The genotype of the NK cell receptor, KIR2DL4, influences INFγ secretion by decidual natural killer cells


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ABSTRACT: Natural killer (NK) cells are the predominant leukocyte in first trimester decidua and play a role in vascular remodelling through interferon gamma (IFNγ) secretion. Membrane expression of the killer immunoglobulin-like receptor (KIR) KIR2DL4 on peripheral blood NK (pNK) cells is controlled by the 9A/10A transmembrane genetic polymorphism. On peripheral NK cells (pNK), KIR2DL4 can only be detected on the membrane of cells from individuals with at least one copy of the 10A allele and ligation of KIR2DL4 results in IFNγ secretion. In this study, we assessed KIR2DL4 expression and IFNγ secretion as a result of KIR2DL4 ligation, by decidual NK (dNK) cells. The 9A/10A transmembrane polymorphism was shown to control KIR2DL4 expression by dNK, as previously shown for pNK cells. Freshly isolated dNK cells from subjects with at least one 10A allele expressed KIR2DL4 whereas those from 9A homozygous subjects did not. Although freshly isolated dNK did not secrete IFNγ in response to KIR2DL4 ligation regardless of KIR2DL4 genotype, activation by in vitro culture with IL-2 enabled dNK cells from individuals with at least one 10A allele, but not those without a 10A allele, to secrete IFNγ in response to KIR2DL4 ligation. This study confirms that expression of KIR2DL4 by dNK is dependent on the 9A/10A polymorphism and that this polymorphism influences IFNγ secretion by dNK cells.

Key words: CD158d / cell surface molecules / cytokines / KIR2DL4 / natural killer cells

Introduction

NK cells are the predominant lymphoid cell population at the site of implantation, and constitute 70% of the infiltrating CD45+ leukocytes in first trimester decidua (Moffett-King, 2002). Receptors that enable decidual NK (dNK) cells to recognize HLA class I molecules include the lectin-like family (CD94/NKG2), leukocyte Ig-like receptor-I, and the immunoglobulin-like receptor (KIR) family (Bashirova et al., 2006). It has been postulated that activation of dNK cells via these receptors may be an important event in human placentation. Indeed, particular combinations of maternal KIR and fetal HLA-C alleles have been shown to predispose to pre-eclampsia and recurrent miscarriage (Hiby et al., 2004, 2008), syndromes characterized by shallow placentation. The immunological events leading to pathological syndromes of pregnancy such as pre-eclampsia are not well-defined. In the mouse, it has been demonstrated that interferon gamma (IFNγ) secretion by dNK cells is an important event in placentation (Croy et al., 2002) although there is as yet no evidence of a role for IFNγ in humans. As ligation of KIR2DL4 on peripheral blood NK (pNK) cells by anti-KIR2DL4 monoclonal antibody (mAb) results in IFNγ secretion (Rajagopalan et al., 2001; Goodridge et al., 2007), KIR2DL4 expression on dNK cells may play a role in placentation. Little is known about the expression of KIR2DL4 on dNK cells, although it has been detected on such cells using a polyclonal antibody in a single report (Ponte et al., 1999). In contrast, much is now known about the expression of KIR2DL4 on pNK cells. KIR2DL4 is only expressed on the surface of freshly isolated CD56bright pNK cells, although it can be induced on CD56dim pNK cells by culture (Goodridge et al., 2003; Kikuchi-Maki et al., 2003). Based on the presence of a sequence of 9 or 10 adenine residues at the end of exon 6, two common allele groups, 9A and 10A, each having a gene frequency of approximately 0.5, have been described (Witt et al., 2000, 2002). The deletion of one adenine in 9A alleles results in a frame-shift and the production of either a protein with a truncated cytoplasmic tail...
or one lacking the transmembrane region, both of which are not expressed at the cell surface. In contrast, the 10A alleles, which can be further divided into the 10A-A and 10A-B subgroups, encode receptors that may be expressed at the cell surface (Goodridge et al., 2003). Monoclonal anti-KIR2DL4 antibodies detect KIR2DL4 expression on the CD56bright population of freshly isolated pNK cells from individuals who have at least one copy of the 10A-allele. The receptor produced by the 10A-B allele is expressed on these cells but cannot be detected because the available mAbs react with the D0-domain of KIR2DL4 and the mRNA produced by the 10A-B allele in freshly isolated CD56bright NK cells omits the D0-domain (Goodridge et al., 2003). However, after in vitro culture of pNK cells, the full-length 10A-B receptor, including the D0 domain, is expressed and can be detected.

