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KIR2DS1-Positive NK Cells Mediate Alloresponse against the C2 HLA-KIR Ligand Group In Vitro[1]

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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 allocytotoxicity in IL2-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 receptors 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 $Ser^{77}Asn^{80}$ 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 $Asn^{77}Lys^{80}$ 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 allotypes, 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 *KIR2DL2/3-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

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[3] Abbreviations used in this paper: LILR, leukocyte Ig-like receptor; BLCL, B lymphoblastoid cell line; HCT, hemopoietic stem cell transplantation; KIR, killer cell Ig-like receptor; SSP, sequence-specific primer; C1, HLA-KIR ligand group C1; C2, HLA-KIR ligand group C2; Bw4, HLA-KIR ligand group Bw4; KIR-A, KIR A haplotype; KIR-B, KIR B haplotype.

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 have 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-2D^d 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 allogeneic 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 B 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 40-μl tissue culture plates (Robbins Scientific, Sunnyvale, CA), and were cocultured with 1×10^6/ml irradiated allogeneic PBMC and 1×10^5/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 B 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-G (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/entrez/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 ^{51}Cr-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 the 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 ^{51}Cr-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 2×10^5 NK cells were then added to wells in a volume of 200 μl with GolgiPlug according to the

Table I. *Monoclonal Abs*

Molecule (CD Marker)	Clone(s)	Fluorophore(s)	Source
FACS phenotyping[a]			
CD3	HIT3a, SK7	FITC, PE, PerCP	BD Biosciences
CD20	L27	PerCP	BD Biosciences
CD56	B159, NCAM16.2	FITC, PE, PE-Cy7	BD Biosciences
KIR2DL1, 2DS1 (CD158a/h)	HP3E4	FITC, PE	BD Biosciences
KIR2DL1, 2DS1 (CD158a/h)	EB6	Allophycocyanin	Beckman Coulter
KIR2DL2/3, 2DS2 (CD158b/j)	CH-L	FITC, PE	BD Biosciences
KIR2DL2/3, 2DS2 (CD158b/j)	GL183	Allophycocyanin	Beckman Coulter
KIR3DL1 (CD158e1)	DX9	FITC, PE	BD Biosciences
KIR3DL1 (CD158e1)	DX9	Allophycocyanin	Miltenyi Biotech
KIR3DL1, 3DS1 (CD158e1/e2)	Z27.3.7	PE	Beckman Coulter
NKG2A (CD159a)	Z199	PE	Beckman Coulter
LILRB1 (CD85j)	HP-F1	PE	Beckman Coulter
NKp30 (CD337)	Z25	PE	Beckman Coulter
NKp44 (CD336)	Z231	PE	Beckman Coulter
NKp46 (CD335)	BAB281	PE	Beckman Coulter
NKG2C/CD94 (CD159c)	134591	PE	R&D Systems
IFN-γ	B27	FITC	BD Biosciences
Functional Assays			
CD16	3G8 (IgG1)	Purified	Beckman Coulter
CD56	N901 (IgG1)	Purified	Beckman Coulter
KIR2DL1, 2DS1 (CD158a/h)	EB6 (IgG1)	Purified	Beckman Coulter
KIR2DL2/3, 2DS2 (CD158b/j)	GL183 (IgG1)	Purified	Beckman Coulter
HLA-B, Cw	4E (IgG2a)	$(Fab')_2$	MSKCC[b] Monoclonal Core Facility

[a] Isotype controls were obtained from BD Biosciences.
[b] Memorial Sloan-Kettering Cancer Center, New York, NY.

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, $\sim 2 \times 10^5$ 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 II. *Target HLA class I*

Cell ID[a]	HLA-A	HLA-A	HLA-B	HLA-B	B Group	HLA-Cw	HLA-Cw	Cw Group
C1 (N80)[b]								
001	A*0203	A*2402	B*1301	B*3802	Bw4	C*0702	C*0102	C1
9004	A*0201	A*0201	B*2705	B*2705	Bw4	C*0102	C*0102	C1
9026	A*2601	A*2601	B*3801	B*3801	Bw4	C*1203	C*1203	C1
9027	A*2902	A*2902	B*4403	B*4403	Bw4	C*1601	C*1601	C1
9035	A*3201	A*3201	B*3801	B*3801	Bw4	C*1203	C*1203	C1
002	A*0101	A*2501	B*0801	B*0801	Bw6	C*0701	C*0701	C1
9013	A*0301	A*0301	B*0702	B*0702	Bw6	C*0702	C*0702	C1
9031	A*0201	A*0201	B*1501	B*1501	Bw6	C*0304	C*0304	C1
9032	A*0201	A*0201	B*1501	B*1501	Bw6	C*0304	C*0304	C1
9038	A*0201	A*0201	B*1801	B*1801	Bw6	C*0701	C*0701	C1
9087	A*0101	A*0101	B*0801	B*0801	Bw6	C*0701	C*0701	C1
C1/C2[c]								
028	A*0201	A*0201	B*1302	B*2705	Bw4	C*0102	C*0602	C1/C2
9205	A*0201	A*2402	B*4001	B*4002	Bw6	C*0202	C*0304	C1/C2
C2 (K80)[b]								
9010	A*6802	A*6802	B*5301	B*5301	Bw4	C*0401	C*0401	C2
9046	A*0201	A*0201	B*1302	B*1302	Bw4	C*0602	C*0602	C2
9201	A*0201	A*0301	B*2705	B*5101	Bw4	C*0202	C*1602	C2
011	A*2407	A*2402	B*3501	B*3505	Bw6	C*0401	C*0401	C2
9018	A*0301	A*2402	B*1801	B*1801	Bw6	C*0501	C*0501	C2
9025	A*3101	A*3101	B*3501	B*3501	Bw6	C*0401	C*0401	C2
9202	A*0205	A*2901	B*3503	B*4102	Bw6	C*0401	C*1701	C2

[a] 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).
[b] Homozygous.
[c] Heterozygous.

