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KIR2DL4 (CD158d) Genotype Influences Expression and Function in NK Cells[1]

Jodie P. Goodridge,*† Campbell S. Witt,[2]* Frank T. Christiansen,*† and Hilary S. Warren‡

The expression and function of the NK cell receptor KIR2DL4 are controversial. Two common alleles of the transmembrane domain of KIR2DL4 exist. The 10A allele with 10 adenines at the end of the transmembrane exon encodes a full length receptor, whereas the 9A allele has only 9 adenines resulting in a frame shift which in turn generates a stop codon early in the first cytoplasmic exon. The possibility that the 10A and 9A alleles might result in differences in expression and function of KIR2DL4 was explored using mAbs to KIR2DL4. Transfection experiments with cDNA from the 10A and 9A alleles revealed significant membrane expression only with the protein encoded by the 10A allele. Analysis of peripheral blood NK cells demonstrated that only in subjects with at least one 10A allele was cell surface expression of KIR2DL4 detectable, and then only on the minor CD56^{bright} NK cell subset. The major CD56^{dim} NK cell subset did not cell surface express KIR2DL4 but, interestingly, did so after in vitro culture. Functional analysis using cultured NK cells in redirected lysis assays demonstrated that KIR2DL4 is an activating receptor for NK cells with at least one 10A allele. No significant activity was detected for NK cells generated from subjects homozygous for the 9A allele. These data show that genotype influences cell surface expression and function of KIR2DL4 which may account for reported differences in KIR2DL4 expression and function. ***The Journal of Immunology*****, 2003, 171: 1768–1774.**

Natural killer cells express receptors for class I HLA molecules that inhibit or activate their effector functions (1, 2). These receptors are encoded by families of genes, which include the killer Ig-like receptors (*KIR*,[3] CD158) and leukocyte Ig-like receptors (*LILR*, CD85) on chromosome 19 and the *CD94/NKG2* receptors on chromosome 12. Various KIR recognize allelic epitopes on HLA-A, HLA-B, and HLA-C molecules, whereas LILRB1, the only member of the *LILR* family on NK cells, recognizes the nonpolymorphic α-3 domain of all HLA molecules. The CD94/NKG2 receptors recognize class I HLA molecules indirectly by binding HLA-E that is surface expressed when it binds class I HLA signal sequence peptides. These families of receptors either inhibit function through immunotyrosine-based inhibitory motifs (ITIMs) or activate function through adaptor molecules (DAP-12) that associate with a charged residue in the transmembrane domain. The inhibitory receptors in these receptor families provide the mechanism by which NK cell effector function is prevented against cells expressing normal levels of class I HLA molecules, yet is permitted against cells expressing reduced levels of class I HLA molecules, such as on virally infected or tumor cells. The activating receptors provide a mechanism by which NK cell function is stimulated. Although HLA-E is known to be the ligand for the activating CD94/NKG2C receptor (3), there is doubt as to the effectiveness of class I HLA molecules as ligands for activating KIR (4).

KIR2DL4 (CD158d) is a member of the *KIR* gene family yet is unique in its genomic organization, expression, and function compared with other KIR (5). *KIR2DL4* is considered a framework *KIR* gene, being present in almost all genomes. It is expressed in a codominant manner contrasting with at least some other KIR that may be monoallelically expressed (6). *KIR2DL4* is reported to be expressed at the mRNA level, by all or most NK cells (7–9), contrasting with the expression of other KIR that is clonally distributed (7). Using polyclonal antisera, KIR2DL4 protein has been variously reported to be present on the surface of all peripheral NK cells (10) or restricted to uterine NK cells (11). Functionally, KIR2DL4 has been reported to be an inhibitory receptor for peripheral NK cells (12) and uterine NK cells (11), but ligation of KIR2DL4 with mAb results in activation of IFN-γ secretion and cytotoxicity in peripheral NK cells (13). These different functional activities of KIR2DL4 are consistent with the fact that KIR2DL4 has both an ITIM in its cytoplasmic domain and a charged residue in the transmembrane domain. Both these domains in KIR2DL4 have been shown to be functional using chimeric receptors (14, 15). These data suggest KIR2DL4 may mediate different functions under different circumstances.

Any study of KIR2DL4 must take into account *KIR2DL4* gene polymorphisms. We have previously reported two genetic variants of the transmembrane exon of the *KIR2DL4* gene (16, 17). One allele (10A) produces only a single mRNA that appears to code for a classical membrane bound receptor that includes an inhibitory ITIM motif in its cytoplasmic tail. The alternate allele (9A) has a deletion of 1 adenine in a series of 10 adenines at the end of the transmembrane exon.[4] The 9A allele produces a small amount of mRNA that includes the transmembrane exon (9ATr). However,

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[3] Abbreviations used in this paper: KIR, killer Ig-like receptors; LILR, leukocyte Ig-like receptors; ITIM, immunotyrosine-based inhibitory motifs; HA, hemagglutinin.

[4] In different alleles, the end of exon 6 in the genomic sequence consists of either 9 or 10 adenines. However, because the first nucleotide of exon 7 is also an adenine, the 9A and 10A genomic sequences result in a series of 10As and 11As, respectively, in the mRNA. Furthermore, in some alleles, the last genomic adenine may be replaced with a guanine.