Although controversial (Allan et al., 1999; Boyson et al., 2002), there is evidence that HLA-G is the ligand for KIR2DL4 (Ponte et al., 1999; Rajagopalan and Long, 1999; Rajagopalan et al., 2005). Since IFNγ is an important mediator of vascular changes associated with placenta, interaction between KIR2DL4 on dNK cells and HLA-G on trophoblast cells may be important for IFNγ secretion and placental development. If KIR2DL4 is not expressed on the membrane of dNK cells from individuals who are homozygous for the 9A allele, then these cells may secrete less IFNγ, thereby affecting placental development. The aims of this study were to determine whether KIR2DL4 is expressed on dNK cells using anti-KIR2DL4 mAbs and to determine whether the 9A/10A polymorphism influences membrane expression and IFNγ secretion by dNK cells.

Materials and Methods

Cells

Peripheral blood and placental tissue samples were obtained with consent from 56 donors undergoing elective first trimester termination of normal pregnancy at the Marie Stopes International Clinic (Perth, Australia). Ethics approval was obtained from the Human Ethics Committee, University of Western Australia. pNK cells were isolated from blood samples by Ficoll density gradient centrifugation with RosetteSep human NK cell enrichment cocktail (Stem Cell Technologies, Vancouver, Canada). Decidual tissue was macroscopically dissected from non-decidual tissue, and mononuclear cells were isolated by mechanical disruption, followed by filtration through a 70 μm sieve and centrifugation over Ficoll. dNK cells were then obtained by incubating the decidual mononuclear cells with the RosetteSep human NK cell enrichment antibody cocktail and 200 μl peripheral blood (γ-irradiated to 30 Gy to prevent proliferation of contaminating peripheral NK cells) from the tissue donor as a source of red blood cells (a specific ratio between mononuclear cells and red blood cells is required for the purification of NK cells via the RosetteSep procedure), followed by a second centrifugation over Ficoll. Due to limited cell numbers, not all of the assays described below were performed on all samples.

Antibodies

Expression of KIR2DL4 on the cell surface and stimulation of NK cells through KIR2DL4 was examined using several different anti-KIR2DL4 mAbs. MAb #33 (IgG1) and #64 (IgM), were both kindly provided by Dr E. Long and S. Rajagopalan (Laboratory of Immunogenetics, NIH, MD, USA). MAb mAb #2238 was purchased from R&D Systems (IgG2a, clone 181703, R&D Systems, USA). Isotype controls included IgG1 and IgM (Beckman Coulter, Australia) and IgG2a (R&D Systems), Leaf-purified anti-human CD16 (IgG1, clone 3G8, Biolegend, USA) and an equivalent IgG1 isotype control (clone MGl-45, Biolegend) were used for the in vitro stimulation of NK cells. The secondary antibody used with mAb #33 to detect KIR2DL4 was FITC-conjugated Affinipure F(AB')2 fragment goat anti-mouse IgG (Fcγ specific, Beckman Coulter, Australia). The secondary antibody used with mAb #64 was FITC-conjugated goat anti-mouse IgM (μ-chain specific, Beckman Coulter) and when mAb #2238 was used to detect KIR2DL4, biotinylated goat anti-mouse IgG2a (Southern Biotechnology Associates, USA), followed by streptavidin-DTAF (Beckman Coulter) was used. Expression of CD9, a dNK cell marker (Koopman et al., 2003), was identified using FITC-conjugated antibody (clone P1/33/2, Dako Cytomation, Australia).

