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Endometrial NK Cells Are Special Immature Cells That Await Pregnancy[1]

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NK cells populate the human endometrium before pregnancy. Unlike decidual NK cells that populate the decidua during pregnancy, the NK cells present in the human endometrium, before pregnancy, have not been fully characterized. In this study, we provide a detailed analysis of the origin, phenotype, and function of endometrial NK cells (eNK). We show that eNK cells have a unique receptor repertoire. In particular, they are negative for NKp30 and chemokine receptor expression, which distinguishes them from any other NK subset described so far. We further show that eNK cells lack NK-specific functional phenotype and activity such as cytokine secretion and cytotoxicity, before IL-15 stimulation. Following such stimulation, endometrial NK cells acquire phenotype and function that are similar to those of decidual NK cells. We therefore suggest that eNK cells are inactive cells (before IL-15 activation and in relation to the known NK activity) that are present in the endometrium before conception, waiting for pregnancy. ***The Journal of Immunology,*** **2008, 181: 1869–1876.**

Natural killer cells comprise ~5–15% of peripheral blood lymphocytes and populate different lymphoid and nonlymphoid tissues, including lymph nodes, thymus, tonsils, spleen, and uterus (1, 2). In humans, NK cells are found in the uterine mucosa, before and during pregnancy, in the endometrium (eNK),[3] and in the decidua tissues (dNK) (3). While dNK cells were extensively investigated, the study of eNK cells still lacks comprehensive research as to their origin and properties (4). Furthermore, many questions regarding eNK cells are still left unanswered. What are the cytotoxic capabilities of eNK cells? Do they play the "classical" role of NK cells and eliminate virally infected and transformed cells or do they have tissue remodeling properties, like dNK cells, which prepare the uterus for blastocyst implantation? In this article, we are trying to answer these critical questions.

In the peripheral blood there are two NK subpopulations, the $CD56^{bright}CD16^{-}$ and the $CD56^{dim}CD16^{+}$ NK cells. These two NK cell subsets are considered to have relatively distinct functions. The $CD56^{bright}CD16^{-}$ population has an increased ability to secrete cytokines (1, 5, 6), while the $CD56^{dim}CD16^{+}$ population specializes in killing tumor and virally infected cells. The killing by NK cells is mediated through several activating NK receptors (7). Among these are NKG2D and the three natural cytotoxicity receptors (NCRs), NKp30, NKp44, and NKp46 (7).

During pregnancy, dNK cells comprise ~70–80% of the decidual lymphocytes and are considered to be a special NK subset (1, 6, 8) that does not exert cytolytic functions against trophoblast cells (9) and has generally reduced cytotoxicity (1, 10). Surprisingly, dNK cells express activating NK receptors, including all the NCRs and NKG2D (6). However, instead of triggering cytotoxicity, these receptors act to stimulate secretion of cytokines and angiogenic factors, which are essential for trophoblast invasion and vascular modifications (6).

As stated above, in humans, NK cells can also be found in the endometrium before pregnancy, but these eNK cells have been barely studied. Research done so far has indicated that eNK cells proliferate, especially in the secretory phase of the menstrual cycle, as they stain positive for the proliferation marker Ki67 (11). The currently established immune-marker phenotype of eNK cells includes $CD56^{+}$, $CD3^{-}$, $CD16^{-}$, $CD94^{+}$, $CD9^{+}$, $CD57^{-}$, $HLA\text{-}DR^{+}$, $CD69^{+}$, $CD158b^{+}$, $NKB1^{+}$, and L-$selectin^{-}$. The expression of the major activating receptors has not yet been determined (12, 13).

In this article, we provide the first evidence that eNK cells are a unique subset of cells, never observed before in any other tissue, and demonstrate that these special NK cells are inactive in the endometrium before conception and merely await pregnancy.

Materials and Methods

Patients

Twenty-five naturally cycling ovulatory women aged 30–43 were recruited from the in vitro fertilization (IVF) clinic. Informed consent was obtained from each woman. Endometrial samples were obtained by Pipelle biopsy of functional endometrium. Our possible sample area is from the fundas to the internal os. Two samples were collected from most of the women, one from the proliferative phase and one from the secretory phase. The procedure was performed during either the proliferative phase (day 9–11) and/or

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[3] Abbreviations used in this paper: eNK, endometrium NK cell; dNK, decidual NK cell; NCRs, natural cytotoxicity receptor; IVF, in vitro fertilization; pb, peripheral blood; VEGF, vascular endothelial growth factor; PLGF, placental growth factor; MICA, MHC class I chain-related chain A; MICB, MHC class I chain-related chain B; ULBP, UL-16 binding protein.

during the secretory phase (day 20–23) in the month before the IVF treatment cycle. This procedure was performed to enhance implantation in the next cycle (14). The secretory phase was confirmed by endometrial appearance on ultrasound examination and by serum progesterone levels.

Isolation of endometrial and peripheral blood lymphocytes and NK cells

Endometrial tissues were trimmed into 1-mm pieces and enzymatically digested for 20 min with 1.5 mg type I DNase (Roche) and 15 mg type IV Colagenase (Worthington) present in 15 ml RPMI 1640 medium, using vigorous shaking. This procedure was repeated three times. After additional 5-min incubation at room temperature, without shaking, the supernatants were passed through a 40-μm cell strainer. The flow through was centrifuged at 1600 rpm for 5 min, resuspended in DMEM (supplemented with 10% FCS, and 1% sodium-pyruvate, glutamine, nonessential amino acids, and pen-strep solution), and incubated overnight in a tissue culture dish. Adherent cells were collected and used, as nonlymphocyte-control cells, to detect the expression of the NCRs and NKG2D ligands. Nonadherent cells were collected and loaded on a Ficoll density gradient to purify the lymphocyte population. (The yield obtained from one sample was $\sim 6\times10^4$-2×10^6 cells.) Peripheral blood (pb) lymphocytes were isolated from different healthy donors (men and women) using Ficoll gradient. Cells were further analyzed by flow cytometry. eNK cells and pbNK cells were purified using the human NK cell isolation kit and the autoMACS instrument (Miltenyi Biotec), according to the manufacturer's instructions. Identity of NK cells was confirmed as they stained positive for CD56 and negative for CD3.

Immunohistochemistry

Immunohistochemistry was conducted using mouse anti-CD56(N-CAM), clone 123C3 (Zymed Laboratories) at a dilution of 1/50 on paraffin-embedded sections as previously described (6). Ag retrieval was performed using citrate buffer (pH 6.0); color was developed with diaminobenzidine and counterstained with Meyer's hematoxylin solution.

Flow cytometry of endometrial and peripheral blood lymphocytes

The following mouse anti-human conjugated mAbs were used: PE-conjugated anti-CD56 (BD Pharmingen), FITC-conjugated anti-CD56 (Sigma-Aldrich), PE-conjugated anti-NKp46 (R&D Systems), PE-conjugated anti-NKG2D (R&D Systems), PE-conjugated anti-NKp44 (Southern Biotechnology Associates), PE-conjugated anti-NKp30 (BioLegend), PE-conjugated anti-CXCR3 (R&D Systems), PE-conjugated anti-CXCR4 (R&D Systems), biotinylated anti-CD3 (clone T3D), and Cy5 conjugated sreptavidin as a secondary Ab (Jackson Immunoresearch Laboratories). All fluorochrome-conjugated mAbs had matching conjugated isotype controls (DakoCytomation). For triple FACS staining, cells ($5–10\times10^4$) were first incubated with saturating amounts of the biotinylated anti-CD3 Ab for 1 h, washed, and incubated with two additional different fluorochrome-conjugated Abs (0.2– 0.5 μg) for 20 min at 4°C. FACS analysis was performed using a FACScan flow cytometer (BD Biosciences). First, all viable cells were selected by a widely set gate on a two-parameter plot of side-scatter vs forward-angle-scatter. Among these cells, a second gate was set to include all NK cells ($CD56^+CD3^-$) or T cells ($CD56^-CD3^+$) and the expression of the investigated receptors expressed in these populations was assessed.

