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Three common alleles of KIR2DL4 (CD158d) encode constitutively expressed, inducible and secreted receptors in NK cells

Jodie P. Goodridge[1], Louise J. Lathbury[1], Noriko K. Steiner[2], Christine N. Shulse[2], Philomena Pullikotil[3], Nabil G. Seidah[3], Carolyn K. Hurley[2], Frank T. Christiansen[1,4] and Campbell S. Witt[4]

[1] School of Surgery and Pathology, University of Western Australia, Australia
[2] Department of Oncology Research Building, Georgetown University, Washington DC, USA
[3] Laboratories of Biochemical Neuroendocrinology, Clinical Research Institute of Montreal, Montreal, Quebec, Canada
[4] Department of Clinical Immunology and Biochemical Genetics, PathWest, Royal Perth Hospital, Western Australia

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Genetic polymorphism of KIR2DL4 results in alleles with either 9 or 10 consecutive adenines in exon 6, which encodes the transmembrane domain. “10A” alleles encode a membrane-expressed receptor that is constitutively expressed on resting CD56^{bright} NK cells and on CD56^{dim} cells after culture. However, in some individuals with the 10A allele, KIR2DL4 cannot be detected on their resting CD56^{bright} NK cells. "9A" alleles have been predicted to encode a secreted receptor due to the splicing out of the transmembrane region. In this publication, we show that those individuals with a 10A allele who lack detectable KIR2DL4 on CD56^{bright} NK cells express a KIR2DL4 receptor in which the D0-domain is excised. This Δ-D0 receptor cannot be detected by the available anti-KIR2DL4 monoclonal antibodies. In such individuals, KIR2DL4 becomes detectable on cultured NK cells due to up-regulation of the full-length KIR2DL4 transcript. In all individuals with 10A alleles, KIR2DL4 ceases to be expressed at the cell surface 16 days after activation, despite the maintenance of maximal levels of KIR2DL4 mRNA transcription, suggesting the existence of a negative regulator of cell surface expression. Finally, we show that the 9A allele can produce a secreted KIR2DL4 receptor.

Key words: KIR2DL4 · NK cells · Receptors

Introduction

KIR2DL4 is a divergent member of the killer cell immunoglobulin-like receptor (KIR) family (reviewed in [1]). KIR2DL4 is unusual among KIR in having a single ITIM in its cytoplasmic tail and a positively charged arginine in the transmembrane region [2, 3], suggesting that it may be capable of both activation and inhibition. Although it has been difficult to show binding of HLA-G to KIR2DL4 [4, 5], evidence is now mounting that HLA-G is indeed the ligand [6–11].

Despite the apparent transcription of KIR2DL4 in all cultured NK cells [12, 13], surface expression of KIR2DL4 is restricted to the CD56^{bright} subset of resting peripheral blood NK cells and up-regulated on IL-2-cultured CD56^{dim} NK cells [14, 15]. However, KIR2DL4 is polymorphic [16–19], and alleles may differ in the number of adenines in exon 6 encoding the transmem-

Correspondence: Dr. Campbell Witt, Department of Clinical Immunology and Biochemical Genetics, Royal Perth Hospital, Wellington St, Perth 6000, Western Australia
Fax: +61-8-92242920
e-mail: Campbell.Witt@health.wa.gov.au

Abbreviations: KIR: killer cell immunoglobulin-like receptor · **SSCP:** single-stranded conformational polymorphism

brane domain [16]. It has been demonstrated that surface expression of KIR2DL4 is only observed on freshly isolated CD56^{bright} or cultured NK cells from subjects with at least one "10A" allele [14, 15]. Individuals homozygous for the "9A" allele (25% of the population) cannot express KIR2DL4 as a membrane-bound receptor. In these individuals, the frame shift caused by the nucleotide deletion results in two transcripts, one missing the transmembrane domain, which potentially produces a soluble receptor, and another that is truncated and not expressed properly on the cell surface [14]. Two groups have reported that even some individuals with a 10A allele do not express KIR2DL4 at the cell surface of their freshly isolated CD56^{bright} cells [14, 15], and this observation remains unexplained.

In this report, we provide new insights into the mechanisms by which genetic polymorphism of the KIR2DL4 gene influences its expression. First, we show that the subgroup of 10A alleles that appear not to express KIR2DL4 at the cell surface can be identified by additional DNA polymorphisms that result in a transcript in which the D0-domain is excised (ΔD0). Secondly, we show that the available anti-KIR2DL4 monoclonal antibodies (mAb) do not detect the product of this transcript, suggesting that they react with the D0-domain. Thirdly, we show that the transcript putatively encoding a secreted receptor produced by the 9A allele (ΔTM) does produce a secreted molecule and that the secreted receptor is not produced by 10A alleles. Finally, we provide evidence for a mechanism that prevents KIR2DL4 cell surface expression despite robust KIR2DL4 mRNA production.

Results

Reactivity of anti-KIR2DL4 antibodies with the 10A allele

We previously demonstrated that, when tested on freshly isolated NK cells, the KIR2DL4-specific mAb 33 and 64 stained only CD56^{bright} NK cells from individuals with at least one 10A allele [14]. However, following *in vitro* culture, the majority of NK cells from such individuals express KIR2DL4. Before embarking on further studies, we wished to evaluate the reactivity of a commercially available anti-KIR2DL4 mAb, 2238, and of mAb 53.1. As the 9A allele of KIR2DL4 is not well expressed on the membrane, blood from a 10A subject was used to assess KIR2DL4 reactivity with the mAb 33, 2238, 53.1 and 64. All mAb were initially titrated to determine their optimal concentration for use in flow cytometry. All four mAb stained the CD56^{bright} population and not the CD56^{dim} population of freshly isolated NK cells (Fig. 1A; day 0). mAb 33, 64 and 2238 gave similar levels of staining, while mAb 53.1 gave weaker staining. After culture with IL-2 for 14 days, most NK cells acquired the CD56^{bright} phenotype and all antibodies stained the majority of NK cells, but once again, mAb 53.1 gave weaker staining (Fig. 1A; day 14). In contrast, mAb 53.1 is the only mAb that detects KIR2DL4 protein in Western blots (Fig. 1B), although limited availability of mAb 64 prevented its testing. We concluded that mAb 33, 64 and 2238 were all suitable for the purposes of membrane staining of KIR2DL4.

10A subtypes and membrane expression

Although membrane staining of KIR2DL4 is visible on freshly isolated NK cells only in subjects with a 10A allele, we previously demonstrated that mAb 33 and 64 failed to stain a subset of such individuals [14]. Two common subtypes of 10A alleles (10A-A and 10A-B) can be distinguished by our routine single-stranded conformational polymorphism (SSCP) typing method [17] due to differences at positions 149, 177 and 440 in intron 6, which precedes the transmembrane exon (Table 1). A review of CD56^{bright} surface expression data from our earlier report revealed that only the 10A-A subtype is expressed by resting CD56^{bright} NK cells (Fig. 1C). Individuals homozygous for the 10A-B subtype or 10A-B/9A heterozygotes do not express KIR2DL4 on CD56^{bright} cells. Using a second mAb, 64, the same phenomenon was shown in a prospective study of an additional set of NK donors (Fig. 1C). Examples of strongly and weakly staining CD56^{bright} NK cells are shown in Fig. 1D.