Table III. *NK donor HLA class I*

Donor ID	HLA-A	HLA-A	HLA-B	HLA-B	B Group	HLA-Cw	HLA-Cw	Cw Group	KIR Haplotype[a]	Autologous BLCL[b]
C1 (N80)[c]										
Donor 001	A*0203	A*2402	B*1301	B*3802	Bw4	C*0702	C*0102	C1	B5, B6	Yes
Donor 013	A*1101	A*2601	B*5101	B*5301	Bw4	C*0801	C*1402	C1	A2, B6	Yes
Donor 016	A*0301	A*0301	B*3801	B*4402	Bw4	C*0704	C*1203	C1	B5, B9	No
Donor 002	A*0101	A*2501	B*0801	B*0801	Bw6	C*0701	C*0701	C1	A1, B5	Yes
Donor 021	A*1101	A*2402	B*1502	B*4002	Bw6	C*0304	C*0801	C1	A2, A2	No
Donor 022	A*0201	A*0301	B*1801	B*1501	Bw6	C*0701	C*0304	C1	A2, B33	Yes
Donor 023	A*0201	A*2402	B*0702	B*3906	Bw6	C*0701	C*0702	C1	B21, Bx[d]	No
Donor 025	A*1101	A*2402	B*1505	B*5501	Bw6	C*0305	C*0313	C1	A1, B12	No
Donor 003	A*2402	A*2902	B*1801	B*4403	Bw4/w6	C*0701	C*1601	C1	A1, B6	Yes
Donor 018	A*0206	A*2402	B*3902	B*5101	Bw4/w6	C*0304	C*0801	C1	B4, B12	No
Donor 024	A*3301	A*6601	B*1402	B*3801	Bw4/w6	C*0802	C*1203	C1	B12, B14	No
Donor 027	A*0206	A*3101	B*5101	B*5401	Bw4/w6	C*0102	C*0304	C1	A1, A2	No
C1/C2[e]										
Donor 028	A*0201	A*0201	B*1302	B*2705	Bw4	C*0102	C*0602	C1/C2	A2, A2	Yes
Donor 029	A*0201	A*0301	B*4901	B*7801	Bw4/w6	C*0602	C*1203	C1/C2	A1, A2	No
C2 (K80)[c]										
Donor 012	A*0201	A*0201	B*1302	B*4405	Bw4	C*0602	C*0202	C2	B9, B13	Yes
Donor 031	A*0201	A*0201	B*4402	B*4402	Bw4	C*0202	C*0501	C2	B7, B29	No
Donor 011	A*2407	A*2402	B*3501	B*3505	Bw6	C*0401	C*0401	C2	A1, B12	Yes
Donor 014	A*1101	A*1101	B*1501	B*1501	Bw6	C*0401	C*0401	C2	A2, B6	No
Donor 015	A*1101	A*2901	B*0705	B*5001	Bw6	C*1504	C*0602	C2	A2, B28	No
Donor 020	A*2901	A*3001	B*3501	B*4501	Bw6	C*0602	C*1701	C2	A1, A1	No
Donor 030	A*0201	A*0301	B*3501	B*4402	Bw4/w6	C*0401	C*0501	C2	A1, A1	No

[a] KIR haplotype numbers from Carrington and Norman (35).
[b] Autologous BLCL generated from indicated donors (see *Materials and Methods*).
[c] Homozygous.
[d] The letter "x" indicates a rare B haplotype.
[e] Heterozygous.

supernatants 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, $1–2 \times 10^6$ NK IL-2-propagated NK cells or $0.5–0.7 \times 10^6$ 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 with lysis and wash buffers to remove rRNA and DNA. The cDNA was synthesized in the column using the μMACS one-step cDNA kit (Miltenyi Biotec). The 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′-GCAGCACCAT GTCGCTCT; 2DL1 reverse, 5′-GTCACTGGGAGCTGACAC-3′; 2DS1 forward, 5′-TCTCCATCAGTCGCATGA(G/A)-3′; and 2DS1 reverse, 5′-AGGGCCCAGAGGAAAGTT-3′) as described previously (30). The *KIR2DL4* forward and reverse primers (2DL4 forward, 5′-GGTGGTCAG GACAAGCCCTTCTGC-3′; and 2DL4 reverse, 5′-GGGGTTGCT GGGTGCCGACCACTC-3′) 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 6× loading dye and electrophoresed on 2% Tris-borate-EDTA agarose gel containing ethidium bromide. Previously genotyped samples were used as positive controls.

Generation of EBV-BLCL blasts

A total of 2×10^6 PBMCs were isolated from selected donors and incubated for 3–4 days with the EBV-containing supernatant of the marmoset 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 (Invitrogen 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 groups, C1, C2, and Bw4, for inhibition of cytotoxicity against allogeneic BLCLs. Polyclonal IL-2-stimulated NK effector cells were prepared from six *KIR-A* homozygous donors. Two 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. 1*A*). Three of the donors were heterozygous *Bw4*/*Bw6*, one was *Bw4* homozygous, and two were *Bw6* only (Table III and Fig. 1*C*). As shown in Fig. 1*B*, none of the effector cells displayed a clear cytotoxic response in dose titration studies against a panel of BLCLs representing all combinations of

