Unique Receptor Repertoire in Mouse Uterine NK cells[1]

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Uterine NK (uNK) cells are a prominent feature of the uterine mucosa and regulate placentation. NK cell activity is regulated by a balance of activating and inhibitory receptors, however the receptor repertoire of mouse uNK cells is unknown. We describe herein two distinct subsets of $CD3^{-}CD122^{+}$ NK cells in the mouse uterus (comprising decidua and mesometrial lymphoid aggregate of pregnancy) at mid-gestation: a small subset indistinguishable from peripheral NK cells, and a larger subset that expresses NKp46 and Ly49 receptors, but not NK1.1 or DX5. This larger subset reacts with *Dolichus biflores* agglutinin, a marker of uNK cells in the mouse, and is adjacent to the invading trophoblast. By multiparametric analysis we show that the phenotype of uNK cells is unique and unprecedented in terms of adhesion, activation, and MHC binding potential. Thus, the Ly49 repertoire and the expression of other differentiation markers strikingly distinguish uNK cells from peripheral NK cells, suggesting that a selection process shapes the receptor repertoire of mouse uNK cells. *The Journal of Immunology,* **2008, 181: 6140–6147.**

Uterine NK (uNK)[3] cells are a prominent feature of the pregnant uterine mucosa (decidua) and are thought to regulate endometrial remodeling, angiogenesis, and placentation. They are always found in the early stages of pregnancy during decidualization in species that have an invasive form of hemochorial placentation (1). Although the origin and the exact functions of uNK cells are not clear, recent work suggests that they cooperate with the invading trophoblast to release factors that modify the uterine spiral arteries, an event that is necessary to increase the blood supply to the fetus (2–4). An important insight into uNK cell function came from the Croy laboratory that showed uNK-cell derived IFN-γ was necessary for vascular modifications to occur during pregnancy (2). Although mice lacking uNK cells or elements of the IFN-γ pathway are fertile, they consistently share abnormalities associated with pregnancy, which include hypocellular decidua and insufficient spiral artery modifications (5). Moreover, pups born from NK-cell deficient *Il15*$^{-/-}$ mice have low birth weights (6).

Several studies have helped define the kinetics and morphology of uNK cells in virgin and pregnant mice (5, 7, 8). uNK cells are first found in 2-wk-old mice, when they appear as small and agranular (9). Following fertilization and implantation, which occurs around gestation day (gd) 4, they start to accumulate first in the decidua basalis, subjacent to the trophoblast layer, and later also in the mesometrial lymphoid aggregate of pregnancy (MLAp), which is a transient lymphoid structure that forms between the two layers of myometrial smooth muscle between gd 8 and gd 14 (5). Peaking in number at mid-gestation (9–10 days), uNK cells decline in the last 5 days of pregnancy (10). uNK cells are reactive to a number of lectins (11), and, in particular, they are the only uterine leukocyte population that binds *Dolichus biflores* agglutinin (DBA) (12), which stains specifically uNK cells of all tested mouse strains, but not peripheral NK cells, nor uNK cells of other species (8), and it is thus a useful marker to distinguish mouse uNK cells from those in the periphery.

NK cell activation is regulated by the integration of positive and negative signals generated by activating and inhibitory receptors. The best characterized NK cell receptors belong to the Ig-like superfamily in humans (KIR) and to lectin-like families: Ly49 in mice and CD94/NKG2 in both species. Despite being structurally different, these receptor families share intracellular signal transduction pathways (13). While the inhibitory receptors within these families bind to classical and nonclassical MHC class I Ags, the ligands for activating receptors are not fully defined (14). Other receptors on NK cells include, among others, NKG2D, natural cytotoxicity receptors, and the low-affinity FcR CD16 (14).

How is uNK cell activation regulated to allow for normal placentation and a successful pregnancy? Immunogenetics studies of human populations suggest that interactions between maternal KIR receptors and fetal HLA-C molecules could set the threshold for NK cell activation, and this ultimately participates in reproductive success (15). Thus, interactions that favor uNK cell activation are less frequently associated with pre-eclampsia, a syndrome characterized by defective placentation due to insufficient vascular modification by trophoblasts (16). Although the population genetics data are consistent with the hypothesis that uNK cells and paternal Ags form a partnership in pregnancy (17), for obvious reasons the functional biological mechanisms underlying these findings cannot be directly tested in humans. Mice can be used to model the biology of uNK cells. Despite differences in placentation between humans and mice (1), the availability of inbred and genetically modified strains makes the mouse a useful tool to study the biology of uNK cells (5). An important limitation to date has been the lack of knowledge of the receptor repertoire expressed by mouse uNK cells. This has precluded the use of mice to model both the interactions between maternal lymphocytes and paternal Ags, as well as

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Received for publication May 19, 2008. Accepted for publication August 27, 2008.

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[1] This work was supported by grants to F.C. from the Medical Research Council, United Kingdom, and a Babraham Institute Synergy Grant to M.H. and F.C.

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[3] Abbreviations used in this paper: uNK, uterine NK; DAPI, 4′,6-diamidino-2-phenylindole; gd, gestation day; DBA, *Dolichos biflores* agglutinin; Lin, lineage; MLAp, mesometrial lymphoid aggregate of pregnancy; PAS, periodic acid-Schiff.

the impact that the receptor repertoire might have on reproductive success.

We set out to use multiparametric flow cytometry analysis to define the receptor repertoire of uNK cells at mid-gestation in C57BL/6 mice. We describe here two distinct $CD3^-CD122^+$ NK cell subsets, one of which appears indistinguishable from peripheral NK cells, whereas the other, larger DBA^+ subset has an unusual $NKp46^+NK1.1^-DX5^-NKG2D^+$ phenotype and a unique receptor repertoire.

Materials and Methods

Animals

C57BL/6, BALB/c, and DBA/1 mice were maintained at the Babraham Institute, (Cambridge, U.K.). C57BL/10 and B10.D2 mice were from Harlan. Mice older than 6 wk of age were used for experiments. All animal experiments were approved by the local Ethical Committee and performed in accordance with United Kingdom Home Office and institutional guidelines.