Incubation of endometrial lymphocytes

One $\times 10^6$ endometrial lymphocytes were incubated in DMEM containing supplements, 10% human serum (Sigma-Aldrich) and 30% F-12 medium (Invitrogen Life Technologies) in 37°C for 72 h with or without 40 ng/ml recombinant IL-15 (PeproTech).

Following incubation, cells were either subjected to flow cytometry to assess the cell surface expression of NKp46, NKp30, NKp44, and NKG2D or to a redirected killing assay to evaluate their cytotoxic abilities. Supernatants were collected and assessed for cytokines that were secreted into the medium (see ELISA).

Redirected killing assay

The cytotoxic activity of endometrial NK cells was assessed in a redirected killing assay, in which endometrial lymphocytes were cocultured with ^{35}[S]-labeled P815 (murine mastocytoma) target cells, that were precoated with 0.1 μg per well of various mAbs: anti-CD56 (DakoCytomation), anti-NKp46, anti NKp30, anti-NKp44, anti-NKG2D (obtained from R&D Systems) and anti-CD16 (clone B73.1.1). E:T ratio was 1:1. The level of cytotoxicity was determined, as previously described (15).

ELISA

The medium in which the endometrial lymphocytes were incubated (as described above) was analyzed for secreted cytokines. The ELISA for the detection of human IFN-γ (BioLegend), IP-10 (R&D Systems), vascular endothelial growth factor (VEGF) (R&D Systems), and placental growth factor (PLGF) (R&D Systems) were performed in accordance with the manufacturer's protocol and reagents.

Flow cytometry of adherent cell fractions

To determine the expression of the NCR ligands, the following Ig-fusion proteins were used: NKp46-Ig, NKp44-Ig, NKp30-Ig, and CCM1-Ig (which served as a negative control). The Ig-fusion proteins were cloned, generated in COS-7 cells, and purified on a protein G column, as previously described (16). For flow cytometry experiments, cells, obtained from the adherent cell fraction, were incubated with 4 μg of the Ig-fusion protein for 2 h on ice. Cells were then washed and incubated for 30 min with Cy5-conjugated $F(ab')_2$ goat anti-human IgG secondary Ab (Jackson Immunoresearch Laboratories). For the detection of the NKG2D ligands, the following mAb were used: anti-MHC class I chain-related chain A (MICA), anti- MHC class I chain-related chain B (MICB), anti-UL16-binding proteins (ULBP) 2, anti-ULBP3, all obtained from R&D Systems and anti-MHC class I hybridoma, W6/32. Cells were incubated with these mAbs for 1 h on ice, washed, and then incubated with FITC-conjugated $F(ab')_2$ goat anti-mouse IgG as a secondary Ab (ICN Biomedicals) for 30 min.

Results

NK cells comprise up to 30% of endometrial lymphocytes

Several recent reports demonstrated an increase in successful pregnancies following Pipelle biopsy (14). The reasons for this phenomenon are still unknown, but because of this fortunate coincidence, we now have access to endometrial tissues. Our initial aim was to determine the number of NK cells in the tissue and the percentages of various lymphocyte populations during the menstrual cycle. To do that, we analyzed endometrial tissues obtained from women undergoing Pipelle biopsy before IVF treatments due to male infertility problems.

To gain insight into the importance of NK cells in the nonpregnant endometrium, we first performed an immunohistochemistry analysis, which confirmed previous results and showed an increase in NK cell numbers during the secretory phase (Fig. 1*A*). The NK cells were distributed throughout the tissue and we could not detect consistent variability in the position of NK cells at the different cycle days. Next, we wanted to determine the percentage of NK cells within the total endometrial lymphocyte population and therefore performed a flow cytometry analysis on endometrial lymphocytes. As in the peripheral blood, the major endometrial lymphocyte subpopulation was that of T cells, which comprised >50% of the endometrial lymphocytes (Fig. 1, *B* and *C*). However, ~30% of the endometrial lymphocytes were NK cells (gated $CD56^+CD3^-$ cells), as opposed to the peripheral blood, where they comprised only 5–15% (Fig. 1, *B* and *C*). These eNK cells were characterized by intermediate expression levels of CD56 and could not be subdivided into the two "classical" NK subpopulations that exist in the peripheral blood, i.e., $CD56^{bright}$ and $CD56^{dim}$. Since the definition of bright and dim NK cells is subjective and might also depend on the Ab clone used, we think that it is better to identify eNK cells as CD16 negative cells (Fig. 2) and not to categorize them based on the levels of CD56 expression.

Remarkably, the percentage of eNK cells remained constant during the menstrual cycle, in both the proliferative and the secretory phases of the cycle (Fig. 1*C*). In addition, we did not observe any variations in the percentage of eNK cells among different age groups.

eNK cells are a unique NK subset

The phenotype and function of eNK cells have been poorly characterized so far. We noted, in agreement with other studies, that