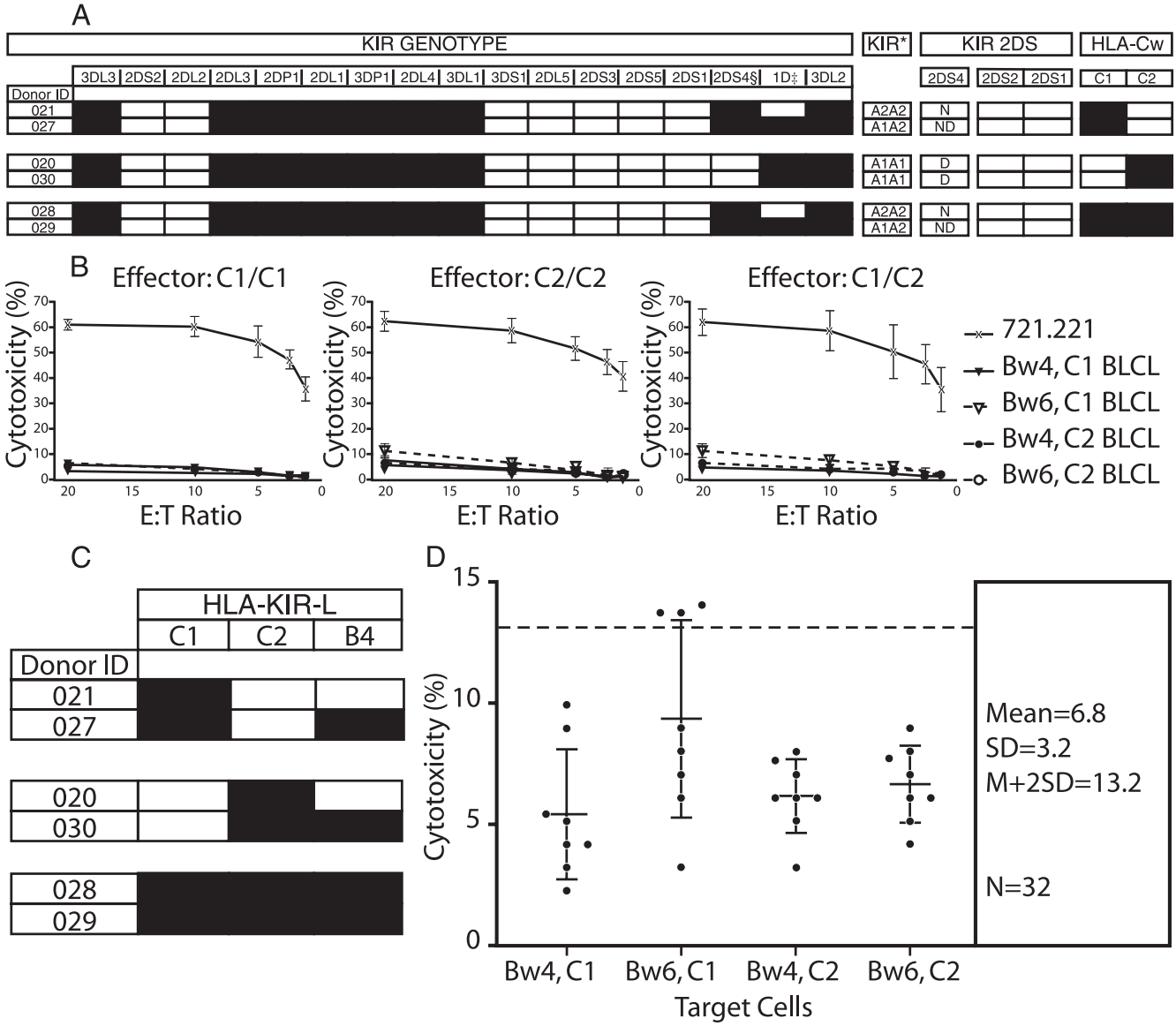

FIGURE 1. Polyclonal, IL-2-propagated NK cells from *KIR-A* homozygous donors demonstrate lack of cytotoxicity against most HLA-KIR ligand-incompatible allogeneic EBV-BLCLs. A panel of six donors (donors no. 020, 021, 027, 028, 029, and 030) homozygous for *KIR-A* haplotypes (A1A1, A2A2, or A1A2) (29, 34) and possessing differing combinations of HLA-Cw C group alleles (*C1* or *C2* group homozygous or *C1/C2* heterozygous) was selected. *A*, KIR genotype is shown for each of the six *KIR-A* homozygous donors (*left portion*). A filled box indicates the presence of a gene and an open box indicates the absence of a gene. The asterisk (*) denotes the donor KIR haplotype combinations as described by Carrington and Norman (35) (see http://ncbi.nlm.nih.gov/entrez/query.fcgi?db=Books). The section symbol (§) denotes a gene encoding normal full-length *KIR2DS4* alleles, and the double dagger (‡) denotes a deletion mutant allele of *2DS4* (29, 34). A summary of *KIR2DS4*, *2DS2*, and *2DS1* and HLA-Cw *C1* and *C2* group status for each donor is also shown (*right portion*). N denotes homozygosity for the normal full-length *2DS4* alleles, and D denotes homozygosity for the deletion mutant of *2DS4*. *B*, Polyclonal, IL-2-activated NK cells were generated from donors and used as effectors in ^{51}Cr release assays against a panel of eight allogeneic BLCL target cells (9010, 9018, 9032, 9035, 9201, 001, 002, and 011) in addition to 721.221 positive control. Individual graphs contain data from two *KIR-A* homozygous donors possessing the same HLA C groups. Each graph represents at least two independent experiments. BLCL target cells were classified according to the HLA-KIR ligand groups *C1*, *C2*, and *Bw4*. Mean cytotoxicity is shown as a line with error bars indicating SEM. *C*, Summary of HLA-KIR ligand groups for each *KIR-A* homozygous donor. *D*, The individual data points for the E:T ratio of 20:1 from all of the experiments represented by the curves in *B* are shown, grouped according to target cell HLA-KIR ligand group. Mean cytotoxicity and SD for the entire data set are shown in the box to the right of graph. The uppermost level of nonspecific cytotoxicity was defined as the mean + 2SD and is represented by the stippled line at 13.2%.

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. 1*D*). 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

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

NK Effector	N[a]	+/+[b]	+/−[c]	−/+[d]	−/−[e]	*p* Value[f]
KIR-A homozygous	32	10	0	19	3	0.534
2DS1-positive C1 group homozygous	60	22	1	8	29	<0.001
2DS1-positive C2 group homozygous	44	15	4	13	12	0.128

[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.

