Human Decidual Natural Killer Cells Are a Unique NK Cell Subset with Immunomodulatory Potential

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Abstract

Natural killer cells constitute 50–90% of lymphocytes in human uterine decidua in early pregnancy. Here, $CD56^{bright}$ uterine decidual NK (dNK) cells were compared with the $CD56^{bright}$ and $CD56^{dim}$ peripheral NK cell subsets by microarray analysis, with verification of results by flow cytometry and RT-PCR. Among the ~10,000 genes studied, 278 genes showed at least a threefold change with $P \leq 0.001$ when comparing the dNK and peripheral NK cell subsets, most displaying increased expression in dNK cells. The largest number of these encoded surface proteins, including the unusual lectinlike receptors NKG2E and Ly-49L, several killer cell Ig-like receptors, the integrin subunits α^D, α^X, $\beta 1$, and $\beta 5$, and multiple tetraspanins (CD9, CD151, CD53, CD63, and TSPAN-5). Additionally, two secreted proteins, galectin-1 and progestagen-associated protein 14, known to have immunomodulatory functions, were selectively expressed in dNK cells.

Key words: pregnancy • maternal-fetal relations • natural killer cells • gene expression profiling • lymphocyte subsets

Introduction

In pregnancy, hemiallogeneic fetal cells invade the maternal decidua but remain spared from attack by the maternal immune system, posing a great unsolved paradox of immunology (1). Although several factors have been proposed to explain or contribute to maternal tolerance, our understanding of the immunobiology of normal pregnancy and its implications for pregnancy-related pathologies is still limited (2). One prominent feature of the pregnant human decidua is the striking abundance of NK cells, which constitute ~70% of resident lymphocytes (3). In contrast, NK cells in peripheral blood comprise <15% of circulating lymphocytes (4). Thus, human uterine decidual NK (dNK) cells have been thought to play a role in implantation and pregnancy, at least in early gestation. Although the precise functions of dNK cells in vivo are still unknown, their proximity to the invading trophoblasts, which lack expression of classical HLA-A and -B antigens (5) but selectively express HLA-C and the nonclassical HLA-E, -G, and CD1d molecules (6–9), has led to the proposal that these MHC antigens on trophoblasts interact with NK cell receptors (3, 10). Human NK cell receptors include the C-type lectinlike NKG2 receptors that associate with CD94 and the killer cell Ig-like receptors (KIRs), each of which have both inhibitory and activating isoforms that depend on the presence of immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in their cytoplasmic tails or immunoreceptor tyrosine-based activating motifs in associated molecules, such as DAP12. Moreover, the KIR locus is polygenic and polymorphic, giving rise to variable KIR repertoires that are expressed on sub-

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Abbreviations used in this paper: dNK, decidual NK; ITIM, immunoreceptor tyrosine-based inhibitory motif; KIR, killer cell Ig-like receptor; pNK, peripheral blood NK; PP14, progestagen-associated protein 14; PSG, pregnancy-specific glycoprotein.

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sets of NK cells among individuals. However, it is still unclear if and which receptors on dNK cells interact with trophoblast-expressed HLA molecules, and whether such interactions inhibit NK cell lysis, or lead to production of cytokines that favor normal placental development and maintenance of pregnancy (11). Moreover, the interactions between NK cell receptors and their ligands have been investigated mostly on peripheral blood NK (pNK) cells, but the relationship between dNK and pNK cells is unclear.

pNK cells comprise two different subsets, the predominant $CD56^{dim}$ NK cell subset and the much smaller $CD56^{bright}$ NK cell subset, constituting ~95 and 5% of pNK cells, respectively (4). $CD56^{dim}$ pNK cells express high levels of CD16 and both CD94-associated lectinlike NKG2 receptors and KIRs, are granular, and known to be cytotoxic. In contrast, $CD56^{bright}$ pNK cells are mostly devoid of granules, CD16, and KIRs, but express higher amounts of other markers (12–15), such as CD94 (16) and L-selectin (17). Recent papers demonstrate greater cytokine production capacity of $CD56^{bright}$ than $CD56^{dim}$ pNK cells and, thus, suggest an immunoregulatory role for the $CD56^{bright}$ pNK subset (18, 19). The resemblance of dNK cells to the $CD56^{bright}$ pNK subset, at least in terms of their $CD56^{bright}$ $CD16^{neg}$ phenotype, has suggested that dNK cells are derived from $CD56^{bright}$ pNK cells that are seeded in the uterus and undergo further differentiation in the decidual microenvironment. For instance, they both exhibit low cytotoxicity against the NK cell target K562 (20), and contain cytoplasmic CD3ε mRNA (21), in contrast to $CD56^{dim}$ pNK cells. On the other hand, like $CD56^{dim}$ pNK, dNK cells are granulated and express KIRs (22, 23). Yet, other properties seem unique to dNK cells, such as the expression of the activation marker CD69 (24) and the absence of L-selectin (25). Despite some additional characterization of phenotypic and functional differences between dNK and pNK cells (26), a comprehensive comparison of dNK cells in relation to both $CD56^{bright}$ and $CD56^{dim}$ pNK cell subsets is lacking.

In this work, patterns of gene expression in freshly isolated $CD56^{bright}$ pNK, $CD56^{dim}$ pNK, and dNK cells were compared using oligonucleotide microarrays. The transcriptional profiles provide detailed information on the differences between dNK and pNK cells, as well as between the peripheral $CD56^{dim}$ and $CD56^{bright}$ subsets. Using a stringent statistical approach, a large number of differentially expressed genes (197) were identified between dNK and pNK cells, whereas only ~40 genes distinguished the two subsets of pNK cells from each other. Multiple genes were expressed in dNK cells whose transcripts were completely absent in pNK cells. A number of differentially expressed genes/proteins were validated by RT-PCR or flow cytometry. Several genes differentially and/or uniquely expressed in dNK cells encode proteins that are likely to be involved in maternal-fetal tolerance and provide potential critical targets for further study of dNK cells.

Materials and Methods

Samples

Decidual samples from patients undergoing elective abortion in the first trimester between 6 and 12 wk of gestation were collected at the New York University Medical Center. Decidual tissue was washed extensively in PBS supplemented with 50 μg/ml gentamicin before mincing with sterile scissors. Decidual lymphocytes were released by digesting the tissue with 0.1% collagenase type IV and 0.01% DNase I (both from Sigma-Aldrich) in RPMI 1640 medium for 30 min at 37°C. Decidual stromal cells and macrophages were allowed to adhere to tissue culture plates for 2 h at 37°C in a humidified 5% CO_2 incubator. Lymphocytes from the overlaying solution were subsequently purified by density gradient centrifugation (Ficoll-Hypaque; Amersham Biosciences) and used for flow cytometric staining and cell sorting. Peripheral blood lymphocytes were isolated from peripheral blood from anonymous healthy donors using Ficoll either directly or after enrichment for NK cells using Rosettesep according to the manufacturer's instructions (StemCell Technologies Inc.).

Flow Cytometry

The following murine mAbs, purified or directly conjugated with FITC, PE, or Cychrome, were used in FACS® analysis: anti-CD3, anti-CD56, anti-CD16, anti-CD9, anti-KIR2DL1/S1 (CD158a; EB6), anti-KIR2DL2/2DS2/2DL3 (CD158b; GL183), and anti-KIR3DL1 (CD158e; NKB1), which were all from BD Biosciences; anti-CD62L (DakoCytomation); anti-KIR2DL4 (KIR2DL4-specific mAbs 33, 36, and 64, supplied by E. Long [National Institutes of Health, Bethesda, MD; reference 27]), and IgG and IgM isotype controls (BD Biosciences). Secondary anti–mouse Ig fluorochrome conjugates used were goat F(ab′)2 anti–mouse IgG-FITC, goat F(ab′)2 anti–mouse IgG + IgM-FITC, and goat anti–mouse IgG-PE (Caltag). For FACS® staining and sorting, cells were washed in PBS supplemented with 2% FCS and incubated with mAb on ice for 30 min, followed by washing twice with PBS, pH 7.2, and 2% FCS. Cell sorting and fluorescence measurements were performed on a MoFLo high performance cell sorter (DakoCytomation). For fluorescence measurements only, data from 10,000 to 50,000 single cell events were collected using a standard FACScalibur™ flow cytometer (Immunocytometry Systems; Becton Dickinson). Data were analyzed using CELLQuest™ (Becton Dickinson) or Flow Jo (Treestar).

Preparation of Labeled RNA and Microarray Hybridization

Flow-sorted cells were washed with PBS, and the cell pellets were frozen in TRIzol (GIBCO BRL). Total RNA was isolated according to the TRIzol manufacturer's instructions, with addition of 5 μg linear polyacrylamide (Genelute LPA; Sigma-Aldrich) to aid visualization of RNA pellets. RNA was subjected to the clean-up protocol as per the manufacturer's instructions (Rneasy mini RNA isolation kit; QIAGEN). The subsequent RNA amplification and clean-up protocol was adapted from Baugh et al. (28). Biotinylated cRNA was hybridized to human genome HGU95Av2 chips displaying probes for ~10,000 full-length genes for expression analysis. Chips were processed according to manufacturer's instructions (for further experimental details see *Online Supplemental Material*). Gene transcript levels were determined using algorithms in the Microarray Suite 5.0 software (Affymetrix, Inc.). Based on Affymetrix, Inc. decision matrices, each probe was assigned a call of present (expressed) or absent (not expressed). 19 samples out of 22 samples were of suf-

ficient quality to be further analyzed (nine dNK, five CD56^{bright} pNK, and five CD56^{dim} pNK cells; Table SI available at http://www.jem.org/cgi/content/full/jem.20030305/DC1).