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the frame shift caused by the deletion results in a stop codon 4 codons into exon 7, the first cytoplasmic exon, suggesting a protein with a truncated cytoplasmic tail. The protein product from this mRNA could conceivably be expressed on the membrane but would lack the inhibitory ITIM motif. The other mRNA transcript produced by the 9A allele (9A ΔTM) is more abundant but omits the transmembrane exon, making it unlikely that any translated protein could be expressed on the membrane. Because the 9A and 10A alleles of *KIR2DL4* each have a population frequency of 50% (16), this suggests balancing selection and functional importance. Apparent differences in expression and function reported for this receptor could be explained by different groups having studied subjects with different transmembrane genotypes. This study reports on the expression and function of KIR2DL4 on NK cells in individuals expressing the 9A and/or 10A alleles. We show that KIR2DL4 cell surface expression is restricted to individuals with at least one 10A allele and that KIR2DL4 is constitutively expressed by only the minor $CD56^{bright}$ peripheral blood NK cell subset but can be induced on a majority of NK cells following in vitro culture. Furthermore, we show KIR2DL4 transmembrane genotype influences activation of cytotoxic function.

Methods and Materials

Blood samples

Blood samples for expression of KIR2DL4 on peripheral NK cells were obtained from randomly selected donors attending the Australian Red Cross Blood Service in Perth and Canberra. Whole blood was obtained by venipuncture and collected into heparinized tubes. For some experiments, whole blood was treated with NH_4Cl to lyse erythrocytes and immediately used for analysis by flow cytometry. For other experiments, mononuclear cells were obtained by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density gradient centrifugation, stored frozen in aliquots in liquid nitrogen, and thawed as required.

mAbs and immunofluorescence flow cytometry

An IgG1 mAb (No. 33) and IgM mAb (No. 64) to KIR2DL4 were produced as previously described (13) and kindly provided by Drs. E. Long and S. Rajagopolan (Laboratory of Immunogenetics, National Institutes of Health, Bethesda, MD). These Abs were used in the Perth laboratory in conjunction with a FITC-conjugated goat anti-mouse Ig(H + L) (Caltag Laboratories, Burlingame, CA) for staining of peripheral NK cells. In the Perth laboratory, the following mAbs were obtained from commercial sources: PE-Cy5-conjugated CD3 (UCHT1, IgG1) and PE-conjugated CD56 (N901, IgG1), both from BD PharMingen (San Jose, CA). In the Canberra laboratory, the following Abs were obtained from commercial sources: PE-conjugated CD56 (IgG2a) from Chemicon Europe (Chandlers Ford, UK); FITC-conjugated CD3 and FITC-conjugated CD19 from BD PharMingen. The NKG2D mAb 149804 (IgG1) was a kind gift from Dr. J. P. Houchins (R&D Systems, Minneapolis, MN). In the Canberra laboratory, mAb 64 was detected using biotinylated goat anti-mouse IgM (Chemicon, Boronia, Australia) and streptavidin-Cy-Chrome (BD PharMingen). Reagents were incubated with cells for 30 min on ice, centrifuged, and washed twice between incubations. All reagents were titrated and used at optimum concentrations. Cells were analyzed using either a BD Biosciences FACScan (San Jose, CA) in Canberra or a Coulter EPICS XL-MCL flow cytometer (Beckman Coulter, Fullerton, CA) in Perth. Lymphocytes were gated electronically on the basis of forward and side scatter parameters. NK cells were identified as $CD3^-$ $CD19^-$ lymphocytes.

Isolation and culture of NK cells

PBMC were labeled with mAbs as described above, and NK cells were sorted using a BD Biosciences FACStar gating electronically on lymphocytes and selecting cells that were CD3-$FITC^-$ and CD19-$FITC^-$. For sorting $CD56^{bright}$ or $CD56^{dim}$ NK subsets, NK cells were first isolated from PBMC using the RosetteSep (Stemcell Technologies, Vancouver, Canada) method of negative selection adapted for use with frozen PBMC (18) before labeling with CD56-PE. Sorted NK subsets were expanded in culture using previously described methods (19). Briefly, NK cell proliferation was initiated using gamma-irradiated MM-170 malignant melanoma cells or JY B-lymphoblastoid cells (19) with medium containing 200 U/ml rIL-2 (generously provided by Dr. G. Zurawski, DNAX Research Institute of Cellular and Molecular Biology, Palo Alto, CA). In some experiments, sorted NK cells were labeled with CFSE (Molecular Probes, Eugene, OR) before culture to monitor cell division (20). Proliferating NK cells were maintained after day 8 by subculturing as required in medium containing 50 U/ml rIL-2. Cultured cells were verified as NK cells ($CD3^-CD94^+$) before functional analysis.

Cytotoxicity assays

Cytotoxicity was measured in a 4-h ^{51}Cr release assay using 5000 ^{51}Cr-labeled target cells as described previously (19). The $FcRII^+$ mouse mastocytoma cell line P815 was used as the target cell. The E:T ratio was 2:1. mAb concentrations that were optimal in the assay were usually one-tenth of that giving maximum staining by flow cytometry.

cDNA production

Total cellular RNA was extracted from 2×10^6 cells using Trizol reagent (Life Technologies, Gaitherburg, MD) according to the manufacturer's instructions. First strand cDNA was synthesised from 5 μg of total RNA using oligo$(dT)_{12-18}$ primer and 200 U of Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA). The reaction was incubated for 1 h at 42°C and then terminated by heating to 65°C for 15 min. Samples were digested with 1 U of RNase H (Roche Diagnostic Australia, Castle Hill, Australia) to remove the complementary RNA strand.