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

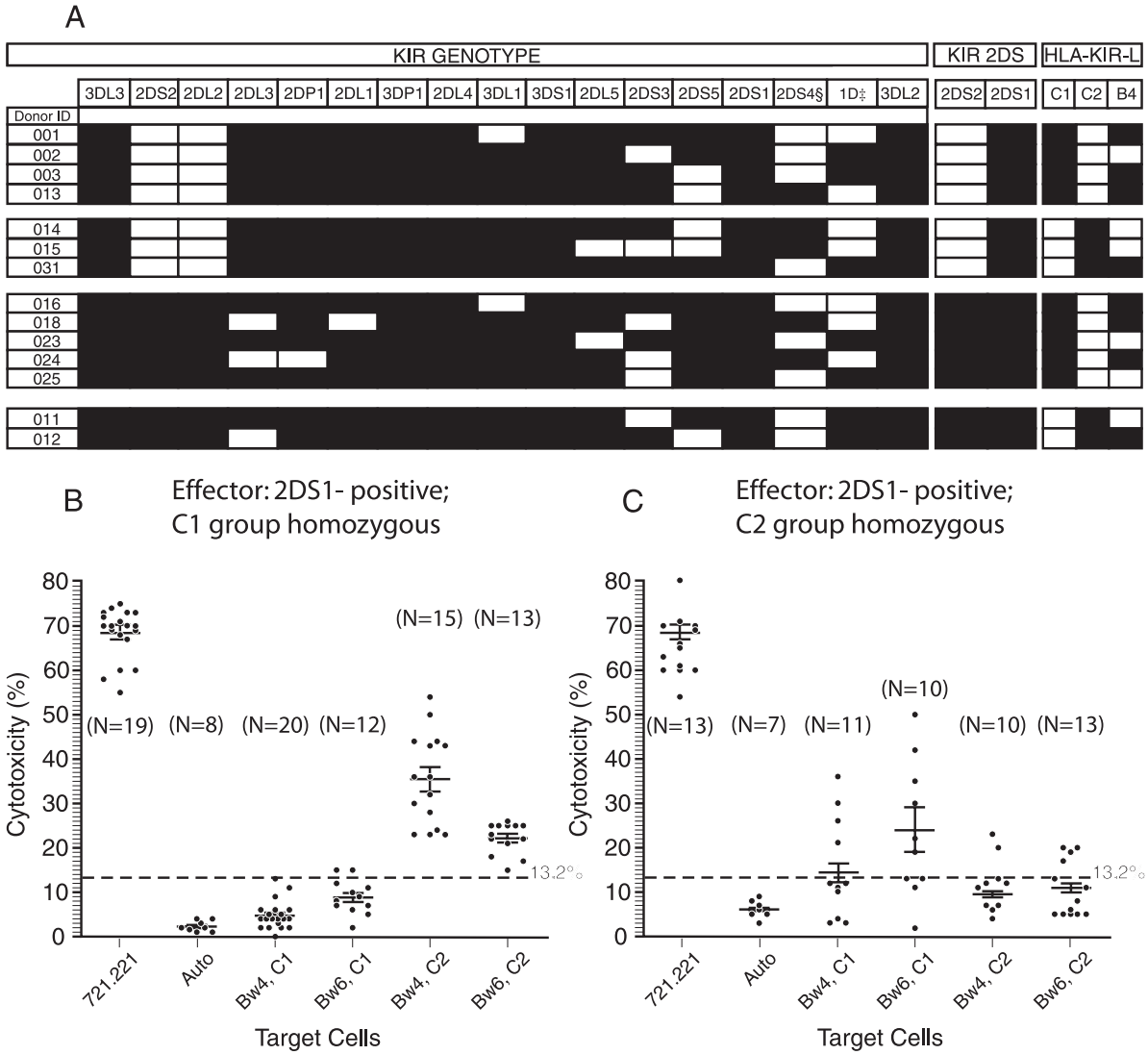

FIGURE 2. Polyclonal, IL-2-propagated NK cells from *2DS1*-positive, *C1* group homozygous donors mediate cytotoxicity against most HLA-KIR ligand-incompatible BLCLs, but only when the C2 group is an alloantigen. IL-2-propagated NK cells were generated from a panel of donors possessing *KIR2DS1* and homozygous for the HLA-Cw *C1* or *C2* group and used in ^{51}Cr-release assays against a panel of BLCL targets. *A*, Complete KIR genotyping results for 14 *KIR2DS1*-positive donors (*left portion*) and a summary of donor *2DS1* and *2DS2* and HLA-KIR ligand groups *C1*, *C2*, and *Bw4* (*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 ^{51}Cr-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, 9032, 9035, 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. 2*A*. 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. 2*B*). 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. 2*A*) (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 ^{51}Cr-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 plus Cw4 compared with 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).

Table V. *NK cell clone phenotype and function*

Donor Identification	Total NK Clones[a]	EB6 Positive NK clones (2DL1/S1) Total EB6$^+$ clones[b]	Cytotoxicity[c] Target: C2 group BLCL	Inhibition[d] Target: 721.221 plus C2 group
KIR2DS1-positive, C1 homozygous donors				
001	93	46	45 (98%)	1 (2%)
002	21	9	2 (22%)	7 (78%)
016	23	7	3 (43%)	4 (57%)
KIR2DS1-positive, C2 homozygous donors				
011	31	13	0 (0%)	13 (100%)
012	7	3	0 (0%)	3 (100%)
031	6	3	0 (0%)	3 (100%)

[a] Total number of NK clones obtained and phenotyped from indicated donors.
[b] Total number of NK clones phenotypically-positive for EB6 mAb (2DL1/2DS1).
[c] Number (and percentage) of EB6-staining NK clones that demonstrated specific cytotoxicity to C2 group-positive BLCL.
[d] Number (and percentage) of EB6-staining NK clones that demonstrated specific inhibition against C2 group-transfected 721.221.