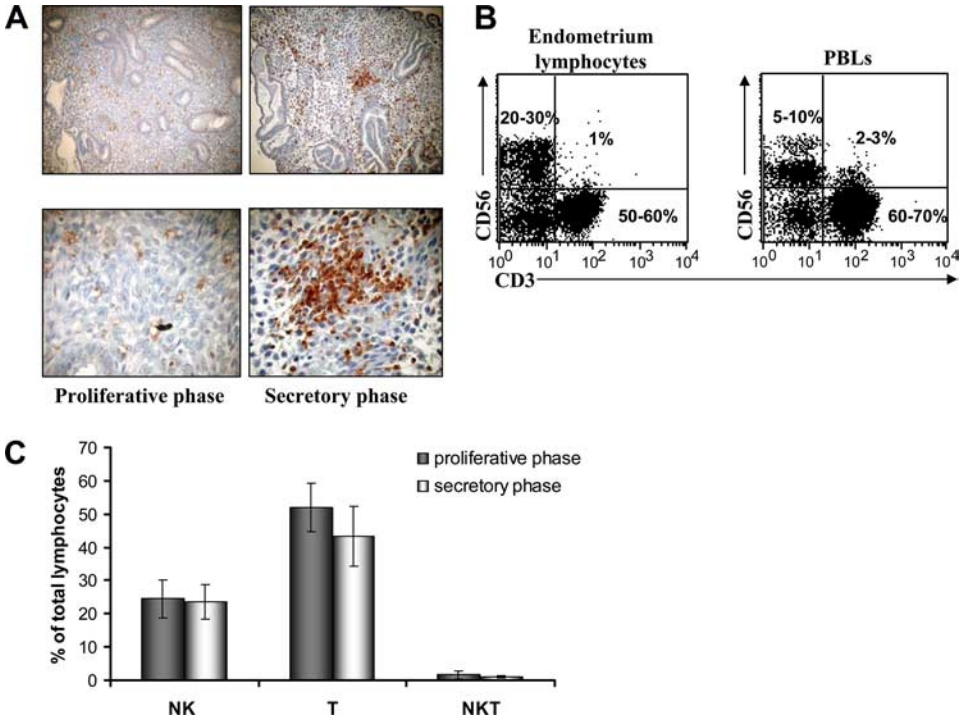

FIGURE 1. Lymphocyte composition of human endometrium and peripheral blood. *A*, Representative immunostaining of CD56 on paraffin sections of proliferative phase (one of five sampled) and secretory phase (one of six sampled) endometrium. Positive staining for NK cells is reddish brown (DAB). Upper panels show a ×100 magnification, 1.6× zoom. Lower panels show a higher magnification (×400 with 1.6× zoom) of the upper panels. *B*, Flow cytometry analysis of endometrial and peripheral blood lymphocytes. Percentages of NK cells ($CD56^+CD3^-$) NKT cells ($CD56^+CD3^+$) and T cells ($CD56^-CD3^+$), as a percentage of total lymphocytes, are indicated in the figure. One representative experiment out of ten performed is shown. *C*, Differences in the percentages of lymphocyte subpopulations in the human endometrium during the menstrual cycle. Values are mean ± SD for more than ten samples analyzed.

eNK cells express inhibitory receptors (our unpublished data and Ref. 12). However, the expression of the NK activating receptors, including the NCRs and NKG2D, on eNK cells has so far never been examined. Flow cytometry analysis showed that both proliferative and secretory phase eNK cells express NKp46 and NKG2D, but do not express, or express only low levels of the activating receptors CD16, NKp44, and NKp30 (Fig. 2). The results were consistent and were observed in all women tested. This expression pattern makes eNK cells quite different from pbNK cells, which express NKp30 (Ref. 17 and Fig. 2). Furthermore, it distinguishes eNK cells from dNK cells, which express relatively high levels of NKp30, NKp44, and NKG2D (6). Thus, the observed expression of activating receptors may allow us to regard eNK cells as a unique subset of NK cells, different from the other characterized NK subsets, including those of the blood, decidua, spleen, and tonsils (18). It should be noted, however, that eNK cells resemble, at least in these features, lymph node NK cells (18), although other features, such as the expression of killer cell Ig-like receptors and chemokine receptors (see below), may point out the differences between these two organ-based NK subsets (12).

Expression of chemokine receptors on eNK cells

Several studies have suggested that some of the dNK cells probably originate from NK cells that migrated from the peripheral blood to the decidua, where they underwent further tissue-specific differentiation (6, 8). The percentages of NK cells in the endometrium, though they did not reach that of the decidua (around 70%; Ref. 1), were still relatively high compared with the peripheral blood (Fig. 1). To test the possibility that endometrial NK cells arise from recruitment of peripheral blood NK cells to the endometrium, we assessed the expression of chemokine receptors on eNK cells (Fig. 3).

Flow cytometry analysis showed that both proliferative and secretory phase eNK cells did not express any of the chemokine receptors tested (Fig. 3*A*), including CXCR1, CXCR2, CXCR3, CXCR4, CCR1, CCR2, CCR3, CCR5, and CCR7. Importantly,

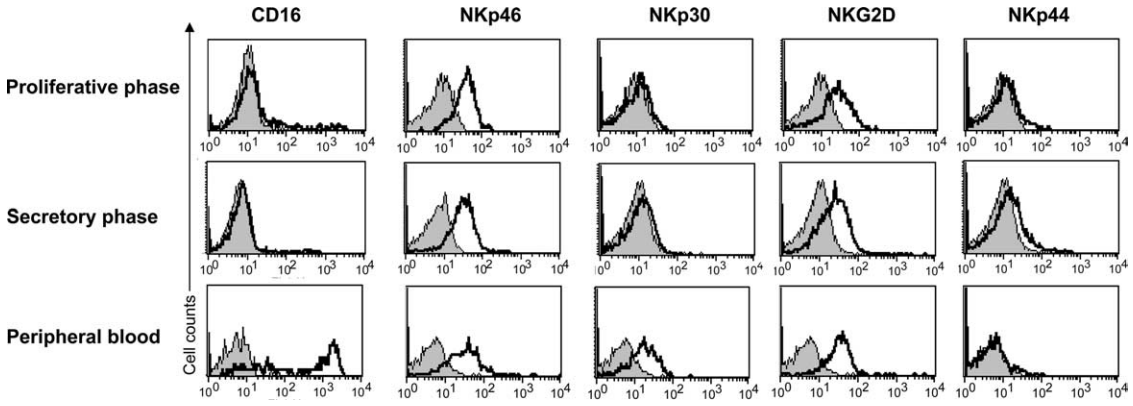

FIGURE 2. Expression of NK-activating receptors on endometrial and peripheral blood NK cells. Flow cytometry analysis of different NK-activating receptors on gated $CD56^+CD3^-$ NK cells from proliferative phase and secretory phase endometrium and from the peripheral blood. The gray filled histograms represent background staining of a corresponding isotype-matched control. One representative is shown of nine experiments performed.

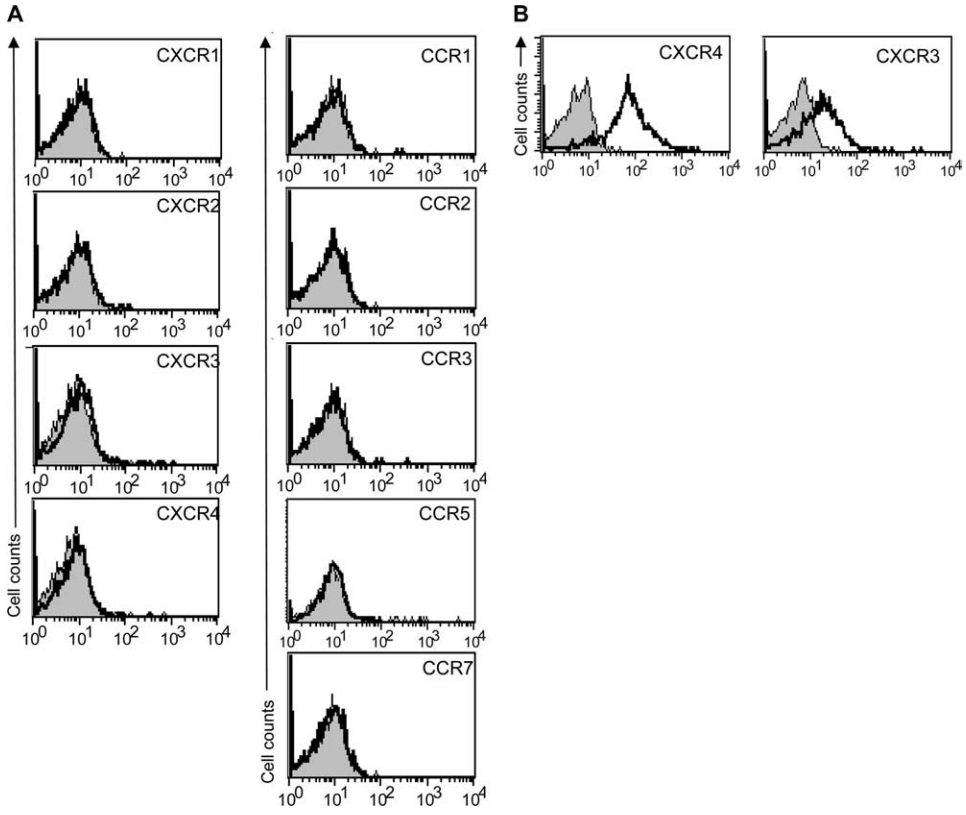

FIGURE 3. Expression of the chemokine receptors on endometrial NK and T cells. Flow cytometry analysis on gated $CD56^{+}CD3^{-}$ NK cells (*A*) or gated $CD56^{-}CD3^{+}$ T cells (*B*). The results presented are similar between the proliferative and the secretory phases of the menstrual cycle. Figure shows the results obtained from secretory phase endometrium. The gray filled histograms represent background staining of a corresponding isotype-matched control. One representative is shown of five experiments performed.