The polymorphisms in intron 6 that distinguish the 10A-A, 10A-B and 9A alleles are in linkage disequilibrium with common polymorphisms in exons 3 and 4 that encode the D0 and D2 Ig domains. Population studies [20] and Genbank entries indicate that the 10A-A intron 6 sequence is usually associated with KIR2DL4*00102, the 10A-B sequence with KIR2 DL4*005, and the 9A sequence with KIR2DL4*008 or *011. We considered whether the apparent lack of expression associated with the 10A-B allele might be due to the mAb being unable to recognise KIR2DL4*005 Ig domains associated with the 10A-B polymorphism. However, we have previously excluded this possibility by showing that the mAb 33 and 64 bind equally well to 293T cells transfected with the full-length cDNA from either KIR2DL4*00102 (10A-A) or *005 (10A-B) [14].

mRNA splicing in cells with different transmembrane genotypes

As the 9A allele results in excision of exon 6, the transmembrane domain, we tested whether the 10A-B

allele also results in excision of the transmembrane domain, thereby explaining the lack of expression of this allele on resting CD56^{bright} NK cells. cDNA was prepared from resting NK cells of KIR2DL4 10A-A, 10A-B and 9A homozygous individuals. PCR products were generated from the cDNA using primers in exon 5 (D2 domain) and exon 7 (cytoplasmic domain), thereby spanning exon 6 encoding the transmembrane region (Fig. 2B). As shown in Fig. 2A, both the 10A-A and 10A-B homozygotes generated an mRNA transcript of approximately 400 bp, the expected size for a transcript that includes the transmembrane region. Thus, splicing out

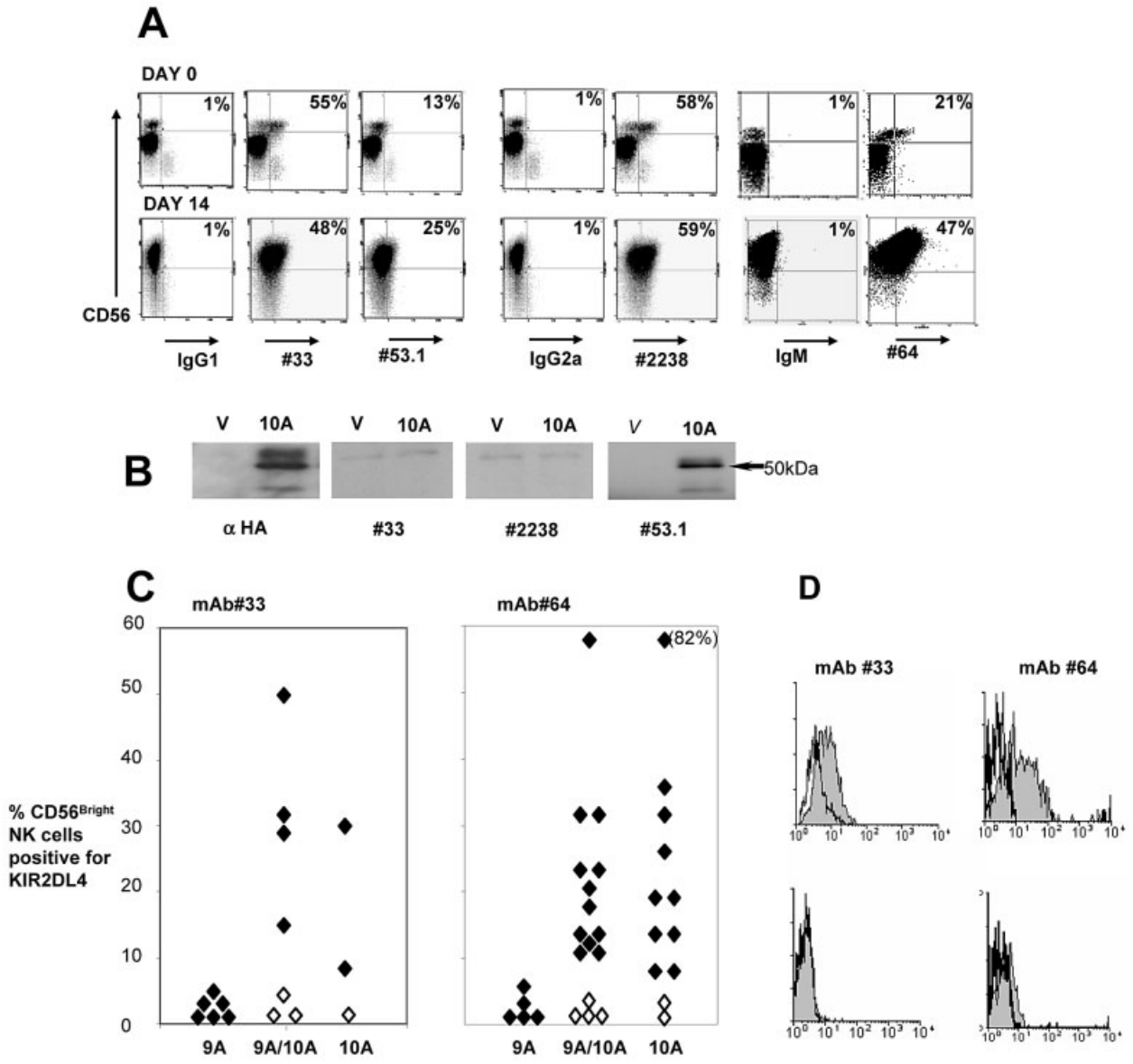

Figure 1. (A) Staining of KIR2DL4 on freshly isolated (upper) and 14-day-cultured NK cells (lower) using mAb 33, 53.1 (IgG1), 2238 (IgG2a), 64 (IgM) and isotype controls. All dot plots are for the same individual, except those for 64 which are from a different individual but have the same KIR2DL4 transmembrane genotype. On fresh NK cells (day 0), staining is restricted to the CD56^{bright} subset of NK cells, while on cultured cells, a proportion of the entire population, which becomes CD56^{bright}, stains positively for KIR2DL4. The percentages represent the proportion of cells with staining brighter than the horizontal dotted line that have KIR2DL4 staining brighter than the vertical dotted line. (B) Western blot of lysates of 293T cells transfected with KIR2DL4 transcript encoding HA-tagged full-length receptor. Only anti-HA antibody and mAb 53.1 detect a protein product of the correct size for full-length receptor. (C) Percentage of CD56^{bright} NK cells expressing KIR2DL4 as detected by mAb 33 (left) or mAb 64 (right) on freshly isolated CD56^{bright} cells from subjects with different transmembrane genotypes. Open symbols represent 10A-B homozygotes and 10A-B/9A heterozygotes. Closed symbols represent all other genotypes. Among subjects with at least one 10A allele, only those with the 9A/10A-B heterozygous or 10A-B homozygous genotype fail to express detectable KIR2DL4. (D) Examples of strong (upper) and weak (lower) staining of CD56^{bright} populations.

Table 1. KIR2DL4 transmembrane genotype nomenclature

Transmembrane allele	Intron 6			SSCP[b] Pattern	Gene[b] Frequency
	149[a]	177[a]	440[a]		
10A-A	C	A	T	A	0.23
10A-B	A	A	C	B	0.24
9A	C	G	T	C	0.53

KIR2DL4 transmembrane genotype nomenclature used in this publication with associated intron 6 single-nucleotide polymorphism (SNP), SSCP patterns and gene frequencies in the Western Australian population. The two 10A transmembrane alleles are distinguishable as different SSCP patterns due to the distinct SNP in intron 6.

