode an activating receptor (14, 15), but no ligand specificity for the HLA-KIR ligand group Bw4 could be detected (15). The activating KIRs 2DS1, 2DS2, and 3DS1 are all associated with DAP12, which participates in the signaling events (9, 15, 16).

Activating NK cell receptors of the Ly49 gene family have been described in the mouse (17–19). The activating function of multiple murine Ly49 receptors has been described, but possible ligand specificity for MHC class I Ags has been difficult to establish (17–19). Ly49D activation of NK cells by H-2D4 has been demonstrated in some studies (18, 20, 21), but ligand specificity could not be demonstrated in another study (19). Ly49H and Ly49P have been shown to be involved with the recognition of murine CMV-infected cells, either through direct binding to virally encoded proteins or in the context of host H-2 molecules (22, 23). These and other studies have led to the concept that activating KIR genes and activating Ly49 possibly have evolved from the homologous inhibitory receptors as recognition receptors for pathogens (24).

We have in this study investigated NK cell activation by allelic target cells expressing different combinations of the HLA-KIR ligand groups. We demonstrate that freshly isolated and IL-2 propagated NK cells from donors that are positive for activating KIR2DS1 and homozygous for the HLA-KIR ligand group C1 are activated in vitro by lymphoblastoid cell lines (BLCLs) expressing the HLA-KIR ligand group C2. This response is due to both the absence of C1 group-mediated inhibition by KIR2DL2/3 (i.e., “missing self”) and a direct activation of the 2DL1/2DS1 population. Allorecognition of the C2 group was rarely observed in NK cells from donors lacking 2DS1. This study provides support for the concept that incompatibility for the HLA-KIR ligand C1, C2, and Bw4 groups dominates NK alloactivation in vitro and probably also in vivo.

Materials and Methods

Cells

PBMCs were obtained from 106 normal volunteer donors. The studies were approved by the Institutional Review Board of Memorial Sloan-Kettering Cancer Center and all blood samples were obtained with consent. After Ficoll-Hypaque density centrifugation of anticoagulated whole blood, NK cells were obtained by negative selection of CD56+ cells from CD3+ T cells, CD20+ B cells, and CD14+ monocytes with mAb-coated immunomagnetic beads (MACS; Miltenyi Biotec). The postsort purity of NK cells (CD56+ CD3−) was determined by FACS and was >90% for all experiments. Following isolation, NK cells were either used in functional assays as fresh NK cells or cocultured with irradiated allogeneic PBMC and an EBV-transformed allogeneic BLCL (JY) and activated on day 5 with 300 IU/ml IL-2 (provided by the National Cancer Institute/Biological Response Modifiers Program, Frederick, MD). IL-2-propagated, polyclonal NK cells were studied after 3–5 wk of culture (25, 26). After MACS sorting for the CD56+, CD3− subpopulation of freshly isolated PBMC from selected donors, NK clones were generated as previously described (25, 26). Briefly, NK cells were plated in limiting dilution in 48-well tissue culture plates (Robbins Scientific, Sunnyvale, CA), and were cocultured with 1 × 105/ml irradiated allogeneic PBMC and 1 × 105/ml irradiated BLCL (JY). NK cell clones were cultured in IMDM with 10% FCS containing heat-inactivated human AB serum (Pel-Freez Biologicals) and 300 IU/ml IL-2. NK clones were replated into 48-well plates along with additional feeders as described above and allowed to grow until an adequate numbers of cells were reached. Clones were then characterized for receptor phenotype by FACS.

BLCLs were either obtained from the International Histocompatibility Working Group (Seattle, WA) or generated in our laboratory. Cell lines were grown in RPMI 1640 with 10% FCS. All cell lines tested negative for mycoplasma by the mAb Core Facility, Sloan-Kettering Institute (New York, NY) and were grown in culture for up to 3 mo continuously before being discarded.

The P815 murine mastocytoma cell line was obtained from American Type Culture Collection. The class I negative cell line 721.221 and 721.221 transfected with HLA-Cw C*0304 and C*0401 were gifts from P. Parham (Stanford University, Palo Alto, CA).

Antibodies

mAbs for NK cell phenotyping and functional assays are shown in Table I. The mAb 4E is a mouse Ab with specificity for human HLA class I and Cw Ags (27).

HLA and KIR genes for NK cell donors and BLCL target cells

HLA typing was performed on genomic DNA using a combination of sequence-based amplification (PCR amplification sequence-specific primer [SSP]) and oligonucleotide probing of genomic DNA (PCR sequence-specific oligonucleotide probe) as previously reported (28). Characterization of HLA-B and HLA-Cw alleles allowed for assignment of the major HLA-KIR ligand groups C1, C2, and Bw4 to each NK cell donor and each EBV-BLCL. The HLA class I genotypes and HLA-KIR ligand groups for each of the EBV-BLCLs are displayed in Table II. The target cell panel includes 11 C1 group homozygous cells, seven C2 group homozygous cells, and two C1/C2 heterozygous cells. Both target cells and NK donors represent a variety of HLA-A allele combinations. KIR genotyping was performed on genomic DNA using PCR-SSP typing according to previously described methods (28–30). The most common KIR haplotype in most populations is the KIR-A haplotype (KIR-A), which contains the inhibitory KIR genes for the C1 group, the C2 group, and the Bw4 group (30), and only two activating KIR genes, 2DL4 (31) and 2DS4 (30). The ligand specificity for 2DL4 is probably an endocytosed receptor for soluble HLA-A (32), whereas the C2 group molecules have been proposed as ligands for 2DS4 (33). In contrast to the KIR-A haplotype, the KIR-B haplotype (KIR-B) contains different combinations of other activating KIR genes, including KIR2DS1 and/or 2DS2 (4, 29, 30, 34). KIR-A and KIR-B haplotypes were assigned to each NK donor according to the classification previously described (34). Haplotype numbering is adapted from Hsu et al. (34) and Carrington and Norman (35) (for the latter, see http://ncbi.nlm.nih.gov/htbin/uhtbin/query.fcgi?db=Books).

The HLA class I genotype, the HLA-KIR ligand group, and the KIR genotype for each NK cell donor is shown in Table III.

Cytotoxicity assay

Cytotoxicity assays were performed using IL-2-propagated NK cells and NK clones as effectors against a panel of 51Cr-labeled target cells, including EBV-BLCL and 721.221 alone and 721.221 transfected with HLA class I. Specific target cells used in each experiment are indicated in the text. Assays were performed in duplicate or triplicate for 4 h at the indicated E:T ratios. The percent specific lysis was calculated as previously described (26). Where indicated, targets were tested in the presence (10 μg/ml) or absence of anti-HLA-B and HLA-Cw mAb, 4E (Fab′)2, and control sheep anti-mouse (Fab′)2. For Ab-mediated receptor-blocking experiments, effector cells were incubated in the presence (10 μg/ml) or absence of the mAbs EB6 (IgG1), GL183 (IgG1), and CD56 (IgG1) or isotype control.