Flow cytometry

When unconjugated primary antibodies were used, incubations with the primary and secondary antibody were completed prior to staining with directly conjugated antibodies. Each tube of cells was stained for three markers, including directly conjugated anti-CD56 and anti-CD3 antibodies (Beckman Coulter) for NK cell identification. The third antibody to detect a third marker was either a directly conjugated antibody or an unconjugated anti-KIR2DL4 antibody that was detected as described above. All antibodies were incubated for 30 min on ice, followed by two washes. NK cells were identified as CD56+, CD3− lymphocytes and CD56bright and CD56dim subsets were clearly distinguished. CD158a (clone HP-3E4), CD158b (clone CH-L) and CD158e (clone DX9) were detected using PE-labelled antibodies (BD Biosciences, Franklin Lakes, USA). Flow cytometry was performed on a Coulter EPICS XL-MCL flow cytometer (Beckman Coulter).

NK cell culture

RosetteSep-purified, freshly isolated dNK or pNK cells (5 × 10⁵ cells per well) were cultured for 8 days with γ-irradiated Daudi cells (3 × 10⁴ cells per well, 60 Gy) in the presence of 200 U/ml recombinant human IL-2 (Chiron, USA). These proliferating, primary cultures of NK cells were maintained by subculture every second day with fresh medium. Secondary NK cell cultures were established by restimulating primary cultured NK cells (3 × 10⁵ cells per well) with 3 × 10⁴ γ-irradiated Daudi cells per well in the presence of 200 U/ml rIL-2 for 12 days. Prior to any functional assay, flow cytometric analysis of both primary and secondary cultured NK cells for CD56, CD3 and KIR2DL4 expression was performed.

SSCP for KIR2DL4 9A/10A transmembrane genotype

DNA was extracted from 200 μl ofuffy coat cells using a QIAamp DNA Blood Mini Kit (Qiagen, Australia). Single stranded conformational polymorphism (SSCP) was used to distinguish the three different transmembrane genotypes of exons 6–7 as described previously (Witt et al., 2000).

Stimulation of cells for IFNγ production and IFNγ ELISA

Non-tissue culture-treated, flat-bottomed, 96 well plates (BD Biosciences) were coated overnight with antibody or isotype control diluted in bicarbonate buffer (pH 9.6) and then washed twice with tissue culture medium. Freshly isolated, purified NK cells were added directly to the stimulation plates. Secondary cultured NK cells were harvested from their original culture wells, washed and then added to the stimulation plates. The plates were then incubated for 20 h at 37°C. In some experiments, soluble mAb #33 or IgG1 isotype control antibodies were included in the tissue culture medium at a concentration of 1 μg/ml.
previously shown to be optimal for IFNγ stimulation. Cell viability was not altered as a result of the stimulation. Supernatants were harvested and IFNγ was quantified using the OPTeia Human IFNγ ELISA (BD Pharmingen, USA).

**Statistics**

Comparisons of the percentage of KIR2DL4 positive cells between individuals with different KIR2DL4 genotypes were performed by 2-tailed t-tests. Comparisons of fold-increase in IFNγ secretion were between cell types, between KIR2DL4 genotypes and between different stimulating antibodies were performed by 2-tailed t-tests.