Cloning and transfections

KIR2DL4 transcripts were amplified using a forward primer specific for exon 3 (D0) of *KIR2DL4* and a reverse primer specific for exon 8 of *KIR2DL4* (10). PCR products were ligated into pGEM-T EasyVectorII (Promega, Annandale, Australia) and cloned into JM109 cells. Individual clones were sequenced to determine the transmembrane type and Ig domain sequences. Clones representing different transmembrane types and Ig domain sequences were excised from pGEM-T using *Bgl*II and *Sal*I (Promega) and inserted into the pDisplay expression vector (Invitrogen) immediately downstream and in frame with the hemagglutinin (HA) tag sequence. The expression constructs were cloned in TOP10F′OneShotTM bacteria (Invitrogen). Equal amounts of cloned pDisplay containing different *KIR2DL4* inserts were transfected into 293T Phoenix cells (courtesy of Dr. D. Eisen, Institute of Child Health Research, Perth, Western Australia) using LipoFectamine-2000 (Invitrogen) and the expression tested using anti-HA ascites (courtesy of Dr. E. Ingley Medical Research Foundation, Royal Perth Hospital, Perth, Australia), KIR2DL4 mAbs 33 or 64, and IgG or IgM isotype controls (Beckman Coulter) followed by a FITC-conjugated anti-mouse Ig (H + L) (Caltag Laboratories). Untransfected cells and control transfections using the pDisplay vector without a *KIR2DL4* insert were included. Transfection efficiency was monitored by cotransfection with a pCDNA3.1/lacZ (courtesy of Dr. Degli-Esposti, Department of Microbiology, QEII Medical Center, Perth, Western Australia) and staining of transfected cells with the fluorescent LacZ substrate resorufin D-galactosylpyranoside (Sigma-Aldrich, Sydney, Australia). KIR2DL4 expression was compared in cells with equal levels of LacZ expression 48 h after transfection. Fig. 1 shows the cDNAs used, the allele from which they were derived, the structure of the protein predicted from the cDNA, and the GenBank accession number corresponding to the cDNA sequence.

Sequencing for genotyping

Exons 6 through 7 were amplified from genomic DNA using primers and method previously described (16). PCR products were sequenced in both directions using M13F and M13R primers and big-dye primer chemistry to determine the transmembrane genotype. Individuals homozygous for the 9A genotype and 10A genotype were in all cases shown to have a series of 9 and 10As, respectively, and the entire sequence was homozygous. The sequence of heterozygotes was homozygous until the *9th A* (forward primer) or *9th T* (reverse primer), after which it became heterozygous.

Results

KIR2DL4 mAbs react with receptors encoded by both common alleles of the Ig domains

In addition to the transmembrane polymorphism, we have previously reported the existence of two common alleles of exons 3 and 4 encoding the Ig domains of KIR2DL4. Before using mAb 33 as a reagent for the investigation of KIR2DL4 expression, the reactivity of mAb 33 for the receptors encoded by each of the two common alleles of the Ig domains was tested. cDNAs Ig1-Tm10A and Ig2-Tm10A of the two common Ig domain alleles (alleles 1F

TM Genotype	[1]Allele	cDNA	Structure (D0 D2 TM CY)	Accession #
10A	1F	Ig1-Tm10A	Y A P	AY223513
10A	1F	Ig1-Tm10ATr[2]	Y A P	AY223514
10A	2F	Ig2-Tm10A	C T A	AY250088
9A	2Δ	Ig2-Tm9ATr	C T A	AY223512
9A	2Δ	Ig2-Tm9ADel	C T A	AY223515

FIGURE 1. Nomenclature and structure of cDNAs transfected into 293T cells. A linker exon is present following the D2 domain in all transcripts but has been omitted to improve clarity. Superscript 1, Allele name given by Witt et al. (16) from which the cDNA was derived. Y/C represents the amino acid polymorphism at aa 53 (D0 domain) of the full length cDNA, including exons 1 and 2, and is due to a G/A substitution at nt 158. A/T represents the amino acid polymorphism at aa 138 (D2 domain) of the full length cDNA and is due to a G/A substitution at nt 412. P/A represents the amino acid polymorphism at aa 209 (D2 domain) of the full length cDNA and is due to a C/G substitution at nt 625. Superscript 2, This transcript is derived from a 10A cDNA but was created by in vitro polymerase slippage at the end of the transmembrane exon during PCR amplification before cloning. It does not occur naturally.

and 2F, respectively, in the paper of Witt et al. (16)) were cloned into the pDisplay expression vector, which added an HA tag to the N-terminal end of the protein. This allowed the use of an anti-HA Ab to check expression of the different KIR2DL4 proteins after transfection of 293T cells. Both cDNAs had 10A sequences in the transmembrane exon and were therefore expected to code for full length receptors. The transfected 293T cells were then stained with an anti-HA Ab and mAb 33. The receptors encoded by both alleles were equally well stained with anti-HA Ab (data not shown). As shown in Fig. 2, the receptors encoded by both alleles were also stained equally well with mAb 33, indicating that mAb 33 is a valid reagent with which to investigate KIR2DL4 expression on peripheral NK cells. Identical results were obtained with KIR2DL4 mAb 64. These mAb have been shown previously not to react with other members of the KIR family (13).