Dissection

Female mice were time mated with syngeneic males; identification of copulation plug was defined as gd 0.5. Mice were killed at the indicated gestation day by cervical dislocation. The uterus was first cleaned of surrounding fat and blood vessels, and then explanted by cutting below the ovaries on each uterine horn and distal to the cervix. The isolated uteri containing the implants were kept on ice in media containing 10% FBS and antibiotics until dissected using a stereomicroscope. Any remaining fat and blood vessels were cut away from the uterus. Then the cervix was removed by cutting the uterine horns proximal to the first implant. A cut was made down the lumen of the antimesometrial side of the uterus. The implants were then stripped away from the uterine wall. The MLAps were individually dissected away from the wall of the uterus and collected on ice-cold media containing 10% FBS. We generally recovered more uNK cells with 10% FBS as compared with 2% or 5%. The decidua was isolated from the implants by cutting the mesometrial pole away from the implant. Since DBA^+ cells are present both in the MLAp and decidua (8), the two tissues were pooled together for maximal recovery of uNK cells. We refer to the MLAp and decidua tissues as uterine tissues. Care was taken not to include tissue from the trophoblast, developing fetus, or embryonic sac. The uterus of nonpregnant females was isolated in a similar way, except that the whole uterine wall was processed because decidua and MLAp are absent in virgin female mice. Uterine tissues were cut into pieces of roughly 1 mm^2 each and mechanically disrupted. We avoided using enzymatic digestion because preliminary experiments showed that the expression of some cell surface receptors could be altered by the treatment.

Cell preparation

Single-cell suspensions of mononuclear cells from spleen, bone marrow, and uterine tissues were prepared by forcing the tissues through a 100-μm cell strainer. Lymphocytes were isolated using Lympholyte-M cell preparation (Cederlane Laboratories). The number of recovered cells per implant at gd 9.5 was $2 \times 10^4 \pm 1 \times 10^4$ ($n = 26$). Cells from multiple implants of 1–10 individual pregnant mice at the same stage of gestation were pooled together for each experiment.

Flow cytometry

The lymphocyte-rich interface was collected, washed, and cells were counted by trypan blue exclusion. Freshly isolated cells were blocked with purified anti-CD16 (clone 2.4G2) for 10 min at 4°C and washed in FACS buffer (1× PBS, 10% FBS). The following mAbs were purchased from BD Biosciences: FITC- and biotin-anti-CD49b (clone DX5), FITC-anti-Ly49G2 (4D11), FITC-anti-Ly49C/I (5E6), FITC-anti-Ly49D (4E5), FITC-anti-NKG2A/C/E (20d5), FITC-anti-CD16 (2.4G2), FITC-anti-CD43 (S7), PE-anti-CD122 (IL2βB, TMβ1), PerCP-anti-NK1.1 (PK136), allophycocyanin-anti-CD19 (1D3), allophycocyanin-anti-CD3ε (145-2C11) and streptavidin Pe-Cy7. FITC-anti-CD127 (IL7R, A7R34), FITC-anti-CD69 (H1.2F3), biotin-anti-KLRG1 (2F1), biotin-anti-CD11b (Mac-1, M1/70), biotin-anti-CD27 (LG.7F9), biotin-anti-CD9 (KMC8), and allophycocyanin-anti-NKG2D (CX5) were obtained from eBioscience. Biotin-anti-NKp46 was purchased from R&D Systems. Purified and FITC-conjugated anti-Ly49H (3D10) was generously donated by Dr. Wayne Yokoyama (Washington University, St. Louis, MO). Biotin-cojugated lectin from *Dolichos biflorus* was purchased from Sigma-Aldrich. The following allophycocyanin-conjugated mAbs were used to electronically gate out lineage-committed cells; anti-Gr-1 (RB6-8C5), anti-TER-119 (TER-119), anti-CD3ε (145-2C11), anti-CD4 (GK1.5), and anti-CD8α (Ly-2), which were purchased from eBioscience, and anti-CD19 (1D3), which was purchased from BD Pharmingen. DAPI (4′,6-diamidino-2-phenylindole) dilacetate (D3571) to exclude dead cells was purchased from Invitrogen. Ab cocktails were made up in FACS buffer. Incubations were performed on ice for 30 min, and stained cells were then analyzed by six-color immunofluorescence using a BD Biosciences LSRII. Percentages of positive cells and mean fluorescence intensity of positive cells were calculated against the background set on the fluorescence-minus-one negative control. Analysis of data was performed using FloJo (Tree Star) software.

Histology

E9.5 implantation sites were collected from C57BL/6 mice, fixed with 4% paraformaldehyde, and processed for paraffin histology. The sections were cut at 7-μm intervals. Before staining, the sections were deparaffinized and rehydrated. The sections were washed with water and oxidized for 5 min in 1% aqueous periodic acid (BDH 29460 3C), washed again in running water for 5 min, and rinsed in distilled water. The sections were then placed in Schiff's reagent (BDH 19120 3S) for 20 min and rinsed three times in 0.5% aqueous sodium metabisulfate. This step was followed by a 10-min wash in running water. The nuclei were counterstained with Harry's hematoxylin. Finally, the sections were dehydrated in alcohol, cleared in xylene, and mounted. The consecutive sections to the ones used for the periodic acid-Schiff (PAS)-glycogen staining were stained with FITC-labeled DBA (Sigma-Aldrich, L9142–1MG). The incubation was performed overnight at 4°C. The implantation sites were also stained with anti-cytokeratin primary Ab (Dako, Z0622) overnight at 4°C. The secondary Ab was incubated for 3 h at room temperature (Alexa Fluor 568, Molecular Probes). Ag retrival on paraffin-embedded material was performed by boiling in 0.01 M sodium citrate (pH 6) and the sections were blocked in PBT for 5 min.

Stastistical analysis

One-way ANOVA followed by Games-Howell posthoc test was used to analyze the statistical significance of differences between groups.

Results

Pregnancy does not have any obvious effect on the phenotype of peripheral NK cells

The systemic effects of pregnancy on the NK cell receptor repertoire are unknown. We compared the expression of several cell surface receptors in splenic NK cells from nonpregnant and pregnant mice at gd 9.5 to assess for any pregnancy-related effect. To identify NK cells we gated on $CD3^-CD122^+$ cells. CD122 is the β-chain of the receptors for IL-2 and IL-15 and is a useful pan-NK lineage marker because, when expressed on hematopoietic precursors, it marks the commitment to the NK cell lineage (18, 19) and is subsequently expressed on all stages during mouse NK cell development (20). However, CD122 is expressed also on other lymphocytes, mainly T cells, and thus it is a better NK cell marker if associated with CD3, which excludes T cells. We saw no difference in the percentage of $CD3^-CD122^+$ NK cells in the spleen of pregnant ($2.4 \pm 0.8\%$) vs nonpregnant ($2.5 \pm 0.6\%$) mice (data not shown). Later stages of NK cell development are marked by the sequential acquisition of NK1.1 and DX5, which are expressed on the vast majority of mature NK cells (18, 19). Within the $CD3^-CD122^+$ NK cells, the percentages of $NK1.1^+DX5^+$ NK cells in the spleen of pregnant ($82 \pm 4\%$) and nonpregnant ($90.5 \pm 7.5\%$) mice were similar (data not shown). Analysis of the blood of pregnant and nonpregnant mice also showed no significant differences (data not shown). No significant differences were found in the expression patterns of NK1.1, DX5, CD43, CD11b, NKG2A, Ly49C/I, and Ly49D (data not shown), suggesting that there are no obvious pregnancy-related systemic effects on the receptor repertoire of peripheral NK cells at mid-gestation.