[a] Nucleotide number from the beginning of intron 6.

[b] As described in [16].

the transmembrane region does not explain the lack of surface expression in resting 10A-B cells. As previously demonstrated [17], the 9A allele does produce a 400-bp transcript, but it has a single nucleotide deletion and produces a truncated protein lacking the cytoplasmic tail. The approximately 300-bp transcript produced by 9A homozygotes is missing the transmembrane exon [14, 17].

KIR2DL4 transcripts lacking the D0 domain have been described [21]. To test whether the 10A-B genotype produces predominantly a transcript that lacks the D0 domain, PCR primers annealing to exon 1 and exon 8 were used to amplify the entire KIR2DL4 cDNA (Fig. 2D). This revealed additional splice variants (Fig. 2C). The homozygous 10A-A genotype produced predominantly a single band of 1.1 kb, consistent with the full-length transcript (1134 bp) of KIR2DL4. The homozygous 10A-B genotype also produced the 1.1-kb transcript, but produced relatively more of a shorter transcript of approximately 0.9 kb. Cloning and sequencing of the 0.9-kb band revealed that the majority of the D0 domain (nucleotide 90 in exon 3 to the end of exon 3) had been excised, and that nucleotide 90 was joined in frame with the first codon of exon 4, resulting in a transcript of 945 bp (Genbank accession number DQ272466). This transcript is henceforth referred to as the Δ-D0 transcript. The 9A homozygote produced four transcripts consistent with all possible combinations of alternate splicing of the D0-domain and the previously reported alternate splicing of the transmembrane domain. The close relationship between the transmembrane genotype and D0 splicing is illustrated in Fig. 2E, in which the splicing patterns for multiple examples of each homozygous genotype are shown. Fig. 2C, D suggests that at least 50% of KIR2DL4 mRNA is of the Δ-D0 type in 10A-B homozygotes. We therefore considered the possibility that most anti-KIR2DL4 mAb react with the D0-domain, thereby explaining the poor staining of KIR2DL4 on CD56^{bright} cells from 10A-B homozygotes. 293T cells were transiently transfected with full-length or Δ-D0 transcripts tagged with the HA-peptide and tested for staining with several

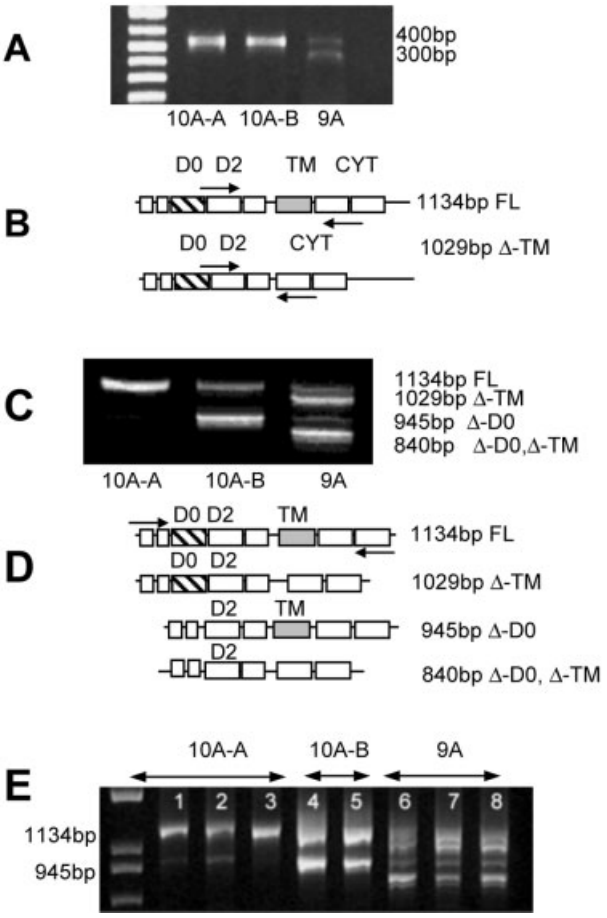

Figure 2. KIR2DL4 mRNA transcripts from freshly isolated NK cells that were homozygous for different transmembrane genotypes were amplified using primers the positions of which are indicated by the horizontal arrows in the schematics. (A) cDNA PCR products detected using primers annealing either side of the transmembrane region. The 10A-A and 10A-B alleles produce a single transcript (400 bp) which includes the transmembrane region, whereas the 9A allele produces both a transcript which includes the transmembrane region (400 bp) and a transcript in which the transmembrane region (shaded in Fig. 2B) is excised. (B) Schematic representations of mRNA sequences determined by cloning and sequencing of PCR products in Fig. 2A. The 10A-B allele produces a transcript lacking the D0 domain. (C) KIR2DL4 mRNA transcripts detected in freshly isolated NK cells that were homozygous for different transmembrane genotypes using primers designed to detect the entire coding region. (D) Schematic representations of mRNA sequences determined by cloning and sequencing of PCR products in Fig. 2C. Only the sequence of the 840-bp transcript was not sequenced and is assumed. (E) KIR2DL4 mRNA transcripts detected in multiple examples of homozygotes for each transmembrane genotype, as determined by SSCP. Primer locations were the same as in (D).

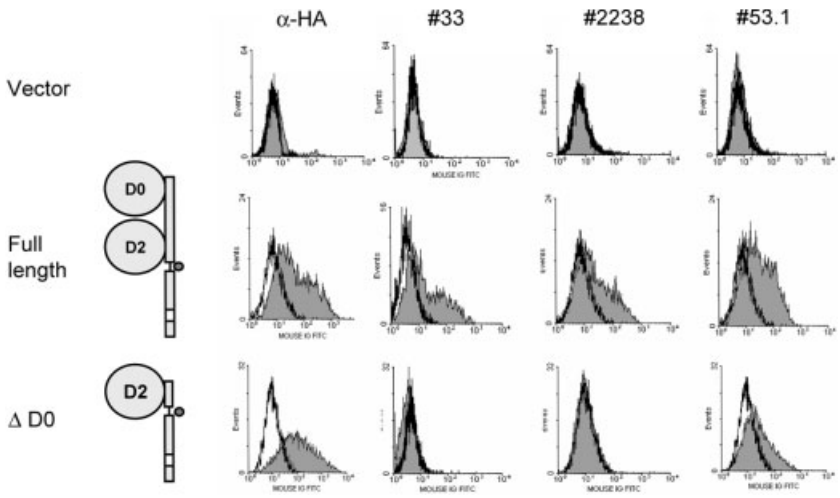

Figure 3. Staining of two alternative Ig domain structures with anti-KIR2DL4 mAb. 293T cells were transfected with either empty vector, full-length KIR2DL4 (10A-B) or an alternative transcript lacking the D0 domain (ΔD0). Transfectants were stained with either anti-HA antibody or one of the three anti-KIR2DL4 mAb.

monoclonal anti-KIR2DL4 antibodies. Fig. 3 shows that receptors produced by both the full-length and Δ-D0 transcripts were successfully expressed, as determined by staining with anti-HA mAb. mAb 33 and 2238 stained the full-length receptor but not the Δ-D0 receptor. mAb 53.1 also stained the full-length receptor and appeared to detect the Δ-D0 receptor, but very weakly. Thus, the available anti-KIR2DL4 mAb cannot detect the Δ-D0 receptor. The membrane staining of KIR2DL4 on resting NK cells from 10A-A homozygotes is relatively weak compared to most surface markers. Therefore, in 10A-B homozygotes, the diversion of some mRNA to the transcript lacking the D0 domain may be sufficient to make staining undetectable.