endometrial T cells showed high expression levels of CXCR3 and CXCR4 (Fig. 3*B*). These results suggest that endometrial NK cells are possibly a local population of innate lymphocytes within the endometrium and are not recruited to this tissue. The high expression levels of CXCR3 and CXCR4 on endometrial T cells rules out the possibility that the lack of chemokine receptor expression on eNK cells was due to internalization following ligand binding (19).

These findings, together with the observations that the percentages (Fig. 1) and the expression of the various activating receptors (Fig. 2) are similar in the two phases of the menstrual cycle, indicate that the same NK subset resides in the endometrium throughout the menstrual cycle.

Up-regulation of eNK cell functions following IL-15 activation

To test whether the phenotype of eNK cells changes upon stimulation, we isolated endometrial lymphocytes and incubated the cells both in the presence or in the absence of IL-15, a cytokine that is important for NK cell differentiation (20, 21) and is known to be important during pregnancy (22, 23).

As can be seen in Fig. 4, we did not observe a significant change in the expression levels of NKp46 and NKG2D. However, there was a striking increase in the expression of NKp30 and NKp44 (Fig. 4). These results suggest that eNK cells are perhaps an immature NK cell subset and are not fully differentiated.

Different NK subsets specialize in different functions. For example, pbNK cells specialize in killing tumor and virally infected cells and in secreting a variety of cytokines, lymph node NK cells possibly act as APCs (24), and dNK cells possess tissue remodeling properties that are essential for successful implantation of the embryo (6). Therefore, we decided to investigate the functional properties of eNK cells. Seeing that IL-15 activation of eNK cells resulted in an up-regulation of two of the major activating receptors (NKp30 and NKp44), we incubated endometrial lymphocytes or highly purified eNK cells in the presence or absence of IL-15 and following this incubation, we preformed two functional assays. In the first assay we cross-linked specific activating receptors to assess the role of specific activating receptors in eNK cytotoxicity (Fig. 5*A*). In the second assay, we investigated the cytokine

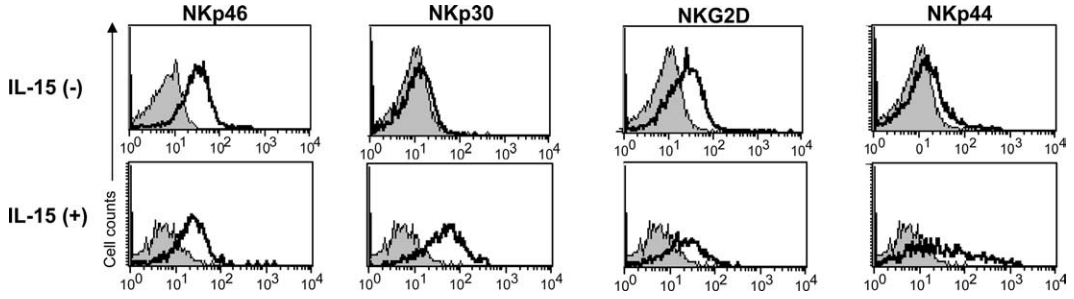

FIGURE 4. Up-regulation of NKp30 and NKp44 following in vitro IL-15 activation. Flow cytometry analysis of gated $CD56^{+}CD3^{-}$ NK cells, following incubation for 72 h in the presence or absence of 40 ng/ml IL-15. The gray filled histograms represent background staining of a corresponding isotype-matched control. Figure shows one representative experiment out of three experiments performed.

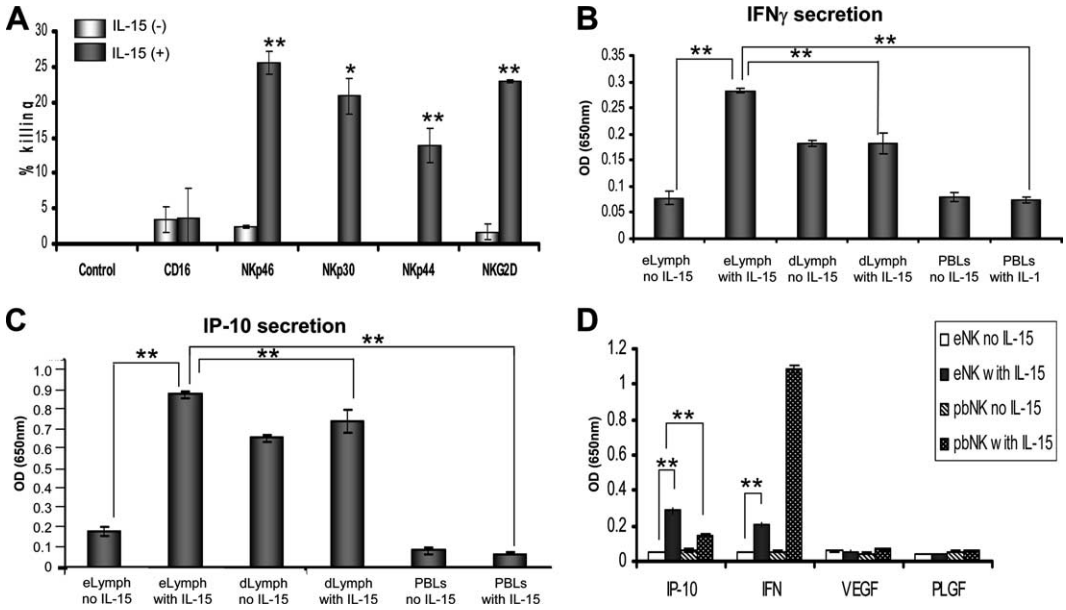

FIGURE 5. Functions of eNK cells are up-regulated following IL-15 activation. *A*, The cytotoxicity of endometrial NK cells was determined by a redirected killing assay using the indicated Abs in the *x*-axis. CD56 served as a control Ab. The E:T ratio was 1:1. Values are mean ± SD for triplicate samples. *, $p < 0.05$; **, $p < 0.01$ (Student's *t* test). Where there is no column presented, the killing was 0%. One representative data set is shown out of three experiments performed. *B* and *C*, Secretion of IFN-γ (*B*) and IP-10 (*C*) by endometrial lymphpcytes (eLymp), decidual lymphocytes (dLymph), and peripheral blood lymphocytes following a 72 h incubation in the presence or absence of IL-15, as indicated. Values are mean ± SD for triplicate samples. **, $p < 0.001$ (Student's *t* test). One representative data set is shown out of four experiments performed. *D*, Secretion of IFN-γ, IP-10, VEGF, and PLGF by highly purified eNK and pbNK cells following a 72-h incubation in the presence or absence of IL-15, as indicated. Values are mean ± SD for triplicate samples. **, $p < 0.001$ (Student's *t* test). One representative data set is shown out of three experiments performed.

production by endometrial lymphocytes and by purified eNK cells following this activation (Fig. 5, *B–D*).