Two NK cell subsets in the mouse uterus at gd 9.5

To analyze the phenotype of uNK cells, we chose gd 9.5. At this point in gestation NK cells have increased in size to reach 40 μm

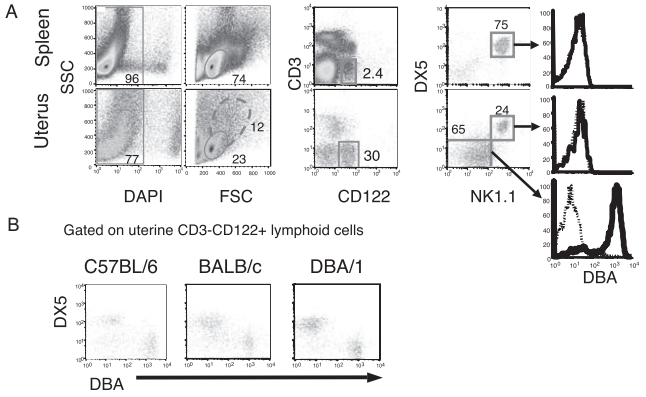

FIGURE 1. Two major NK cell subsets in the mouse uterus. *A*, Dead cells were excluded by DAPI. The unbroken light scatter gates indicate lymphoid cell populations in the uterus and spleen, whereas the dotted line gate indicates the granular cell population in the uterus. NK cells within the two light scatter gates were defined as $CD3^-CD122^+$ cells, and the expression of NK1.1 and DX5 on these cells was evaluated. While most splenic $CD3^-CD122^+$ cells were $NK1.1^+DX5^+$, the uterine $CD3^-CD122^+$ cells contained two subsets, with the predominant one being negative for both NK1.1 and DX5. This major subset appears to overlap with the bona fide uNK cells, as most of the cells in the $NK1.1^-DX5^-$ subset stain positive for DBA, whereas the uterine $NK1.1^+DX5^+$ cells are DBA^- and therefore resemble splenic NK cells. One representative of 4 (for DBA staining) or 15 (for the other markers) independent experiments is shown. *B*, The two subsets of uterine NK cells are readily detectable also in BALB/c and DBA/1 mice that lack NK1.1 reactivity. C57BL/6 mice are shown for comparison. Uterine $CD3^-CD122^+$ NK cells from 9.5 gd pregnant mice were stained with DX5 and DBA lectin. As in C57BL/6 mice, DBA^+ NK cells are $DX5^-$ and, conversely, the DBA^- NK cells are $DX5^+$. One representative of three independent experiments for each strain is shown.

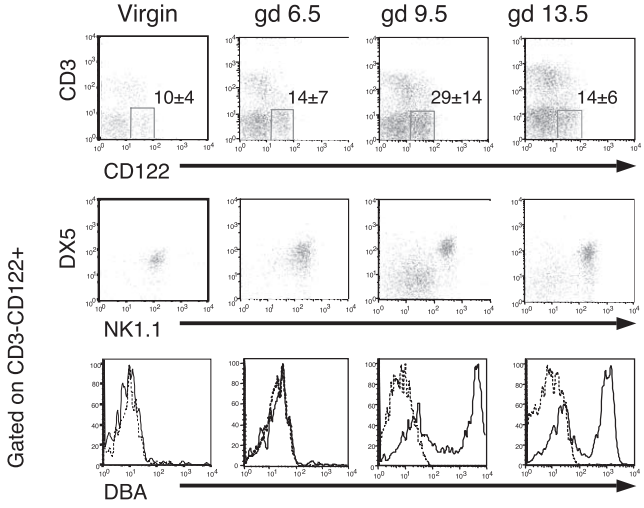

FIGURE 2. Phenotypic changes of uterine NK cells during gestation. Uterine cells of virgin mice, as well as cells from the uterus of pregnant mice at the indicated gestation day, were gated on $CD3^-CD122^+$ cells and further analyzed for expression of NK1.1 and DX5 (middle row) and for DBA (bottom row). The numbers within the FACS plot in the top row indicate mean percentages ± SDs of live $CD3^-CD122^+$ cells within the lymphoid gate. The bottom row shows a bimodal distribution of DBA staining within the $CD3^-CD122^+$ cells (at gd 9.5 and gd 13.5) because it includes both the $NK1.1^+DX5^+$ cells, which are DBA^-, and the $NK1.1^-DX5^-$ cells, which are DBA^+ uNK cells. One representative of three (for virgin and for gd 13.5 pregnant mice) or four (for gd 6.5 and gd 9.5 pregnant mice) independent experiments is shown.

and are present in both decidua basalis and the MLAp (5, 7). By analyzing the light scatter parameters of live cells from both of these sites, two populations were found in the uterine tissues, compared with the spleen (Fig. 1). One population had size and granularity similar to splenic lymphocytes, whereas the other was composed of larger and more granular cells. The lymphoid-like population was more abundant than the more granular population (23 ± 10% vs 12 ± 5%, $n = 11$) (Fig. 1). When we analyzed cells in a gate that included both populations in the uterine tissues, 30 ± 10% were $CD3^-CD122^+$ NK cells. Most (65 ± 6%) of these $CD3^-CD122^+$ cells were $NK1.1^-DX5^-$, with only 24 ± 7% being $NK1.1^+DX5^+$ ($n = 15$, Fig. 1). When the lymphoid population and the more granular population were analyzed separately, we found similar frequency of $CD3^-CD122^+$ cells (29 ± 14% and 30 ± 13%, respectively), as well as similar proportions of the $NK1.1^-DX5^-$ subset (62 ± 10% and 57 ± 12%) and the $NK1.1^+DX5^+$ subset (24 ± 10% and 13 ± 8%). These results show that there are two subsets of NK cells in the mouse uterus at gd 9.5: a major subset characterized by the absence of the conventional NK markers NK1.1 and DX5, and a smaller one with a similar phenotype to NK cells in the periphery. These two subsets are found in similar frequencies within both the lymphoid and the more granular populations of uterine cells.