Interestingly, one of the 10A-A samples (sample 2) exhibited a significant amount of D0 splicing. In order to determine which polymorphism controls excision of the D0-domain, we sequenced the entire KIR2DL4 gene of samples 2, 3, 4, 5, 6 and 7. As both the 10A-B and 9A alleles splice out the D0 domain, we searched for polymorphic sites that shared the same nucleotide in the 10A-B and 9A samples and which had a different nucleotide in both 10A-A samples. None were found. However, as shown in Table 2, there were numerous positions at which the nucleotides were homozygous and shared by all 10A-B and 9A samples (samples 4–7; Fig. 2E) but homozygous for a different nucleotide in one of the 10A-A samples (sample 3; Fig. 2E), and heterozygous in the other 10A-A sample (sample 2; Fig. 2E). In fact, the sequence of sample 2 was consistent with heterozygosity for KIR2DL4*00102 (usually associated with 10A-A) + KIR2DL4*005 (usually associated with 10A-B), with the exception of the intron 6 polymorphisms that contribute to the SSCP pattern that we routinely use to assign the transmembrane genotype, and nucleotide 920 in exon 8. Thus, the small amount of D0 splicing in sample 2 was probably due to the KIR2DL4*005-like sequence at all other positions. Any of these heterozygous nucleotides could be responsible for the D0 (exon 3) splicing, but it would seem most likely that nucleotides prior to exon 4 (*i.e.* those found in introns 1 and 3) would be involved.

The 10A-B allele is expressed on cultured NK cells

We have previously shown that culture of NK cells with IL-2 and feeder cells results in up-regulation of CD56 and *de novo* expression of KIR2DL4 on previously $CD56^{dim}$ cells [14]. To determine whether KIR2DL4 can be expressed on cultured NK cells from subjects with the 10A-B genotype, NK cells from subjects with different transmembrane genotypes were purified and cultured with γ-irradiated Daudi feeder cells and IL-2 for 12 days. As shown in Fig. 4, KIR2DL4 was detectable on NK cells with at least one 10A-A allele and also on 10A-B homozygotes, while 9A homozygotes remained negative. After culture, 40–73% of NK cells from five 10A-A/9A heterozygotes expressed KIR2DL4. (Only three are shown in Fig. 4 as the other two were stained using a different mAb.) Surprisingly, however, only 0–6% of NK cells from four heterozygous 10A-B/9A individuals expressed KIR2DL4 after culture. (Only the two stained with mAb 33 are shown in Fig. 4.) In order to determine why KIR2DL4 could not be detected on 10A-B/9A heterozygotes, the DNA transcripts produced in the heterozygotes were examined. PCR of cDNA prepared from a cultured 9A/10A-B heterozygote showed the full-length 1134-bp transcript (which should be expressed and detectable by flow cytometry) and 945-bp transcripts expected from the 10A-B allele, and the 1029-bp

Table 2. Polymorphic nucleotides in KIR2DL4 sequences of samples 2–7

Sample ID[a)]	9A/10A	Putative genotype	Exon				4															8
			Intron	1	3	3		4	4	4	4	4	4	4	4	4	5	5	5	5	5	5	
			Nucleotide[b)]	161	493	568	446	772	930	972	1354	1504	1905	2306	2506	2546	531	1175	2353	2409	3263	3693	1057
2	10A-A	?2DL4*00102 + *005-like[e)]		S	M	Y	R	4/5[c)]	Y	S	M	Y	K	Y	K	2/4A[d)]	Y	R	W	R	S	K	R
3	10A-A	2DL4*00102		G	A	T	A	5/5	T	C	A	C	T	T	G	4A	T	A	A	A	C	G	A
4	10A-B	2DL4*005		C	C	C	G	4/4	C	G	C	T	G	C	T	2A	C	G	T	G	G	T	G
5	10A-B	2DL4*005		C	C	C	G	4/4	C	G	C	T	G	C	T	2A	C	G	T	G	G	T	G
6	9A	(2DL4*0080101 or 0080103) + *011		C	C	C	G	4/4	C	G	C	T	G	C	T	2A	C	G	T	G	G	T	G
7	9A	2DL4*0080102 + *011		C	C	C	G	4/4	C	G	C	T	G	C	T	2A	C	G	T	G	G	T	G

The table shows all nucleotide positions that are homozygous and shared by both examples of the 10A-B and 9A genotypes but which are homozygous for a different nucleotide in sample 3 (10A-A).

a) Sample numbers are the same as shown in Fig. 2E.

b) Intronic nucleotides are numbered from the first nucleotide in that intron. Exonic nucleotides are numbered according to the mRNA sequence, with the first nucleotide being the A of the ATG start codon.

c) Number of AATA repeats.

d) Number of adenine repeats.

e) The sequence for sample 2 (10A-A) is consistent with 2DL4*00102 (found in sample 3) and KIR2DL4*005, except for nucleotide 920 in exon 8 (not shown) which is heterozygous for C/G. All known alleles have a C at 920. For sample 2, heterozygous positions have been represented by IUB letter codes for heterozygous positions (S = G/C, M = A/C, Y = T/C, R = A/G, K = T/G, W = A/T).

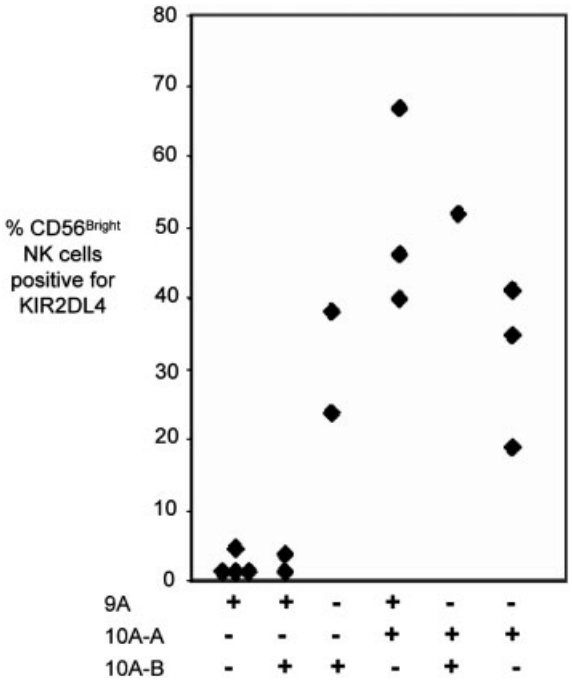

Figure 4. Expression of KIR2DL4 in cultured NK cells of different transmembrane genotypes stained with mAb 33. KIR2DL4 is detectable on 10A-B homozygotes but not on 9A/10A-B heterozygotes.

and 840-bp transcripts expected from the 9A allele (data not shown). Furthermore, expression of the full-length 1134-bp 10A-B receptor in 293T cells was not significantly reduced by co-transfection with either the 9A truncated or 9A ΔTM transcript (data not shown). This suggests that expression of the 10A-B full-length receptor in 10A-B/9A NK cells is suppressed by a mechanism specific to NK cells.