Our results showed that non-activated eNK cells had no cytotoxic activity. However, following IL-15 activation, their receptor-mediated cytotoxicity toward their target cells increased dramatically (Fig. 5*A*). This effect was specific, as cross-linking of other receptors (such as CD56, marked as control), or crosslinking of CD16, which is absent in eNK, did not alter eNK cells cytotoxicity (Fig. 5*A*).

It has been previously shown that $CD16^-$ cells specialize in cytokine secretion and are less cytotoxic compared with $CD16^+$ NK cells (5, 25). Hence, we speculated that the primary function of eNK might be cytokine secretion. We first analyzed the cytokine secretion by the entire endometrial lymphocyte population and compared it to that of the decidua and peripheral blood. Remarkably, before IL-15 activation, endometrial lymphocytes did not secrete any of the cytokines tested including IFN-γ (Fig. 5*B*), IFN-inducible protein-10 (IP-10, Fig. 5*C*), VEGF, and placental growth factor (PLGF, our unpublished data). Real time RT-PCR confirmed that endometrial lymphocytes, in contrast to decidual lymphocytes, did not contain VEGF and PLGF transcripts (data not shown). In marked contrast, non-activated decidual lymphocytes secreted IP-10 and IFN-γ, as we and others previously showed (6, 26) and as was shown regarding IFN-γ in mouse dNK cells (27), thus indicating that decidual lymphocytes, as opposed to endometrial lymphocytes, are already activated in the tissue. Furthermore, in vitro IL-15 activation significantly up-regulated the secretion of IP-10 and IFN-γ by endometrial lymphocytes to levels significantly higher than those of decidual and peripheral blood lymphocytes, whether activated or not (Fig. 5, *B* and *C*). The secretion of the angiogenic factors VEGF and PLGF by eNK cells was not evident even after IL-15 activation (our unpublished data). To test whether eNK could secrete all these cytokines, we analyzed the cytokine secretion by highly purified eNK and pbNK cells, before or following IL-15 activation (Fig. 5*D*). Before IL-15 activation, eNK cells did not secrete IP-10 or IFN-γ, however following this activation there was a significant increase in the secretion of these cytokines by eNK cells.

IL-15-activated eNK cells secreted higher amounts of IP-10 than IL-15-activated pbNK cells, however, they secreted less IFN-γ than IL-15-activated pbNK cells. We did not observe any secretion of VEGF or PLGF by eNK cells, whether activated or not (Fig. 5*D*). Importantly and similar to the above observations (Fig. 1–4), no major differences in cytokine secretion or cytotoxicity were observed between proliferative and secretory phase eNK cells.

Therefore, our results suggest that eNK cells are inert lymphocytes in the endometrium that are unable to kill target cells or to secrete NK known cytokines and growth factors, before IL-15 activation.

Activating receptors of eNK cells recognize endometrial cells

We demonstrated above that eNK cells express the activating receptors NKp46 and NKG2D, but do not express NKp30 and NKp44. The ligands recognized by these receptors are either stress-induced proteins (ligands for NKG2D, i.e., MICA, MICB, and ULBPs 1–4; Ref. 28, 29), viral proteins (such as the hemagglutinins and the HCMV protein pp65; Ref. 30, 31), or as yet unidentified ligands (32). To see whether ligands for these receptors are expressed in the endometrial tissue, we stained the adherent endometrial cell fraction (see *Materials and Methods*) with soluble NKp30, NKp44, and NKp46-Fc fusion proteins (which can detect known as well as unknown ligands for these receptors) and with Abs against the known NKG2D ligands. Staining with an Ab against MHC class I (W632) was used as a positive control (Fig. 6, *A* and *B*). In both proliferative phase and secretory phase-cell fractions there was no expression of NKp46-binding ligands. However, there was a substantial expression of NKp30 and NKp44-binding ligands, at variable expression levels (Fig. 6*A*).

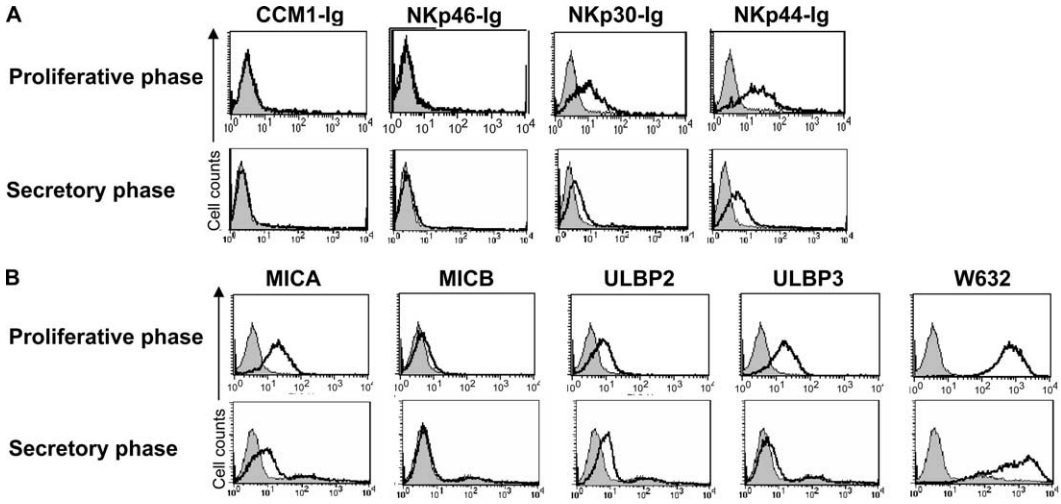

FIGURE 6. Expression of NCR and NKG2D ligands in the adherent cell fraction of the endometrium. *A*, Expression of NCR ligands in the adherent cell fraction of proliferative phase (*top panel*) and secretory phase (*bottom panel*) endometrium using different NCR-Ig fusion proteins. CCM1-Ig serves as a control. *B*, Expression of NKG2D ligands in the adherent fraction of proliferative phase (*top panel*) and secretory phase (*bottom panel*) endometrium. The gray filled histograms represent background staining of a corresponding isotype-matched control. One representative staining is shown of six performed.

Throughout the menstrual cycle we detected the expression of the NKG2D ligands MICA and ULBP2 and the lack of expression of MICB (Fig. 6*B*). The ligand ULBP3 was expressed in the proliferative endometrial cell fraction, but its expression was downregulated in the secretory phase endometrium (Fig. 6B).

Discussion

The study of dNK cells has revealed the unique functional activity of these cells, showing their ability to support the implantation of the embryo through tissue remodeling activity and the secretion of various growth factors (6, 33). Since, in humans, the preparation for blastocyst implantation begins after ovulation, during the secretory phase of the menstrual cycle, it was tempting to assume that the population of eNK cells during the secretory phase of the menstrual cycle possesses similar phenotype and functional properties as dNK cells. In this study, we characterized the phenotype and function of eNK cells and studied the relations between eNK cells and dNK cells.