Microarray Data Analysis

Sample Clustering. Sample clustering analysis was performed with D-chip software (http://www.dchip.org). Intensity values were normalized by the model-based expression analysis method (29). Genes with variable expression levels across the 19 samples were selected by applying two criteria as follows: genes should be expressed (have presence calls) in at least 20% of the 19 samples, and σ_i/μ_i ratio should be >0.5 and <25, where σ_i and μ_i are the standard deviation and mean of the hybridization intensity values of each particular gene across all samples, respectively. An unsupervised hierarchical clustering algorithm (30) was applied to group the 19 samples based on the similarity of the expression profiles of the selected genes.

Student's T Test Comparisons and Identification of Differentially Expressed Genes. The 19 samples were normalized by setting a trimmed mean (using middle 95% of the distribution) to be the same; there were no noticeable problems with saturation or other nonlinear effects. To reduce the number of genes for subsequent analysis, a conservative requirement was applied that a gene must be present (according to the Affymetrix, Inc. algorithm) in at least 5 of the 19 samples, given the smallest sample size of 5 in both pNK cell groups.

Samples were grouped according to their biological origin in dNK (nine samples), CD56^{dim} pNK (five samples), and CD56^{bright} pNK cells (five samples). Three pairwise comparisons (dNK vs. CD56^{bright} pNK, dNK vs. CD56^{dim} pNK, and CD56^{bright} pNK vs. CD56^{dim} pNK) were performed to identify differentially expressed genes using the Student's *t* test with unequal variances. At $P \leq 0.01$ (unadjusted for multiple testing), >1,100 genes were significantly differentially expressed in the pairwise comparisons involving dNK cells, whereas ~300 were differentially expressed in the CD56^{bright} pNK versus CD56^{dim} pNK cell comparison. To rule out the possibility that this result was generated by the larger sample size in the case of dNK (nine samples vs. five of each pNK subset), the analysis was repeated with all possible sets of five samples from the nine original decidual samples. Although the total number of differentially expressed genes between all three subsets was slightly less, the number of differentially expressed genes between dNK and pNK^{bright} or pNK^{dim} cells was still nearly three times the number of such genes between pNK^{bright} and pNK^{dim} cells (Table SII available at http://www.jem.org/cgi/content/full/jem.20030305/DC1).

278 genes showing at least a threefold change in transcript levels at Student's *t* test $P \leq 0.001$ in at least one of the three pairwise comparisons were considered for further analysis. For genes represented by multiple probesets, the result for only one representative probeset is shown. Genes were classified, based on information available on the worldwide web (see *Online Supplemental Material*).

For Venn diagrams, genes were considered to be up-regulated in a particular NK subset if they showed a significant fold increase ($P \leq 0.001$) in at least one of the Student's *t* test comparisons involving that particular subset. Venn diagrams were constructed by intersecting the set of genes considered up-regulated in each of the three NK cell groups.

RT-PCR. RT-PCR was performed using equal amounts of RNA available from three dNK cell samples and CD56^{dim} and CD56^{bright} pNK cells. First strand cDNA was synthesized from 300 ng total RNA using Superscript II Reverse transcriptase for RT-PCR, according to the manufacturer's protocol (Life Technologies). PCR was done with 2 μl cDNA template in a total volume of 50 μl containing 1.5 mM $MgCl_2$, 10 mM dNTP, 2U Taq DNA polymerase, and 10 μM of each primer in 10× PCR buffer (Taq DNA polymerase PCR kit; QIAGEN). The PCR reaction profile consisted of 1 min at 95°C, followed by 30 cycles of 30 s at 94°C, 30 s at 60°C, and extension for 30 s at 72°C, with a final 5-min extension at 72°C. 10 μl of each product was visualized on 1–2% agarose gels. Primers used were as follows: NKG2E-Fw (5′-AGAATTAAACCTTCAAAATGCTTCT-3′) and NKG2E-Rev (5′-GATTCTTGAAGATCCACACTGGT-3′) for specific amplification of a 553-bp NKG2E product; NKG2C-Fw (5′-GAAGAGAGTTTGCTGGCCTGTA-3′) and NKG2C-Rev (5′-CACTGGGCTGATTTAAGTCGAT-3′) for a 242-bp NKG2C product, β actin–Fw (5′-AACTGGGACGACATGGAGAAAA-3′) and β actin–Rev (5′-TCGGTGAGGATCTTCATGAGGT-3′) for a 353-bp β-actin product; progestagen-associated protein 14 (PP14)/GdA-Fw (5′-AGAGCTCAGAGCCACCCAC-3′) and PP14/GdA-Rev (5′-GTGGGAGTCTGGTCTTCC-3′) for a 544-bp PP14/GdA; and GAL1-Fw (5′-CCCACGGCGACGCCAAC-3′) and GAL1-Rev (5′-AGAGGCAGCTGCCTTTATTG-3′) for a 295-bp galectin-1 product.

Online Supplemental Material. The supplemental material, data sets, Tables S1 and S2, and Figs. S1 and S2 are available at http://www.jem.org/cgi/content/full/jem.20030305/DC1. The supplemental material provides further details about cRNA preparation and hybridization, and gene classification, as well as explanatory comments for supplemental microarray Data Sets 1–3. Table S1 lists samples used for hybridization to genechips. Table S2 summarizes the number of differentially expressed genes in pairwise comparisons. Fig. S1 supplements Fig. 3. Fig. S2 shows FACS® and RT-PCR results for relevant markers after applying the procedure used for isolating dNK cells to pNK cells.

Results and Discussion

Comparison of Gene Expression in Human dNK and pNK Cells by Microarray Analysis

CD56^{bright} dNK cells and the two subsets of pNK cells (CD56^{bright} and CD56^{dim}) have been distinguished by only a few differentially expressed surface markers (3). The functional differences among them are still unclear. To further characterize dNK cells in relation to pNK cells and to discover genes with relative overexpression that could provide clues to their function, the gene expression profiles of freshly isolated NK cells from decidual and peripheral blood were compared. Cells obtained as described in Materials and Methods were flow sorted as illustrated in Fig. 1 A. The hybridization results, listing the number of present calls, the average hybridization intensities, and standard deviations for each NK cell subset (nine dNK, five CD56^{bright} pNK, and five CD56^{dim} pNK cell samples) and all 12,558 probe sets are available in Data Set S1 (available at http://www.jem.org/cgi/content/full/jem.20030305/DC1).

The 19 samples were organized on the basis of overall similarity in their gene expression patterns by an unsupervised hierarchical clustering algorithm of variable genes (30). A dendrogram, in which the pattern and length of the