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. 3*A*). 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. 3*A*). 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. 3*A* 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 LILRB1 (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

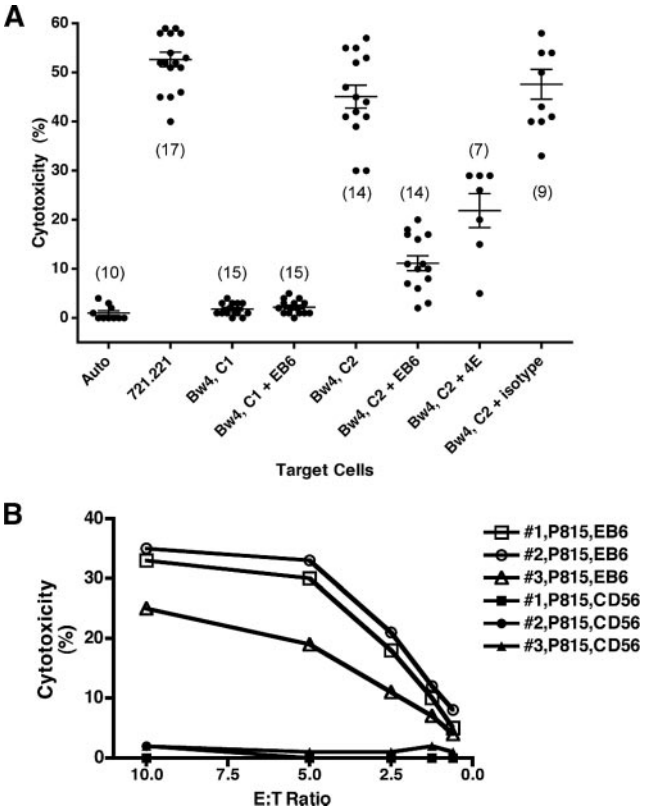

FIGURE 3. Selected EB6-positive NK clones derived from *KIR2DS1*-positive, *C1* group homozygous donors are activated by C2 group-positive allogeneic BLCLs and receptor cross-linking. NK clones were derived from donors no. 001, 002, and 016 and used in ^{51}Cr-release assays against BLCL targets or P815 redirected assays. *A*, Fourteen EB6-expressing clones, selected for cytotoxic function to C2 group (as described in text), were tested against 10 allogeneic (9004, 9018, 9025, 9031, 9032, 9087, 9201, 001, 002, and 011) and two autologous (001 and 002) ^{51}Cr-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 4E $(Fab')_2$), 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.

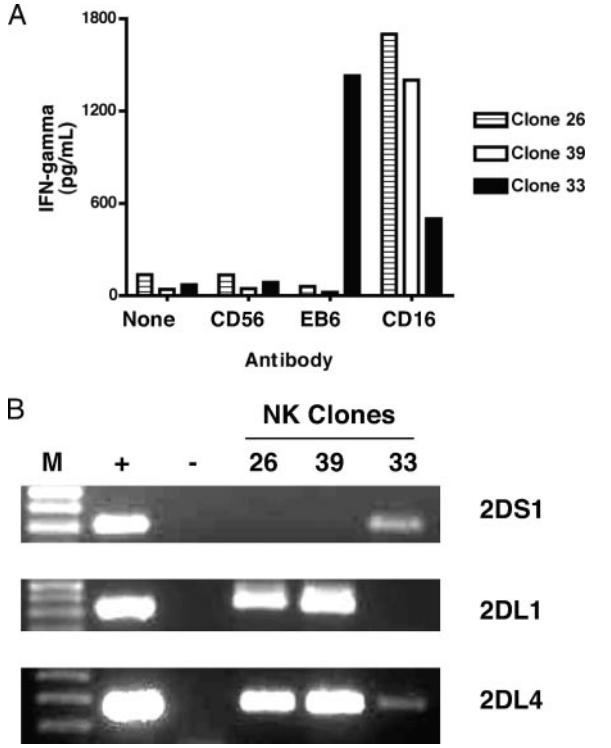

FIGURE 4. EB6-positive NK clones possessing 2DS1 mRNA, and not 2DL1 mRNA, that are activated following receptor cross-linking can be obtained from *KIR2DS1*-positive, *C1* group-positive donors. NK clones were generated from a *KIR2DS1*-positive, *C1* group homozygous donor (donor no. 023), and three clones phenotypically positive for EB6 (2DL1/2DS1) by FACS were selected for receptor cross-linking experiments. Cells were added to plates prelabeled with the indicated Abs and incubated for 12 h at 37°C. Supernatants were collected and tested for individual cytokines by ELISA. *A*, IFN-γ results are shown in for all clones (individual clone numbers shown in the legend on the *right*). Total mRNA was isolated from clones no. 26, 33, and 39 and cDNA was generated as described (see *Materials and Methods*). All clones were analyzed for the presence of KIR2DS1, KIR2DL1, and the ubiquitous KIR2DL4 PCR product. *B*, Results for each NK clone. M indicates marker lane, the plus sign (+) is the positive control, and the minus sign (−) is the negative control. The figure is representative of data obtained with 10 EB6-positive NK clones.

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

These studies demonstrate that EB6 (2DL/S1)-positive NK clones with anti-C2 group cytotoxicity can be readily generated from *2DS1*-positive, *C1* homozygous donors. In contrast, such NK clones have not been generated from *2DS1*-positive, *C2* homozygous donors (Table V). Selected EB6-positive NK clones were inhibited by anti-HLA class I and by EB6 and demonstrated redirected cytotoxicity with EB6-labeled P815. EB6 and GL183 dual positive NK clones from donor no. 001 were also tested on P815. Although EB6 mediated redirected cytotoxicity, GL183 did not. These clones were inhibited by 721.221 plus Cw3 and mediated cytotoxicity against C2 homozygous targets (data not shown). 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. 4*A*). Clone no.33 also produced increased levels of TNF-α and GM-CSF (data not shown). KIR2DS1 mRNA was present and 2DL1 mRNA was absent in clone no.33 (Fig. 4*B*). In contrast, the NK clones no.26 and 39 that were not activated by EB6 cross-linking possessed