Culture-induced changes in KIR2DL4 mRNA

As KIR2DL4 was not expressed on resting CD56^{bright} NK cells from 10A-B homozygotes but appeared after culture, we monitored KIR2DL4 mRNA and surface expression in the same cells during culture, to determine whether quantitative or qualitative changes occurred in mRNA production and splicing. NK cells from 10A-A, 10A-B and 9A homozygotes were purified and cultured for 16 days with feeder cells and IL-2. Fig. 5 shows that prior to culture, as previously shown, KIR2DL4 was expressed by CD56^{bright} NK cells of 10A-A homozygotes only. However, following culture, KIR2DL4 was equally well expressed by 10A-A and 10A-B homozygotes at days 10 and 13, and became essentially negative again on day 16 for both genotypes. As expected, the 9A homozygotes were unable to express KIR2DL4 at any time. In mRNA extracted from freshly isolated NK cells (mostly CD56^{dim}), the KIR2DL4 mRNA of the full-length transcript was barely detectable in all genotypes when standardised against the β-actin gene, but a weak band was just visible in the 10A-A cells. The increase in KIR2DL4 expression detected by flow cytometry after culture was accompanied by a marked increase in transcription of KIR2DL4 in all genotypes. In particular, the 10A-B genotype produced a substantial quantity of the full-length transcript that included the D0-domain (1134 kb) and which is detectable by the anti-KIR2DL4 mAb. An unexpected finding, however, was that on day 16 cell surface expression had disappeared from both the 10A-A and 10A-B genotypes, despite mRNA transcription of the full-length transcript being as high as at any time. This suggests that transcription alone is not sufficient for surface expression of KIR2DL4. As FcεRI-γ has been reported to be a co-receptor for KIR2DL4, we examined the possibility that a decrease in transcription of FcεRI-γ was responsible for the decreased expression of KIR2DL4 on day 16. The same cDNA samples used to examine KIR2DL4 transcription were used for amplification of FcεRI-γ. No variation in FcεRI-γ expression was observed between days 10, 13 and 16 (data not shown).

The 9A allele produces a soluble receptor

To determine whether the putative soluble receptor produced by the 9A allele might actually be secreted, the different HA-tagged transcripts of KIR2DL4 were transiently expressed in 293T and CHOK-1 cells. A Western blot of the 293T cell lysates (Fig. 6A) revealed a protein band corresponding to the 49-kD full-length receptor in cells transfected with the full-length transcript produced by the 10A-A genotype. The same-sized transcript from a 10A-B NK cell also produced a protein of 49 kD. A protein of similar size was also identified in lysates of cells transfected with the cDNA encoding the putative soluble receptor lacking the transmembrane region (ΔTM) from 9A cells. The predicted size of the soluble receptor is only 35 amino acids shorter than the full-length receptor and therefore indistinguishable from the full-length receptor in this Western blot. A much shorter protein band (34 kD) can be seen for the 9A transcript encoding the predicted truncated protein (9A-Tr). The culture supernatants from the same transfectants showed that protein corresponding to KIR2DL4 is secreted only by cells transfected with the transcript encoding the putative soluble receptor lacking the transmembrane region (9A-ΔTM) produced by the 9A allele. The weaker 38-kD band seen in both the 293T and CHOK cells with both the 10A-A full-length and 10A-B full-length transcripts probably represents unglycosylated receptor. The intense KIR2DL4 band observed in the unconcentrated 9A-ΔTM culture supernatant suggests that the secreted receptor is produced in significant quantities. The

production of the secreted receptor was not peculiar to 293T cells, as CHOK cells transfected with the 9A-ΔTM cDNA also produced the secreted receptor (Fig. 6B).

Influence of KIR2DL4 TM genotypes on IFN-γ secretion

We have previously shown that expression of at least one 10A allele is necessary for activation of redirected lysis of anti-KIR2DL4-coated targets. Others have shown that stimulation of KIR2DL4 using anti-KIR2DL4 antibodies also results in IFN-γ secretion [22]. We therefore compared the ability of NK cells with different KIR2DL4 transmembrane genotypes to secrete IFN-γ in response to anti-KIR2DL4 antibody. Day 10 secondary cultured NK cells from subjects homozygous and heterozygous for different KIR2DL4 transmembrane alleles were stimulated with solid-phase-immobilised anti-KIR2DL4 mAb 2238. At 20 h, culture supernatants were assayed for IFN-γ. No obvious relationship was observed between the KIR2DL4 genotype and IFN-γ secretion expressed as pg/mL (Fig. 7A). However, the IFN-γ response to the IgG2a isotype control was highly variable, in some cases substantial, and correlated with