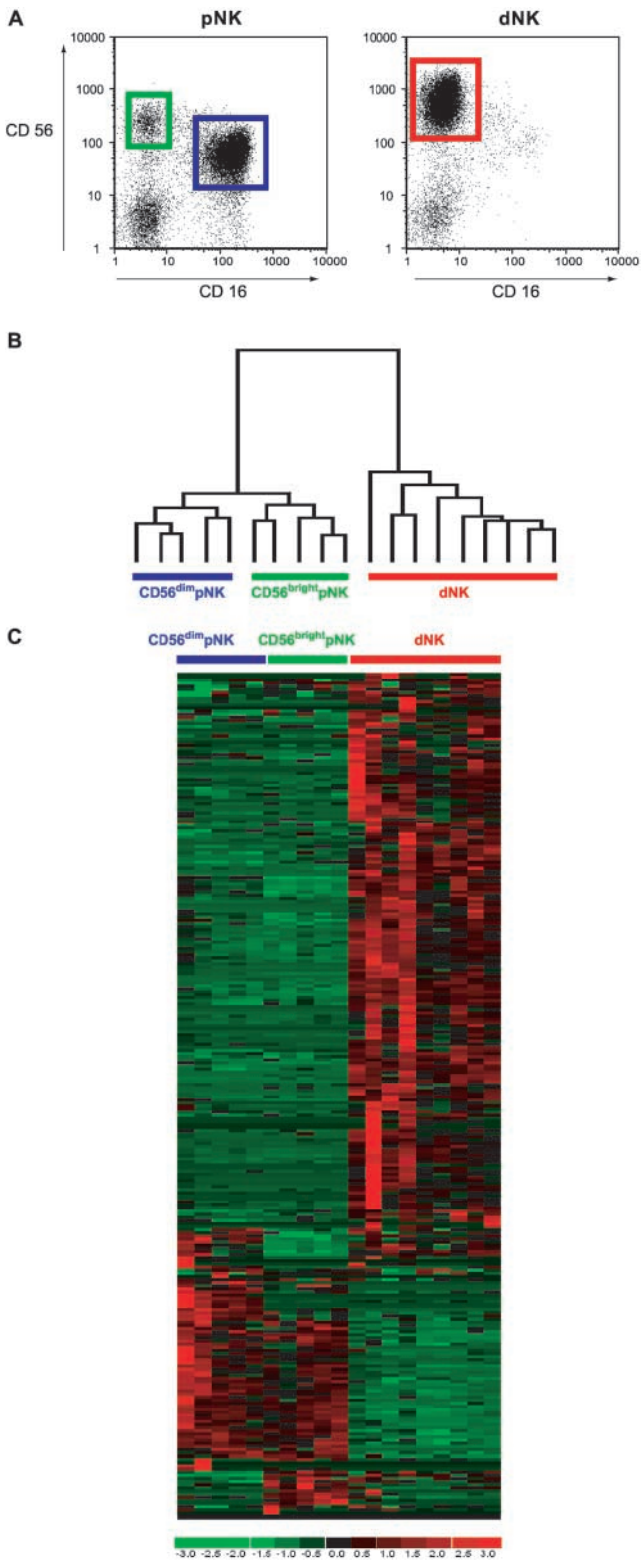

Figure 1. dNK CD56^{bright}, pNK CD56^{bright}, and pNK CD56^{dim} cells represent three different NK cell subsets. (A) Typical gates used for flow sorting dNK (red), pNK CD56^{bright} CD16- (green), and CD56^{dim} CD16- (blue) NK cell subsets. Decidual and peripheral lymphocyte suspensions were triple stained with directly conjugated mAbs reactive with CD3, CD16, and CD56. Lymphocytes were first gated by forward scatter/side scatter characteristics. After setting a gate on CD3 negative cells (NK-enriched peripheral blood preparations were typically already >99% CD3-negative), cells were sorted on the basis of CD56 and CD16 expression. (B) Unsupervised hierarchical clustering of 19 freshly sorted NK cell samples based on the expression profile of genes with variable expression levels across all samples. The organization and length of the branches in the resulting dendrogram reflect the similarity in gene expression profiles between each of the samples. No correlation between gestational age (range, 6–12 wk) and clustering pattern was observed. (C) Relative intensity profiles of 278 genes differentially expressed with at least a threefold change at $P \leq 0.001$ in at least one of the three comparisons (dNK vs. CD56^{bright} pNK; dNK vs. CD56^{dim} pNK; CD56^{bright} vs. CD56^{dim} pNK). Each row represents relative hybridization intensities of a particular gene across different samples. Each column represents one sample. Colors reflect the magnitude of relative expression of a particular gene across samples. Brighter red means higher expression, brighter green means lower expression, and black means average intensity across samples.

branches reflect the comparative difference in gene expression profiles between each of the dNK and pNK cell samples is shown in Fig. 1 B. dNK cells are clearly distinct from both subsets of pNK cells, as illustrated by the length of the two terminal branches. Within the group of pNK cells, a secondary branching point separates the CD56^{bright} and CD56^{dim} samples. At the transcriptional level, dNK cells differ notably from both subsets of pNK cells and are more distinct than the two peripheral CD56^{bright} and CD56^{dim} subsets are from each other. In other words, the two subsets of pNK cells are far more closely related to each other than either is to dNK cells.

Pairwise Student's *t* test comparisons of the hybridization data with the three NK cell subsets using $P \leq 0.01$ subsequently revealed differential expression of 1,785 genes, most of which were found in the comparisons involving dNK cells. Even after adjusting for the uneven sample sizes, the number of genes differentially expressed between the pNK subsets was about three times smaller (Table SII).

To reduce the number of differentially expressed genes to a manageable number, genes with a fold change of three or greater in at least one of the three pairwise comparisons at $P \leq 0.001$ were considered. These stringent criteria were met by 278 genes. An image of these genes clustered by similar expression patterns is represented in Fig. 1 C. Overall, ~70% of the 278 differentially expressed genes were overexpressed in dNK cells (197 genes) as compared with either CD56^{bright} or CD56^{dim} pNK cells, whereas roughly 30% of genes were overexpressed in pNK cells versus dNK cells, as illustrated by a Venn diagram (Fig. 2 A).

As an internal control, CD56, a prototypical NK cell marker, was first considered. CD56 (NCAM-1) appeared in the list of 278 genes, and revealed higher intensities in dNK as compared with both pNK subsets. This was also observed by FACS® analysis (Fig. 1 A and see Fig. 4 A). CD16 identifies CD56^{dim} NK cells and was absent in CD56^{bright} pNK and dNK cells (see Fig. 4 B). The latter result was foreseen because cell separation included CD16 expression as one parameter (Fig. 1 A). After classification of the genes into functional categories, the largest number of transcripts enriched in dNK cells were those encoding

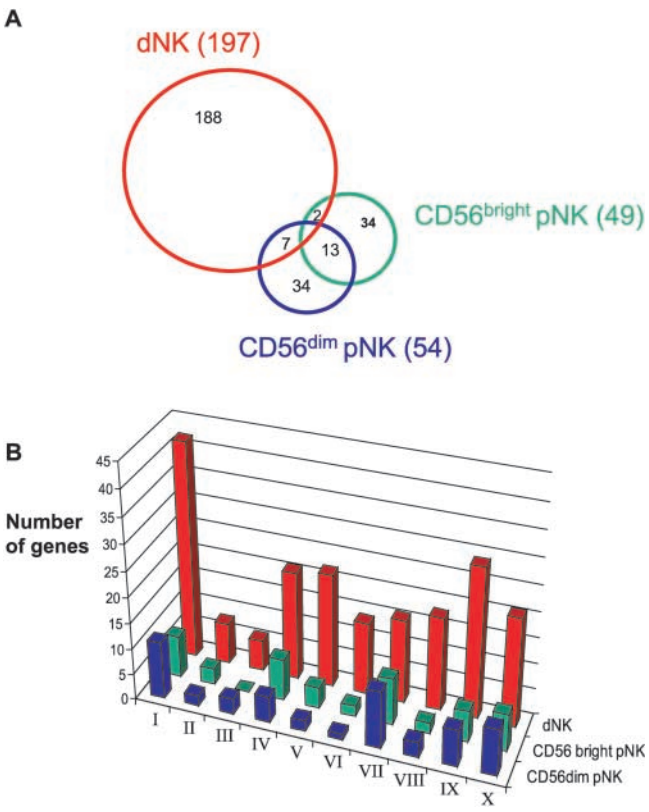

Figure 2. The majority of differentially expressed genes are up-regulated in dNK cells. (A) Venn diagram generated by the intersection of the list of genes up-regulated by each NK cell subset. Genes were considered to be overexpressed in a particular NK cell subset if they showed higher expression relative to at least one of the other subsets in the pairwise Student's *t* test comparisons at $P \leq 0.001$. For example, a total of 197 genes are overexpressed in dNK versus at least one of the two pNK subsets, 188 of which were overexpressed in dNK only. Only 2 of the 197 genes are overexpressed in both dNK and $CD56^{bright}$ pNK cells versus $CD56^{dim}$ pNK cells (intersection of red and green), and 7 of the 197 genes are overexpressed in dNK and $CD56^{dim}$ pNK cells versus $CD56^{bright}$ pNK cells (intersection of red and blue). (B) Distribution by functional category of genes with significant up-regulation in each NK cell type. I, surface molecules/receptors/adhesion; II, chemokines/cytokines/immunomodulatory and other secreted proteins; III, immune effector molecules and apoptosis related; IV, signal transduction related; V, cytoskeleton related; VI, cell cycle + stress; VII, DNA binding/transcription/translation; VIII, metabolism; IX, other genes; and X, genes with unknown function.

membrane proteins (Fig. 2 B). Additionally, the 278 differentially expressed genes are shown with fold changes for each comparison in Fig. 3 (also see Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20030305/DC1). Expression of several of these genes was examined by either flow cytometry (Fig. 4) or RT-PCR (Fig. 5).

Membrane Proteins Overexpressed in dNK Cells

Tetraspanins and Integrin Subunits. Among the 278 differentially expressed genes, a large number of genes were surface molecules and receptors (Fig. 2 B and Fig. 3 A). Among the genes in this category, three members of the transmembrane 4 superfamily (CD151, CD9, and tetraspan-5; also known as tetraspanins) were exclusively expressed in dNK cells. Two integrin subunits, the αD (ITGAD;CD11d) and the β5 (ITGB5) subunits were also highly overexpressed by dNK. Other tetraspanins (CD53 and CD63) and integrin subunits (β1 [ITGB1] and αx [ITGAX;CD11c]) were relatively overexpressed in dNK cells in relation to at least one of the peripheral subsets. Integrins are involved in cell–cell and cell–extracellular matrix interactions and influence diverse processes such as cell adhesion, migration, activation, and interaction with target cells. Many of the tetraspanins may facilitate these processes by assembling molecules into membrane clusters, distinct from lipid rafts (e.g., by participating in functional signaling complexes with other integrins, lineage-markers, or other tetraspanins; references 31–36). The overexpression of several of these proteins in dNK cells may be related to the mechanisms used by dNK cells for migration to, retention within, and/or their effector function within the pregnant uterine mucosa. FACS® analysis confirmed surface expression of both CD9 and CD151. CD9 is exclusively expressed on dNK cells and emerges as a specific marker for these cells ex vivo (Fig. 4, C and D).