Redirected cytotoxicity assays were performed using IL-2-propagated NK cells and NK clones as effectors against 51Cr-labeled P815 cells. The P815 cell line was incubated in the presence (10 μg/ml) or absence of the mAbs EB6, GL183, and CD56 or isotype control. Assays were performed in triplicate for 4 h and the percentage of specific lysis was calculated.

NK cell stimulation

For detection of intracellular IFN-γ production by multicolor flow cytometry following receptor cross-linking, 96-well enzyme immunoassay/radioimmunoassay, high-binding plates (Fisher Scientific) were prelabeled with the indicated Abs for 4–6 h at 4°C. A total of 2 × 105 NK cells were then added to wells in a volume of 200 μl with GolgiPlug according to the
manufacturer’s recommendations (BD Biosciences). Plates were incubated at 37°C on a continuous shaker for 12–16 h. Cells were fixed and stained with the indicated Abs.

For BLCL assays, 2 × 10^5 NK cells were plated into 96-well round-bottom plates with indicated target cells (E:T ratio of 1:1) in a final volume of 250 µl. GolgiPlug was added as above, and plates were incubated at 37°C for 12–16 h. Cells were fixed and stained with the indicated Abs.

For the detection of cytokine production by ELISA, 2 × 10^5 NK cells were incubated in precoated plates or with the indicated target cells as described above. Plates were incubated at 37°C for 12–16 h and

Table I.  **Monoclonal Abs**

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<th>Molecule (CD Marker)</th>
<th>Clone(s)</th>
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<td>FTTC, PE, PerCP</td>
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<td>FITC</td>
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</table>

**Functional Assays**

| CD16                  | 3G8 (IgG1) | Purified | Beckman Coulter |
| CD56                 | N901 (IgG1) | Purified | Beckman Coulter |
| KIR2DL1, 2DS1 (CD158a/b) | EB6 (IgG1) | Purified | Beckman Coulter |
| KIR2DL2/3, 2DS2 (CD158b/j) | GL183 (IgG1) | Purified | Beckman Coulter |
| HLA-B, Cw            | 4E (IgG2a) | (Fab')2 | MSKCC Monoclonal Core Facility |

<sup>a</sup> Isotype controls were obtained from BD Biosciences.

<sup>b</sup> Memorial Sloan-Kettering Cancer Center, New York, NY.

Table II.  **Target HLA class I**

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<sup>a</sup> Cell identification for those cells obtained from the International Histocompatibility Working Group is based on a four-digit identifier beginning with “90” (i.e. 90xx). Cells prepared locally are labeled according to another four-digit identifier beginning with “92” (i.e. 92xx). Autologous cells generated from donors included in this study are labeled according to the three-digit donor identifier (e.g., 001).

<sup>b</sup> Homozygous.

<sup>c</sup> Heterozygous.
superantigens were harvested. IFN-γ, TNF-α, or GM-CSF levels were measured by ELISA (R&D Systems) according to manufacturer’s recommendations.

**KIR mRNA isolation and cDNA preparation**

mRNA from NK cell clones was prepared using μMAC mRNA isolation (Miltenyi Biotec) according to the manufacturer’s instructions. Briefly, 1–2 × 10⁶ NK IL-2-propagated NK cells or 0.5–0.7 × 10⁶ NK clones were washed twice with PBS and lysed with 1 ml of lysis buffer with vigorous vortexing. The lysate was mixed with 50 μl of oligo(dT) microbeads and transferred to the μMACS column in a magnetic field after rinsing with lysis buffer. The column was washed and cDNA was released using cDNA release solution. The temperature was set at 42°C for 1 h using a thermoMACS magnet. The MACS column was equilibrated with equilibration buffer and reverse transcriptase was added. The temperature was set at 42°C for 1 h using a thermomACS magnet. The column was washed and cDNA was released using cDNA release solution. After 10 min, the cDNA was eluted using elution buffer.

**RT-PCR amplification**

**KIR2DL1, KIR2DS1, and KIR2DL4** expressions were determined directly from cDNA with SSP amplification (2DL1 forward, 5'-GGGTGGTCACCACTGTGCTC; 2DL1 reverse, 5'-GGGTGGTCACCACTGTGCTC; 2DS1 forward, 5'-TCTTCCCATGCTGCTGATA(G/A)-3'; and 2DS1 reverse, 5'-AAGGCCCAGCAGAAGTTTCC-3') as described previously (30). The **KIR2DL4** forward and reverse primers (2DL4 forward, 5'-GGGTGGTCACCACTGTGCTC; and 2DL4 reverse, 5'-GGGTGGTCACCACTGTGCTC) were designed for specific amplification of 2DL4 exon 3 (36). Five microliters of cDNA was used as a template with the described PCR conditions of 94°C for 2 min followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s followed by an extension of 72°C for 7 min. For the **KIR2DL4** annealing temperature 65°C for 30 s was used. The amplified products were mixed with 6x loading dye and electrophoresed on 2% Tris-borate-EDTA agarose containing ethidium bromide. Previously genotyped samples were used as positive controls.

**Generation of EBV-BLCL blasts**

A total of 2 × 10⁶ PBMCs were isolated from selected donors and incubated for 3–4 days with the EBV-containing supernatant of the mammoset cell line B95-8 (provided by R. J. O’Reilly, Memorial Sloan-Kettering Cancer Center, New York, NY) in the presence of 1 μg/ml cyclosporin in RPMI 1640 (In vitro Use Life Technologies), 20% heat-inactivated FCS, and 1% L-glutamine. Subsequently, the cells were washed and recultured with RPMI 1640, 20% FCS, and L-glutamine and expanded according to the growth and cell number. The generation of EBV-BLCL was performed for donor nos. 001, 002, 003, 011, 012, 013, 022, and 028 (Table III).

**Statistical analysis**

Fisher’s exact test was applied for analysis of the effects of HLA-KIR ligand group compatibility between NK effectors and allogeneic target cells. The Wilcoxon rank sum test was used to compare relative NK cell cytotoxicity results against target cells. Paired (sign test) analysis was used to test IFN-γ response in Ab cross-linking and EBV-BLCL assays. p < 0.05 was considered significant.