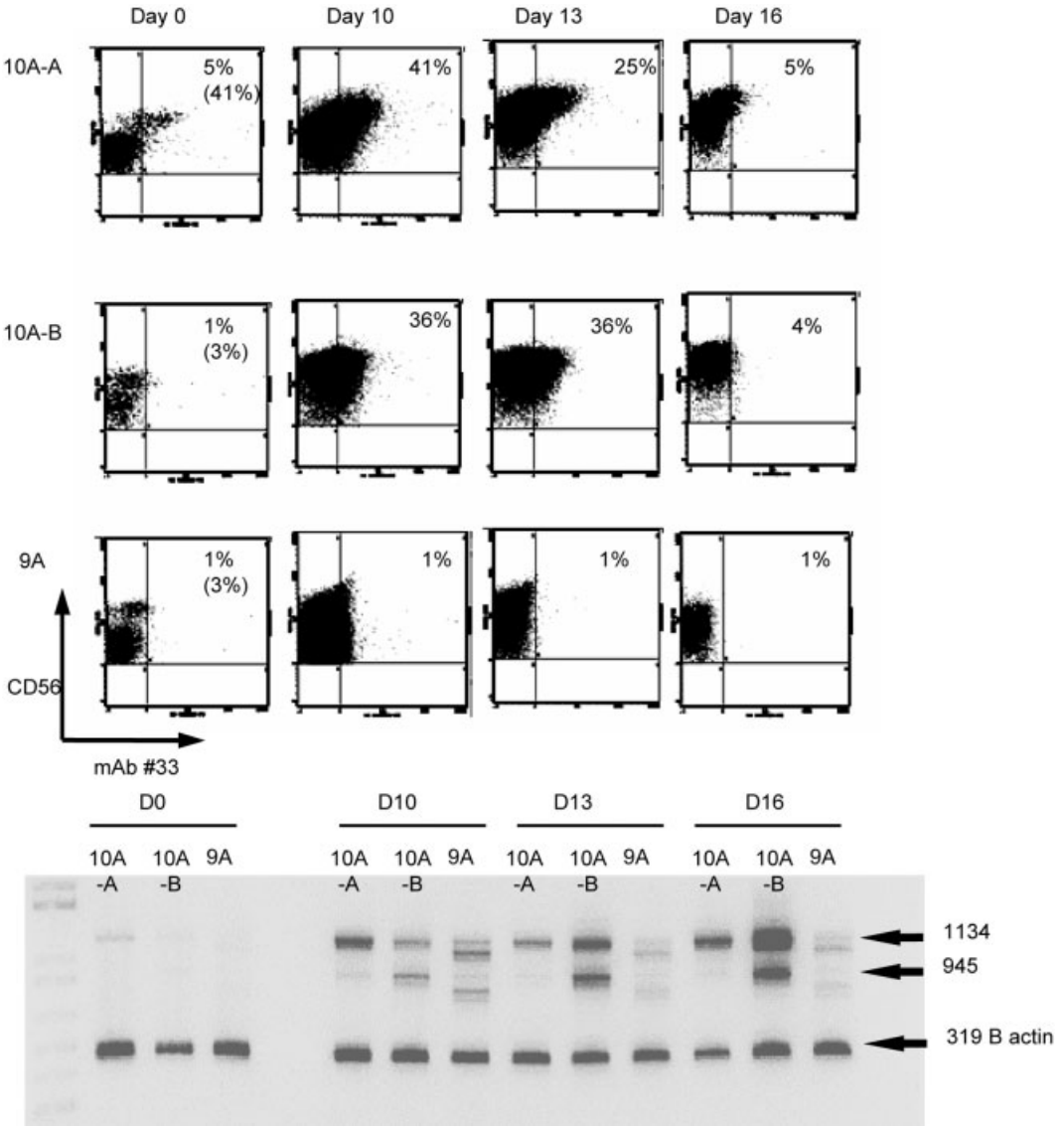

Figure 5. KIR2DL4 expression as detected by flow cytometry (scattergrams) and mRNA expression (gel) at various time points in NK cells of different transmembrane genotype (homozygous 10A-A, homozygous 10A-B, homozygous 9A) cultured for 16 days with Daudi feeder cells and rIL-2. The percentage of either NK cells or $CD56^{bright}$ (in parentheses for day 0) cells that are KIR2DL4 positive appears in the top right-hand corner of all scattergrams. Increased surface expression of KIR2DL4 in the 10A homozygotes at day 13 is associated with increased transcription of the 1134-bp mRNA band, but KIR2DL4 surface expression is down-regulated on day 16 whilst mRNA transcription remains high. The 9A homozygote was unable to express KIR2DL4 at any time despite producing increased amounts of various KIR2DL4 transcripts.

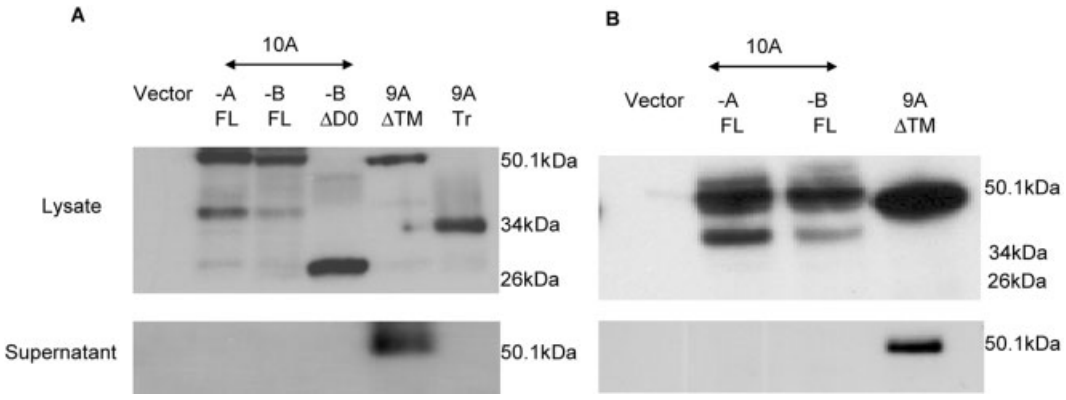

Figure 6. Western blots of KIR2DL4 proteins present in (A) 293T or (B) CHOK cell lysates (upper) and culture supernatants (lower) after transfection with various HA-tagged KIR2DL4 transcripts detected with anti-HA antibody. Lysates and supernatants were tested without concentration. A secreted receptor is observed only in the ΔTM transcript supernatants. FL = full-length, Tr = truncated, ΔD0 = transcript without D0-domain.

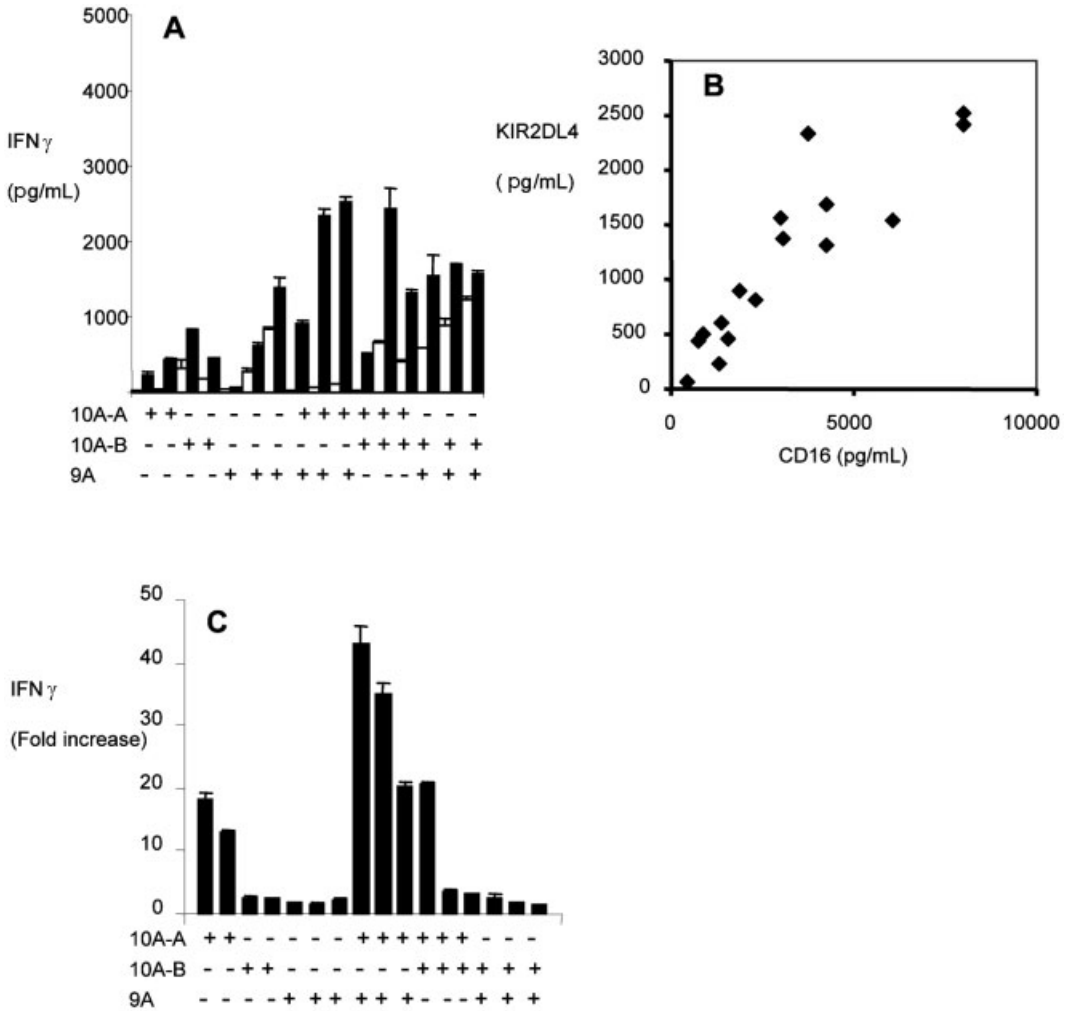

Figure 7. IFN-γ production by day 10 secondary cultured NK cells of different transmembrane genotypes after 20 h of stimulation with solid-phase-bound mAb. KIR2DL4 alleles present are indicated by "+" next to the relevant allele. Means of triplicates ± SEM are shown. (A) IFN-γ secretion expressed as pg/mL in response to anti-KIR2DL4 mAb 2238 (solid bars) or IgG2a isotype control (open bars). (B) Correlation between IFN-γ secretion expressed as pg/mL in response to anti-KIR2DL4 and anti-CD16 antibodies. (C) IFN-γ secretion expressed as fold-increase over isotype control in response to anti-KIR2DL4 antibody.