DBA staining distinguishes the two uNK subsets

Mouse uNK cells are known to react with the DBA lectin, which binds to glycoconjugates containing *N*-acetyl-D-galactosamine in the terminal position (8, 12). DBA selectively decorates the plasma membrane as well as the cytotoxic granules of uNK cells, but not peripheral NK cells (8). Previous studies have described the use of DBA to mark uNK cells in tissue sections, but DBA is also effective to stain uNK cells by flow cytometry (A. Yamada, unpublished oberservations). Thus, we reasoned that DBA could be used to determine which of the two subsets within the $CD3^-CD122^+$ cells belonged to "true" uNK cells. Approximately 95% of $NK1.1^-DX5^-$ uterine cells were DBA^+, and therefore they seem to be the bona fide uNK cells (Fig. 1). In contrast, both uterine and splenic $NK1.1^+DX5^+$ cells were negative for DBA and are thus indistinguishable. The two subsets of uterine NK cells were found also in $NK1.1^+$ strains other than C57BL/6, such as C57BL/10 and B10. D2 (not shown) and in $NK1.1^-$ strains such as BALB/c and DBA/1, in which we readily distinguished $DX5^+DBA^-$ and $DX5^-DBA^+$ NK cells (Fig. 1*B*). We focused our attention on the DBA^+ NK cells, as they have a unique phenotype and are only found in the uterus. In subsequent experiments DBA^+ or $CD3^-CD122^+NK1.1^-DX5^-$ were used as interchangeable criteria to electronically gate on uNK cells.

Kinetics of uNK cells during gestation

Uterine NK cells, defined by immunohostological staining with LGL-1 (also known as Ly49G2), are a transient and dynamic cell lineage, as they are rare in virgin mice, dramatically expand up to mid-gestation, and suddenly contract during the second third of gestation (5, 9). We therefore determined the percentages of $CD3^-CD122^+$ NK cells in the uterus of virgin mice and of pregnant females at gd 6.5, gd 9.5 (as above), and gd 13.5. To directly compare lymphoid cells of similar size and granularity, we chose to focus on the lymphoid population (as shown in Fig. 1). The percentages of $CD3^-CD122^+$ NK cells were lowest in virgin mice and highest at mid-gestation (Figs. 2 and 3), confirming previously published data (reviewed in Ref. 5). We then sought to determine whether the relative frequencies of the two subsets found at gd 9.5 would vary with the progression of pregnancy. True uNK cells, as defined by DBA^+ or $NK1.1^-DX5^-$, were virtually absent within

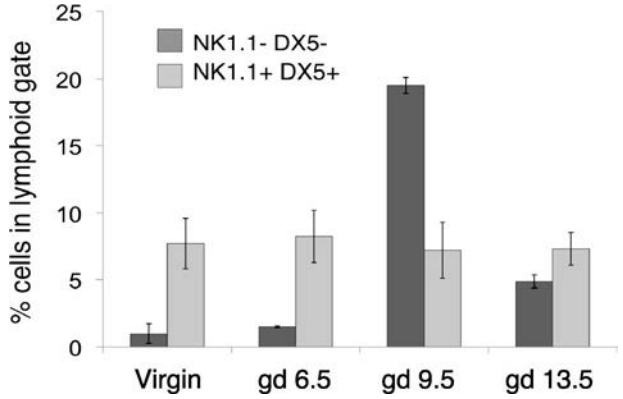

FIGURE 3. Kinetics of uNK cells during gestation. Uterine cells of virgin mice, as well as cells from the uterus of pregnant mice at the indicated gestation day, were stained as in Fig. 2. The data are mean percentages ± SDs of the live NK1.1$^-$DX5$^-$ and NK1.1$^+$DX5$^+$ subsets within the lymphoid gates pooled from the same independent experiments described in Fig. 2. The frequency of the NK1.1$^+$DX5$^+$ subset was similar in virgin and in gd 6.5, gd 9.5, or gd 13.5 pregnant mice. The frequency of the NK1.1$^-$DX5$^-$ subset at gd 9.5 was significantly greater than that in virgin ($p = 0.0001$) or gd 6.5 ($p = 0.0001$) and gd 13.5 ($p = 0.002$) pregnant mice.

the CD3$^-$CD122$^+$ uterine cells in virgin mice (Figs. 2 and 3). These results are consistent with previous immunohistological studies, in which no DBA$^+$ cells were found in the uterus of virgin mice (8). Thus, the unusual NK1.1$^-$DX5$^-$ subset of uNK cells we find in the uterus of pregnant mice is essentially absent from nonpregnant endometrium, where 90% of the CD3$^-$CD122$^+$ NK cells are NK1.1$^+$DX5$^+$, similar to the spleen. At gd 6.5 we did find a few uterine CD3$^-$CD122$^+$ cells having the unusual NK1.1$^-$DX5$^-$ phenotype (2–10%), but most were NK1.1$^+$DX5$^+$ (60–70%) and some were positive for NK1.1 but negative for DX5 (10–35%) (Figs. 2 and 3). At this stage of pregnancy, uterine CD3$^-$CD122$^+$ cells were consistently negative for DBA, suggesting that, in our analysis, DBA reactivity is acquired between gd 6.5 and gd 9.5. These results are consistent with immunohistological studies by Paffaro et al., who showed only a few DBA cells at gd 6 (8). Fig. 2 also shows the distribution of DBA$^+$ and DBA$^-$ cells at gd 9.5, which we have discussed above. Briefly, 65–70% of the CD3$^-$CD122$^+$ cells were DBA$^+$ and NK1.1$^-$DX5$^-$ uNK cells, with the rest being DBA$^-$ and NK1.1$^+$DX5$^+$. Later in gestation, at day 13.5, the CD3$^-$CD122$^+$ NK cells contained a sizable NK1.1$^-$DX5$^-$ DBA$^+$ population, accounting for about one-third of the CD3$^-$CD122$^+$ cells. The loss of NK1.1$^-$DX5$^-$ uNK cells at day 13.5 compared with day 9.5 is consistent with the initial decline of uNK cells as reported previously (7). These results collectively indicate that the unusual NK1.1$^-$DX5$^-$ uNK cell subset is dynamic and increases relative to the NK1.1$^+$DX5$^+$ subset through pregnancy, peaking at mid-gestation (Fig. 3). The temporal change of the NK1.1$^-$DX5$^-$ subset partially overlaps with that described for the typical DBA$^+$ uNK cells identified by immunohistology (8), but the appearance of the unusual NK1.1$^-$DX5$^-$ phenotype may occur earlier than the acquisition of DBA reactivity.