**Results**

**Allocytotoxicity and “HLA-KIR ligand groups” in IL-2-stimulated polyclonal NK cells**

**KIR-A homozygous NK cell donors.** It is well established that inhibitory receptors for “self” MHC class I are responsible for the lack of NK cell cytotoxicity in vitro against autologous BLCLs (25), whereas NK cells from some patients with a lack of HLA class I caused by homozygous TAP deficiency are autoreactive (37, 38). We first investigated the role of the three major HLA-KIR ligand, two were homozygous, and two were heterozygous (A1/C2 (Table II and Fig. 1A). Three of the donors were homozygous for the C1 HLA-KIR ligand, two were homozygous for the C2 HLA-KIR ligand, and two were heterozygous C1/C2 (Table II and Fig. 1A). Three of the donors were homozygous **Bw4/Bw6**, one was **Bw4** homozygous, and two were **Bw6** only (Table III and Fig. 1C). As shown in Fig. 1B, none of the effector cells displayed a clear cytotoxic response in dose titration studies against a panel of BLCLs representing all combinations of
the HLA-KIR ligand groups. We therefore analyzed all cytotoxicity responses at a single E:T ratio (20:1). Of the 32 tests, only three demonstrated specific cytotoxicity of 13.2% cytotoxicity (mean ± 2SD) (Fig. 1D). We then compared all combinations of effector cells and target cells for HLA-KIR ligand compatibility (39–42). The absence of at least one HLA-KIR ligand group on a target cell compared with the NK effector cell occurred in 22 of 32 combinations, and all three positive responses were in that group. In contrast, none of the 10 effector combinations with target cells expressing the same HLA-KIR ligand groups as the NK effector cells resulted in positive cytotoxicity. The results are consistent with the HLA-KIR ligand compatibility concept. There were, however, 19 combinations that lacked at least one HLA-KIR ligand group and surprisingly did not result in cytotoxicity. These results were analyzed for statistical significance based on the HLA-KIR ligand incompatibility model (Table IV). The pattern of in vitro
Cytotoxicity by polyclonal, IL-2-propagated NK cells from KIR-A homozygous donors did not demonstrate a significant association with incompatibility for the HLA-KIR ligand groups Bw4, C1, and C2 (Table IV) (*p* = 0.534).

**2DS1-positive NK cell donors.** Because the activating KIR2DS1 is known to have detectable affinity in vitro for the C2 HLA-KIR ligand group (10, 12, 13), we hypothesized that the C2 group Ags could contribute to alloactivation of NK cells in 2DS1-positive donors lacking C2 group Ags. The KIR-B haplotype contains different combinations of activating KIR genes including KIR2DS1 and/or 2DS2. Fourteen NK cell donors positive for 2DS1 but with different combinations of KIR-B genotypes were selected for further studies (Fig. 2). HLA class I and KIR haplotype designations were obtained for 14 KIR2DS1-positive donors (left portion) and a summary of donor KIR2DS1 and HLA-KIR ligand groups (right portion). A filled box indicates presence of a gene and an open box indicates the absence of a gene. The section symbol (§) denotes a gene encoding normal full-length KIR2DS4 alleles and the double dagger (‡) denotes a deletion mutant allele of 2DS4. Because the activating KIR2DS1 is known to have detectable affinity in vitro for the C2 HLA-KIR ligand group (10, 12, 13), we hypothesized that the C2 group Ags could contribute to alloactivation of NK cells in 2DS1-positive donors lacking C2 group Ags. The KIR-B haplotype contains different combinations of activating KIR genes including KIR2DS1 and/or 2DS2. Fourteen NK cell donors positive for 2DS1 but with different combinations of KIR-B genotypes were selected for further studies (Fig. 2). HLA class I and KIR haplotype designations were obtained for 14 KIR2DS1-positive donors (left portion) and a summary of donor KIR2DS1 and HLA-KIR ligand groups (right portion). A filled box indicates presence of a gene and an open box indicates the absence of a gene. The section symbol (§) denotes a gene encoding normal full-length KIR2DS4 alleles and the double dagger (‡) denotes a deletion mutant allele of 2DS4.

**Table IV.** HLA-KIR ligand group and NK cell allocytotoxicity

<table>
<thead>
<tr>
<th>NK Effector</th>
<th>N*</th>
<th>+/+</th>
<th>+/-</th>
<th>-/-</th>
<th>--/+</th>
<th>--/-</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>KIR-A homozygous</td>
<td>32</td>
<td>10</td>
<td>0</td>
<td>19</td>
<td>3</td>
<td>0.534</td>
<td></td>
</tr>
<tr>
<td>2DS1-positive</td>
<td>60</td>
<td>22</td>
<td>1</td>
<td>8</td>
<td>29</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>C1 group homozygous</td>
<td>13</td>
<td>4</td>
<td>1</td>
<td>12</td>
<td>0.128</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2DS1-positive</td>
<td>44</td>
<td>15</td>
<td>4</td>
<td>13</td>
<td>12</td>
<td>0.128</td>
<td></td>
</tr>
<tr>
<td>C2 group homozygous</td>
<td>13</td>
<td>4</td>
<td>1</td>
<td>12</td>
<td>0.128</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Number of test combinations.

b Ligands present on both effector and target without cytotoxicity.

c Ligands present on both effector and target with cytotoxicity.

d Ligands absent on target, present on effector without cytotoxicity.

e Ligands absent on target, present on effector with cytotoxicity.

f Calculated by Fisher’s exact test.

FIGURE 2. Polyclonal, IL-2-propagated NK cells from 2DS1-positive, C1 group homozygous donors mediate cytotoxicity against most HLA-KIR ligand-incompatible BLCLs, but only when the C2 group is an alloantigen. A, Complete KIR genotyping results for 14 KIR2DS1-positive donors (left portion) and a summary of donor KIR2DS1 and HLA-KIR ligand groups (right portion). A filled box indicates presence of a gene and an open box indicates the absence of a gene. The section symbol (§) denotes a gene encoding normal full-length KIR2DS4 alleles and the double dagger (‡) denotes a deletion mutant allele of 2DS4. B, Polyclonal, IL-2-expanded NK cells from six 2DS1-positive, C1 group homozygous donors (donors no. 001, 002, 003, 013, 016, and 018) were used as effectors in 51Cr-release assays against 17 allogeneic BLCLs (9004, 9010, 9013, 9018, 9025, 9026, 9027, 9031, 9035, 9038, 9046, 9087, 9201, 9202, 001, 002, and 011) and four autologous BLCLs (001, 002, 003, and 013) shown according to HLA-B and HLA-Cw group. C, Polyclonal NK cells from four 2DS1-positive, C2 group homozygous donors (donors no. 011, 012, 014, and 015) were used as effectors against a panel of twelve allogeneic BLCLs (9004, 9010, 9018, 9025, 9027, 9031, 9035, 9038, 9046, 9087, 9201, 001, 002, and 011) and two autologous BLCLs (011 and 012). The stippled line at 13.2% in B and C represents the uppermost level of nonspecific cytotoxicity (described previously). E:T ratio of 20:1 was used in all experiments, and the number of experiments with each target group is shown in parentheses.
for all NK donors are shown in Table III, and their complete KIR genotype and HLA-KIR ligand groups are summarized in Fig. 2A. IL-2-propagated polyclonal NK cells were generated from six 2DS1-positive, C1 group homozygous donors and tested against a panel of 17 allogeneic and four autologous BLCL target cells (Fig. 2B). All combinations of effector cells and target cells were compared for HLA-KIR ligand groups on NK effector cells and target cells. The absence of at least one HLA-KIR ligand group on the target cell compared with the effector cell occurred in 37 of 60 combinations, and 29 had a positive cytotoxicity response of \( >13.2\% \). In contrast, only one of 23 effector combinations with target cells expressing all corresponding HLA-KIR ligand groups resulted in positive cytotoxicity (\( p < 0.001 \)) (Table IV). The HLA-KIR ligand incompatibility model for explaining NK cytotoxicity against allogeneic BLCL targets (39–42) is strongly supported by these results. The results are in contrast to those obtained in studies of KIR-A homozygous effectors, where 19 of 22 combinations in the HLA-KIR ligand incompatible group did not mediate a cytotoxic response. The results also support the hypothesis that the presence of 2DS1 in NK donors lacking C2 HLA-KIR ligands contributes to NK allocytotoxicity.