**Results**

**Credentials of anti-KIR2DL4 mAbs**

The reactivity of the anti-KIR2DL4 mAbs has been previously published and is summarized here. According to the manufacturer, mAb #2238 detects KIR2DL4 on transfected BaF/3 cells and does not recognize transfectants expressing KIR2DL1, 2DL2, 2DL3, 2DL5, 2DS1, 2DS2, 2DS4, 3DL1, 3DL2 or 3DS1. MAb #33 and #64 have also been shown to be specific for KIR2DL4 in similar transfection experiments (Rajagopalan et al., 2001). We have compared the reactivity of the monoclonal anti-KIR2DL4 antibodies, mAb #33, #64 and #2238 by testing them on freshly isolated pNK cells from subjects representing all KIR2DL4 genotypes (homozygous 10A-A, 10A-B, 9A and all heterozygous combinations) (Goodridge et al., 2007 and unpublished observations). All three antibodies gave equivalent results staining only the CD56bright pNK cells of individuals with at least one 10A-A allele. None of the mAb stained freshly isolated CD56dim pNK cells. All three antibodies also gave equivalent staining of in vitro activated pNK cells from donors representing all genotypes (Goodridge et al., 2007 and unpublished observations). That is, the majority of in vitro activated NK cells became KIR2DL4 positive but this only occurred for pNK cells from donors with at least one 10A allele. In all cases staining was of only moderate intensity with no clear bimodal staining of distinct positive and negative cell populations. The only difference between the mAbs was that mAb #33 and mAB #2238 differed in their ability to induce IFNγ secretion from pNK cells (unpublished observations). Although both antibodies induced IFNγ secretion from NK-92 and cultured pNK cells when solid-phase bound (SPB), only mAb #33 induced IFNγ from freshly isolated peripheral NK cells when used as a soluble ligand. MAb #64 has also been reported to stimulate IFNγ when used as a soluble ligand (Rajagopalan et al., 2001) but has not been tested in this laboratory. As none of the available mAb are immunoprecipitating, it is not possible to formally exclude cross reactivity with other surface antigens on primary NK cells.

**Identification of dNK cells**

To confirm the identity of the cells isolated from the decidual tissue following the RosetteSep NK cell separation, samples were stained for CD56 and CD9, a marker that has recently been found on freshly isolated endometrial NK cells (Eriksson et al., 2004) and dNK cells (Koopman et al., 2003). Representative examples are presented in Fig. 1. Most dNK cell preparations (Fig. 1A), in agreement with previously published data (Trundley and Moffett, 2004), contained a majority of cells that were CD56bright and expressed high levels of CD9. However, a few dNK preparations (Fig. 1B), had a significant number of cells that were not CD56bright and which were CD9+. dNK cell preparations with <50% CD56bright cells were considered to be possibly contaminated with peripheral blood cells and were excluded from functional studies.

To further verify the identity of the cells in the RosetteSep purified populations, we examined the expression of three KIR that are known to be expressed on dNK cells; namely CD158a (KIR2DL1/2DS1), CD158b (KIR2DL2/3/2DS2) and CD158e (KIR2DL3). Expression on freshly isolated CD56bright dNK was compared with that of CD56dim pNK cells from the same donors. As shown in Fig. 2, the proportion of cells expressing each of these receptors, particularly CD158a and CD158b, on freshly isolated CD56bright dNK cells was generally higher than that observed for their matched CD56dim pNK cells. These results are in agreement with previous reports (Hiby et al., 1997; Verma et al., 1997) and are consistent with the majority of cells in these preparations being dNK cells (Verma et al., 1997).

**Freshly isolated dNK cells express KIR2DL4 in accordance with their transmembrane genotype**

We have previously shown that membrane expression of KIR2DL4 is relatively weak compared with other NK cell markers and is restricted to the CD56bright subpopulation of freshly isolated pNK cells from subjects with at least one 10A-A allele of the transmembrane domain (Goodridge et al., 2003). We therefore investigated whether this polymorphism also influences KIR2DL4 membrane expression on dNK cells. The level of KIR2DL4 expression by CD56bright dNK cells isolated from 35 decidual tissue samples were determined and related to transmembrane genotype.