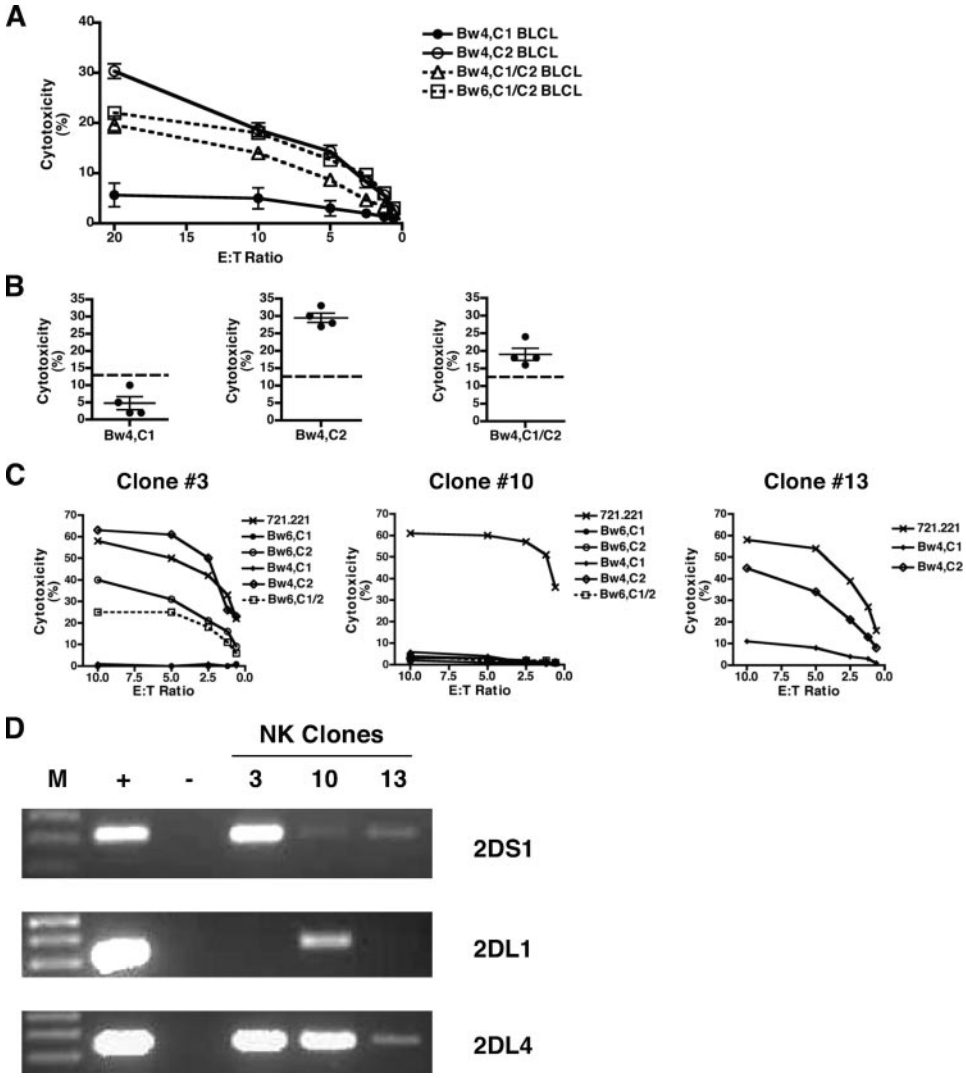

FIGURE 5. HLA C2 group-directed cytotoxicity by *KIR2DS1*-positive, *C1* group homozygous donors is present against C1/C2 heterozygous targets. IL-2-propagated, polyclonal NK cells from three donors positive for *KIR2DS1* and homozygous for both HLA-*Bw4* and the HLA-Cw *C1* group (donors no. 001, 013, and 016) were used as effectors against four BLCL targets (9004, 9201, 9205, and 028) in ^{51}Cr -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 ^{51}Cr-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.

only 2DL1 mRNA and not 2DS1 mRNA (Fig. 4*B*). Seven additional EB6-positive NK clones from two *2DS1*-positive, *C1* homozygous donors (donors no. 016 and 023) were tested. Three of these were positive for 2DS1 mRNA and lacked 2DL1 mRNA (data not shown). We did not test mRNA expression of 2DL2/3 and 2DS2 in these clones. 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 can be activated by EB6 receptor cross-linking.

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. 5*A*). Individual data points from the E:T ratio of 20:1 for four independent experiments are displayed in Fig. 5*B*. Because all three donors were HLA-*Bw4* homozygous, only the Bw4 group-positive target cells are shown. The positive cytotoxic response