We therefore predict that a contribution of 2DS1 to the activation of polyclonal NK cells would be diminished in NK donors homozygous for the C2 HLA-KIR ligand group. IL-2 stimulated NK cells were prepared from four donors with these combinations of HLA and KIR genotypes and used as NK effectors against the allogeneic target cells (Fig. 2, A and C). The absence of at least one HLA-KIR ligand group on target cells compared with effector cells occurred in 25 of 44 combinations, and 12 had a positive cytotoxicity response of \( >13.2\% \). In contrast, only four of 19 effector combinations with target cells expressing all corresponding HLA-KIR ligand groups resulted in positive cytotoxicity (\( p = 0.128 \)) (Table IV). The HLA-KIR ligand incompatibility model for explaining NK cytotoxicity against allogeneic BLCL targets is therefore not supported when the NK donor has the activating 2DS1 but is homozygous for the C2 HLA-KIR ligand group. Thus, polyclonal NK cells from 2DS1-positive donors mediate allocytotoxicity against C2 group-positive BLCL, which can readily be detected in donors lacking the C2 group. The results support that incompatibility for HLA-KIR ligands is an important component in NK activation in such donors. The effect of the presence of 2DS1 disappears when the C2 group is “self.” These results do not address the mechanisms involved in NK alloreactivity. Furthermore, our results do not address whether a direct interaction between a C2 group Ag and 2DS1 also contributes to NK activation against C2 group-positive target cells.

**NK clones demonstrating activating function against C2 group-positive BLCL can be generated from 2DS1-positive, C1 group homozygous donors**

In our studies of IL-2-propagated polyclonal NK cells we have demonstrated that NK cytotoxicity against allogeneic BLCLs is at least in part mediated by the incompatibility for HLA-KIR ligands for inhibitory receptors that have ligand specificity for C1, C2, or Bw4. We also determined that this model for explaining NK allocytotoxicity in vitro was statistically significant for NK effector cells from 2DS1-positive, C1 homozygous donors. We therefore hypothesize that the frequency of NK clones positive for the KIRs detected by mAb EB6 (2DL1/S1) and activated by C2-positive BLCLs was higher in NK donors homozygous for C1 than in donors homozygous for C2 group.

NK clones were generated from three donors that fulfilled the criteria of being 2DS1 gene positive and homozygous for C1 group (donors no. 001, 002, and 016). All three donors also lacked the expressed alleles of KIR2DS4 (Fig. 2A) (29, 34). NK clones were phenotyped by FACS staining for the presence of KIR2DL1/2DS1, KIR2DL2/2DL3/2DS2, and KIR3DL1. NK clones phenotypically positive with the mAb EB6 were then screened in functional 51Cr-release assays against allogeneic BLCL homozygous for the C1 and C2 groups as well as 721.221 untransfected and transfected with HLA-Cw4 (721.221 plus Cw4) (Table V). NK clones demonstrating inhibitory function against 721.221 alone and lacking cytotoxicity against C2 group-positive BLCL were considered inhibitory (inhibition). Those clones cytotoxic to C2 group-positive BLCL and demonstrating cytotoxicity against 721.221 alone and 721.221 plus Cw4 were considered to have C2 group-associated activation (cytotoxicity). Those clones not demonstrating definitive cytotoxicity against 721.221 were not studied further. Cytotoxic EB6-positive NK clones were obtained from all three 2DS1-positive and C1 group homozygous donors. As shown in Table V, the frequency of NK clones demonstrating cytotoxicity against the C2 group varied among the C1 group homozygous donors. NK clones were also generated from three 2DS1-positive C2 group homozygous donors (donors no. 011, 012, and 031). All EB6-positive NK clones obtained from these donors demonstrated C2 group-directed inhibition (cytotoxicity against 721.221 alone and inhibition against 721.221 plus Cw4) (Table V).

Fourteen EB6 clones from donors no. 001, 002, and 016 displaying specific cytotoxicity against C2 group-positive target cells as described in Table V were chosen for receptor- and ligand-blocking studies (Fig. 3A). Cytotoxicity against C2 group homozygous BLCL was significantly reduced after receptor (mAb EB6) (\( p < 0.01 \)) or ligand (mAb 4E (Fab’\(_{2}\)) (\( p < 0.01 \)) blockade (Fig. 3A). EB6-negative NK clones from these donors were not cytotoxic to C1 or C2 group homozygous BLCL but did lyse the HLA class I-negative 721.221 (data not shown). All clones shown in Fig. 3A were positive for the inhibitory NKG2A/CD94 receptor as well as the natural cytotoxicity receptors (NK p46, p44, and p30) and NKG2D. The presence or absence of the inhibitory receptor LILR81 (ILT2) was not correlated with C2 group-directed cytotoxicity (data not shown).

Additional EB6-expressing NK clones generated from donor no. 001 demonstrating C2 group allocytotoxicity were tested in redirected cytotoxicity assays using the murine mastocytoma cell line

<table>
<thead>
<tr>
<th>Donor Identification</th>
<th>Total NK Clones(^{a})</th>
<th>Total EB6(^{+}) clones(^{b})</th>
<th>Cytotoxicity Target: C2 group BLCL</th>
<th>Inhibition Target: 721.221 plus C2 group</th>
</tr>
</thead>
<tbody>
<tr>
<td>KIR2DS1-positive, C1 homozygous donors</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>001</td>
<td>93</td>
<td>46</td>
<td>45 (98%)</td>
<td>1 (2%)</td>
</tr>
<tr>
<td>002</td>
<td>21</td>
<td>9</td>
<td>2 (22%)</td>
<td>7 (78%)</td>
</tr>
<tr>
<td>016</td>
<td>23</td>
<td>7</td>
<td>3 (43%)</td>
<td>4 (57%)</td>
</tr>
<tr>
<td>KIR2DS1-positive, C2 homozygous donors</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>011</td>
<td>31</td>
<td>13</td>
<td>0 (0%)</td>
<td>13 (100%)</td>
</tr>
<tr>
<td>012</td>
<td>7</td>
<td>3</td>
<td>0 (0%)</td>
<td>3 (100%)</td>
</tr>
<tr>
<td>031</td>
<td>6</td>
<td>3</td>
<td>0 (0%)</td>
<td>3 (100%)</td>
</tr>
</tbody>
</table>

\(^{a}\) Total number of NK clones obtained and phenotyped from indicated donors.