As we have shown for CD56bright pNK cells (Goodridge et al., 2003), dNK cells also exhibited low level expression of KIR2DL4 and the level of expression varied between individuals. In Fig. 3 dot plots of representative examples showing low (upper panels) and
high (lower panels) KIR2DL4 expression by dNK and pNK cells are presented. As shown, even in those individuals with high expression, there is only a modest shift in the mean fluorescent intensity with non-bimodal distribution between positive and negative cells. The small population of CD56 negative cells apparently staining for KIR2DL4 is also present in the isotype control tube (data not shown) and therefore represent non-specific staining. In Fig. 4, the percentage of CD56bright dNK and matched CD56bright pNK cells from the same donors (except for three 10A-B/9A dNK donors for whom matched pNK cells were not available) that was KIR2DL4 positive (as shown by mAb #33) is plotted for each KIR2DL4 genotype. Positivity was defined as having fluorescence intensity >99% of the same cells stained with an isotype control antibody. The 9A/10A transmembrane genotype influenced KIR2DL4 expression by dNK in the same way, we have previously reported for pNK (Goodridge et al., 2007). Individuals with at least one 10A-A allele had a significantly higher percentage of KIR2DL4 positive dNK cells (ranging from 8 to 66%), than dNK cells derived from 9A homozygous individuals (ranging from 0.7 to 9.0%) \( (P < 0.00001) \). This was also true for pNK cells \( (P = 0.03) \). Homozygous 10A-A individuals had the highest percentage of KIR2DL4 positive dNK and pNK cells. The single example of a homozygous 10A-B individual had <5% positive dNK and pNK, consistent with the inability of anti-KIR2DL4 antibodies to react with the protein lacking the D0-domain made by freshly isolated pNK from such donors.

We have previously shown that pNK cells from 9A/10A-B heterozygous individuals have low KIR2DL4 expression (Goodridge et al., 2007). This was also true for pNK in this study \( (P = 0.03 \) for genotypes with a 10A-A allele versus 10A-B/9A), and this genotype was also generally associated with low surface expression by dNK cells \( (P = 0.02 \) for genotypes with a 10A-A allele versus 10A-B/9A). However, three individuals with this genotype formed a distinct cluster in which ~40% of their dNK cells expressed KIR2DL4 (Fig. 4), despite their pNK cells being negative when stained with the same antibody (mAb #64). Further investigation is required to explain this heterogeneity. Thus, with the exception of three 9A/10A-B heterozygotes, KIR2DL4 membrane expression by dNK cells was determined by the 9A/10A transmembrane genotype in the same way as for pNK cells.

**Freshly isolated dNK and pNK cells secrete little IFNγ in response to stimulation through KIR2DL4**

To test the ability of dNK cells to secrete IFNγ when stimulated via KIR2DL4, 8 pNK and 11 freshly isolated dNK cell samples were stimulated for 20 h with SPB anti-KIR2DL4 (mAb #33), anti-CD16 as a positive control (for the pNK cells) or an isotype control and the IFNγ content of the supernatants was measured by ELISA. Mean
background levels of IFNγ secretion in response to isotype control antibody were similarly low for both populations (pNK: 1.35 pg/10⁵ cells; dNK: 1.94 pg/10⁵ cells). pNK cells stimulated via CD16 had a mean of a 43-fold-increase in INFγ secretion, with all eight samples producing at least a 5-fold increase over the isotype control (range: 6.2–165-fold increase) (Fig. 5). Stimulation of pNK via KIR2DL4 produced a relatively small response with a mean 9-fold increase, significantly lower than for CD16 (P < 0.05). This was true even for the freshly isolated pNK cells from individuals whose CD56 brightly NK cells expressed high levels of KIR2DL4. Although the one pNK cell sample with a substantial IFNγ responses (60-fold increase) had high KIR2DL4 expression, a further three individuals with high KIR2DL4 expression failed to make a greater than 5-fold increase in IFNγ. The failure to detect large INFγ responses from pNK cells, even from individuals with high expressing KIR2DL4 genotypes, is perhaps not surprising. Given that only the CD56bright pNK express KIR2DL4 and these cells constitute only 5–10% of the pNK cell population, the number of responding cells may have been too low to allow detection of INFγ secretion.