Transmembrane genotype influences KIR2DL4 expression in 293T cells

To determine whether the receptors produced by different transmembrane alleles of KIR2DL4 can be expressed on the membrane, 293T cells were transfected with full length cDNA having a transmembrane domain, long cytoplasmic tail, and the Ig domains of alleles 1F or 2F (Ig1-Tm10A and Ig2-Tm10A), a cDNA postulated to encode a truncated receptor having the Ig domains of allele 2Δ (Ig2-Tm9ATr), or a cDNA having the Ig domains of allele 2Δ but lacking a transmembrane domain (Ig2-Tm9ADel). Transcripts representing a truncated receptor and having the Ig domains of allele Ig1 do not occur naturally, but such a transcript was discovered during cloning of the Ig1 transcripts, probably created by in vitro polymerase slippage. This artificial transcript having the Ig domains of allele Ig1 but postulated to encode a truncated cytoplasmic tail (Ig1-Tm10ATr) was also transfected. Transfected cells were tested with anti-HA Ab and KIR2DL4 mAb 33. Fig. 3 shows that the protein encoded by the transcript postulated to encode a truncated receptor (Ig2-Tm9ATr) is expressed but relatively weakly compared with that encoded by the full length transcript (Ig2-Tm10A), whereas the receptor encoded by the transcript lacking the transmembrane exon (Ig2-Tm9ADel) is not detectable. Cells transfected with the cDNA encoding a truncated receptor of the 1F allele (Ig1-Tm10ATr) produced by in vitro artifact stained with the same intensity as cells transfected with cDNA encoding the naturally occurring truncated receptor derived from the Ig2 allele (data not shown). Identical results were obtained using either anti-HA Ab (Fig. 3) or KIR2DL4 mAb 33 (data not shown).

KIR2DL4 is expressed on CD56^{bright} peripheral NK cells and expression correlates with transmembrane genotype

The transfection data suggested that individuals with different transmembrane genotypes may differ in their expression of KIR2DL4. Having established that the KIR2DL4 mAbs react with receptors encoded by both common Ig domain alleles, we examined KIR2DL4 expression on peripheral NK cells from 27 individuals. Interestingly, when expressed, positive staining for KIR2DL4 was weak and observed only on the minor CD56^{bright} population of NK cells. Representative data (Fig. 4) show that expression of KIR2DL4 on CD56^{bright} NK cells varied between different individuals (Fig. 4, *A* and *B*), and in some cases KIR2DL4 was not detectable (Fig. 4*C*). Staining of CD56^{dim} NK cells was not observed in any of the 27 subjects tested. To determine

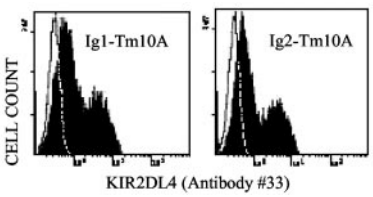

FIGURE 2. Staining of 293T cells transfected with cDNA from *KIR2DL4* alleles 1F (*left*, Ig1-Tm10A) and 2F (*right*, Ig2-Tm10A) by KIR2DL4 mAb 33. Alleles 1F and 2F differ by 1 amino acid in the D0 domain and 2 amino acids in the D2 domain as shown in Fig. 1. mAb 33 reacts equally well with the Ig domains of two common alleles of *KIR2DL4*.

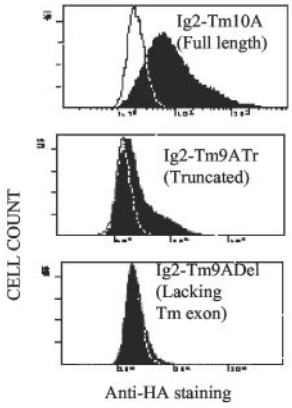

FIGURE 3. The receptor encoded by the full length transcript of *KIR2DL4* is expressed more strongly on the cell membrane than either receptor encoded by the transcripts from the 9A allele. Anti-HA staining of 293T cells transfected with *KIR2DL4* cDNA tagged with HA representing full length membrane receptor (*top*), truncated receptor (*middle*), or "soluble" receptor (*bottom*).