the anti-KIR2DL4 response, suggesting that the total IFN-γ response was influenced by the activation state of the NK cells. We therefore compared the total IFN-γ response to anti-KIR2DL4 antibody with the total IFN-γ response to anti-CD16 antibody, another potent activator of IFN-γ production, without regard to the isotype controls. As shown in Fig. 7B, the two responses were strongly correlated, suggesting that absolute IFN-γ responses are strongly influenced by the activation state of the cells. We therefore examined whether expressing the IFN-γ response as fold-increase over that elicited by the isotype control might be more closely related to the KIR2DL4 genotype. Fold-increase did appear to relate to the KIR2DL4 genotype (Fig. 7C). In particular, homozygous 10A-A individuals gave large fold-increases and homozygous 9A individuals gave small fold-increases. In fact, the possession of at least one 10A-A allele appeared necessary for a good response. The response attributable to the 10A-B allele was less clear, with homozygotes and 10A-B/9A heterozygotes giving poor responses, but one 10A-B/10A-A heterozygote responding well while two others responded poorly.

Discussion

This study clarifies our understanding of the influence of genetic polymorphism on the expression of KIR2DL4, which can now be summarised as follows. Six different KIR2DL4 transcripts resulting in six different proteins are possible through differential splicing of the KIR2DL4 mRNA. The 10A-A allele produces almost exclusively the full-length transcript (1134 bp) which is expressed at the membrane even in resting CD56^{bright} NK cells. In resting CD56^{bright} NK cells, the 10A-B allele produces both some full-length transcript but relatively more of the Δ-D0 transcript (945 bp), the latter being also expressed on the membrane but not detectable by available antibodies. The 9A allele produces four transcripts: Two produce soluble proteins – one including the D0 domain (ΔTM, 1029 kb) and one missing the D0 domain (ΔTM, ΔD0, 840 kb), whilst the other two produce receptors with truncated cytoplasmic tails, neither of which are expressed well on the cell membrane – one including the D0 domain (1133 bp) and one missing the D0 domain (944 bp).

The 10A-B allele can be distinguished from the 10A-A allele by polymorphisms in intron 6 at positions 149, 177 and 440 which are characteristic of the KIR2DL4*005 allele. Others have previously cloned a ΔD0 transcript [7]. Thus, resting CD56^{bright} NK cells with the 10A-B genotype probably do express KIR2DL4 – although mainly the Δ-D0 receptor – and the apparent lack of expression reflects the specificity of the antibodies used for detection.

The nucleotides controlling splicing out of the D0 domain could not be identified in this study due to the tight linkage disequilibrium between the polymorphisms that distinguish the 10A-A and 10A-B genotypes and other polymorphic nucleotides. Nevertheless, a short list of candidate nucleotides has been identified. Intuitively, one might expect nucleotides in intron 2 immediately preceding exon 3 (which encodes the D0 domain) or perhaps in intron 3 to be more likely to influence splicing out of the D0 domain. No such polymorphisms were identified in intron 2, but nucleotides 493 and 568 in intron 3 would be worthy of further investigation.

In vitro culture of NK cells has been shown to increase the level of surface expression of KIR2DL4 on NK cells with at least one 10A allele, both on CD56^{dim} and CD56^{bright} NK cell subsets [14]. We have now shown that NK cells with the 10A-B genotype express detectable KIR2DL4 after culture due to up-regulation of KIR2DL4 mRNA production, including that for the full-length receptor which is detectable by the available mAb. Cultured NK cells with the 9A genotype remain negative for KIR2DL4 expression. Although the "full-length" transcript is also up-regulated in cultured cells of the 9A genotype, we have shown previously that this transcript has a single nucleotide deletion that results in a poorly expressed, truncated receptor. The lack of KIR2DL4 expression on 10A-B/9A heterozygotes, even after culture, is surprising. The mRNA species produced by heterozygotes are as expected for co-dominantly expressed alleles [23], and we could not show any protein level interaction by co-transfection of 293T cells with 9A and 10A-B cDNA. This suggests an NK cell-specific mechanism that prevents surface expression of the full-length 10A-B protein in the presence of the 9A proteins. The existence of a negative regulator of surface expression is also suggested by the decline in expression of KIR2DL4 on cultured NK cells at day 13, despite the maintenance of high levels of mRNA transcription. A negative regulator would also explain why surface expression is restricted to the CD56^{bright} population in freshly isolated NK cells, despite similar levels of mRNA in CD56^{dim} cells [24]. It is possible that down-regulation of an adaptor molecule could prevent surface expression of KIR2DL4. Indeed, co-transfection of FcεRI-γ has been shown to enhance KIR2DL4 expression in Jurkat cells [25]. However, we were unable to show any difference in transcription of FcεRI-γ between day 10 and day 16 of culture.

Since the discovery of KIR2DL4 transcripts lacking the transmembrane region (Δ-TM) in NK cells of the 9A genotype, we have hypothesised that these splice products may encode soluble receptors that could be secreted from the cell [17], since the correct reading frame is maintained in the cytoplasmic region of the

Δ-TM transcript. In this report, we have confirmed that this is possible. Expression of the HA-tagged ΔTM transcript in 293T and CHOK cells resulted in a sufficient concentration of the secreted protein such that strong bands were detectable by Western blot without concentration of the supernatant. Whether this soluble receptor is produced *in vivo* and its possible functional significance remain to be determined.

Ligation of KIR2DL4 has been shown to result in weak activation of cytotoxicity [14] and strong IFN-γ secretion [26]. In the current study, fold-increases in IFN-γ responses differed according to the KIR2DL4 transmembrane genotype, largely as predicted from the surface expression data. Thus, 10A-A homozygotes produced the strongest IFN-γ response to KIR2DL4 ligation, and 9A homozygotes produced the weakest response. Although we show data for a limited number of each genotype, we are now assaying IFN-γ responses in uterine NK cells, and these samples confirm the effect of genotype as indicated in Fig. 7 (manuscript in preparation) including the variability associated with 10A-A/10A-B heterozygotes. While IFN-γ responses were tested using an anti-KIR2DL4 antibody, it seems likely that the natural ligand, HLA-G, would give similarly genotype-dependent effects. Evidence has recently emerged [10] that demonstrable surface expression may not be necessary for KIR2DL4-mediated IFN-γ secretion induced by soluble HLA-G. Although surface expression was not required for HLA-G binding in that system, the truncated protein produced by the 9A allele was unable to mediate IFN-γ secretion in a transfection system. We have begun to investigate the influence of KIR2DL4 polymorphism on the response of freshly isolated NK cells to soluble HLA-G.