The percentage of NK cells in the nonpregnant endometrium may reflect their importance to the drastic periodical changes that occur during the menstrual cycle. To test this idea, we performed flow cytometry analysis on freshly isolated endometrial lymphocytes. The results showed that the major lymphocyte population in the endometrium was that of T and not NK cells. Furthermore, the percentage of NK cells remained constant during the menstrual cycle. Although our immunohistochemistry analysis and other studies showed that the absolute numbers of eNK cells during the menstrual cycle increase dramatically during the secretory phase (Fig. 1*A* and Ref. 34), we think that the important parameter that should be considered when evaluating the importance of this population is that of percentage, since other lymphocyte populations can also increase in numbers during this period. Our results tie in with other early studies, which demonstrated that the percentage of eNK cells during the menstrual cycle remains constant and is around 30% and that the major lymphocyte subpopulation in the endometrium is that of T cells (35, 36). It will be interesting to study in the future the properties of the endometrial T cells.

The marked increase in NK cell numbers during the late secretory phase that was reported by others and was also observed by us (Fig. 1*A*) was attributed to migration of NK cells from the peripheral blood to the endometrium via interactions between chemokine receptors expressed on the NK cells and their ligands, expressed in the tissue (37, 38). However, no expression of chemokine receptors (including CXCR1, 2, 3, and 4 and CCR1, 2, 3, 5, and 7) was observed on eNK cells derived from the proliferative or secretory phase. The differences between the results presented in this study and those obtained by Sentman et al. (37) could be attributed to the differences in the methods used. The analysis of eNK cells that is shown in this study was performed on freshly isolated, nonmanipulated endometrial lymphocytes, whereas the Sentman et al. analysis was performed on IL-2-activated eNK clones. Furthermore, IL-2 is a cytokine that was shown not to be expressed in the tissue and therefore is not suitable for in vitro activation of eNK cells (39).

The lack of expression of chemokine receptors indicates that eNK cells do not migrate to the endometrium from other tissues or from the blood, but rather originate from local hematopoietic progenitor cells. Indeed, several studies support the presence of hematopoietic progenitor cells in the endometrium (40, 41). In contrast, in mice, LGL1-positive NK cells reside in the nonpregnant uterus, however, they do not self-renew and differentiate to mature uterine NK cells (42, 43).

We have also characterized the expression pattern of the NK-activating receptors on eNK cells and demonstrated that eNK cells lack the expression of CD16, but express relatively high levels of NKp46 and NKG2D (as do dNK cells). However, in contrast to dNK cells, eNK cells also lack the expression of NKp30 and NKp44. This unusual repertoire of activating and chemokine receptors makes eNK cells unique among other known NK subsets. The lack of expression of NKp30 and NKp44 could hypothetically be a result of sustained activation of the receptors by their unknown ligands which are expressed in tissue (Fig. 6), as was previously shown regarding NKG2D (44). Indeed, we show in this study that endometrial cells express ligands for NKp30 and NKp44 and that an inverse correlation exists between the expression of these ligands and their cognate receptors. However, until the identity of these unknown ligands is revealed and the nature of their expression discovered, we cannot deduce for their role in the regulation of eNK cell activity.

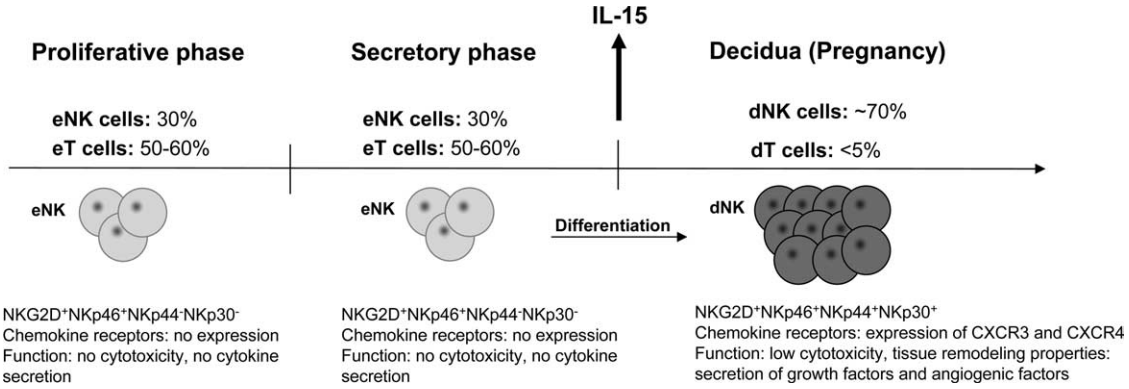

FIGURE 7. Schematic diagram summarizing the NK cell phenotype and function in the endometrium and in the decidua. During the menstrual cycle, eNK cells compose 30% of the endometrial lymphocytes and display an immature form: they possess no apparent functional activity, do not express the major activating receptors NKp30 and NKp44, and lack the expression of chemokine receptors. Following conception, the levels of IL-15 rise in the tissue (decidua) and promote the differentiation of eNK cells toward dNK cells. This differentiation includes up-regulation of NKp30 and NKp44, up-regulation of cytotoxicity (although it is still low), and up-regulation in the secretion of growth factors.

eNK cells also exhibited extremely low levels of cytotoxicity and failed to produce cytokines such as IFN-γ, IP-10, VEGF, and PLGF (even when the entire endometrial lymphocyte population was cultured). Following in vitro stimulation with IL-15, whose receptor is expressed on eNK cells (45), the expression of the activating receptors NKp30 and NKp44 was up-regulated, making the activating receptor-expression profile of eNK cells similar to that of dNK cells. Moreover, eNK cells resemble dNK cells in their high expression levels of killer cell Ig-like receptors, CD94, and CD9 (12, 46), in the expression of the activation marker CD69 and in the lack of expression of the adherent molecule L-selectin (13, 35, 46). Thus, following IL-15 activation, the phenotype of eNK cells resembles that of dNK cells, inferring that eNK cells are perhaps an early, undifferentiated form of some of the dNK cells.

Indeed, we show that following in vitro IL-15 activation, eNK cell cytotoxicity was up-regulated, as was reported for dNK cells (10, 22). We also observed a marked increase in the secretion of IFN-γ and IP-10 by endometrial lymphocytes, and specifically by eNK cells. This observation is supported by the research of Eriksson et al. (12) who showed up-regulation of IFN-γ and IL-10 secretion following in vitro activation with IL-12 and IL-15. Importantly, these cytokines are also secreted by dNK cells (6, 26, 27) and were shown to regulate trophoblast invasion. Although dNK cells were also shown to secrete the angiogenic factors VEGF and PLGF, we did not observe mRNA transcripts or any secretion of these factors before or following IL-15 activation. It is possible that other decidual factors besides IL-15 can drive further differentiation of dNK cells that enables the secretion of these angiogenic factors.