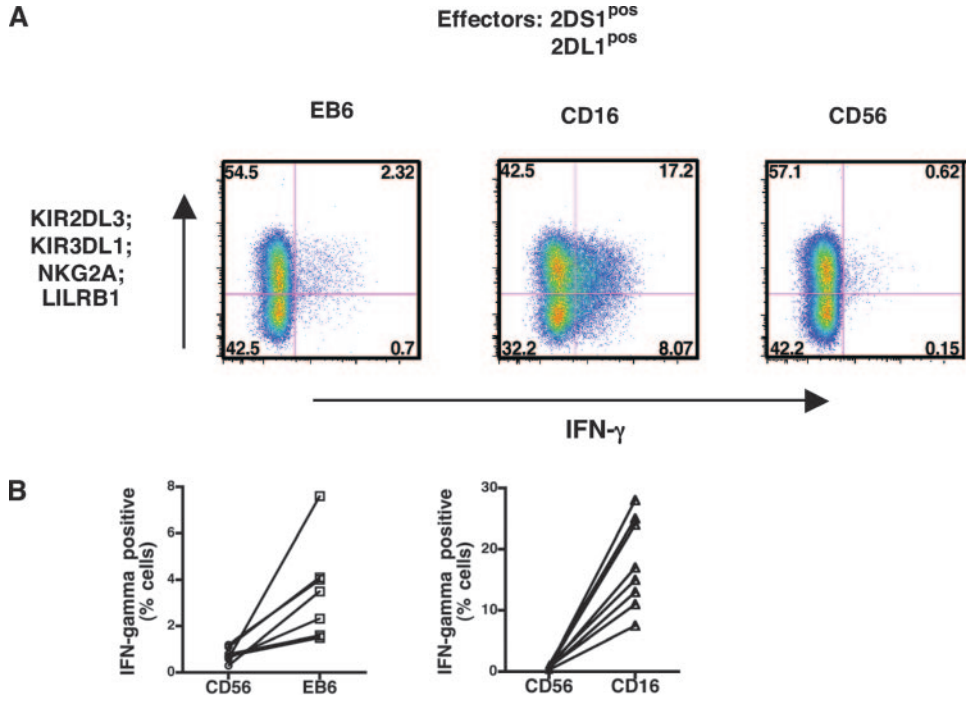

FIGURE 6. Freshly isolated NK cells from *KIR2DS1*-positive, *C1* group homozygous donors produce IFN-γ following EB6 (2DL1/S1) receptor cross-linking. Freshly isolated NK cells were isolated as described (see *Materials and Methods*) from *2DS1*-positive donors (donors no. 001, 002, 016, 023, 024, and 025) homozygous for the HLA-Cw *C1* group. Cells were plated in unlabeled plates or plates prelabeled with the indicated Abs for 12 h. *A*, Representative results of IFN-γ staining following receptor cross-linking with the mAb EB6, CD16, or CD56 (Ab is identified above individual figures) with NK cells from a *2DS1*-positive, *2DL1*-positive, *C1* group homozygous donor (donor no. 024). The figures depict live, lymphocyte-gated followed by CD3-negative, CD56-positive-gated NK cells. Inhibitory receptors recognizing "self" HLA class I for this donor are displayed on the *y*-axis and IFN-γ staining is displayed on the *x*-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. 5*D*). 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 cytolytic 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 by multicolor FACS analysis. Shown in Fig. 6*A* is a representative experiment from a *2DS1*-positive, *2DL1*-positive, *C1* group homozygous donor (donor no. 024). 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

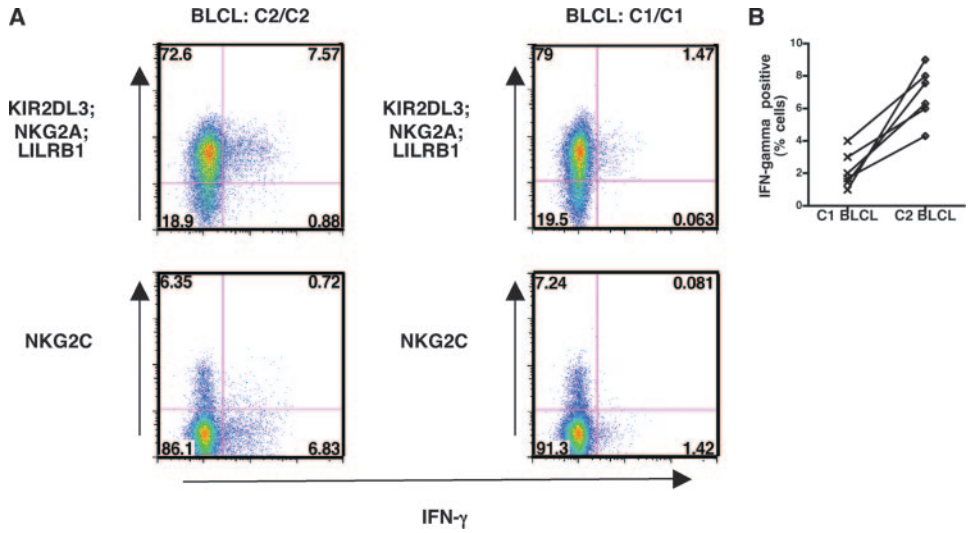

FIGURE 7. Allorecognition of HLA C2 group-positive target cells by freshly isolated NK cells from *2DS1*-positive, *C1* homozygous donors occurs predominantly within the NK subset expressing inhibitory receptors for self-HLA. Freshly isolated NK cells were obtained from *2DS1*-positive, *C1* homozygous donors (donors no. 001 and 016) and cocultured with allogeneic BLCL target cells matched for the HLA-Bw4 group (9004, 9010, and 9201) in addition to 721.221 as positive control at E:T ratios of 1:1 for 12 h. *A*, Representative experiment using donor no. 001 NK effector cells cocultured with C2 group (C2/C2) or C1 group (C1/C1) homozygous target cells. Cells were first gated for live lymphocytes followed by the CD3-negative, CD56-positive population. IFN-γ staining results are shown on the *x*-axis plotted against either inhibitory receptors recognizing donor HLA class I (*top sections*) or NKG2C (*bottom sections*) on the *y*-axis. As described previously, KIR3DL1 staining using the DX9 clone was negative for both donors. Results are representative of six independent experiments obtained with two donors (no. 001 and 016). *B*, Summarized results of IFN-γ staining against BLCL targets for all six independent experiments.

less responsive to EB6 or CD16 cross-linking (Fig. 6*A*). No significant IFN-γ response was seen in NK cells following GL183 cross-linking in any of these donors (data not shown).

Data from eight experiments measuring IFN-γ response by *2DS1*-positive, *C1* group homozygous donor NK cells following EB6 cross-linking, along with positive control response to CD16 cross-linking, are shown in Fig. 6*B*. 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. 7*A* 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. 6*A*) 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 LILRB1). 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. 7*A*, *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. 7*A* (*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 Z27 (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. 2*A*) (29, 34).

The results of six independent experiments using NK cells from donors no. 001 and 016 are shown in Fig. 7*B*. 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 3DL1/3DS1 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