Interestingly, a ligand for murine CD9, murine pregnancy-specific glycoprotein (PSG) 17, was recently identified (37). This prompts the interesting hypothesis that dNK cells, through their CD9 receptor, may interact with placental PSGs and consequently produce cytokines that support successful pregnancy. PSGs are a family of highly similar, placentally secreted proteins, originally isolated from the circulation of pregnant women. In humans, PSG concentration in the bloodstream increases exponentially until term (38) and low PSG levels are associated with recurrent spontaneous abortions (39). Administration of anti-PSG Abs induced spontaneous abortion in primates, indicating that PSGs are essential for successful pregnancy (40). In addition, PSGs may induce production of antiinflammatory cytokines by monocytes (41).

Lectinlike Receptors. Of particular interest, NKG2C and NKG2E genes both were highly significantly overexpressed in dNK cells. Both showed increased hybridization in the three- to fivefold range (Fig. 3 A and Fig. 5). The specificity of this result was confirmed by RT-PCR. NKG2C and NKG2E are CD94-associated members of the C-type lectin like receptors with presumed activating functions. The CD94/NKG2C heterodimer associates with DAP12 and binds to HLA-E (42, 43). Although the resemblance of NKG2E to NKG2C in terms of an ITIM-lacking cytoplasmic domain (both containing a Lys residue in the transmembrane region that may lead to association with DAP12) suggests an activating function, the ligand and functions of NKG2E have not been described previously (44). Moreover, detection of NKG2E protein has not been reported.

The CD94-associated lectinlike inhibitory receptor NKG2A, which bears two ITIMs in the cytoplasmic domain and for which the ligand is HLA-E, was not represented on the chip because the polymorphism and isoforms of this gene makes it difficult to select specific probes. Its expression on the three NK cell subsets in association with CD94 was detected by FACS® analysis using the NKG2A-

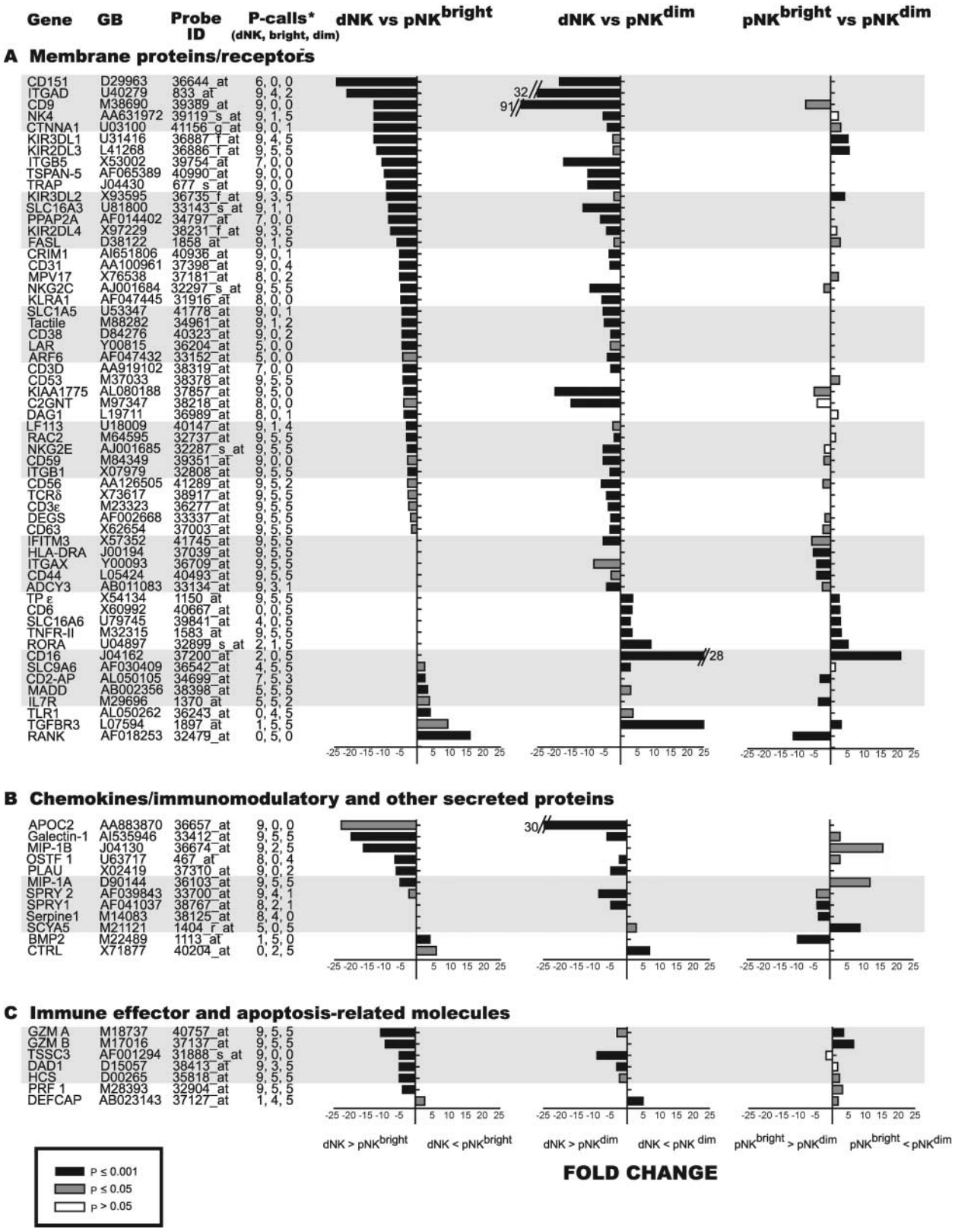

Figure 3. Differentially expressed genes in dNK and pNK cells. Fold changes of genes that showed greater than or equal to threefold change. $P \leq 0.001$ (black bars) in at least one of the three pairwise comparisons: dNK versus pNK^{bright} (left diagrams); dNK versus pNK^{dim} (middle diagrams); and pNK^{bright} versus pNK^{dim} (right diagrams) are presented. The 278 transcripts that met these criteria were classified into the following 10 categories: I, surface molecules/receptors/adhesion (A, 58 genes); II, chemokines/cytokines/immunomodulatory and other secreted proteins (B, 12 genes); III, immune effector molecules and apoptosis related (C, 7 genes); IV, signal transduction related (32 genes); V, cytoskeleton related (26 genes); VI, cell cycle + stress (15 genes); VII, DNA- binding/