Tissue distribution of uNK cells

Flow cytometry analysis of isolated MLAp and decidua basalis showed that the two subsets of NK1.1$^+$DX5$^+$ and NK1.1$^-$DX5$^-$ NK cells are found in both sites, although the NK1.1$^-$DX5$^-$ NK cells are underrepresented in the MLAp (~30% as opposed to 65% in deciduas basalis, data not shown). To define the relative location of the two subsets of uterine NK cells we analyzed tissue sections of gd 9.5 implantation sites. The universal NK cell marker for

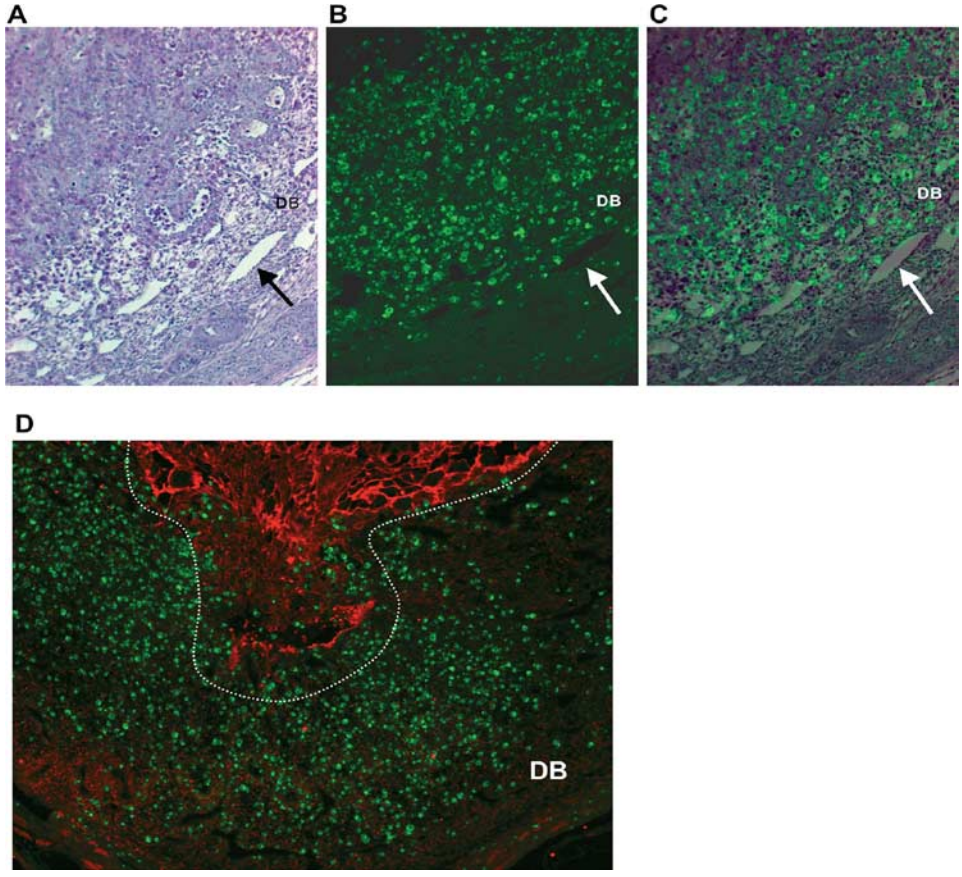

FIGURE 4. PAS and DBA lectin localization in the implantation site. Two consecutive paraffin-embedded sections of the same implantation site were stained with (*A*) PAS-glycogen stain marking the localization of granules (purple) and (*B*) anti-DBA lectin Ab showing the localization of the uNK cells (green fluorescence). *C*, Overlay of *A* and *B*. The arrows indicate the same blood vessel. *D*, The location of DBA$^+$ uNK cells (green) within the decidua basalis (DB) can be seen in relation to the invasive trophoblast, which is stained with anti-cytokeratin Ab (red). Magnification for *A*, *B*, and *C*, ×20; *D*, ×10.

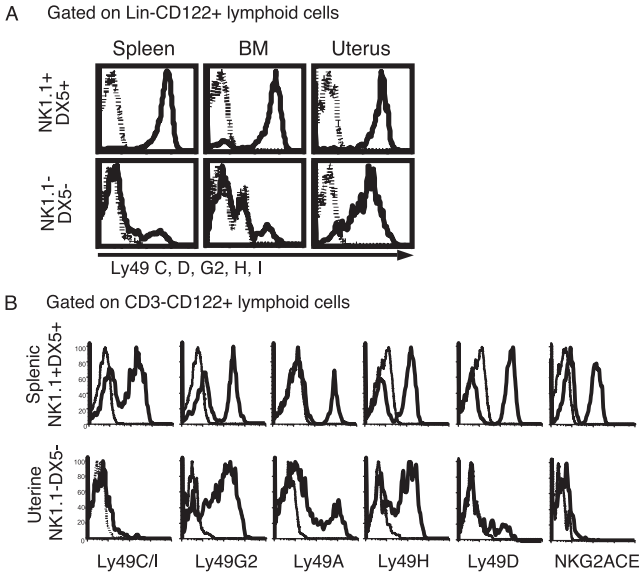

FIGURE 5. uNK cells express Ly49 receptors but no CD94/NKG2ACE. *A*, Live cells from spleen, bone marrow, and uterus of gd 9.5 pregnant mice were gated on Lin^- (CD3, CD4, CD8α, CD19, Gr-1, TER-119) $CD122^+$ cells. The expression of multiple Ly49s was compared between $NK1.1^+$ $DX5^+$ and $NK1.1^-DX5^-$ cells in the three tissues. Most $NK1.1^+DX5^+$ cells from the three tissues were $Ly49^+$, but only the uterine $NK1.1^-DX5^-$ cells express Ly49s, whereas splenic and marrow $NK1.1^-DX5^-$ cells are largely negative for Ly49s. One representative of two independent experiments that gave similar results is shown. *B*, Live cells from spleen and uterus of gd 9.5 pregnant mice were gated on $CD3^-CD122^+$, and the expression of individual Ly49s and combined NKG2ACE receptors was compared between splenic $NK1.1^+DX^+$ cells and uterine $NK1.1^-DX5^-$ cells. One representative of at least three independent experiments for each marker is shown.

usage in histology is NKp46; however, we failed to obtain satisfactory stainings in uterine tissue sections, possibly due to the low expression of the Ag (see Fig. 6*A*). An alternative, yet not specific staining for uterine NK cells, is the PAS reaction, which detects glycoproteins in the granules of mature NK cells. Consecutive serial sections of gd 9.5 implantation sites revealed that PAS^+ and DBA^+ cells do not clearly segregate into different locations (Fig. 4*A–C*). Nevertheless, the DBA^+ cells do appear to be the ones in contact with the invading trophoblast (Fig. 4*D*), suggesting that their function may be influenced by interactions with fetal tissues.