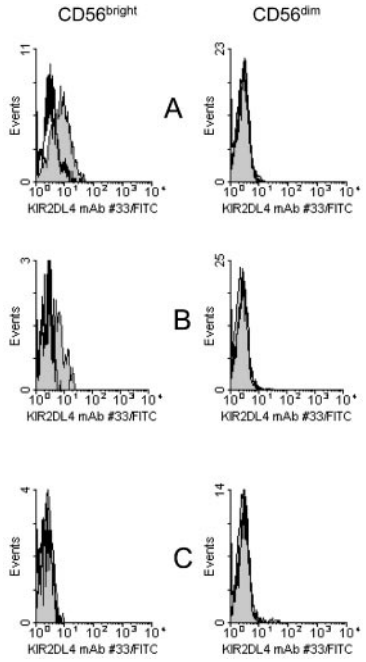

FIGURE 4. KIR2DL4 is expressed only on CD56^{bright} peripheral blood NK cells of some individuals. PBMC were stained with CD3-PE-Cy5 to identify NK cells (CD3$^-$) and with CD56-PE to identify the CD56^{bright} and CD56^{dim} subsets. KIR2DL4 was stained with mAb 33 detected with anti-mouse Ig-FITC. Weak KIR2DL4 staining was observed on only the CD56^{bright} NK cells of some individuals (*A* and *B*), whereas for other individuals NK cells were KIR2DL4 negative (*C*).

whether the presence of KIR2DL4-positive NK cells correlates with transmembrane genotype, the proportion of KIR2DL4-positive cells among CD56^{bright} NK cells of 27 individuals was determined and related to their transmembrane genotype (Fig. 5). Although Fig. 4 shows that there is a shift of the entire CD56^{bright} population when Ab to KIR2DL4 is used, we assessed staining intensity by measuring the proportion of cells with an intensity greater than an arbitrary fluorescence cutoff. The results in Fig. 5 are therefore likely to be an underestimate of the proportion of CD56^{bright} NK cells expressing KIR2DL4. The data show that most subjects with the 10A/10A or 9A/10A genotypes had at least 10% of CD56^{bright} cells staining for KIR2DL4, whereas there was little or no KIR2DL4 staining for any of the subjects homozygous for the 9A allele. Age and sex did not appear to correlate with low numbers of KIR2DL4 expressing cells in those subjects who had the 9A/10A or 10A/10A genotype. Health status is also unlikely to be relevant to low staining as all subjects tested were healthy blood donors.

KIR2DL4 is expressed after in vitro culture of CD56^{dim} cells

NK cells were purified by negative selection from subjects with different *KIR2DL4* transmembrane genotypes and established in culture with IL-2 and irradiated feeder cells. After 16 to 20 days of culture, KIR2DL4 expression was assessed using indirect staining with the IgM mAb 64, biotinylated anti-mouse IgM and streptavidin-Cy-Chrome. In contrast to uncultured peripheral NK cells among which only a small percentage of all NK cells have detectable staining for KIR2DL4, a considerable proportion of cultured cells from 10A/10A or 9A/10A subjects stained positively for KIR2DL4 (Fig. 6). In contrast, for cultured cells from 9A/9A subjects, much weaker staining (donor 2458) or no staining (donor 27629) was observed. Thus, for individuals in whom genotype analysis predicts that KIR2DL4 will be poorly expressed, the culture conditions were unable to induce cell surface expression of KIR2DL4.

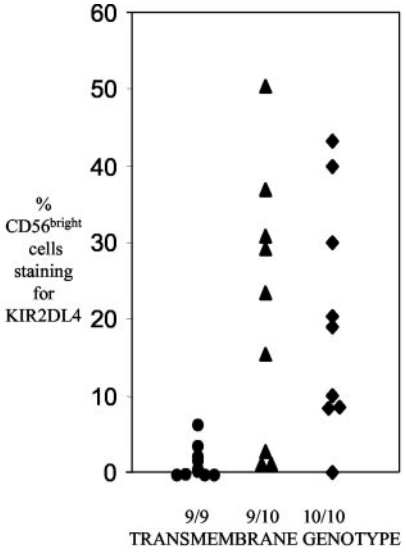

FIGURE 5. KIR2DL4 expression is detectable only on the CD56^{bright} cells of individuals with at least one 10A allele. PBL from 27 individuals with different transmembrane genotypes (9A/9A, 9A/10A, 10A/10A) were stained with CD3-PE-Cy5 to identify NK cells (CD3$^-$) and with CD56-PE to identify the CD56^{bright} and CD56^{dim} subsets. KIR2DL4 was stained with mAb 33 detected with anti-mouse Ig-FITC. The percentage of CD56^{bright} NK cells staining for KIR2DL4 was determined by comparison with a cut off set on cell stained with an isotype control Ab.

To determine whether KIR2DL4 expression on cultured NK cells was a result of outgrowth of KIR2DL4 positive CD56^{bright}

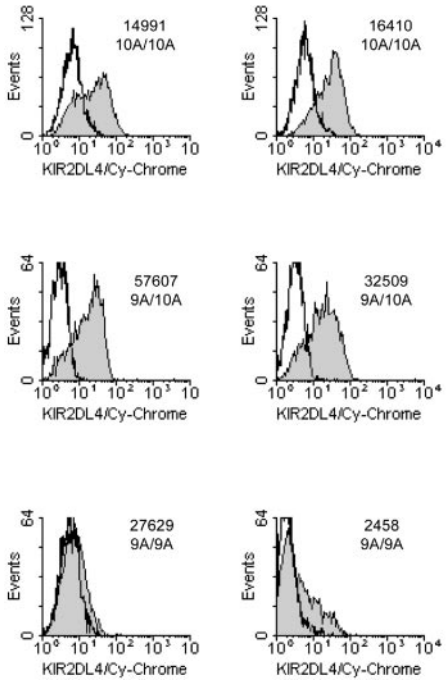

FIGURE 6. Expression of KIR2DL4 on cultured NK cells is dependent on transmembrane genotype. NK cells were generated in culture from different donors by stimulation with rIL-2 and gamma-irradiated MM-170 cells. On day 16, NK cells were stained for KIR2DL4 (mAb 64) detected with biotinylated anti-mouse IgM and streptavidin-Cy-Chrome. Empty histograms show control staining using mouse IgM and the secondary detection reagents. Donor identity numbers are shown above the transmembrane genotype.