As expected, freshly isolated dNK cells, which do not express CD16, responded poorly to stimulation via CD16 with a mean fold increase in INFγ secretion of 7. They also responded weakly to stimulation via KIR2DL4 with a mean fold-increase of 4 (Fig. 5). Thus, although the majority of dNK cells is CD56 brightly and expresses KIR2DL4 in donors with the appropriate KIR2DL4 genotype, dNK failed to make a vigorous response to stimulation via KIR2DL4. dNK from only two donors made a greater than 5-fold increase in IFNγ secretion. Although one of these had high KIR2DL4 expression, dNK cells from a further four donors with high levels of KIR2DL4 failed to show such an increase.
Cultured dNK cells and pNK cells are able to secrete IFNγ in response to SPB anti-KIR2DL4 mAb

As freshly isolated dNK at best made minimal IFNγ in response to SPB anti-KIR2DL4, we determined whether their activation by culture with interleukin (IL)-2 would enable them to respond to this stimulus. In order to obtain sufficient cell numbers for these experiments it was necessary to expand the dNK cells by primary and secondary culture as described in the Materials and Methods. The cells expanded by such culture were confirmed to be NK cells, i.e. they expressed CD56, and not CD3, on their surface. Both primary and secondary cultured pNK (data not shown) and dNK cells (Fig. 6) continued to express KIR2DL4 in accordance with their transmembrane genotypes, a finding we have previously shown for primary cultures of pNK cells (Witt et al., 2002). KIR2DL4 expression was generally higher on cultured dNK cells from individuals with at least one 10A-A allele, although those from 9A homozygous and 9A/10A-B individuals remained essentially KIR2DL4 negative.

We next tested whether activation by in vitro culture would enable dNK cells stimulated with SPB anti-KIR2DL4 to produce IFNγ. Although the majority of dNK cells from all donors uniformly acquired CD16 following culture, the cultured dNK cells retained other characteristics of their decidual phenotype remaining predominantly CD9 positive (mean of 75%), whereas the cultured pNK cells remained predominantly CD9 negative (mean of 6%) (data not shown). As expected, cultured pNK cells responded strongly to stimulation via CD16 with a mean 46-fold-increase in IFNγ secretion whereas cultured dNK also responded strongly with a mean 37-fold-increase (Fig. 7A). When stimulated via KIR2DL4, pNK had a mean 15-fold-increase and dNK had a mean 8-fold-increase (Fig. 7A). The IFNγ response to KIR2DL4 was influenced by KIR2DL4 genotype. Cultured pNK cells from individuals with a membrane expressing KIR2DL4 allele (10A-A or homozygous 10A-B) produced a mean 23.0-fold-increase in IFNγ compared with a mean 3.5-fold-increase from pNK cells from individuals without a 10A-A allele (P < 0.03) (Fig. 7C), a result consistent with our previous report (Goodridge et al., 2007). Similarly, dNK from six donors with a membrane expressing KIR2DL4 genotype had a mean 11.4-fold-increase, compared with an increase of only 2.3-fold for the four donors that lacked a 10A-A allele (P < 0.02) (Fig. 7B). Thus, for cultured pNK and dNK cells, the 9A/10A transmembrane polymorphism affects IFNγ production in response to stimulation with SPB anti-KIR2DL4.

Freshly isolated dNK cells do not respond to stimulation with soluble mAb #33

As Rajagopalan et al. (2005) have shown that freshly isolated pNK cells secrete IFNγ in response to stimulation with soluble mAb #33 via an unusual mechanism not requiring membrane expression of KIR2DL4, we investigated whether freshly isolated dNK cells can respond to soluble mAb #33. As shown in Fig. 8, the dNK cell preparations studied produced no IFNγ in response to stimulation with soluble mAb #33 with none of the mAb #33 stimulated preparations producing more IFNγ than the isotype control. In contrast, and in agreement with Rajagopalan et al. (2005), freshly isolated pNK cells were capable of producing IFNγ in response to stimulation with soluble mAb #33 with a mean fold-increase of 44 (P < 0.01 for pNK versus dNK). However, the pNK cells from only half of the donors responded (Fig. 8B). The ability to respond did not appear to be related to the 9A/10A transmembrane genotype (data not shown).