Uterine $CD3^-CD122^+NK1.1^-DX5^-$ cells express a distinct Ly49 receptor repertoire

The results indicate that the unusual $NK1.1^-DX5^-$ cells in the uterus of pregnant mice are the true uNK cells and thus they may be a differentiated cell lineage. However, the lack of NK1.1 and DX5 on CD122 expressing cells is reminiscent of the phenotype of NK precursors, the earliest committed NK cell precursors found mainly in the bone marrow (18), but also in other lymphoid tissue such as the spleen and lymph nodes. NK precursors do not express markers of late NK cell differentiation, including MHC receptors CD94/NKG2ACE and Ly49s, which are acquired after NK1.1 and DX5. To test whether the phenotype of uNK cells overlapped with that of NK precursors, we directly compared the expression of Ly49 receptors on spleen, bone marrow, and uterine $CD122^+$ cells among cells negative for lineage (Lin)-specific markers, including Gr-1, TER-119, CD3ε, CD4, CD8α, and CD19. To directly compare lymphoid cells of similar size and granularity between spleen, marrow, and uterus, we chose to focus on the lymphoid population (as shown in Fig. 1*A*), with the composition of cell surface receptors on the lymphoid and granular uterine populations being very similar (see below). As reported previously (18), we found that the $NK1.1^-DX5^-$ cells within the spleen and marrow Lin^-CD122^+ cells were mostly negative for Ly49 receptors. In striking contrast, a large portion of the $NK1.1^-DX5^-$ cells within the uterine Lin^-CD122^+ cells did express Ly49 receptors (Fig. 5*A* and Table I). The $NK1.1^+DX5^+$ cells within the Lin^-CD122^+ showed a similar pattern of Ly49 expression, regardless of whether they were isolated from spleen, bone marrow, or uterus (Fig. 5*A* and Table I). We next analyzed the frequencies of individual $Ly49^+$ subsets on $NK1.1^-DX5^-$ uNK cells and found these do not reflect those of splenic cells. Thus, $Ly49C/I^+$ and $Ly49D^+$ uNK cells were reduced 3-fold, $Ly49G2^+$ uNK cells were increased 1.5-fold, while $Ly49A^+$ and $Ly49H^+$ cells were essentially equivalent to the splenic subsets (Fig. 5*B* and Table I). The mean intensity of Ly49A, Ly49G2, and Ly49H expression was similar on uNK and splenic NK cells, whereas the mean intensity of Ly49D and Ly49C/I expression was reduced on uNK cells, although the reduction of Ly49C/I was not statistically significant. In the classical scheme of NK cell development, Ly49 receptors are expressed after CD94/NKG2ACE receptors (20), but, surprisingly, uNK cells expressed virtually no NKG2ACE receptors (Fig. 5*B*). These results argue against a potential immature phenotype of uNK cells, confirm that uNK cells have an unprecedented phenotype, and show for the first time that

Table I. *Frequency of $Ly49^+$ subsets at gd 9.5*[a]

	$Ly49A^+$	$Ly49C/I^+$	$Ly49G2^+$	$Ly49D^+$	$Ly49H^+$	All[b]
$NK1.1^+DX5^+$						
Spleen	17 ± 5	47 ± 5	43 ± 6	47 ± 4	56 ± 13	90 ± 0.5
Marrow	26 ± 9	30 ± 4	46 ± 13	29 ± 5	40 ± 10	82 ± 2
Uterus	12 ± 2	47 ± 6	45 ± 10	43 ± 5	54 ± 7	90 ± 1.5
$NK1.1^-DX5^-$						
Spleen	ND	ND	ND	ND	ND	5 ± 7
Marrow	ND	ND	ND	ND	ND	4 ± 4
Uterus	18 ± 3	17 ± 5	68 ± 6	14 ± 6	41 ± 11	69 ± 18

[a] Cell suspensions were prepared from spleen, bone marrow, and uterus of pregnant mice at gd 9.5 and stained for FACS analysis. Live cells gated by light scatters were further gated on $CD3^-$(or Lin^-) $CD122^+$ NK cells and subdivided in $NK1.1^+DX5^+$ and $NK1.1^-DX5^-$ subsets as in Fig. 4, *A* and *B*. The percentage of cells positive for the indicated Ly49s receptors was compared between the two subsets within the indicated tissues as in Fig. 4*B*. The data show mean percentages ± SDs of cells staining positive for the indicated Ly49s of at least three measurements for each Ly49.

[b] Indicates cells positive for either of Ly49C, D, G, H, I, as determined by a staining where all mAbs specific for these Ly49 were pooled together in one single channel as in Fig. 5*A*.

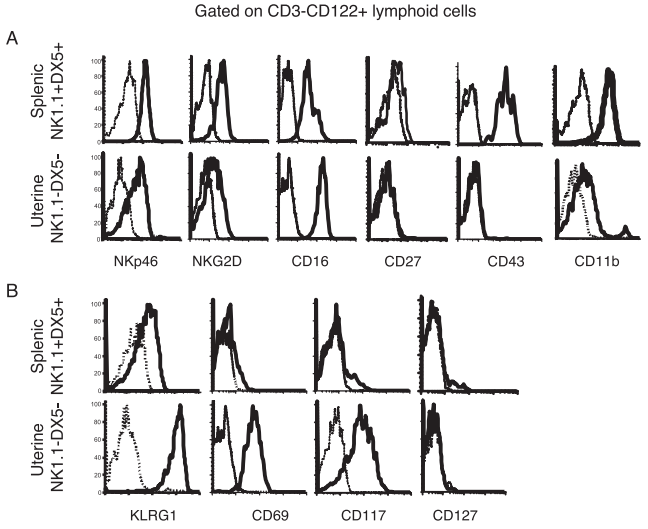

FIGURE 6. Unique phenotype of uNK cells. Live cells from spleen and uterus of gd 9.5 pregnant mice were gated on $CD3^-CD122^+$ and the expression of (*A*) activating receptors and markers of lineage and differentiation, and (*B*) markers of activation or thymic origin was compared between splenic $NK1.1^+DX5^+$ cells and uterine $NK1.1^-DX5^-$ cells. One representative of at least three independent experiments for each marker is shown.

mouse uNK cells do express multiple Ly49 receptors. Moreover, they suggest that uNK cells follow a unique developmental pathway, whereby Ly49 receptors are expressed independently of the expression of differentiation markers NK1.1 and DX5 and they are uncoupled from the expression of CD94/NKG2ACE receptors.