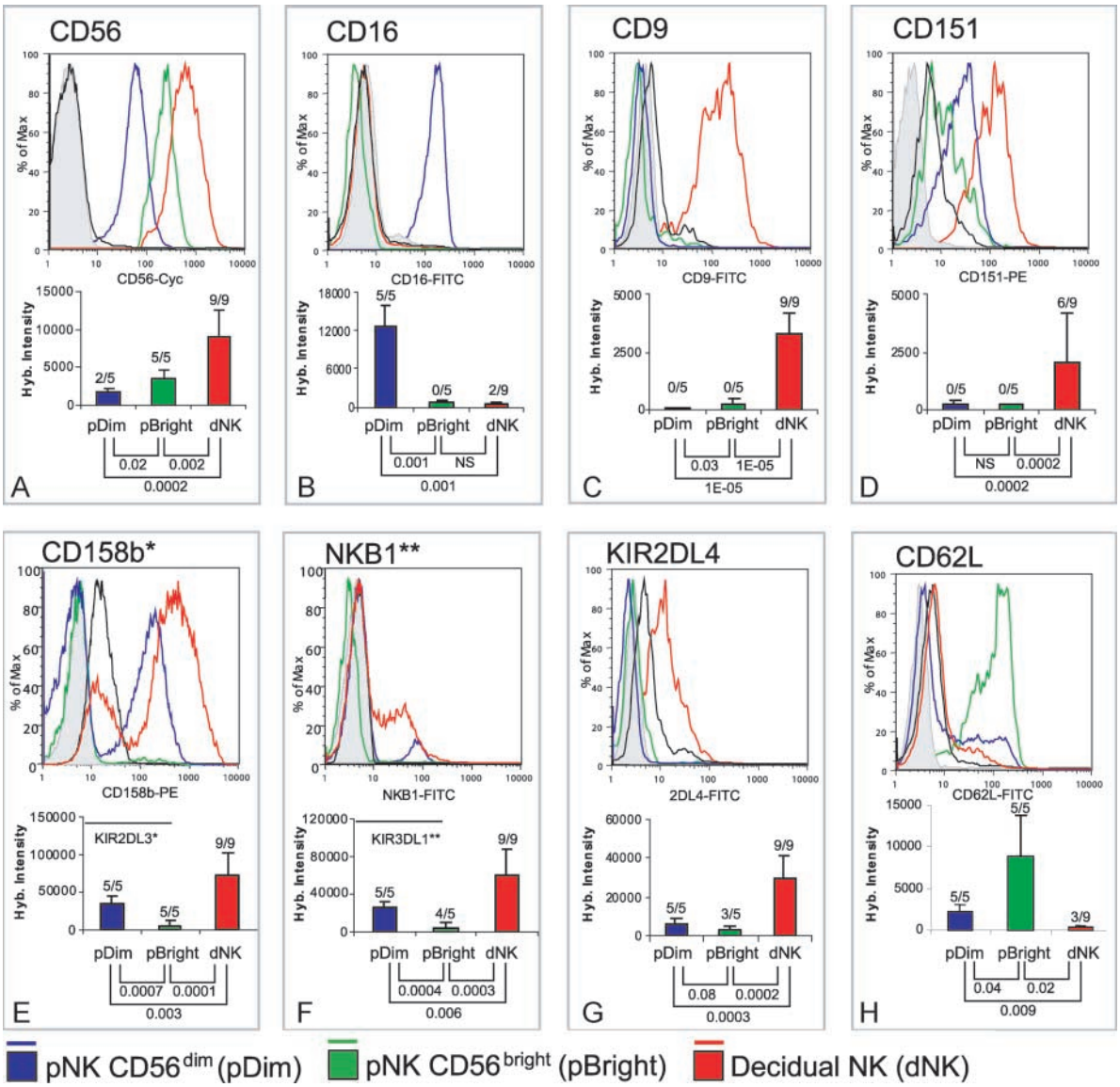

Figure 4. Verification of selected surface expressed molecules by flow cytometry. To compare protein expression on the different NK cell populations (gated as in Fig. 1 A), triple or quadruple [CD56 + CD16 + (CD3) + (marker of interest) on NK-enriched peripheral blood preparations; CD56 + CD3 + (CD16) + (marker of interest) on decidual samples] staining was performed. Histogram overlays (representative of at least five experiments) demonstrating the expression of various markers are shown together with genechip hybridization intensity data (bars) for the probeset of the corresponding gene. Gray and black histograms represent the isotype controls for pNK and dNK cells, respectively. Lines and bars are color-coded for dNK (red), $CD56^{bright}$ pNK (green), and $CD56^{dim}$ pNK cells (blue). Numbers on bars represent number of "present" calls (according to Affymetrix, Inc. algorithms) per number of samples hybridized. CD56 (a), CD16 (b), CD9 (c), CD151(d), KIR2DL3* (e), KIR3DL1** (f), and KIR2DL4 (g) were among the list of 278 differentially expressed genes fitting the stringent criteria (threefold change and $P \leq 0.001$ in any of the comparisons). *KIR2DL3 was verified with mAb CD158b, (reactive besides KIR2DL3, with KIR2DS3 and KIR2DL2/S2 that were not represented on the chip) **KIR3DL1 was verified with mAb NKB1 (also reactive with KIR3DS1). CD62L (h), shown as an example of a gene differentially expressed in $CD56^{bright}$ pNK cells (17), was differentially expressed resulting from pairwise Student's *t* test comparisons at $P < 0.01$, but did not fit the more stringent criteria of threefold change and $P < 0.001$. Levels of significance per comparison are indicated. NS, not significant.

specific mAb Z199 and anti-CD94. CD94 was not differentially expressed in the chip analyses (unpublished data). The manner in which signals mediated through activating and inhibitory receptors on the same cell are integrated remains to be explored.

Ly-49L (Fig. 3 A, KLRA1) was present and overex-

transcription/translation (33 genes); VIII, metabolism (23 genes); IX, other genes (40 genes); and X, genes with unknown function (33 genes). The first three categories are shown in this figure (A–C); data for the remaining categories are shown as Fig. S1. Black and gray bars represent fold changes in pairwise comparisons with significance levels ≥0.001 and ≤0.05, respectively. White bars represent statistically nonsignificant changes. Gene, gene name, or gene symbol obtained from Locuslink or Netaffx (Affymetrix, Inc.). GB, GenBank accession number. Probe ID, Affymetrix probeset designation. P-(Presence) calls (dNK, bright, and dim), number of present calls by Affymetrix algorithms in nine dNK, five pNK $CD56^{bright}$, and five $CD56^{dim}$ NK cells, respectively.

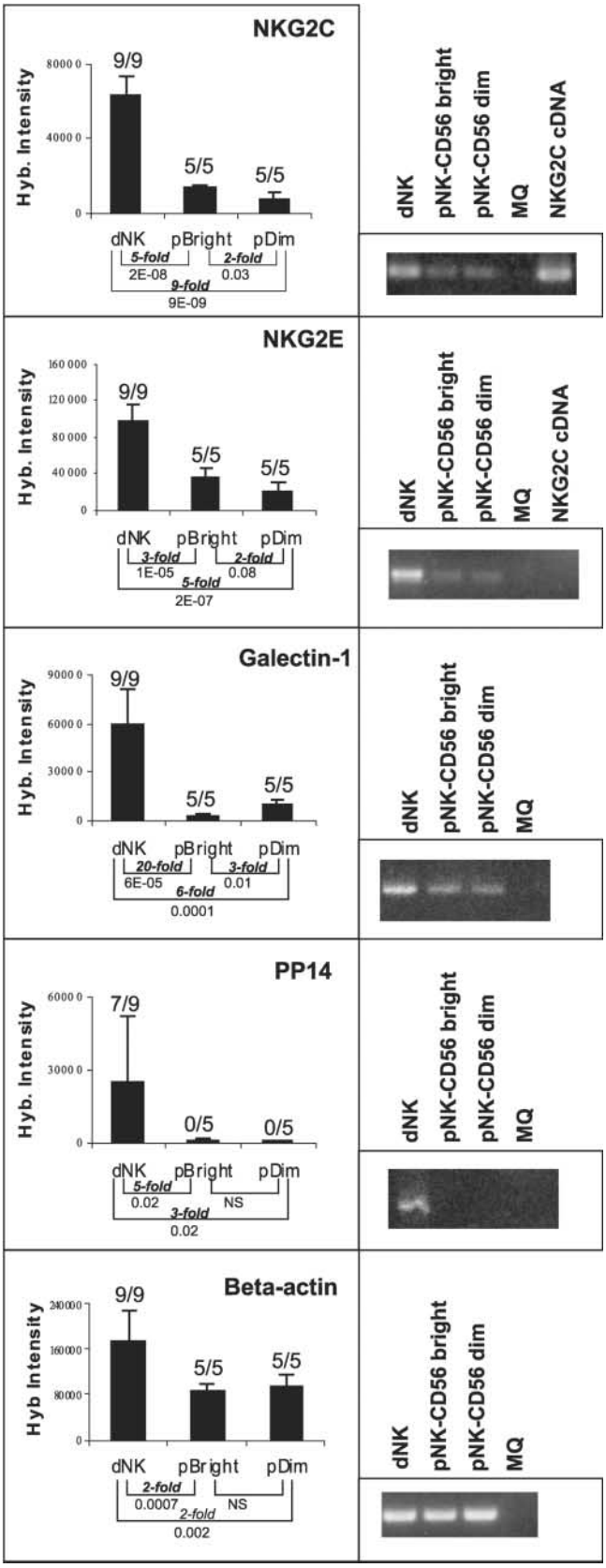

Figure 5. Confirmation of transcript expression by RT-PCR analysis. RT-PCR was performed on total RNA isolated from human dNK, CD56^{dim}, and CD56^{bright} pNK cells. Results shown are representative of three different samples for each of these NK cell types (right). Genechip hybridization data for the 19 samples and statistical significance levels of pairwise comparisons are presented (left). Numbers on bars represent number of present calls (according to Affymetrix, Inc. algorithms) per number of samples hybridized to genechips. Fold changes and levels of significance per comparison are indicated. NS, not significant. Specific transcripts for NKG2C, NKG2E, galectin-1, and PP14 were detected in dNK cell samples by RT-PCR, whereas their presence appeared to be relatively less (or absent in the case of PP14) in both pNK cells subsets. In the genechip experiments, these transcripts were found to be overexpressed in dNK cells with fold differences, 3- to 9-fold (NKG2s and PP14) and 6- to 20-fold (galectin-1). MQ, Millipore Q water.

pressed in nearly all of the dNK cells but absent from both sets of pNK cells (Fig. 3 A, P-calls). This unusual transcript is derived from a gene that encodes a member of the lectin superfamily closely related to the large family of murine Ly-49 genes. The single human gene has been found expressed only as transcripts that would lead to a truncated protein, due to a single nucleotide substitution preceding a splice donor site in Exon 5 (45, 46). However, the full-length transcript was found in both baboons and the orangutan (47, 48). It remains to be seen whether the full-length transcript and the corresponding protein can be produced in human materials, particularly in dNK cells, and if so, what its function might be.