Discussion

In this study, using two different mAbs, we have shown that KIR2DL4 is expressed on dNK cells, confirming the earlier report using a polyclonal antibody (Ponte et al., 1999). However, as for CD56bright pNK cells, the intensity of KIR2DL4 expression on dNK cells was low, such that distinct KIR2DL4 positive and negative populations reported for the polyclonal antibody, were not seen. Rather, there appears to be low level expression on the majority of the dNK cells. The low level of surface expression may be related to the report that KIR2DL4 resides predominantly in intracytoplasmic endosomes that cycle between the cytoplasm and cell surface (Rajagopalan et al., 2005). KIR2DL4 expression by freshly isolated CD56bright dNK was controlled by the 9A/10A transmembrane polymorphism in the same way that we have previously reported for the CD56bright pNK cells (Goodridge et al., 2003). Thus, women who were homozygous for the 9A transmembrane allele or heterozygous for the 9A/10A-B alleles lacked detectable KIR2DL4 surface expression on their dNK cells and this was not changed by activation (culture with IL-2). In subjects with a 10A-A allele, the majority of freshly isolated dNK cells expressed KIR2DL4, and 10A-A homozygous individuals tended to have higher expression than heterozygous individuals. In the single 10A-B homozygote, detectable KIR2DL4 expression was induced by in vitro culture on both dNK cells and pNK cells, as previously reported for pNK (Goodridge et al., 2007). The fact that membrane expression of KIR2DL4 is found on freshly isolated CD56bright pNK cells or cultured CD56dim pNK cells (which become CD56bright after culture), and on freshly isolated and cultured dNK cells, all of which have the CD56bright phenotype, suggests that membrane expression...
of KIR2DL4 is associated with bright expression of CD56. Our current study confirms the lack of detectable KIR2DL4 expression on both pNK and dNK cells from 9A/10A-B heterozygotes even after culture, a phenomenon that currently lacks explanation, given that there is up-regulation of the same mRNA species as is seen in 10A-B homozygotes following culture (Goodridge et al., 2007).

Despite membrane expression of KIR2DL4, stimulation of freshly isolated dNK cells with SPB mAb #33 resulted in very little IFNγ secretion. However, responsiveness to KIR2DL4 stimulation was recovered by in vitro culture. Both cultured dNK cells and cultured pNK cells secreted IFNγ in response to SPB anti-KIR2DL4 providing at least one 10A-A allele was present. It is not clear whether SPB or soluble ligand is more relevant to dNK cell activation in vivo. Several studies have suggested that the non-classical class I molecule, HLA-G, is a ligand for KIR2DL4 (Ponte et al., 1999; Rajagopalan and Long, 1999), although this has not been confirmed by all groups (Allan et al., 1999; Ponte et al., 1999; Boyson et al., 2002). Rajagopalan et al. (2005) recently demonstrated that in freshly isolated pNK cells soluble HLA-G can be taken up by intracellular KIR2DL4 via an unusual mechanism, resulting in IFNγ secretion. We were able to confirm that soluble mAb #33 elicits IFNγ production by freshly isolated pNK cells from some but not all individuals but responsiveness was not related to KIR2DL4 genotype. However, freshly isolated dNK cells from the same women were in all cases completely unresponsive to this stimulus. The responsiveness of pNK to soluble mAb #33 does not appear to be related to the 9A/10A genotype and further experiments are in progress to clarify this point and to determine the basis for the differences between pNK and dNK in their response to soluble ligands.

Our data suggest that freshly isolated dNK cells, in contrast to freshly isolated pNK cells, cannot produce IFNγ in response to stimulation through KIR2DL4, regardless of whether the stimulus is SPB or soluble in nature. Basal production of IFNγ by freshly isolated dNK cells has previously been reported to be very low (Vigano et al., 2005).