Uterine $CD3^-CD122^+NK1.1^-DX5^-$ cells have a unique phenotype

To gain further insights into the phenotype of uNK cells, we evaluated the expression of a number of markers on the uterine $CD3^-CD122^+$ cells in the lymphoid gate and compared it to that in the spleen of pregnant mice. For each of the markers analyzed, the $NK1.1^+DX5^+$ cells from both the uterus and spleen were indistinguishable (data not shown), and thus we only show the comparison between splenic $NK1.1^+DX5^+$ and uterine $NK1.1^-DX5^-$ NK cells. The expression of NKp46 is restricted to virtually all NK cells across species (21), and thus NKp46 is possibly the only NK-specific marker. Notably, uterine $NK1.1^-DX5^-$ cells were $NKp46^+$, although the expression intensity was lower on these cells compared with splenic NK cells (Fig. 6*A*). Similarly, NKG2D, which is also present on virtually all NK cells, was expressed, although at low levels, on $NK1.1^-DX5^-$ cells (Fig. 6*A*). Furthermore, $NK1.1^-DX5^-$ cells expressed unusually high amounts of CD16. The latter strikingly distinguishes mouse from human uNK cells, since human $CD56^{bright}$ uNK cells are $CD16^-$ (22, 23). Mouse peripheral NK cells can be subdived in functional subsets by virtue of CD27 and CD11b expression (24). Furthermore, most mature, peripheral NK cells express CD43. Fig. 6*A* shows that uNK $NK1.1^-DX5^-$ cells expressed essentially no CD27, CD11b, or CD43, which distinguishes them further from splenic NK cells. Fig. 6*B* shows that uNK cells are most likely activated as they express CD69, stem cell factor receptor CD117, and KLRG1. KLRG1 expression was nearly 10-fold greater than the splenic NK cells (geometric mean fluorescence intensities of 1030 ± 141 and 112 ± 9, respectively). Human $CD56^{bright}$ peripheral NK cells share some features with thymically derived mouse NK cells because both are marked by the preferential expression of the α-chain of the IL-7 receptor CD127 (25). Because human uNK cells are $CD56^{bright}$, it is possible that they are thymically derived. Fig. 6*B* shows evidence against a thymic origin of mouse uNK cells, as they did not express CD127 (25).

Table II. *Unique phenotype of uNK cells at gd 9.5*

	Uterine		Splenic
	$NK1.1^-DX5^-$	$NK1.1^+DX5^+$	$NK1.1^+DX5^+$
DBA reactivity	+	−	−
NKp46	+	++	++
NKG2D	+	++	++
CD16	++	+	+
CD27	−	+	+
CD43	−	+	+
CD11b	+[a]	++	++
CD69	+	−	−
CD117	+	−	−
KLRG1	++	+	+
CD127	−	−	−
CD9	+	+	+

Cell suspensions were prepared from spleen and uterus of pregnant mice at gd 9.5 and stained for FACS analysis as in Fig. 5, *A* and *B*. Live cells gated by light scatters were further gated on $CD3^-CD122^+$ NK cells and subdivided in $NK1.1^+DX5^+$ and $NK1.1^-DX5^-$ subsets. The table summarizes the expression of each marker so that + or − indicates presence or absence on the majority of the cells in the indicated subsets and ++ indicates higher expression relative to the other subsets.

[a] CD11b was expressed at low levels on the uterine $NK1.1^-DX5^-$ cells in the lymphoid gate, but was expressed at high levels on the same subset within the more granular uNK cells.

uNK cells are reported to increase size and granularity as they mature (5). We therefore compared the expression pattern of most of the above cell surface receptors on the small lymphoid-like cells on the one hand, and the larger, more granular cells on the other hand (as defined in Fig. 1). The assumption was that if these cells were at a different stage of maturation or differentiation, they might display different patterns of receptor expression. With the exception of CD11b, which was positive and expressed at high levels on most of the larger cells, no other differences were found in two independent experiments, which gave similar results (data not shown). At present we do not know the reason of the differential expression of CD11b on the two populations of $NK1.1^-DX5^-$ uNK cells.

In summary, uterine $CD3^-CD122^+$ cells belong to the NK lineage as they express NKp46, NKG2D, and CD16 and have a unique phenotype because they do not express common markers of mature NK cells, such as NK1.1 and DX5, nor differentiation markers CD27 and CD43, and yet they express several Ly49s, KLRG1, CD69, and CD117 (Table II).

Discussion

We report herein the first in-depth multiparametric analysis of mouse uNK cells. Our results show that mouse uNK cells have a unique receptor repertoire and are distinct from peripheral NK cells in several ways. uNK cells do not express the most widely used markers of peripheral NK cells NK1.1 and DX5, yet they are positive for NKp46, which has recently been described as the ultimate NK cell marker across different tissues, mouse strains, and species (21). By showing that uNK cells are $NKp46^+$, we confirm by an unequivocal criterion that the $CD3^-CD122^+NK1.1^-DX5^-$ cells within the uterus are of the NK cell lineage. Although uNK cells appear to have a phenotype consistent with NK cell precursors ($Lin^-CD122^+NK1.1^-DX5^-$), they surprisingly express various Ly49 receptors. Moreover, they show unique expression profiles of integrins and adhesion molecules, as well as markers of

activation and MHC binding capability. Furthermore, as they are $CD127^-$, they are unlikely to be thymically derived.

Alongside this novel NK cell subset, we also found a relatively minor subset of NK cells in the uterus of mice at mid-gestation that mirrored all the phenotypic features of peripheral NK cells. At present we have not established the relationship between these two subsets of NK cells found in the pregnant mouse uterus, and several possible scenarios arise. Because the phenotype of uterine $NK1.1^+DX5^+$ cells overlaps with that of splenic NK cells, they may be peripheral NK cells that have been recently recruited into the uterus. Once homed into the uterus, the effect of local factors may alter their phenotype, making them become "true" uNK cells that acquire all the features of uNK cells, including reactivity to DBA and loss of NK1.1 and DX5 expression. An alternative scenario is that uterine $NK1.1^+DX5^+$ cells are derived from recirculating NK cells, whereas the $NK1.1^-DX5^-$ uNK cells are a separate subset ab initio that develop and expand in situ from resident or recently recruited precursors. Considering the $NK1.1^+DX5^+$ phenotype of resident endometrial NK cells in the uterus of virgin mice, it is also tempting to speculate that these may be the precursors of the true uNK cells, as they may undergo phenotypic changes under the influence of pregnancy. Nevertheless, it is unclear what stage of uNK cell differentiation is defined by the unusual $NK1.1^-DX5^-DBA^+$ phenotype of this novel uNK cell subset. The vicinity of DBA^+ cells to, and, in some cases, the contact with, the invading trophoblast does suggest that these uNK cells are functionally involved in placentation.