There is mounting evidence that inflammation results in the up-regulation of HLA-G in various cell types, including dendritic cells [27]. As inflammation is also likely to activate NK cells and up-regulate KIR2DL4 expression, the resultant stimulation of IFN-γ secretion by HLA-G has the potential to influence the cytokine environment during an immune response when interaction between NK cells and dendritic cells is known to occur. KIR2DL4 polymorphism may therefore influence the type of cellular immune response (T1/T2) that develops. 10A-A alleles, by facilitating IFN-γ production, may promote a T1 bias. In contrast, 9A alleles, in addition to lacking an effective membrane-bound receptor for HLA-G, produce a soluble KIR2DL4 that may act to block HLA-G, thereby introducing a T2 bias. Interestingly, HLA-G is also subject to alternate splicing which results in both membrane-bound and soluble forms [28]. If soluble KIR2DL4 does bind HLA-G, then it may also be able to block interactions between HLA-G and other NK receptors such as ILT-2. Whilst the effects of KIR2DL4 polymorphism on the nature of the immune response remain speculative at this stage, our study has provided a greater understanding of the functional significance of these polymorphisms.

Materials and methods

Cells and antibodies

PBMC and purified NK cells were isolated from blood samples collected from blood donors at the Australian Red Cross Blood Service by Ficoll density gradient centrifugation with or without RosetteSep according to the manufacturer's instructions (StemCell Technologies). Expression of KIR2DL4 on the cell surface was examined using anti-KIR2DL4 mAb 33 (IgG1) and 64 (IgM, kindly provided by Dr. E. Long and S. Rajagopalan, Laboratory of Immunogenetics, NIH, MD), clone 2238 (R&D Systems, Minneapolis, MN) and mAb 53.1 (IgG1, kindly provided by Dr. M. Colonna, Department of Immunology and Pathology, Washington University School of Medicine). Appropriate isotype controls included IgG1 and IgM (Coulter Immunotech, Marseille, France) and IgG2a (R&D Systems). Isotype-specific secondary antibodies were FITC-conjugated Affinipure $F(ab')_2$ fragment goat anti-mouse IgG (Fcγ specific) and R-PE-conjugated Affinipure $F(ab')_2$ fragment goat anti-mouse IgM (μ chain specific) from Coulter Immunotech. NK cells were identified by incubation with primary antibody on ice for 30 min, washed two times and then incubated for a further 30 min with the appropriate secondary antibody. NK cells were identified as $CD56^+$ $CD3^-$ lymphocytes with clearly visible $CD56^{bright}$ and $CD56^{dim}$ subsets, using CD56-PC5 and CD3-FITC or CD3-PE (Coulter Immunotech). Flow cytometry was performed on a Coulter EPICS XL-MCL flow cytometer (Beckman Coulter, Fullerton, CA).

NK cell culture

RosetteSep purified NK cells (5×10^3 cells/well) were cultured in a total volume of 200 μL with γ-irradiated Daudi cells (3×10^4 cells/well, 6000 rad) in the presence of 1000 U/mL recombinant human IL-2 (Chiron, Emeryville, CA), with feeding every 2–3 days by replacement of 75% fresh medium per well. Staining of cultured NK cells for flow cytometry was always performed before feeding and prior to any functional assay. Secondary culture of NK cells involved restimulating frozen quiescent NK cultures by plating 3×10^3 freshly thawed NK cells cultured with 3×10^4 γ-irradiated Daudi cells per well in the presence of 200 U recombinant IL-2.

DNA and RNA

DNA extraction from 200 μL of buffy coat cells was performed using QIAamp DNA Blood Mini Kit (Qiagen, Doncaster, VIC). Total RNA was extracted from 0.5×10^6 NK cells using an RNeasy Protect Mini Kit (Qiagen). RT-PCR was performed using Qiagen Omniscript with Oligo(dT)15 primer and recombinant RNasin ribonuclease inhibitor (Promega, Annandale, Australia) according to the manufacturer's instructions.

Cloning and expression

KIR2DL4 transcripts were amplified using the primers KIR2DL4 IGF (5′-GGGAGATCTCACGTGGGTGGTCAGGAC-3′) and KIR2DL4SR (5′-GACTGGTCGACGCTAGCTCAGATTC-CAGCTG-3′), which are located at the beginning of exon 3 and the end of exon 8, respectively. The underlined sequences in the primers represent Bgl II and Sal I restriction sites, respectively. cDNA amplicons were inserted into pGEM-T EasyVectorII (Promega) and cloned into JM109 competent *E. coli*. Colonies were screened using the original primers and sequenced using primers M13 forward and reverse and Big Dye Terminator Automated sequencing on a 3100 DNA sequencer (ABI, Foster City, CA). Clones containing appropriate inserts were digested with Bgl II for 4 h and Sal I (Promega) for the final 2 h to release the inserts. Inserts were separated and excised from a 1% TAE gel and purified using the QiaEx II Gel Extraction kit (Qiagen). Purified inserts were ligated into the mammalian expression vector pDisplay and cloned into TOP10F'OneShot bacteria (Invitrogen, Carlsbad, CA). Colonies were screened as above and sequenced for correct orientation and sequence, using primers T7 forward and BGH reverse. Equal amounts of plasmid DNA were transfected into 90% confluent 293T cells using LipoFectamine-2000 (Invitrogen) and surface expression tested using anti-HA ascites and anti-KIR2DL4 mAb as described above.

Western blot analysis

293T cells or CHOK-K1 cells that had been transfected with an HA-tagged KIR2DL4 transcript or vector alone were cultured for 24 h, washed and cultured for a further 24 h in serum-free medium (Gibco, Mount Waverley, VIC). After the second 24 h, supernatants and cell lysates were prepared from 2×10^6 of these cells. Of cell lysates and neat culture supernatants, 15 μL were separated by electrophoresis on 6% SDS-PAGE and transferred to Hybond-C nitrocellulose membranes (Amersham, Piscataway, NJ). The membranes were reacted with a primary monoclonal anti-HA antibody (clone 16B12; Covance, Berkeley, CA) or monoclonal anti-KIR2DL4 antibodies and then a secondary sheep anti-mouse IgG-HRP (Chemicon, Temecula, CA). Membranes were washed and developed with enhanced chemiluminescence (ECL; Amersham).

SSCP for transmembrane genotype

SSCP was used to distinguish the three different transmembrane genotypes on transcripts of exons 6–7, as described previously [16]. The frequency of the 10A-A, 10A-B and 9A alleles shown in Table 1 was determined using the Western Australian controls described in [16].

KIR2DL4 genomic sequencing

Both alleles of KIR2DL4 were sequenced simultaneously as previously described [19].

Detection of KIR2DL4 and FcεRI-γ transcripts

To investigate alternate splicing of the transmembrane domain, KIR2DL4 exons 4–7 were amplified from cDNA using the primers KIR103IG3F and KIR103TCR, as described [17]. Full-length transcripts were detected using the primers KIR103FLF (5′-GCGCGCGGCGGCATGTCCATGTCACCCACGG-TCATCATCCTGGC-3′) located at the start codon of exon 1 [2] and KIR2DL4SR (see above). Transcription of FcεRI-γ was detected using primers and amplification conditions described in [25].

IFN-γ ELISA

Non-tissue culture-treated 96-well plates were coated with antibodies overnight using bicarbonate buffer (pH 9.5) and washed two times prior to use in an assay with culture medium. *In vitro* secondary cultured NK cells (2×10^5) were incubated in triplicate wells for 20 h in the presence of solid-phase-bound anti-human CD16 antibody (IgG1, clone 3G8) or IgG1 isotype control (clone MG1-45; Biolegend, San Diego, CA) or anti-KIR2DL4 (IgG2a, clone 2238) or IgG2a isotype control (clone 20102; R&D Systems). After 20 h, the supernatants were harvested and IFN-γ was quantified by OPTeia Human IFN-γ ELISA (Pharmingen, San Diego, CA).

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