KIR Genes. All of the KIR genes for which probes were present on the genechip (KIR3DL1, KIR3DL2, KIR2DL3, and KIR2DL4) were overexpressed by dNK cells (Fig. 3 A). KIRs have been known to be expressed on both dNK and CD56^{dim} pNK cells, but not on CD56 ^{bright} pNK cells. Staining for KIRs using the available mAbs CD158a (unpublished data), CD158b, and NKB1 (Fig. 4, E and F) consistently showed a much higher proportion of positive cells in dNK cells than in CD56^{dim} pNK cells, in keeping with the increased transcript levels found on the average in dNK cells. These data are in accordance with previously published data on KIR expression in peripheral and decidual blood from the same individual (22).

KIR2DL4 expression has been reported on both dNK and pNK cells (49, 50). Although it has been suggested that it is the HLA-G receptor, other recent data do not support this conclusion (51, 52). The present findings indicate that KIR2DL4 is transcribed in dNK cells to a higher degree than in CD56^{dim} or CD56^{bright} pNK cells (Fig. 3 A). Using anti-KIR2DL4–specific antibodies (27), the surface expression of this receptor protein was found in 5 out of 11 tested dNK cell samples, but was not detectable on pNK cells in 5 tested samples (Fig. 4 G). In contrast with many of the other KIRs that are variably present in individual genomes, KIR2DL4 is found in all haplotypes. Yet, a single base deletion in the KIR2DL4 gene, which predicts generation of a molecule with a shortened cytoplasmic tail (53) and possibly a protein which is ultimately not surface expressed, occurs. A considerable number of individuals are homozygous for this deletion (54), which may partly explain the lack of reactivity seen with the anti-KIR2DL4 mAb.

Molecules with Immunomodulatory Potential

Galectin-1 and Core 2 β-1,6-N-acetylglucosaminyl Transferase (C2GNT). Although galectin-1 is expressed by all NK cell types, its transcription level is greatly elevated in dNK cells relative to the other two subsets (Fig. 3 B), a result that was confirmed by RT-PCR (Fig. 5). Galectin-1 is a member of a small family of carbohydrate-binding proteins (galectins 1–14; reference 55). Although galectins recognize *N*-acetyl-lactosamine (galactosyl-*N*-acetylglucosamine), they have a higher affinity for polylactosamine moieties present in *N*-glycosylated and core 2 O-glycosylated glycoproteins. Two enzymes are essential for the presence of these moieties on N- or O-glycan structures. For N-glycans, Mgat 5 initiates a fourth branch to form a tetraantenary glycan, which is elongated with polylactosamine groups. For O-glycans, C2GNT initiates a second branch (core 2) on O-glycans that is also further elongated with polylactosamine residues (56, 57). Divalent galectin-1 homodimers binding to cell surface O- and N-glycosylated glycoproteins mediate cell–cell interactions, and generate lattices that can segregate, group, or affect clustering of cell surface glycoproteins (57). Microarray expression profiles show that Mgat5 is not expressed on any of the three subsets of NK cells studied here. Strikingly, however, C2GNT required for core 2 O-glycan synthesis is expressed on dNK cells but on neither of the two subsets of pNK cells (Fig. 3 B).

Galectins have been implicated in immune maturation and modulation through various mechanisms (55). Mgat5 expression in T cells negatively regulates T cell activation and autoimmunity. Galectin binding restricts TCR recruitment to the synapse by formation of a lattice resulting from its binding to tetraantennary N-glycosylated glycoproteins (58). Most recently, galectin-1 has been implicated in pre–B cell and bone marrow stromal cell synapse formation as a stromal cell ligand of the pre–B cell receptor (59). Core 2 O-glycans are expressed by CD4 + CD8 + T cells in a regulated manner during intrathymic maturation (56). Galectin-1 mediates thymocyte binding to stromal cells. It also induces apoptosis of immature $CD8^{low}$ $CD4^{low}$ thymocytes and peripheral activated T cells through galectin-1–mediated segregation of O-glycosylated CD45 from CD7 and CD43. This process is dependent on C2GNT expression (57, 60). Other effects of galectin-1 on T cells have been reported previously (55). Two hypotheses are suggested by these data as follows: (a) galectin-1 and C2GNT, the enzyme required to initiate formation of its ligand, are expressed by NK cells in the decidua because they are needed to down-modulate cytotoxic activity; and (b) galectin-1 present on trophoblasts in the placental bed (61) may originate from dNK cells and may function on the trophoblast to down-modulate cytotoxicity.

PP14 (Also Called GdA, Glycodelin A). PP14 was uniquely expressed in dNK cells (Fig. 5). Although its expression level was variable (Fig. 5) and, therefore, did not reach the statistical significance level required for inclusion in Fig. 3, it was present and highly expressed in seven of the nine dNK samples but in neither of the pNK cell subsets. Its differential expression was confirmed by RT-PCR. PP14 is a 28-kD glycoprotein with a unique carbohydrate configuration that is very unusual in mammals. Although it was first isolated from human placenta, it was later shown to be synthesized by endometrial glands and gestational deciduas, with the highest production at 10–14 wk of gestation (62). PP14 has been reported to inhibit T lymphocyte proliferative responses and to negatively regulate T cells by localizing to the APC–T cell contact site and elevating activation thresholds (63). It was also reported to inhibit K562 lysis by pNK cells (64).

Granzymes and Perforin

Granularity is a characteristic of both dNK and $CD56^{dim}$ pNK cells, both of which are also called large granular lymphocytes (65), whereas $CD56^{bright}$ pNK cells are known to be less granular (15). Granzyme B and perforin (components of cytotoxic granules) were expressed at similar levels in dNK and $CD56^{dim}$ pNK cells, whereas granzyme A was 3- and 10-fold overexpressed in dNK cells versus the $CD56^{dim}$ and $CD56^{bright}$ pNK cells, respectively (Fig. 3 C). Thus, the potential cytolytic function of dNK cells, probably regulated, cannot be ignored. This nascent activity could be manifest in the decidua under special circumstances (e.g., virus infection of the trophoblast or decidual remodeling).

Many other genes overexpressed in dNK cells that are presented in Fig. 3 (e.g., CD31, CD38, MIP-1 α/β, Syk, LEF1, AARS, arp2/3 subunit, and tubulins) may be of considerable interest but are beyond the scope of the present paper. Likewise, genes that are absent or have lower expression in dNK cells in relation to pNK cells may be relevant to dNK or pNK cell function and interesting candidates for further study. For instance, toll-like receptor 1 (Fig. 3 A) and SH2D1A (Fig. S1 A) are expressed in both pNK subsets but not in dNK cells. CD6 is expressed only in $CD56^{dim}$ pNK cells, whereas the receptor activator of NF-κB, a new member of the TNF superfamily of receptors, transcripts are found only in $CD56^{bright}$ pNK cells (Fig. 3 A). Both may be relevant to interactions between these and other cells (66, 67). Finally, functions other than a role in fetal-maternal tolerance have been suggested for dNK cells. In particular, roles for them in placental development and in vascular remodeling of uterine spiral arteries have been suggested in the mouse (11, 66, 67). dNK cells may have different functions in response to novel ligands in distinct uterine microenvironments. The data presented in Fig. S1, as well as that included in Fig. 3 (A–C), should be particularly useful to those interested in these and other phenomena associated with pregnancy.

It is worthwhile to ask whether the overexpression of a large number of genes in dNK cells may have been due to activation by the procedure used to isolate them (i.e., disaggregation with collagenase and DNase, incubation to allow adhesion of stromal cells and macrophages to plastic and Ficoll density gradient separation, a procedure requiring 4–6 h [Materials and Methods]). The procedure has been applied previously to isolate lymphocytes from other organs such as pancreas, liver, and intestines without apparent activating effects. Nevertheless, pNK cells were put

through a mock isolation procedure and the markers that distinguished dNK cells from their blood counterparts were analyzed. The mock isolation was performed with and without the addition of human decidual stroma.

FACS® and PCR analysis of the most relevant markers (Figs. 3–5; CD69, a marker for activation; CD9, a characteristic marker of dNK cells as shown in this paper; CD16, a specific marker of $CD56^{dim}$ pNK cells; and PP14, a protein exclusively expressed by dNK cells) was performed. No changes in the expression of these markers on $CD56^{dim}$ or $CD56^{bright}$ pNK cells were observed when the isolation procedure was performed in the absence or in the presence of decidual stroma (Fig. S2 available at http://www.jem.org/cgi/content/full/jem.20030305/DC1). Nevertheless, it cannot be excluded that changes in a fraction of the 197 upregulated genes in dNK cells may have been the result of activation during the isolation procedure.