Therefore, our results suggest the following hypothesis: After conception, the expression levels of IL-15 rise in the decidua (19). IL-15 is a cytokine that is known as a key cytokine in the differentiation of hematopoietic progenitor to NK cells (20), it is expressed in the human endometrium (22, 47) and its expression levels are higher in the decidua than in the endometrium (22). The high levels of IL-15 in this developmental niche promote the differentiation of the local eNK cells toward dNK cells. In other words, our results imply that eNK cells might be part of the progenitor cells of dNK cells. This hypothesis is further strengthened by studies suggesting that dNK cells can originate not only from blood NK cells (8), but also from local progenitor cells (40, 41). Finally, the expression profile of chemokine receptors in dNK cells supports this notion: dNK cells express high levels of CXCR3 and intermediate levels of CXCR4, which mediate their migration to the decidua (8). However, since these receptors and others are not expressed on all dNK cells (8), it is possible that some dNK cells, which do not express chemokine receptors at all, originated from local cells, such as eNK cells, and did not migrate to the decidua from the peripheral blood via their chemokine receptors. Fig. 7 summarizes this hypothesis.

It is possible that eNK cells secrete other factors, not tested in this study, that contribute to the preparation of the uterus for blastocyst implantation. However, the lack of known NK-functional activity of eNK, viewed together with our observation that the NK cell proportion within the lymphocyte population is constant during the menstrual cycle and that in fact the major lymphocyte population in the endometrium is that of T cells, suggests that eNK cells have no apparent significant role during the menstrual cycle before conception. It also suggests that the role of eNK cells, which might be regenerated each month in the nonpregnant endometrium, is simply to linger and wait for conception.

Disclosures

The authors have no financial conflict of interest.

References

1. Hanna, J., and O. Mandelboim. 2007. When killers become helpers. *Trends Immunol.* 28: 201–206.
2. Vosshenrich, C. A., M. E. Garcia-Ojeda, S. I. Samson-Villeger, V. Pasqualetto, L. Enault, O. Richard-Le Goff, E. Corcuff, D. Guy-Grand, B. Rocha, A. Cumano, et al. 2006. A thymic pathway of mouse natural killer cell development characterized by expression of GATA-3 and CD127. *Nat. Immunol.* 7: 1217–1224.
3. Croy, B. A., M. J. van den Heuvel, A. M. Borzychowski, and C. Tayade. 2006. Uterine natural killer cells: a specialized differentiation regulated by ovarian hormones. *Immunol. Rev.* 214: 161–185.
4. Manaster, I., and O. Mandelboim. 2008. The unique properties of human NK cells in the uterine mucosa. *Placenta* 29S: 60–66.
5. Hanna, J., P. Bechtel, Y. Zhai, F. Youssef, K. McLachlan, and O. Mandelboim. 2004. Novel insights on human NK cells' immunological modalities revealed by gene expression profiling. *J. Immunol.* 173: 6547–6563.
6. Hanna, J., D. Goldman-Wohl, Y. Hamani, I. Avraham, C. Greenfield, S. Natanson-Yaron, D. Prus, L. Cohen-Daniel, T. I. Arnon, I. Manaster, et al. 2006. Decidual NK cells regulate key developmental processes at the human fetal-maternal interface. *Nat. Med.* 12: 1065–1074.
7. Arnon, T. I., G. Markel, and O. Mandelboim. 2006. Tumor and viral recognition by natural killer cells receptors. *Semin. Cancer Biol.* 16: 348–358.
8. Hanna, J., O. Wald, D. Goldman-Wohl, D. Prus, G. Markel, R. Gazit, G. Katz, R. Haimov-Kochman, N. Fujii, S. Yagel, et al. 2003. CXCL12 expression by invasive trophoblasts induces the specific migration of CD16-human natural killer cells. *Blood* 102: 1569–1577.
9. King, A., C. Birkby, and Y. W. Loke. 1989. Early human decidual cells exhibit NK activity against the K562 cell line but not against first trimester trophoblast. *Cell. Immunol.* 118: 337–344.
10. Kopcow, H. D., D. S. Allan, X. Chen, B. Rybalov, M. M. Andzelm, B. Ge, and J. L. Strominger. 2005. Human decidual NK cells form immature activating synapses and are not cytotoxic. *Proc. Natl. Acad. Sci. USA* 102: 15563–15568.