cells or due to up-regulation of KIR2DL4 on CD56^{dim} cells, peripheral NK cells were sorted into CD56^{bright} and CD56^{dim} subsets before culture and examined for KIR2DL4 expression before culture and at day 13 of culture. Fig. 7 shows data for an individual of 10A/10A genotype. Before culture, the CD56^{bright} and CD56^{dim} NK cells were estimated to be >99% pure, and as expected only the CD56^{bright} NK cells expressed detectable KIR2DL4. After culture, the CD56^{bright} NK cells retained their KIR2DL4 phenotype, and a substantial proportion of CD56^{dim} NK cells acquired KIR2DL4. For the experiment shown, the CD56^{bright} subset had expanded 14-fold during the 13-day culture period, and the CD56^{dim} subset had expanded 123-fold. Interestingly, CD56 expression increased during culture on the majority of CD56^{dim} NK cells, and this correlated with expression of KIR2DL4. The observation that CD56^{dim} NK cells show enhanced CD56 expression and acquire KIR2DL4 following culture was shown in three different experiments and with donors of both 10A/10A and 9A/10A genotype. Induction of KIR2DL4 expression required that NK cells divide, and this did not occur if NK cells were cultured with rIL-2 alone. This was demonstrated using the intracellular fluorescent dye CFSE to monitor cell division (data not shown). In contrast to the results with donors of 10A/10A and 9A/10A genotype, KIR2DL4 expression remained low on cells derived from both CD56^{bright} and CD56^{dim} subsets generated from a donor of 9A/9A genotype (data not shown).

KIR2DL4 transmembrane genotype influences activating function

NK cells that were cultured and tested for KIR2DL4 expression (Fig. 6) were also tested for cytotoxicity in a redirected lysis assay. As a positive control, cytotoxicity stimulated through NKG2D was assessed, because for all donors NKG2D was expressed on all NK cells and at similar levels regardless of *KIR2DL4* genotype (data not shown). The data in Fig. 8 demonstrate that cultured NK cells from subjects of 10A/10A and 9A/10A genotype kill the FcRII^{+} target cell P815 in the presence of KIR2DL4 mAb 33, but cytotoxicity is weak compared with killing in the presence of NKG2D mAb. For cultured NK cells from donors of 9A/9A genotype, killing though KIR2DL4 was almost undetectable above the background killing induced by the control CD56 mAb. The *KIR2DL4* genotype dependency of cytolytic activity was seen also for NK cells generated from the CD56^{bright} and CD56^{dim} subsets (data not shown).

Discussion

The present data show that *KIR2DL4* transmembrane exon genotype influences both levels of membrane expression and activation of cytotoxic function. Transfection studies using 293T cells demonstrated that the protein encoded by transcripts produced by the 9A allele in which the transmembrane region is deleted is not detectable on the cell membrane. However, the receptor produced by transcripts derived from 9A alleles postulated to encode a truncated receptor can be detected on the cell membrane although at lower levels than the receptor derived from full length transcripts produced by 10A alleles. Although the nucleotide deletion is at the end of the transmembrane exon, the last eight amino acids of this exon (HRWCSKKK) are highly charged and likely to be in the cytoplasm. An additional three codons in the next exon would be translated but would result in the hydrophobic amino acid sequence MLL-stop instead of the usual more hydrophilic sequence DAA. This would not disrupt the recently reported protein kinase C substrate site SxK involved in KIR-mediated inhibition of activation-induced cell death (21, 22) but could conceivably interfere with its accessibility. The weak staining of the putative truncated receptor may not be an accurate reflection of in vivo expression for two reasons. Firstly, the amount of transcript encoding truncated receptors that was transfected was the same as that for the full length transcript, whereas the transcript encoding a truncated receptor appears to be a minority transcript in vivo (16). Thus, the transfection data may be an overestimate of in vivo expression of the truncated transcript. On the other hand, it has been suggested that KIR2DL4 associates with an adaptor molecule at the cell membrane (13). It could be argued that association with an adaptor molecule could improve membrane expression of the truncated receptor and if 293T cells did not express the adaptor, then 293T cells might underestimate the in vivo expression. This possibility seems unlikely, however, given that peripheral NK cells from subjects homozygous for the 9A allele had weak membrane expression and that culturing NK cells, which resulted in up-regulation of KIR2DL4 in cells with a 10A allele, did not result in up-regulation of KIR2DL4 in subjects homozygous for the 9A genotype.

In freshly isolated peripheral NK cells, KIR2DL4 expression was found exclusively on a proportion of the minor CD56^{bright} subset. This was demonstrated with the KIR2DL4 mAbs 33 and 64 that were shown to react with the receptors encoded by the two most common Ig domain alleles of *KIR2DL4*, alleles 1 and 2 (16).