The present paper has provided a detailed characterization of gene expression in the different NK cell subsets present in peripheral blood and early pregnancy decidual tissues. First, dNK cells are remarkably different from either subset of pNK cells; $CD56^{bright}$ pNK cells are far more similar to the $CD56^{dim}$ pNK cell subsets than they are to the $CD56^{bright}$ dNK cells. Second, the majority of differentially expressed genes are overexpressed by dNK cells. In contrast, the relatively small number of differentially expressed genes in the pNK cell comparison are equally distributed between those that were overexpressed in $CD56^{dim}$ versus $CD56^{bright}$ pNK cells, and those overexpressed in $CD56^{bright}$ versus $CD56^{dim}$ pNK cells. The observed differences in gene expression may be explained by two alternative hypotheses as follows: (a) dNK cells represent a distinct lineage of NK cells, possibly arising from a distinct hematopoietic precursor; or (b) the differences reflect a maturation of pNK cells (most likely the $CD56^{bright}$ subset) in the decidual microenvironment. Additionally, the nature of the precursors and sites of differentiation, of dNK cells, and, in particular, the effect of hormones and decidual stroma (whose effect on differentiation have been scarcely studied) are of considerable interest. Special attention must be paid to general effects common to many stroma and possible specific effects that may lead to expression of dNK cell–specific molecules. Third, and most interestingly, genes that provide exciting new venues for research were overexpressed in dNK cells. These include CD9 and other tetraspanins (at least four integrin subunits: NKG2C and NKG2E, Ly-49L, KIRs, galectin-1, and PP14), all of which could have immunomodulatory functions during pregnancy. The data strongly suggest a distinct function for dNK cells in pregnancy (i.e., a role in maternal-fetal tolerance).

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References

1. Billingham, R.E., L. Brent, and P.B. Medawar. 1953. Actively acquired tolerance of foreign cells. *Nature*. 172:603–606.
2. Pearson, H. 2002. Reproductive immunology: immunity's pregnant pause. *Nature*. 420:265–266.
3. Moffett-King, A. 2002. Natural killer cells and pregnancy. *Nat. Rev. Immunol*. 2:656–663.
4. Cooper, M.A., T.A. Fehniger, and M.A. Caligiuri. 2001. The biology of human natural killer-cell subsets. *Trends Immunol*. 22:633–640.
5. Faulk, W.P., and A. Temple. 1976. Distribution of beta2 microglobulin and HLA in chorionic villi of human placentae. *Nature*. 262:799–802.
6. King, A., C. Boocock, A.M. Sharkey, L. Gardner, A. Beretta, A.G. Siccardi, and Y.W. Loke. 1996. Evidence for the expression of HLA-C class I mRNA and protein by human first trimester trophoblast. *J. Immunol*. 156:2068–2076.
7. King, A., D.S. Allan, M. Bowen, S.J. Powis, S. Joseph, S. Verma, S.E. Hiby, A.J. McMichael, Y.W. Loke, and V.M. Braud. 2000. HLA-E is expressed on trophoblast and interacts with CD94/NKG2 receptors on decidual NK cells. *Eur. J. Immunol*. 30:1623–1631.
8. Boyson, J.E., B. Rybalov, L.A. Koopman, M. Exley, S.P. Balk, F.K. Racke, F. Schatz, R. Masch, S.B. Wilson, and J.L. Strominger. 2002. CD1d and invariant NKT cells at the human maternal-fetal interface. *Proc. Natl. Acad. Sci. USA*. 99: 13741–13746.
9. Kovats, S., E.K. Main, C. Librach, M. Stubblebine, S.J. Fisher, and R. DeMars. 1990. A class I antigen, HLA-G, expressed in human trophoblasts. *Science*. 248:220–223.
10. King, A., S.E. Hiby, L. Gardner, S. Joseph, J.M. Bowen, S. Verma, T.D. Burrows, and Y.W. Loke. 2000. Recognition of trophoblast HLA class I molecules by decidual NK cell receptors–a review. *Placenta*. 21:S81–S85.
11. Croy, B.A., S. Chantakru, S. Esadeg, A.A. Ashkar, and Q. Wei. 2002. Decidual natural killer cells: key regulators of placental development (a review). *J. Reprod. Immunol*. 57:151–168.
12. Campbell, J.J., S. Qin, D. Unutmaz, D. Soler, K.E. Murphy, M.R. Hodge, L. Wu, and E.C. Butcher. 2001. Unique subpopulations of CD56+ NK and NK-T peripheral blood lymphocytes identified by chemokine receptor expression repertoire. *J. Immunol*. 166:6477–6482.
13. Lima, M., M.A. Teixeira, M.L. Queiros, M. Leite, A.H. Santos, B. Justica, and A. Orfao. 2001. Immunophenotypic characterization of normal blood CD56+lo versus CD56+hi NK-cell subsets and its impact on the understanding of their tissue distribution and functional properties. *Blood Cells Mol. Dis*. 27:731–743.
14. Carson, W.E., T.A. Fehniger, and M.A. Caligiuri. 1997. CD56bright natural killer cell subsets: characterization of distinct functional responses to interleukin-2 and the c-kit ligand. *Eur. J. Immunol*. 27:354–360.
15. Jacobs, R., G. Hintzen, A. Kemper, K. Beul, S. Kempf, G.

Behrens, K.W. Sykora, and R.E. Schmidt. 2001. CD56 bright cells differ in their KIR repertoire and cytotoxic features from CD56dim NK cells. *Eur. J. Immunol.* 31:3121–3127.
16. Voss, S.D., J. Daley, J. Ritz, and M.J. Robertson. 1998. Participation of the CD94 receptor complex in costimulation of human natural killer cells. *J. Immunol.* 160:1618–1626.
17. Frey, M., N.B. Packianathan, T.A. Fehniger, M.E. Ross, W.C. Wang, C.C. Stewart, M.A. Caligiuri, and S.S. Evans. 1998. Differential expression and function of L-selectin on CD56bright and CD56dim natural killer cell subsets. *J. Immunol.* 161:400–408.
18. Cooper, M.A., T.A. Fehniger, S.C. Turner, K.S. Chen, B.A. Ghaheri, T. Ghayur, W.E. Carson, and M.A. Caligiuri. 2001. Human natural killer cells: a unique innate immunoregulatory role for the CD56(bright) subset. *Blood.* 97:3146–3151.
19. Cooper, M.A., T.A. Fehniger, A. Ponnappan, V. Mehta, M.D. Wewers, and M.A. Caligiuri. 2001. Interleukin-1beta costimulates interferon-gamma production by human natural killer cells. *Eur. J. Immunol.* 31:792–801.
20. King, A., P.P. Jokhi, T.D. Burrows, L. Gardner, A.M. Sharkey, and Y.W. Loke. 1996. Functions of human decidual NK cells. *Am. J. Reprod. Immunol.* 35:258–260.
21. King, A., L. Gardner, A. Sharkey, and Y.W. Loke. 1998. Expression of CD3 epsilon, CD3 zeta, and RAG-1/RAG-2 in decidual CD56+ NK cells. *Cell. Immunol.* 183:99–105.
22. Verma, S., A. King, and Y.W. Loke. 1997. Expression of killer cell inhibitory receptors on human uterine natural killer cells. *Eur. J. Immunol.* 27:979–983.
23. Hiby, S.E., A. King, A.M. Sharkey, and Y.W. Loke. 1997. 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.* 34:419–430.
24. Loke, Y.W., and A. King. 1995. Human Implantation: Cell Biology and Immunology. Cambridge University Press, Cambridge. 299 pp.
25. Slukvin, I.I., V.P. Chernyshov, A.A. Merkulova, M.A. Vodyanik, and A.K. Kalinovsky. 1994. Differential expression of adhesion and homing molecules by human decidual and peripheral blood lymphocytes in early pregnancy. *Cell. Immunol.* 158:29–45.
26. Deniz, G., S.E. Christmas, R. Brew, and P.M. Johnson. 1994. Phenotypic and functional cellular differences between human CD3- decidual and peripheral blood leukocytes. *J. Immunol.* 152:4255–4261.
27. Rajagopalan, S., J. Fu, and E.O. Long. 2001. Cutting edge: induction of IFN-gamma production but not cytotoxicity by the killer cell Ig-like receptor KIR2DL4 (CD158d) in resting NK cells. *J. Immunol.* 167:1877–1881.
28. Baugh, L.R., A.A. Hill, E.L. Brown, and C.P. Hunter. 2001. Quantitative analysis of mRNA amplification by in vitro transcription. *Nucleic Acids Res.* 29:E29.
29. Li, C., and W.H. Wong. 2001. Model-based analysis of oligonucleotide arrays: expression index computation and outlier detection. *Proc. Natl. Acad. Sci. USA.* 98:31–36.
30. Eisen, M.B., P.T. Spellman, P.O. Brown, and D. Botstein. 1998. Cluster analysis and display of genome-wide expression patterns. *Proc. Natl. Acad. Sci. USA.* 95:14863–14868.
31. Hemler, M.E. 2001. Specific tetraspanin functions. *J. Cell Biol.* 155:1103–1107.
32. Yauch, R.L., A.R. Kazarov, B. Desai, R.T. Lee, and M.E. Hemler. 2000. Direct extracellular contact between integrin alpha(3)beta(1) and TM4SF protein CD151. *J. Biol. Chem.* 275:9230–9238.
33. Kazarov, A.R., X. Yang, C.S. Stipp, B. Sehgal, and M.E. Hemler. 2002. An extracellular site on tetraspanin CD151 determines alpha 3 and alpha 6 integrin-dependent cellular morphology. *J. Cell Biol.* 158:1299–1309.
34. Yang, X., C. Claas, S.K. Kraeft, L.B. Chen, Z. Wang, J.A. Kreidberg, and M.E. Hemler. 2002. Palmitoylation of tetraspanin proteins: modulation of CD151 lateral interactions, subcellular distribution, and integrin-dependent cell morphology. *Mol. Biol. Cell.* 13:767–781.
35. Zhang, X.A., A.L. Bontrager, and M.E. Hemler. 2001. Transmembrane-4 superfamily proteins associate with activated protein kinase C (PKC) and link PKC to specific beta(1) integrins. *J. Biol. Chem.* 276:25005–25013.
36. Vogt, A.B., S. Spindeldreher, and H. Kropshofer. 2002. Clustering of MHC-peptide complexes prior to their engagement in the immunological synapse: lipid raft and tetraspan microdomains. *Immunol. Rev.* 189:136–151.
37. Waterhouse, R., C. Ha, and G.S. Dveksler. 2002. Murine CD9 is the receptor for pregnancy-specific glycoprotein 17. *J. Exp. Med.* 195:277–282.
38. Lin, T.M., S.P. Halbert, and W.N. Spellacy. 1974. Measurement of pregnancy-associated plasma proteins during human gestation. *J. Clin. Invest.* 54:576–582.
39. Arnold, L.L., T.M. Doherty, A.W. Flor, J.A. Simon, J.Y. Chou, W.Y. Chan, and B.C. Mansfield. 1999. Pregnancy-specific glycoprotein gene expression in recurrent aborters: a potential correlation to interleukin-10 expression. *Am. J. Reprod. Immunol.* 41:174–182.
40. Bohn, H., and E. Weinmann. 1974. [Immunological disruption of implantation in monkeys with antibodies to human pregnancy specific beta 1-glycoprotein (SP1) (author's transl).] *Arch. Gynakol.* 217:209–218.
41. Snyder, S.K., D.H. Wessner, J.L. Wessells, R.M. Waterhouse, L.M. Wahl, W. Zimmermann, and G.S. Dveksler. 2001. Pregnancy-specific glycoproteins function as immunomodulators by inducing secretion of IL-10, IL-6 and TGF-beta1 by human monocytes. *Am. J. Reprod. Immunol.* 45:205–216.
42. Lanier, L.L., B. Corliss, J. Wu, and J.H. Phillips. 1998. Association of DAP12 with activating CD94/NKG2C NK cell receptors. *Immunity.* 8:693–701.
43. Carretero, M., M. Llano, F. Navarro, T. Bellon, and M. Lopez-Botet. 2000. Mitogen-activated protein kinase activity is involved in effector functions triggered by the CD94/NKG2-C NK receptor specific for HLA-E. *Eur. J. Immunol.* 30:2842–2848.
44. Lazetic, S., C. Chang, J.P. Houchins, L.L. Lanier, and J.H. Phillips. 1996. Human natural killer cell receptors involved in MHC class I recognition are disulfide-linked heterodimers of CD94 and NKG2 subunits. *J. Immunol.* 157:4741–4745.
45. Barten, R., and J. Trowsdale. 1999. The human Ly-49L gene. *Immunogenetics.* 49:731–734.
46. Westgaard, I.H., S.F. Berg, S. Orstavik, S. Fossum, and E. Dissen. 1998. Identification of a human member of the Ly-49 multigene family. *Eur. J. Immunol.* 28:1839–1846.
47. Mager, D.L., K.L. McQueen, V. Wee, and J.D. Freeman. 2001. Evolution of natural killer cell receptors: coexistence of functional Ly49 and KIR genes in baboons. *Curr. Biol.* 11: 626–630.
48. Guethlein, L.A., L.R. Flodin, E.J. Adams, and P. Parham. 2002. NK cell receptors of the orangutan (*Pongo pygmaeus*): a