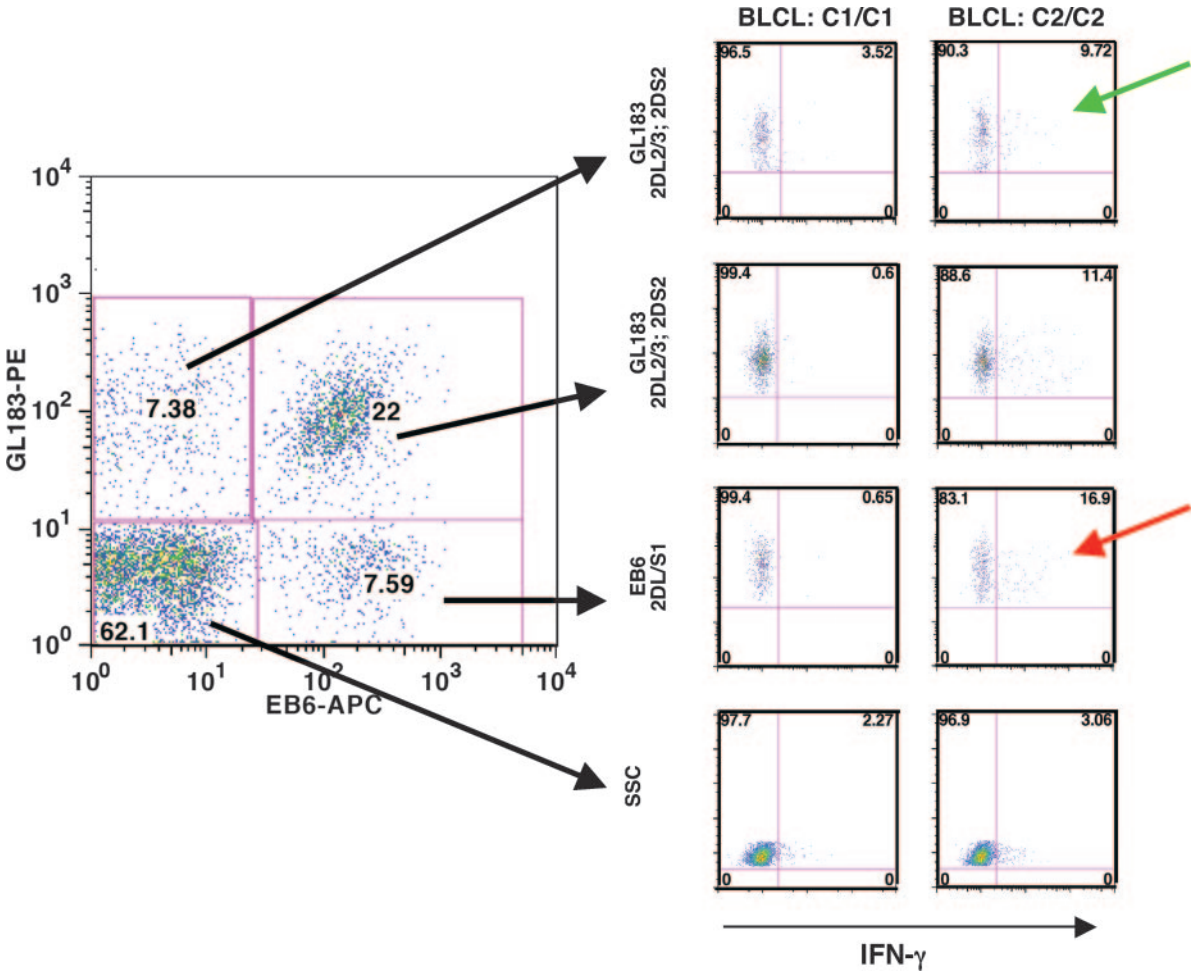

FIGURE 8. IFN-γ alloresponse against C2-positive BLCL by NK cells from *2DS1*-positive, *C1* homozygous donors is observed in both GL183 single-positive NK cells ("missing HLA-KIR ligand group") and in EB6 single-positive cells (direct activation of EB6 (2DL/S1) effector cells). Freshly isolated NK cells from *2DS1*-positive, *C1* homozygous donors (donors no. 001 and 016) were cocultured with allogeneic BLCL target cells matched for the HLA-Bw4 group (9004, 9010, and 9201), in addition to 721.221 as positive control, at E:T ratios of 1:1 for 12 h. Cells were gated for live lymphocytes and the CD3-negative, CD56-positive population followed by additional gating on the EB6- and GL183-staining KIR subsets (*left portion* of figure). These donors were negative for KIR3DL1/3DS1 staining. IFN-γ response to C1 and C2 group homozygous BLCL is shown for each KIR subset, including the subset negative for KIR2DL1, 2DS1; 2DL2/3, 2DS2; and 3DL1, 3DS1 expression (*right portion* of figure). IFN-γ staining is shown on the *x*-axis plotted against GL183 (GL183-positive and EB6, GL183 dual-positive subsets), EB6 (EB6-positive subset), or side scatter (SSC) (negative subset) on the *y*-axis. The green arrow indicates the IFN-γ response against C2 group BLCL by the GL183-positive population, and the red arrow indicates the IFN-γ response against C2 BLCL mediated by the EB6-positive population. Data are representative of four independent experiments performed with two donors (donors no. 001 and 016).

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 allogeneic C2 Ag. Our results support the concept that C2 Ag binding to 2DS1 contributes to the alloactivation of *2DS1*-positive NK cells in addition to "missing self" recognition. We demonstrate that polyclonal IL-2 propagated NK cells derived from *Bw4* homozygous, *C1* 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 allocytotoxicity 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 cytolytic 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 addressed the issues of other receptor-ligand interactions involved in allorecognition by *2DS1*-positive, *C1* group homozygous NK cells. We demonstrate that IFN-γ-producing cells increase following C2 group allorecognition (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 alloresponses. The results we have obtained for NK allorecognition 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 have 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 interaction between the MHC class I Ag and the activating receptor (20, 21, 23).

In this study we have described a model system where the presence of *KIR2DS1* is associated with alloreactivity 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. Multiple mechanisms for self-tolerance by NK cells have been described (reviewed in Refs. 1 and 46). NK cells achieve tolerance to self primarily 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 donors (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 population 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 receptors recognizing MHC molecules. In those NK cells lacking these inhibitory signals the lack of autoreactivity may be achieved by an overall hyporesponsiveness. Our studies using freshly isolated NK cells consistently demonstrated KIR2DS1 activation predominantly 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 hyporesponsive to alloactivation (Figs. 6*A* and 7*A*).

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 indicated that no differences in NK education were observed between *2DL2*-positive, *2DS2*-positive and *2DL2*-positive, and *2DS2*-negative NK cells (44). The only previous studies suggesting a contribution 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 receptors (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 alloreactivity against the C2 group. Six of our nine *KIR2DS1*-positive, *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 following receptor cross-linking (14, 15). There was, however, no evidence for 3DS1 receptor binding to cells transfected with "high binding" Bw4 alleles, and the natural ligand for this receptor remains 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 alloactivation of fresh NK cells (Fig. 7*A*).

Our study was designed to test the hypothesis that NK alloreactivity 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 components 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 hemopoietic 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 alloreactive 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 upon 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 that 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

The authors have no financial conflict of interest.

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