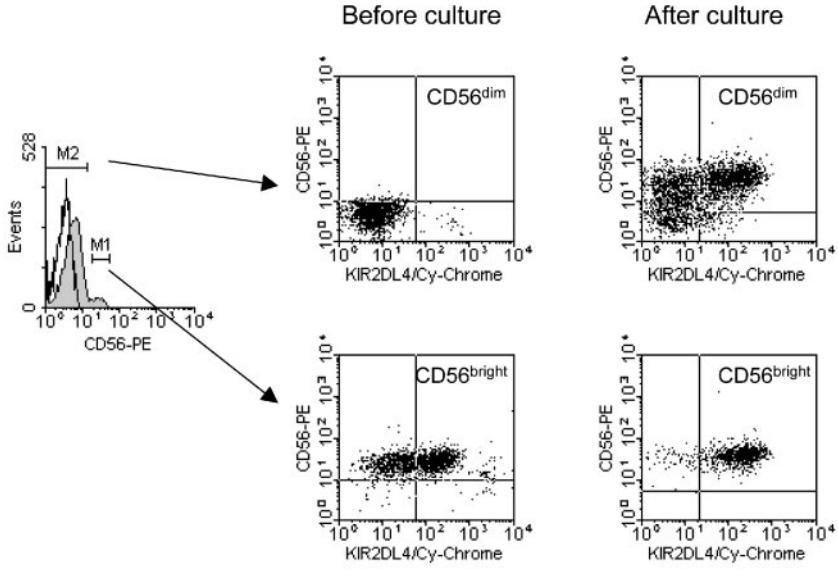

FIGURE 7. KIR2DL4 is expressed on CD56^{dim} NK cells after culture. NK cells isolated from PBMC (donor 14991, 10A/10A genotype) by negative selection were labelled with anti-CD56-PE and sorted into CD56^{bright} (marker, M1) and CD56^{dim} (marker, M2) subsets. KIR2DL4 expression on the subsets is shown before culture and at day 13 of culture after stimulation with rIL-2 and gamma-irradiated JY stimulator cells.

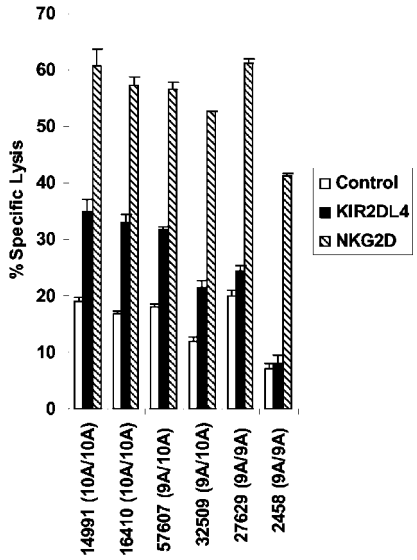

FIGURE 8. Cytotoxicity stimulated through KIR2DL4 is dependent on transmembrane genotype. NK cells were generated in culture from different donors by stimulation with rIL-2 and gamma-irradiated MM-170 cells. NK cells were assayed on day 16 for their ability to kill P815 cells in the presence of isotype control mAb (CD56) and in the presence of KIR2DL4 (mAb 33) or NKG2D mAb. Data are expressed as percent of specific lysis. Donor identity numbers are shown next to their transmembrane genotype.

Selective expression of KIR2DL4 on CD56^{bright} NK cells has not been reported previously but may explain why some groups have found KIR2DL4 to be either not expressed or expressed weakly on peripheral NK cells (11). Expression of KIR2DL4 on CD56^{bright} NK cells is weak relative to other membrane markers, and detection requires attention to the sensitivity of reagents and instrumentation. A second factor complicating the detection of KIR2DL4 on peripheral NK cells is the influence of the transmembrane genotype on membrane expression. KIR2DL4 expression was detectable only on the CD56^{bright} cells of individuals with at least one 10A allele. When peripheral blood NK cells were stimulated to proliferate in culture, KIR2DL4 cell surface expression increased on the CD56^{bright} NK cell subset from individuals with a 10A allele. Importantly, KIR2DL4 was induced on the CD56^{dim} NK cell subset. This is a unique observation, because when studied, expression of other KIR is stable irrespective of the activation status of NK cells (6). It has been reported that KIR2DL4 mRNA is detectable in most peripheral NK cells (7, 9). In the study by Husain (9), resting CD56^{positive} NK cells were analyzed by single-cell PCR, but whether these NK cells were CD56^{bright} or CD56^{dim} was not stated. In the studies by Valiante et al. (7) and Cantoni et al. (8), culture-generated NK cell clones were analyzed, and in this case NK cell proliferation would be expected to induce KIR2DL4 expression. It is also possible that mRNA for KIR2DL4 is present in all NK cells and that its translation into cell surface-expressed protein occurs once NK cell proliferation is initiated.