\(^{b}\) Number (and percentage) of EB6-staining NK clones that demonstrated specific cytotoxicity to C2 group-positive BLCL.

\(^{c}\) Number (and percentage) of EB6-staining NK clones that demonstrated specific inhibition against C2 group-transfected 721.221.

Table V. NK cell clone phenotype and function
In these experiments, EB6-labeled P815 cells were lysed significantly more than CD56-labeled P815 cells (Fig. 3B). These studies demonstrate that EB6-expressing clones, selected for cytotoxic function to C2 group (as described in text), were tested against 10 allogeneic (H9004, H9018, H9025, 9031, 9032, 9087, 9201, 001, 002, and 011) and two autologous (001 and 002) 51Cr-labeled BLCL target cells. The figure only includes data from HLA-Bw4 BLCL, as well as positive and negative controls. All NK clones were positive for the heterodimeric inhibitory receptor NKG2A/CD94. Receptor blockade (mAb EB6), KIR ligand blockade (mAb 4E1 [Fab’2]), and mAb isotype controls are included. The E:T ratio was 10:1 for these experiments. The numbers of NK clones tested on each target cell group are shown in parentheses. B. Three NK clones (clones 1, 2, and 3) from donor no. 001, phenotypically positive for EB6 (2DL1/2DS1) and previously shown to be cytotoxic to C2 group-positive BLCL, were used in redirected cytotoxicity assays against a P815 murine mastocytoma cell line labeled with EB6 or CD56 control. E:T ratio titration curves are shown. Mean cytotoxicity is demonstrated by the symbols and error bars indicate SEM. Results are representative of five independent experiments.

P815. In these experiments, EB6-labeled P815 cells were lysed significantly more than CD56-labeled P815 cells (Fig. 3B).

These results indicate that these clones expressed the inhibitory receptor 2DL3 in addition to 2DS1.

Cytokine release by EB6-positive NK clones following receptor cross-linking is correlated with the presence of KIR2DS1 mRNA

We hypothesized that EB6-positive NK clones releasing increased levels of activating cytokines following receptor cross-linking would possess 2DS1 mRNA. To test this hypothesis, we generated NK clones from a 2DS1-positive, C1 homozygous donor (donor no. 023). Three NK clones phenotypically positive for mAb EB6 were selected and used in receptor cross-linking experiments with plate-immobilized Abs. Supernatants were tested for IFN-γ, TNF-α, and GM-CSF levels. One of the clones produced increased IFN-γ following EB6 cross-linking (clone no.33) while the other two were not activated (clones no.26 and 39) (Fig. 4A). Clone no.33 also produced increased levels of TNF-α (data not shown). KIR2DS1 mRNA was present and 2DL1 mRNA was absent in clone no.33 (Fig. 4B). In contrast, the NK clones no.26 and 39 that were not activated by EB6 cross-linking possessed...
only 2DL1 mRNA and not 2DS1 mRNA (Fig. 4B). Seven additional EB6-positive NK clones from three donors positive for KIR2DS1 and homozygous for both HLA-Bw4 and the HLA-Cw C1 group (donors no. 001, 013, and 016) were used as effectors against four BLCL targets (9004, 9201, 9205, and 028) in 51Cr-release assays. A, E:T ratio titration curves for each target BLCL categorized by the HLA-B and HLA-Cw groups. Symbols represent mean cytotoxicity with error bars indicating SEM. The graph displays results from four independent experiments. B, Individual data points for an E:T ratio of 20:1 in A are shown for experiments against HLA-Bw4 homozygous BLCL. The stippled line at 13.2% indicates nonspecific cytotoxicity as described previously. C, Three NK clones from donor no. 001 were selected based on the presence (clones no. 3 and 13) or absence (clone no. 10) of anti-C2 group cytotoxicity in 51Cr-release assays and used in additional cytotoxicity assays against a panel of five BLCL (9004, 9201, 9205, 002, and 011) targets, in addition to 721.221 as positive control. Clone no. 013 was only tested against two BLCLs (9004 and 9201) secondary to low cell numbers. mRNA was isolated from clones no. 3, 10, and 13 and cDNA was generated as described (see Materials and Methods). D, Analysis for the presence of KIR2DS1, KIR2DL1, and KIR2DL4 according to clone number. M indicates marker lane, the plus sign (+) is positive control, and the minus sign (−) is negative control. Data are representative of 31 EB6-positive NK clones.

C2 group-directed cytotoxicity by 2DS1-positive, C1 homozygous donors is present against HLA-Cw C group heterozygous targets in polyclonal populations

We have so far demonstrated that polyclonal NK cells from 2DS1-positive, C1 homozygous donors mediate allocytotoxicity, which conforms to the “HLA-KIR ligand incompatibility” model (Table IV). We therefore tested whether polyclonal IL-2-propagated NK cells from such donors also generated NK allocytotoxicity against C1/C2 heterozygous BLCL target cells. Polyclonal IL-2-propagated NK cells were generated from three HLA-Bw4 homozygous, C1 homozygous donors (donors no. 001, 013, and 016) (Table III) and tested against a panel of HLA-KIR ligand homozygous and heterozygous targets. No cytotoxicity was obtained against the C1 group homozygous targets, whereas the C2 group homozygous cells were lysed efficiently. Importantly, NK cells from these donors were also cytotoxic against the C1/C2 heterozygous targets (Fig. 5A). Individual data points from the E:T ratio of 20:1 for four independent experiments are displayed in Fig. 5B. Because all three donors were HLA-Bw4 homozygous, only the Bw4 group-positive target cells are shown. The positive cytotoxic response
panel of BLCLs (Fig. 5, against C2 group homozygous targets were tested against the same donor (donor no. 001) demonstrating cytotoxicity. Additional studies were performed on mRNA obtained from cells and Z27 (anti-3DL1/3DS1) and did not stain positive for these receptors recognizing “self” HLA class I for this donor are displayed on the x-axis and IFN-γ staining is displayed on the y-axis. The Abs used for individual receptors are described in Materials and Methods. KIR3DL1 staining was performed using clone DX9 only. B, Summarized IFN-γ staining results following CD56 isotype control and EB6 (left) or CD16 positive control (right) cross-linking for eight experiments with freshly isolated NK cells from these six 2DS1-positive, C1 group homozygous donors.

observed against the Bw4 homozygous, C1/C2 heterozygous target cells mediated by 2DS1-positive, Bw4 positive, C1 homozygous NK effectors is not fully explainable by the missing HLA-KIR ligand group on the target cells. A similar pattern of C2-group directed cytotoxicity was also seen in the HLA-Bw6-positive BLCL targets (data not shown).