**Figure 7** Cultured dNK and pNK cells can produce IFNγ in response to SPB anti-KIR2DL4 and anti-CD16.

Secondary cultures of pNK (samples 1, 3, 5, 6, 8, 9, 13, 14, 16, 17, 19) and dNK (samples 1, 3, 5, 6, 9, 11, 13, 14, 16, 18) cells were harvested, stimulated for 20 h by SPB mAb specific for KIR2DL4 (#2238), CD16, or isotype control and the IFNγ content in the supernatants determined by ELISA. (A) The mean (+SEM) fold-increase in IFNγ secretion when compared with isotype control is shown for pNK and dNK cells when stimulated through SPB anti-KIR2DL4 (shaded bars) or SPB anti-CD16 (white bars). (B) Responses of individual samples after stimulation with isotype control (squares) or anti-KIR2DL4 or anti-CD16 (triangles). (C) The mean (+SEM) fold-increase in IFNγ secretion when stimulated with anti-KIR2DL4 for pNK and dNK cells from donors with expressing KIR2DL4 genotypes (shaded bars) or non-expressing genotypes (white bars). (D) INFγ responses of individual samples to isotype control (squares) and anti-KIR2DL4 (triangles). INFγ responses were greater for pNK cells and dNK cells from individuals with a membrane expressing KIR2DL4 genotype than a non-expressing genotype (P < 0.03, P < 0.02, for pNK and dNK, respectively).
cytokines IL-4 and IL-1β (Lash et al., 2001), although in that report, IFNγ production by dNK cells was increased in the presence of IL-12, either alone or in combination with either IL-2 or IL-10. We found that activation by in vitro culture with IL-2 enabled dNK cells to secrete IFNγ in response to SPB anti-KIR2DL4 mAb, providing the donor had at least one 10A-A allele. Although there is little evidence of significant IL-2 production in the decidua, if dNK cells were activated in vivo by physiological stimuli, their KIR2DL4 genotype might then influence their secretion of IFNγ.

Chemokines, cytokines and growth factors other than IFNγ are thought to play an important role in the development of the placenta and the maintenance of a successful pregnancy (Parmentier et al., 1991). Indeed, various studies have shown that dNK cells can produce, amongst others, the angiogenic factors vascular endothelial growth factor, angiopeitin 2, and angiogenin; the macrophage colony-stimulating factor; the chemokines IL-8, monocyte protein-1 and Regulated upon Activation, Normal T-cell Expressed and Secreted; the regulatory factor, tumour growth factor-beta; and the macrophage inflammatory protein-1-alpha, macrophage inflammatory protein-1-beta and IL-8 (Rajagopalan et al., 2005). Whether freshly isolated dNK cells can produce any of these factors as a result of stimulation through KIR2DL4, and the effect of the 9A/10A transmembrane polymorphism on this function, remains to be established. If HLA-G is indeed the ligand for KIR2DL4, our data suggest that the 9A/10A polymorphism would control the response of dNK cells to membrane-bound HLA-G (equivalent to our solid-phase stimulation), and have the potential to influence placentation.

### Acknowledgements

We would like to acknowledge the cooperation of the manager and staff at Marie Stopes International, Perth. We would also like to thank Drs E. Long and S. Rajagopalan for generously providing antibodies for our studies.

### Funding

This work was funded by the NHMRC (Grant Number 303236) and L. Lathbury was supported by a Child Health Research Foundation of WA Research Fellowship.

### References


Hiby SE, King A, Sharkey AM, Loke YW. Human uterine NK cells have a similar repertoire of killer inhibitory and activatory receptors to those found in blood, as demonstrated by RT–PCR and sequencing. Mol Immunol 1997;34:419–430.


Submitted on January 14, 2009; resubmitted on April 22, 2009; accepted on May 29, 2009.