The observation that KIR2DL4 is constitutively expressed on CD56^{bright} NK cells may account for the observation that KIR2DL4 mAb selectively stimulates IFN-γ production from freshly isolated peripheral blood NK cells yet little cytotoxicity (13). This effector phenotype is characteristic of CD56^{bright} peripheral blood NK cells (23). Uterine NK cells are predominantly CD56^{bright} (24), and it may be that they are derived from CD56^{bright} peripheral blood NK cells. The expression of KIR2DL4 on uterine NK cells (11) and its selective expression on CD56^{bright} peripheral blood NK cells are consistent with this notion. It also has been shown that human CD56^{bright} NK cells harvested from peripheral blood adhere to endothelium of murine uterine tissue sections (25). However, our data with cultured NK cells show that CD56^{dim} NK cells acquire KIR2DL4 and show enhanced expression of CD56. Cultured NK cells express activation markers such as CD45R0 (26), markers that are seen on uterine NK cells (27). Thus, it is also possible that uterine NK cells are derived from the CD16^{-} subset of CD56^{dim} peripheral blood NK cells (23) after activation. That KIR specific for HLA-C alleles are expressed on uterine NK cells (27) yet apparently not on CD56^{bright} peripheral blood NK cells (28) is consistent with the notion that a proportion of uterine NK cells are derived from CD56^{dim} peripheral blood NK cells.

In the present study, redirected lysis assays showed that KIR2DL4 behaves like an activating receptor for cytotoxicity. These findings are consistent with a previous report (13) using cultured NK cells. We observed for NK cells from individuals with at least one 10A allele that the cytotoxic activity stimulated through KIR2DL4 is weak compared with that stimulated through the activating receptor NKG2D. This was the case for 10A homozygote and heterozygote individuals. This weak cytotoxic activity may reflect competing activation signals generated by an adaptor molecule and inhibitory signals produced by the inhibitory ITIM in the cytoplasmic tail. However, interestingly for some heterozygotes, cytotoxicity was weaker than expected given that the level of KIR2DL4 membrane expression was similar to that of homozygotes (data not shown). Peripheral blood NK cells from 9A/9A subjects have little if any membrane expression of KIR2DL4, which was not increased after culture. These cells also had barely detectable cytotoxicity. The present studies did not examine the influence of transmembrane genotype on IFN-γ secretion, but this is clearly of interest in view of the data of Rajagopalan and Long (13) and the role of NK cell-derived IFN-γ in modifying uterine arteries (reviewed by Croy et al. (29)).

Whether KIR2DL4 can behave as an inhibitory receptor under any circumstances is still not clear. The single ITIM in KIR2DL4 has been demonstrated to be functional when uncoupled from the transmembrane arginine (14, 15), and it seems unlikely that this inhibitory potential would be retained in a functional form if it were never used. Whereas we and others (13, 15), using peripheral NK cells and anti-KIR2DL4 Ab in redirected lysis assays, have demonstrated activating activity for KIR2DL4, others using HLA-G expressing cells as the target (11, 12) and uterine NK cells (11) have provided evidence that KIR2DL4 may have an inhibitory function. These contrasting data suggest that KIR2DL4 may be capable of both functions under different circumstances. However, it is clear that the KIR2DL4 receptor encoded by the 9A transmembrane allele cannot mediate an inhibitory function given the absence of a cytoplasmic tail containing an ITIM.

A role for KIR2DL4 in pregnancy is yet to be established. There is evidence that HLA-G is a ligand for KIR2DL4 (8, 10, 11), and HLA-G was originally reported to be expressed uniquely on trophoblasts (30). The absence of a fully functional KIR2DL4 receptor on peripheral blood NK cells in 25% of the population raises doubts as to the importance of KIR2DL4 in pregnancy. Indeed, we have not found any deviation from control frequencies for the *KIR2DL4* transmembrane genotypes in problems associated with pregnancy including pre-eclampsia (16) and recurrent miscarriage (our manuscript in preparation). An essential function for KIR2DL4 in successful pregnancy is also questionable with the recent description of a multiparous woman lacking a *KIR2DL4* gene (31). It is also the case that HLA-G binds the inhibitory LILRB1 and LILRB2 receptors and is indirectly monitored via

HLA-E binding of the CD94/NKG2 receptors, providing alternative means for HLA-G recognition in addition to KIR2DL4. The LILRB1 and CD94/NKG2 receptors as well as other KIR also monitor HLA-C, the only classical class I HLA molecule expressed on trophoblast cells (32). Clearly multiple NK cell receptors and ligands may be involved in regulating the interaction between mother and fetus.

The maintenance of the 9A and 10A alleles of KIR2DL4 at gene frequencies of 0.5 in humans is indicative of balancing selection. In other cases of balancing selection (e.g., β-thalassemia) homozygosity for the wild-type allele and heterozygosity are advantageous genotypes under different circumstances. Homozygosity for the mutant allele is generally disadvantageous, but the mutant allele is maintained in the population due to circumstances that select for the heterozygous genotype. If the 9A allele can be considered the mutant, then this model predicts that homozygosity for the 9A allele will be disadvantageous under some circumstances. The 9A and 10A alleles of KIR2DL4 are present in chimpanzees (33) and pigmy chimps (34), demonstrating that these alleles have been in existence for a long time. It remains to be determined what role if any this polymorphism of the *KIR2DL4* gene has in terms of a selective advantage. Nonetheless, our study has clearly shown that this polymorphism influences both receptor expression and the activation of NK cell cytotoxicity.

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