Three EB6-positive NK clones from a 2DS1-positive, C1 homozygous donor (donor no. 001) demonstrating cytotoxicity against C2 group homozygous targets were tested against the same panel of BLCLs (Fig. 5, C and D). Clone no. 3 was cytotoxic against Bw4 C2 homozygous and Bw6 C2 homozygous target cells but not against Bw4 C1 or Bw6 C1 targets. Cytotoxicity was also detected on Bw6 C1/C2, but this clone was not tested on the Bw4 C1/C2 target. These data support that clone no. 3 is activated by the C2 HLA-KIR ligand presented by the C1/C2 heterozygous target, but we cannot exclude other possibilities. NK cells from donor no. 001 were repeatedly tested with DX9 (anti-KIR 3DL1) and Z27 (anti-3DL1/3DS1) and did not stain positive for these KIRs (14, 15). A possible role for 3DL1 and 3DS1 in the NK alloactivation of this donor can therefore be excluded. The presence or absence of the natural cytotoxicity receptors NKG2C/CD94, NKG2A/CD94, and LILRB1 was not associated with C2 group-directed activation (data not shown). mRNA studies revealed that C2 group cytotoxic NK clones (no. 3 and 13) were positive for 2DS1 mRNA but not for 2DL1 mRNA. The C2 group inhibitory clone no. 10 possessed both 2DS1 and 2DL1 mRNA. Clone no.10 appeared to have a larger amount of 2DL1 mRNA than 2DS1 mRNA, consistent with the inhibitory function (Fig. 5D). Additional studies were performed on mRNA obtained from 28 NK clones from two 2DS1-positive, C1 homozygous donors (no. 001 and 002). Twelve of these NK clones expressed mRNA for 2DS1 and no mRNA for 2DL1 and were cytotoxic to C2-positive targets (data not shown). We can conclude from these studies that NK clones expressing mRNA for only 2DS1 and not 2DL1 can be isolated from 2DS1-positive, C1 group homozygous donors and that such clones are cytotytic to C2-positive targets.

Freshly isolated NK cells from 2DS1-positive, C1 homozygous donors produce increased IFN-γ following EB6 cross-linking

Stimulation of human NK cells with cytokines such as IL-2 is known to affect their function (43, 44). We therefore tested KIR2DS1-mediated IFN-γ production using intracellular FACS following receptor cross-linking of freshly isolated NK cells. NK cells were obtained from six 2DS1-positive, 2DL1-positive donors (donors no. 001, 002, 016, 023, 024, and 025) and two 2DS1-negative, 2DL1-positive donors (donors no. 021 and 022) homozygous for the C1 group. We then performed receptor cross-linking assays using plate-immobilized Abs and assessed NK cell IFN-γ production following EB6 cross-linking using plate-immobilized Abs and assessed NK cell IFN-γ production using multicolor FACS analysis. Shown in Fig. 6A is a representative experiment from a 2DS1-positive, 2DL1-positive donor (KIR2DS1, KIR3DL1, and Z27) with NK cells from a 2DS1-positive, 2DL1-positive donor. As shown, EB6 receptor cross-linking induced IFN-γ in these NK cells. NK cells from 2DS1-negative donors did not produce IFN-γ following EB6 cross-linking but were activated following CD16 cross-linking (data not shown). Importantly, IFN-γ production following EB6 cross-linking in the 2DS1-positive donors, as well as CD16 cross-linking, was predominantly within the NK subset expressing one or more inhibitory receptors for “self” HLA class I (KIR2DL3/2DL2, CD94/NKG2A, LILRB1, and KIR3DL1). Those cells lacking inhibitory receptors recognizing autologous HLA class I were
Freshly isolated NK cells from 2DS1-positive, C1 homozygous donors produce increased IFN-γ. EB6 cross-linking induced significantly increased IFN-γ production (3.3 ± 0.7%, mean ± SEM) in NK cells from 2DS1-positive, C1 homozygous donors when compared with CD56 isotype control (0.7 ± 0.1%, mean ± SEM; p = 0.01 by paired analysis, p < 0.001 by log-rank statistic). CD16 response (17.6 ± 2.6%, mean ± SEM) was also statistically increased compared with CD56 (p < 0.01 by paired analysis, p < 0.001 by log-rank statistic). Therefore, KIR2DS1 function following EB6 cross-linking is present in freshly isolated polyclonal NK cells from 2DS1-positive, C1 group homozygous donors.

**Freshly isolated NK cells from 2DS1-positive, C1 group homozygous donors produce increased IFN-γ against C2 group-positive allogeneic BLCL**

We next used freshly isolated NK cells from two 2DS1-positive, C1 homozygous donors (donors no. 001 and 016) as effectors against C1 group and C2 group allogeneic BLCL and analyzed for IFN-γ alloresponse. A representative experiment is shown in Fig. 7A using donor no. 001 NK cells. As shown, the response to C2 group allogeneic BLCL was increased compared with that to C1 group BLCL. Further, as also observed in the previous cross-linking studies (Fig. 6A) the IFN-γ-positive NK cells were predominantly within the NK cell subsets expressing one or more inhibitory receptors recognizing “self” HLA class I (KIR2DL3/2DL2, CD94/NKG2A, and IL15R). KIR3DL1 cell surface staining in both of these donors was negative. Those NK cells lacking inhibitory receptors to autologous HLA class I demonstrated reduced responsiveness to allogeneic BLCL (Fig. 7A, upper panels).

The heterodimeric activating receptor NKG2C/CD94 associates with DAP12 and mediates NK cell response against allogeneic cells through the recognition of HLA-E. We next analyzed for a possible role of this receptor in the alloresponse against C2 group BLCL. As seen in Fig. 7A (lower panels), NKG2C/CD94 did not play a significant role in the IFN-γ response by KIR2DS1-positive, C1 group homozygous donor NK cells against C2 group homozygous targets. NK cells from donors no. 001 and 016 were tested with mAbs DX9 and ZZ7 (14, 15). We were unable to identify a KIR3DS1-positive subset in either of these donors (data not shown). Furthermore, both donors were chosen for these studies because they lacked expressed alleles of the KIR2DS4 gene (Fig. 2A) (29, 34).

The results of six independent experiments using NK cells from donors no. 001 and 016 are shown in Fig. 7B. The IFN-γ response by fresh NK cells from 2DS1-positive, C1 group homozygous donors against the C2 group BLCL was 6.9 ± 0.7% (mean ± SEM), and the response to the C1 group BLCL was 2.2 ± 0.5% (mean ± SEM; p = 0.03 by paired analysis, p < 0.01 by log-rank statistic). Freshly isolated NK cells from 2DS1-negative, C1 group homozygous donors did not demonstrate a significant IFN-γ response against C2 group-positive BLCL targets (data not shown). We can conclude that freshly isolated polyclonal NK cells from 2DS1-positive, C1 homozygous donors mediate a significant alloresponse against C2 group Ags.