pivotal species for tracking the coevolution of killer cell Ig-like receptors with MHC-C. *J. Immunol.* 169:220–229.
49. Rajagopalan, S., and E.O. Long. 1999. A human histocompatibility leukocyte antigen (HLA)-G-specific receptor expressed on all natural killer cells. *J. Exp. Med.* 189:1093–1100.
50. Ponte, M., C. Cantoni, R. Biassoni, A. Tradori-Cappai, G. Bentivoglio, C. Vitale, S. Bertone, A. Moretta, L. Moretta, and M.C. Mingari. 1999. Inhibitory receptors sensing HLA-G1 molecules in pregnancy: decidua-associated natural killer cells express LIR-1 and CD94/NKG2A and acquire p49, an HLA-G1-specific receptor. *Proc. Natl. Acad. Sci. USA.* 96: 5674–5679.
51. Boyson, J.E., R. Erskine, M.C. Whitman, M. Chiu, J.M. Lau, L.A. Koopman, M.M. Valter, P. Angelisova, V. Horejsi, and J.L. Strominger. 2002. Disulfide bond-mediated dimerization of HLA-G on the cell surface. *Proc. Natl. Acad. Sci. USA.* 99:16180–16185.
52. Allan, D.S., M. Colonna, L.L. Lanier, T.D. Churakova, J.S. Abrams, S.A. Ellis, A.J. McMichael, and V.M. Braud. 1999. Tetrameric complexes of human histocompatibility leukocyte antigen (HLA)-G bind to peripheral blood myelomonocytic cells. *J. Exp. Med.* 189:1149–1156.
53. Witt, C.S., A. Martin, and F.T. Christiansen. 2000. Detection of KIR2DL4 alleles by sequencing and SSCP reveals a common allele with a shortened cytoplasmic tail. *Tissue Antigens.* 56:248–257.
54. Witt, C.S., J.M. Whiteway, H.S. Warren, A. Barden, M. Rogers, A. Martin, L. Beilin, and F.T. Christiansen. 2002. Alleles of the KIR2DL4 receptor and their lack of association with pre-eclampsia. *Eur. J. Immunol.* 32:18–29.
55. Rabinovich, G.A., L.G. Baum, N. Tinari, R. Paganelli, C. Natoli, F.T. Liu, and S. Iacobelli. 2002. Galectins and their ligands: amplifiers, silencers or tuners of the inflammatory response? *Trends Immunol.* 23:313–320.
56. Lowe, J.B. 2001. Glycosylation, immunity, and autoimmunity. *Cell.* 104:809–812.
57. Daniels, M.A., K.A. Hogquist, and S.C. Jameson. 2002. Sweet 'n' sour: the impact of differential glycosylation on T cell responses. *Nat. Immunol.* 3:903–910.
58. Demetriou, M., M. Granovsky, S. Quaggin, and J.W. Dennis. 2001. Negative regulation of T-cell activation and autoimmunity by Mgat5 N-glycosylation. *Nature.* 409:733–739.
59. Gauthier, L., B. Rossi, F. Roux, E. Termine, and C. Schiff. 2002. Galectin-1 is a stromal cell ligand of the pre-B cell receptor (BCR) implicated in synapse formation between pre-B and stromal cells and in pre-BCR triggering. *Proc. Natl. Acad. Sci. USA.* 99:13014–13019.
60. Nguyen, J.T., D.P. Evans, M. Galvan, K.E. Pace, D. Leitenberg, T.N. Bui, and L.G. Baum. 2001. CD45 modulates galectin-1-induced T cell death: regulation by expression of core 2 O-glycans. *J. Immunol.* 167:5697–5707.
61. Vicovac, L., M. Jankovic, and M. Cuperlovic. 1998. Galectin-1 and -3 in cells of the first trimester placental bed. *Hum. Reprod.* 13:730–735.
62. Julkunen, M., M. Seppala, and O.A. Janne. 1988. Complete amino acid sequence of human placental protein 14: a progesterone-regulated uterine protein homologous to beta-actoglobulins. *Proc. Natl. Acad. Sci. USA.* 85:8845–8849.
63. Rachmilewitz, J., Z. Borovsky, G. Mishan-Eisenberg, E. Yaniv, G.J. Riely, and M.L. Tykocinski. 2002. Focal localization of placental protein 14 toward sites of TCR engagement. *J. Immunol.* 168:2745–2750.
64. Logdberg, L., and L. Wester. 2000. Immunocalins: a lipocalin subfamily that modulates immune and inflammatory responses. *Biochim. Biophys. Acta.* 1482:284–297.
65. Gudelj, L., S.E. Christmas, G. Laskarin, P.M. Johnson, E.R. Podack, and D. Rukavina. 1997. Membrane phenotype and expression of perforin and serine esterases by CD3− peripheral blood and decidual granular lymphocyte-derived clones. *Am. J. Reprod. Immunol.* 38:162–167.
66. Gimferrer, I., M. Farnos, M. Calvo, M. Mittelbrunn, C. Enrich, F. Sanchez-Madrid, J. Vives, and F. Lozano. 2002. The accessory molecules CD5 and CD6 associate on the membrane of T lymphoid cells. *J. Biol. Chem.* 278:8564–8571.
67. Anderson, D.M., E. Maraskovsky, W.L. Billingsley, W.C. Dougall, M.E. Tometsko, E.R. Roux, M.C. Teepe, R.F. DuBose, D. Cosman, and L. Galibert. 1997. A homologue of the TNF receptor and its ligand enhance T-cell growth and dendritic-cell function. *Nature.* 390:175–179.