11. Pace, D., L. Morrison, and J. N. Bulmer. 1989. Proliferative activity in endometrial stromal granulocytes throughout menstrual cycle and early pregnancy. *J. Clin. Pathol.* 42: 35–39.
12. Eriksson, M., S. K. Meadows, C. R. Wira, and C. L. Sentman. 2004. Unique phenotype of human uterine NK cells and their regulation by endogenous TGF-β. *J. Leukocyte Biol.* 76: 667–675.
13. Searle, R. F., R. K. Jones, and J. N. Bulmer. 1999. Phenotypic analysis and proliferative responses of human endometrial granulated lymphocytes during the menstrual cycle. *Biol. Reprod.* 60: 871–878.
14. Barash, A., N. Dekel, S. Fieldust, I. Segal, E. Schechtman, and I. Granot. 2003. Local injury to the endometrium doubles the incidence of successful pregnancies in patients undergoing in vitro fertilization. *Fertil. Steril.* 79: 1317–1322.
15. Porgador, A., O. Mandelboim, N. P. Restifo, and J. L. Strominger. 1997. Natural killer cell lines kill autologous β2-microglobulin-deficient melanoma cells: implications for cancer immunotherapy. *Proc. Natl. Acad. Sci. USA* 94: 13140–13145.
16. Mandelboim, O., P. Malik, D. M. Davis, C. H. Jo, J. E. Boyson, and J. L. Strominger. 1999. Human CD16 as a lysis receptor mediating direct natural killer cell cytotoxicity. *Proc. Natl. Acad. Sci. USA* 96: 5640–5644.
17. Pende, D., S. Parolini, A. Pessino, S. Sivori, R. Augugliaro, L. Morelli, E. Marcenaro, L. Accame, A. Malaspina, R. Biassoni, et al. 1999. Identification and molecular characterization of NKp30, a novel triggering receptor involved in natural cytotoxicity mediated by human natural killer cells. *J. Exp. Med.* 190: 1505–1516.
18. Ferlazzo, G., D. Thomas, S. L. Lin, K. Goodman, B. Morandi, W. A. Muller, A. Moretta, and C. Munz. 2004. The abundant NK cells in human secondary lymphoid tissues require activation to express killer cell Ig-like receptors and become cytolytic. *J. Immunol.* 172: 1455–1462.
19. Hoxie, J. A., M. Ahuja, E. Belmonte, S. Pizarro, R. Parton, and L. F. Brass. 1993. Internalization and recycling of activated thrombin receptors. *J. Biol. Chem.* 268: 13756–13763.
20. Becknell, B., and M. A. Caligiuri. 2005. Interleukin-2, interleukin-15, and their roles in human natural killer cells. *Adv. Immunol.* 86: 209–239.
21. Caligiuri, M. A., A. Zmuidzinas, T. J. Manley, H. Levine, K. A. Smith, and J. Ritz. 1990. Functional consequences of interleukin 2 receptor expression on resting human lymphocytes. Identification of a novel natural killer cell subset with high affinity receptors. *J. Exp. Med.* 171: 1509–1526.
22. Verma, S., S. E. Hiby, Y. W. Loke, and A. King. 2000. Human decidual natural killer cells express the receptor for and respond to the cytokine interleukin 15. *Biol. Reprod.* 62: 959–968.
23. Ashkar, A. A., G. P. Black, Q. Wei, H. He, L. Liang, J. R. Head, and B. A. Croy. 2003. Assessment of requirements for IL-15 and IFN regulatory factors in uterine NK cell differentiation and function during pregnancy. *J. Immunol.* 171: 2937–2944.
24. Hanna, J., T. Gonen-Gross, J. Fitchett, T. Rowe, M. Daniels, T. I. Arnon, R. Gazit, A. Joseph, K. W. Schjetne, A. Steinle, et al. 2004. Novel APC-like properties of human NK cells directly regulate T cell activation. *J. Clin. Invest.* 114: 1612–1623.
25. Nagler, A., L. L. Lanier, S. Cwirla, and J. H. Phillips. 1989. Comparative studies of human FcRIII-positive and negative natural killer cells. *J. Immunol.* 143: 3183–3191.
26. Saito, S., K. Nishikawa, T. Morii, M. Enomoto, N. Narita, K. Motoyoshi, and M. Ichijo. 1993. Cytokine production by CD16-CD56bright natural killer cells in the human early pregnancy decidua. *Int. Immunol.* 5: 559–563.
27. Platt, J. S., and J. S. Hunt. 1998. Interferon-γ gene expression in cycling and pregnant mouse uterus: temporal aspects and cellular localization. *J. Leukocyte Biol.* 64: 393–400.
28. Cosman, D., J. Mullberg, C. L. Sutherland, W. Chin, R. Armitage, W. Fanslow, M. Kubin, and N. J. Chalupny. 2001. ULBPs, novel MHC class I-related molecules, bind to CMV glycoprotein UL16 and stimulate NK cytotoxicity through the NKG2D receptor. *Immunity* 14: 123–133.
29. Steinle, A., P. Li, D. L. Morris, V. Groh, L. L. Lanier, R. K. Strong, and T. Spies. 2001. Interactions of human NKG2D with its ligands MICA, MICB, and homologs of the mouse RAE-1 protein family. *Immunogenetics* 53: 279–287.
30. Mandelboim, O., N. Lieberman, M. Lev, L. Paul, T. I. Arnon, Y. Bushkin, D. M. Davis, J. L. Strominger, J. W. Yewdell, and A. Porgador. 2001. Recognition of haemagglutinins on virus-infected cells by NKp46 activates lysis by human NK cells. *Nature* 409: 1055–1060.
31. Arnon, T. I., M. Lev, G. Katz, Y. Chernobrov, A. Porgador, and O. Mandelboim. 2001. Recognition of viral hemagglutinins by NKp44 but not by NKp30. *Eur. J. Immunol.* 31: 2680–2689.
32. Arnon, T. I., H. Achdout, N. Lieberman, R. Gazit, T. Gonen-Gross, G. Katz, A. Bar-Ilan, N. Bloushtain, M. Lev, A. Joseph, et al. 2004. The mechanisms controlling the recognition of tumor- and virus-infected cells by NKp46. *Blood.* 103: 664–672.
33. Ashkar, A. A., J. P. Di Santo, and B. A. Croy. 2000. Interferon γ contributes to initiation of uterine vascular modification, decidual integrity, and uterine natural killer cell maturation during normal murine pregnancy. *J. Exp. Med.* 192: 259–270.
34. Starkey, P. M., L. M. Clover, and M. C. Rees. 1991. Variation during the menstrual cycle of immune cell populations in human endometrium. *Eur. J. Obstet. Gynecol. Reprod. Biol.* 39: 203–207.
35. Ho, H. N., K. H. Chao, C. K. Chen, Y. S. Yang, and S. C. Huang. 1996. Activation status of T and NK cells in the endometrium throughout menstrual cycle and normal and abnormal early pregnancy. *Hum. Immunol.* 49: 130–136.
36. Lachapelle, M. H., P. Miron, R. Hemmings, and D. C. Roy. 1996. Endometrial T, B, and NK cells in patients with recurrent spontaneous abortion: altered profile and pregnancy outcome. *J. Immunol.* 156: 4027–4034.
37. Sentman, C. L., S. K. Meadows, C. R. Wira, and M. Eriksson. 2004. Recruitment of uterine NK cells: induction of CXC chemokine ligands 10 and 11 in human endometrium by estradiol and progesterone. *J. Immunol.* 173: 6760–6766.
38. Kitaya, K., T. Nakayama, N. Daikoku, S. Fushiki, and H. Honjo. 2004. Spatial and temporal expression of ligands for CXCR3 and CXCR4 in human endometrium. *J. Clin. Endocrinol. Metab.* 89: 2470–2476.
39. Tabiasco, J., M. Rabot, M. Aguerre-Girr, H. El Costa, A. Berrebi, O. Parant, G. Laskarin, K. Juretic, A. Bensussan, D. Rukavina, and P. Le Bouteiller. 2006. Human decidual NK cells: unique phenotype and functional properties: a review. *Placenta* 27 (*Suppl.* A): S34–S39.
40. Matsuura-Sawada, R., T. Murakami, Y. Ozawa, H. Nabeshima, J. Akahira, Y. Sato, Y. Koyanagi, M. Ito, Y. Terada, and K. Okamura. 2005. Reproduction of menstrual changes in transplanted human endometrial tissue in immunodeficient mice. *Human reproduction (Oxford, England)*. 20: 1477–1484.
41. Lynch, L., L. Golden-Mason, M. Eogan, C. O'Herlihy, and C. O'Farrelly. 2007. Cells with haematopoietic stem cell phenotype in adult human endometrium: relevance to infertility? *Hum. Reprod.* 22: 919–926.
42. Kiso, Y., B. A. McBey, L. Mason, and B. A. Croy. 1992. Histological assessment of the mouse uterus from birth to puberty for the appearance of LGL-1$^+$ natural killer cells. *Biol. Reprod.* 47: 227–232.
43. Chantakru, S., C. Miller, L. E. Roach, W. A. Kuziel, N. Maeda, W. C. Wang, S. S. Evans, and B. A. Croy. 2002. Contributions from self-renewal and trafficking to the uterine NK cell population of early pregnancy. *J. Immunol.* 168: 22–28.
44. Oppenheim, D. E., S. J. Roberts, S. L. Clarke, R. Filler, J. M. Lewis, R. E. Tigelaar, M. Girardi, and A. C. Hayday. 2005. Sustained localized expression of ligand for the activating NKG2D receptor impairs natural cytotoxicity in vivo and reduces tumor immunosurveillance. *Nat. Immunol.* 6: 928–937.
45. Kitaya, K., T. Yamaguchi, and H. Honjo. 2005. Central role of interleukin-15 in postovulatory recruitment of peripheral blood CD16$^-$ natural killer cells into human endometrium. *J. Clin. Endocrinol. Metab.* 90: 2932–2940.
46. Koopman, L. A., H. D. Kopcow, B. Rybalov, J. E. Boyson, J. S. Orange, F. Schatz, R. Masch, C. J. Lockwood, A. D. Schachter, P. J. Park, and J. L. Strominger. 2003. Human decidual natural killer cells are a unique NK cell subset with immunomodulatory potential. *J. Exp. Med.* 198: 1201–1212.
47. Kitaya, K., J. Yasuda, I. Yagi, Y. Tada, S. Fushiki, and H. Honjo. 2000. IL-15 expression at human endometrium and decidua. *Biol. Reprod.* 63: 683–687.