**Alloreactivity in response to C2 group-positive BLCLs in freshly isolated NK cells from 2DS1-positive, C1 homozygous donors is mediated by “missing HLA-KIR ligand group” on target cells and activation of EB6 (2DL/S1) single-positive effector cells**

We next examined the alloresponse to C2 group-positive targets in NK subsets of freshly isolated NK cells from 2DS1-positive, C1 homozygous donors. Multicolor FACS analysis was performed by gating on live NK cells followed by gating the KIR-expressing subsets GL183 and EB6 (donors no. 001 and 016 were negative for 3DLI/3DS1 staining). Shown in Fig. 8 is a representative experiment using NK cells from donor no. 016 cocultured with allogeneic BLCL target cells matched for the HLA-Bw4 group. The
IFN-γ response was analyzed within each KIR subset against C1 (C1/C1) or C2 (C2/C2) homozygous target cells (Fig. 8). FACS results are shown for the GL183 single-positive subset (Fig. 8, green arrow shows IFN-γ positive cells responding to C2 stimulation) and EB6 single-positive subset (Fig. 8, red arrow shows IFN-γ positive cells responding to C2 stimulation). Data from four independent experiments using fresh NK cells from 2DS1-positive, C1 homozygous donors revealed an increased IFN-γ response against C2 group homozygous BLCL compared with C1 group homozygous BLCL. The GL183 single-positive population showed a mean increase in IFN-γ production of 2.9-fold (range 1.5–4.0), and the EB6 single-positive population demonstrated a mean increase of 10.9-fold (range 1.4–20) against the C2 homozygous BLCL compared with C1 homozygous BLCL. We interpret GL183 single-positive subset data as demonstrating a missing HLA-KIR ligand group C1 on the target cell. The EB6 single-positive subset data would support that direct activation of 2DL/S1 single-positive effector cells can be induced by C2 group, because these NK cells do not express the inhibitory receptors 2DL2/2DL3 recognized by GL183.

Discussion

We demonstrate in this study that fresh NK cells as well as IL-2-propagated NK cells from 2DS1-positive donors that also are homozygous for the C1 HLA-KIR ligand group are activated in vitro by BLCLs expressing the C2 group of HLA-C Ags. This response is in part due to “missing self” recognition of C1 group mediated by the inhibitory receptors 2DL2/3. This “missing self” alloresponse to C2 is rarely observed in NK donors lacking 2DS1. Even in presence of 2DS1, the NK alloresponse is dramatically reduced in NK donors that have the C2 group as “self”.

Recent tetramer binding studies have firmly established that the C2 group of Ags binds in vitro to both 2DL1 and 2DS1 but with low affinity for the C2–2DS1 interaction (10, 12, 13). It is therefore possible that one component of the NK alloresponse observed in
our studies is mediated by the direct activation of 2DS1 by alloge- 
neic C2 Ag. Our results support the concept that C2 Ag binding to 2DS1 contributes to the allovitiation of 2DS1-positive NK cells in addition to “missing self” recognition. We demonstrate that polyclonal IL-2 propagated NK cells derived from Bw4 homozygous, C7 homozygous donors are cytotoxic against C1/C2 heterozygous target cells, whereas no cytotoxicity was obtained against the C1 group homozygous targets. This NK alloresponse cannot be fully explained by the “missing HLA-KIR ligand group” on the target cells because both effector and target express Bw4 and C1. The results do not exclude a contribution by other interactions, because both the effector cells and the target cells in our in vitro assays express additional pairs of activating and inhibiting receptor ligands. We also demonstrate that some EB6-positive NK clones express mRNA for 2DS1 but no mRNA for 2DL1. In addition we present data on 14 EB6 positive NK clones with cytotoxicity against C2 group homozygous BLCLs. This allicytotoxicity was significantly reduced by mAb blocking of receptor (mAb EB6) or ligand (mAb 4E). Some of these NK clones were also tested for mRNA expression of 2DS1 and 2DL1. There were rare clones that were cytotoxic to C2-positive targets and expressed mRNA for both the activating and the inhibiting EB6 positive receptors, but the majority of the EB6-positive clones with anti-C2 group cytotoxicity expressed only mRNA for 2DS1 and not for 2DL1. The combination of (1) EB6 positivity (2), anti-C2 group cytotoxicity (3), mRNA for 2DS1 without mRNA for 2DL1 (4), and the inhibition of cytotoxicity by EB6 and anti-HLA class I strongly supports the concept that C2 Ag binding to 2DS1 contributes to the activation of these NK cells. In the present study we have not investigated the activation signals mediated by this isolated receptor-ligand pair. Such studies are currently in progress.

Our studies of freshly isolated NK cells have specifically ad- 
dressed the issues of other receptor-ligand interactions involved in alloreognition by 2DS1-positive, C1 group homozygous NK cells. We demonstrate that IFN-γ-producing cells increase follow- 
ing C2 group alloreognition (Figs. 7 and 8). We find that IFN-γ producing cells are increased by C2 stimulation relative to C1 and that the alloreactive NK cells are primarily within the NK cell populations expressing at least one inhibitory receptor for “self” HLA class I, including NKG2A/CD94 and LILRB1 (ILT2). These results are in agreement with the general principle previously reported that the activation of NK cells is predominantly mediated by cells that are “licensed” by “self” MHC class I (44, 45). We can now extend these rules to also include NK alloreresponses. The re- 
sults we have obtained for NK allorereognition may be limited to 2DS1-positive, C1 group homozygous NK donors, because C2 group Ags bind directly to 2DS1 (13). In contrast, C1 group Ags do not bind to 2DS2 (10–13) and the HLA-B Ags of the Bw4 HLA-KIR group do not bind to 3DS1 (15). Similarly, detectable binding in vitro between MHC class I and activating Ly49 receptors has provided conflicting results for H2-Dd and the activating Ly49d (18, 19), and no binding has been detected for H-2Dk and the activating Ly49p receptor (23). In both instances, however, functional analysis in vitro and in vivo supports a functional in- 
teraction between the MHC class I Ag and the activating receptor (20, 21, 23).

In this study we have described a model system where the pres- 
ence of KIR2DS1 is associated with allorereognition against C2 group-positive BLCL, but predominantly in those donors lacking this HLA molecule as a “self” Ag. None of the donors displayed autoreactivity by either polyclonal NK cells or NK clones. Multi- 
ple mechanisms for self-tolerance by NK cells have been described (reviewed in Refs. 1 and 46). NK cells achieve tolerance to self pri- 
marily through the expression of MHC class I-recognizing inhibitory receptors (2). Early studies of human NK cells indicated that NK clones lacking inhibitory receptors that recognize class I Ags were not generated from the peripheral blood of normal don- 
ors (25, 30). The concept of “at least one inhibitory receptor for self-MHC” prevailed until recently. Two recent studies of murine NK cells have demonstrated the existence of a significant popula- 
tion of NK cells without inhibitory receptors for self-MHC (45, 47), and the term “licensing” is being used to describe functional NK cells that express inhibitory receptors for self-MHC class I (45). Functional NK cells are also observed in humans primarily within the population that expresses inhibitory receptors for self- 
HLA (44). These studies support the concept that NK tolerance to self is generated predominantly through inhibitory receptor rec- 
ognizing MHC molecules. In those NK cells lacking these inhibi- 
tory signals the lack of autoreactivity may be achieved by an 
overall hyporesponsiveness. Our studies using freshly isolated NK cells consistently demonstrated KIR2DS1 activation predomi- 
nantly within the NK cell subset expressing one or more inhibitory receptors for self-HLA class I. Those NK cells positive for EB6 (2DS1/2DL1) but lacking inhibitory receptors to self-HLA class I were hypo-responsive to alloactivation (Figs. 6A and 7A).