Mature mouse NK cells can be subdivided in functionally distinct phenotypes on the basis of CD11b and CD27 expression (24). All $NK1.1^-DX5^-$ uNK cells were negative for CD27 but they were positive for CD11b, with the lymphoid-like cells being $CD11b^{low}$ and the more granular cells being $CD11b^{high}$. Thus, uNK cells may belong to the $CD27^-CD11b^+$ subset, which is also positive for KLRG1 (24), and indeed uNK cells showed very high expression of KLRG1 compared with splenic NK cells. KLRG1, also known as MAFA-1, is an inhibitory receptor that binds to E-cadherin and is also a marker of active NK cell proliferation (26, 27), which is consistent with the notion that uNK cell rapidly proliferate from gd 6 to 13 (7, 28). Most human uNK cells are $CD69^+$, indicating that they may be activated (23). Similarly, we found that mouse uNK were also $CD69^+$, suggestive of an activated state. Another function that has been attributed to CD69 is in the retention of lymphocytes within lymphoid organs by negatively regulating signaling through the S1P1 receptor (29). It will be interesting to investigate whether CD69 also regulates the S1P5 receptor, which has recently been shown to be selectively expressed on human and mouse NK cells (30), as such a mechanism may be relevant to the recruitment and/or retention of uNK or their precursors to the uterus. CD43 is expressed on most mature NK cells, but uNK cells were completely negative for CD43, thus making it difficult to fit them into any previous scheme of NK cell differentiation. In line with the other unusual features of uNK cell phenotype, they also express high levels of CD117 (the c-kit receptor for stem cell factor), which is a marker of hematopoietic precursors, but is also up-regulated on IL-2 activated mouse NK cells (data not shown) and is found on human uNK cells (31). The expression of the IL-7 receptor α-chain (also known as CD127) on a subset of mouse NK cells marks their thymic origin (25). We found that uNK cells lacked CD127 expression, suggesting that either CD127 is down-regulated in the uterine microenvironment, or that these cells are not thymically derived. Analysis of uNK cell populations found in athymic nude mice or in IL-7Rα-deficient mice will provide definitive evidence. CD9, a member of the tetraspanin family, is selectively expressed on human uNK cells (32). We found that mouse uNK cells also expressed CD9, but so did splenic and bone marrow NK cells (data not shown), and thus CD9 cannot be used as a marker of uNK cells in the mouse. Reactivity to DBA is instead the preferential marker of uNK cells in this species, at least from gd 6 onward.

Our results suggest that the regulation of Ly49 and NKG2 expression may be unique in uNK cells, since it is unconventional for three reasons. First, a subset of $CD3^-CD122^+$ NK cells that express Ly49 receptors before acquisition of CD94/NKG2ACE, NK1.1, and DX5 has never been described before (20) (33). It is possible that this subset of uNK cells does initially follow the canonical sequence of receptor acquisition, but simply down-regulates the other receptors due to local factors. Indeed, activation is one possible cause of down-regulation of both NK1.1 (34) and DX5 (35). We are testing this possibility. Second, the Ly49 repertoire of $NK1.1^-DX5^-$ uNK cells is qualitatively and quantitatively different from that of splenic and uterine $NK1.1^+DX5^+$ cells on one hand, and bone marrow $NK1.1^-DX5^-$ cells on the other hand. Some receptors are selected against (Ly49C/I, Ly49D, and CD94/NKG2ACE), others are preferentially expanded (Ly49G2), and others do not change (Ly49A and Ly49H). These results suggest that a selection process shapes the repertoire of uNK cells. The most striking feature of the skewed Ly49 repertoire is the low Ly49C/I frequency, as well as the low expression intensity of these inhibitory receptors. Ly49C binds to self-MHC in C57BL/6 mice, and a functional interaction between self-MHC and Ly49C is required during development to acquire functional competence (36). It will be interesting to define the factors that regulate "licensing" in uNK cells. We also noted an overrepresentation of $Ly49G2^+$ cells among $NK1.1^-DX5^-$ uNK cells. Ly49G2 (also known as LGL-1) has previously been described on uNK cells. Its expression was lost when cells matured (37) (9, 38), suggesting that Ly49G2 is expressed on immature cells. Our data do not support the notion that $Ly49G2^+$ are immature cells, as they express other Ly49 receptors, as well as other markers of NK cell differentiation. Third, the last unconventional feature of uNK cells is the expression of Ly49 receptors in the absence of CD94/NKG2ACE receptors. In the canonical scheme of NK cell development, the expression of CD94/NKG2ACE receptors anticipates the expression of Ly49 receptors (20). CD94/NKG2ACE receptors are collectively expressed on all NK cells early in ontogeny (39) and, in adult life, are expressed on ~50% of NK cells in both humans and mice. Moreover, CD94/NKG2ACE receptors are expressed at high levels on the vast majority of human uNK cells (23). In contrast, $NK1.1^-DX5^-$ uNK cells do not appear to significantly express any CD94/NKG2ACE receptors. In particular, the observation of low or no expression of NKG2A, which is another inhibitory receptor that binds self-MHC in C57BL/6 mice, combined with that of low Ly49C/I expression, raises the question as to how uNK cells can bind to self-MHC.

Our results show that uNK cells are "armed" with a number of activating receptors, including Ly49H, NKp46, NKG2D, and CD16. The reason why NKp46 and NKG2D are expressed at low levels in uNK cells is unknown. Similarly, we do not yet know why Ly49D is strongly down-regulated in uNK cells. We are currently testing the possibility that relevant ligands expressed in the uterine microenvironment, including maternal stromal and fetal-derived trophoblasts, may shape the expression of uNK cell receptors in terms of cellular selection, receptor modulation, or gene expression. In light of the recent observations by Sharkey et al., who have described that the KIR repertoire on uNK cells in early human pregnancy is different than blood NK cells (40), we suggest that conserved mechanisms may underlie the establishment of

uNK cell repertoire in the two species and have an impact on reproductive success (41).

Acknowledgments

We thank Anne Croy for enthusiastic support and for showing us unique dissection techniques, Wendy Dean and Aureo Yamada for precious advice, Wayne Yokoyama for sharing reagents, William Seaman and Geoff Butcher for sharing unpublished data, Geoff Morgan for FACS, the staff of the Babraham Small Animal Facilities, and Anne Clarke and members of the Moffett laboratory and the Colucci laboratory for suggestions.

Disclosures

The authors have no financial conflicts of interest.

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