It is currently considered unlikely that activating KIR or Ly49 interactions with MHC class I contribute to NK cell education and NK repertoire development. Licensing was also obtained in studies with DAP12-deficient mice (45), and others have recently indi- 
cated that no differences in NK education were observed between 2DL2-positive, 2DS2-positive and 2DL2-positive, and 2DS2-neg- 
avive NK cells (44). The only previous studies suggesting a con- 
tribution by activating NK receptors and MHC class I interactions to NK education and tolerance to self were reported by Bennett and colleagues for the activating Ly49d with H2-Dd (20, 21). Tolerance to “self” in these mice was not achieved through anergy or deletion of anti-H2-Dd-specific NK cells but instead by coexpression of one or more “self”-specific inhibiting recep- 
tors (20, 21).

The activating KIR2DS4 has been suggested to initiate NK cell cytotoxicity against HLA-Cw4 Ags of the C2 group but not HLA- 
Cw6 Ags (33). In our studies KIR2DS4 was not associated with allorereactivity against the C2 group. Six of our nine KIR2DS1-posi- 
tive, C1 homozygous donors were negative for KIR2DS4, and there were no significant differences in C2 group-directed cytotoxicity based on expressed or nonexpressed alleles of KIR2DS4 (29, 34).

Recent studies have revealed that KIR3DS1-encoded receptors bind the mAb Z27 and are capable of activating NK cells follow- 
ing receptor cross-linking (14, 15). There was, however, no evi- 
dence for 3DS1 receptor binding to cells transfected with “high 
binding” Bw4 alleles, and the natural ligand for this receptor re- 
mains unknown (15). Our studies of NK clones or freshly isolated NK cells did not include NK donors that expressed mAb Z27- 
positive NK cells. We are therefore at present not able to evaluate a possible role for 3DS1 in NK alloreactivity. We can, however, exclude the activating NKG2C/CD94 receptor in mediating 2DS1- 
dependent NK allovitiation of fresh NK cells (Fig. 7A).

Our study was designed to test the hypothesis that NK alorreac- 
tivity in vitro is dominated by incompatibility between NK effector cells and target cells for the three HLA-KIR ligand groups, Bw4, 
C1, and C2. Polyclonal IL-2-propagated NK cells are not cytotoxic to autologous BLCLs (25), whereas NK cells from some patients with a lack of HLA class I caused by homozygous TAP deficiency are autoreactive (37, 38). We therefore tested this hypothesis on target cells expressing a full complement of HLA class I Ags by the use of EBV-transformed BLCL. The results presented in Table IV demonstrate the surprising finding that the major genetic com- 
ponents controlling alloantigen activation of polyclonal NK cells
are: 1) the HLA-KIR ligand groups present on the NK cells of the donor; 2) the HLA-KIR ligand groups of the target (recipient); and 3) the presence or absence of activating KIR2DS1 in NK cell donor. Ruggeri and colleagues were the first to study the impact of KIR and HLA genes on outcome in hematopoietic stem cell transplantation (HCT) (39–41). They demonstrated in HLA-mismatched HCT that incompatibility for one of the major HLA-KIR ligand groups C1, C2, and Bw4 between donor and recipient in the graft-vs-host direction resulted in improved overall survival, lower relapse rates, and lower incidence of graft-vs-host disease in acute myelogenous leukemia (40). The patterns of HLA disparity led them to conclude that these effects were mediated by alloloreactive NK cells recognizing “missing self” and proposed a model based on HLA-KIR ligand incompatibility for the C1, C2, and Bw4 groups to describe these findings (39, 40). We and others hypothesized that NK activation could be mediated by a missing HLA-KIR ligand (28, 48, 49). This alternative concept was based on the fact that many individuals in the population would have inhibitory KIR genes and all of the corresponding ligands, whereas others would lack one or more ligands for their endogenous inhibitory KIR genes. Likewise, some allotransplant recipients may lack a HLA ligand for their donor-inhibitory KIR (missing KIR ligand) even among HLA genotypically identical sibling pairs (28). Numerous studies of the effects of KIR and HLA on HCT have provided conflicting results, and neither the HLA-KIR ligand incompatibility model nor the missing KIR ligand model has led to generally acceptable explanations (28, 39–41, 48–51). The present study strongly supports the HLA-KIR ligand incompatibility model as originally presented and applied to HLA class I incompatible, HLA haploidentical donor-recipient pairs (39, 40). In addition, we demonstrate an overwhelming influence on NK alloreactivity in vitro by the presence of activating KIR2DS1 and the absence of C2 group Ags in the donor. These findings support a recent report that HCT recipients homozygous for the C1 group HLA-KIR ligands had favorable outcome compared with those with C2 group (52). The authors also confirm that developing NK cells acquire inhibitory receptors to C1 at earlier time points than the C2-specific receptors (52, 53). Collectively, these studies support the concept that the presence of C2 group Ags in the HCT recipient probably has an impact on NK alloreactivity, and our studies would suggest that the presence of 2DS1 and the absence of C2 Ags in the HCT donor is contributing to this effect.

Several reports have described an association between KIR2DS1 and/or KIR2DS2 in psoriatic arthritis, rheumatoid arthritis, and psoriasis vulgaris (54–56). It is possible that some at-risk individuals express additional ligands for activating coreceptors that, combined with signals propagated by the low-affinity activating KIR, could overcome normal NK self-tolerance. Other studies have described protection against hepatitis C virus infection (57) and delayed progression to AIDS in individuals positive for KIR3DS1 and HLA-Bw4 (58). These findings support the concept that the decreased inhibition of NK cells by the lack of a HLA class I ligand for the corresponding inhibitory KIR, or possibly the ligation of activating KIRs with disease-associated peptides presented by Bw4 alleles (15), may affect the overall balance between activating and inhibitory signals and favor NK cell activation. It is tempting to speculate that HLA ligands for activating KIR have been selected due to their low affinity, and HLA class I restriction will only be displayed in the context of specific pathogens or transformed cells. Such low affinity interactions may require additional costimulating signals that are up-regulated during cellular stress.

The role of activating KIRs in immune response is still enigmatic. The activating 2DL4 receptor, which signals through association with the FcεRIγ-chain, has recently been shown to bind soluble HLA-G leading to cytokine and chemokine production (31, 32). We now demonstrate that 2DS1 contributes to NK alloactivation by the recognition of C2 group Ags. It remains to be determined whether the KIR2DS1 gene contributes to immune recognition during immune response to pathogens.

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Disclosures
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