In vivo generation of decidual natural killer cells from resident hematopoietic progenitors

Laura Chiossone,[1]* Paola Vacca,[2]* Paola Orecchia,[2] Daniele Croxatto,[2] Patrizia Damonte,[3] Simonetta Astigiano,[3] Ottavia Barbieri,[2,3] Cristina Bottino,[1,2] Lorenzo Moretta,[1] and Maria Cristina Mingari[2,3]

[1]Giannina Gaslini Institute, Genova; [2]Department of Experimental Medicine and Center of Excellence for Biomedical Research, University of Genova; and [3]IRCCS AOU San Martino-IST (National Institute for Cancer Research), Genova, Italy

*LC and PV equally contributed to this work

ABSTRACT

Decidual natural killer cells accumulate at the fetal-maternal interface and play a key role in a successful pregnancy. However, their origin is still unknown. Do they derive from peripheral natural killer cells recruited in decidua or do they represent a distinct population that originates *in situ*? Here, we identified natural killer precursors in decidua and uterus of pregnant mice. These precursors underwent rapid *in situ* differentiation and large proportions of proliferating immature natural killer cells were present in decidua and uterus as early as gestation day 4.5. Here, we investigated the origin of decidua- and uterus-natural killer cells by performing transfer experiments of peripheral mature natural killer cells or precursors from EGFP$^+$ mice. Results showed that mature natural killer cells did not migrate into decidua and uterus, while precursors were recruited in these organs and differentiated towards natural killer cells. Moreover, decidua- and uterus-natural killer cells displayed unique phenotypic and functional features. They expressed high levels of the activating Ly49D receptor in spite of their immature phenotype. In addition, decidua- and uterus-natural killer cells were poorly cytolytic and produced low amounts of IFN-γ, while they released factors (GM-CSF, VEGF, IP-10) involved in neo-angiogenesis and tissue remodeling. Our data reveal *in situ* generation of decidual natural killer cells and provide an important correlation between mouse and human decidual natural killer cells, allowing further studies to be carried out on their role in pregnancy-related diseases.

Introduction

Natural killer (NK) cells are lymphoid cells of the innate immune system involved in the elimination of virally infected or tumor cells. NK cells secrete pro-inflammatory cytokines that modulate downstream adaptive immune responses. In turn, NK cell function can be greatly influenced by the microenvironment, i.e. cytokines, chemokines and cell-to-cell interactions.[1-6] NK cells with peculiar features have been identified in different tissues, including liver, mucosal tissues, lymphoid organs and decidua.[7,8] During normal pregnancy, a high number of immune cells, such as NK cells, regulatory T cells and macrophages, accumulate in decidua during the early phase of gestation and are required for a successful pregnancy.[9] Decidual NK (dNK) cells represent as much as 50-70% of lymphoid cells in the human decidua during the first trimester, while their numbers progressively decrease during the second and third trimester of pregnancy.[10,11] Moreover, dNK cells display unique phenotypic and functional properties: a CD56^{bright}, CD16^{neg}, killer immunoglobulin-like receptor positive (KIR$^+$) phenotype and low cytolytic capacity.[12,13] Previous studies in humans revealed the presence of CD34$^+$ hematopoietic precursors in decidual and endometrial tissues able to give rise to NK cells *in vitro*.[14-16] Recent findings, both in humans and mice, have shown that uterine NK cells are involved in endometrial remodeling, spiral artery modifications and placentation.[17-21] Although a large body of information on uterine NK cell function comes from data in mice, our knowledge regarding the presence and the phenotype of murine NK cells in decidua and uterus during early pregnancy (first week) is still incomplete. Another important question concerns their origin: they can be generated *in situ* from precursors or recruited from the periphery into decidua and uterus, where the microenvironment can modulate their phenotypic and functional characteristics.

NK cells originate from hematopoietic stem cells (HSC) in the bone marrow (BM). Their differentiation process leads to a sequential loss of pluripotency paralleled by a progressive commitment to the NK cell lineage. Several NK-committed developmental intermediates have been identified.[22-27] Experimental evidence suggests that a fraction of NK cell precursors (NKP) traffic from the BM to other tissues where they undergo terminal differentiation.[28,29] NKP have been identified in thymus, lymph nodes, tonsils and decidua.[16,30-32] In mouse, the earliest committed NKP is characterized by the expression of the IL-2 receptor β-chain (CD122) and the lack of lineage markers, including CD3, CD19, Ter119, Gr-1 (=Lineage negative, Lin-).[33,34] The acquisition of CD122 on Lin- HSC corresponds with their commitment to the NK cell lineage.[35] NKP progressively acquire the phenotypic and functional

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Correspondence: lorenzomoretta@ospedale-gaslini.ge.it

properties of mature NK cells. Various markers allow the identification of different stages of NK cell maturation. The first to appear are NKG2D and NK1.1, followed by NKp46, CD94/NKG2A/C/E, CD27, DX5, Ly49 receptors and CD11b.[28,36] Recent studies have suggested that CD27 and CD11b identify 4 consecutive maturation stages, namely: $CD27^{low}CD11b^{low}$ (stage I), $CD27^{high}CD11bl^{low}$ (stage II), $CD27^{high}CD11b^{high}$ (stage III) and $CD27^{low}CD11b^{high}$ (stage IV).[37] The first 2 stages are found mostly in the BM, lymph nodes and liver, and display a high rate of homeostatic proliferation. Stages III and IV are prevalent in peripheral sites, such as spleen, lung and peripheral blood. $CD27^{high}CD11b^{high}$ NK cells (stage III) express intermediate levels of the Ly49 receptors while $CD27^{low}CD11b^{high}$ (stage IV) express high levels and correspond to terminally differentiated NK cells.[37] A marker of mouse uterine NK cells is Dolichus biflores agglutinin (DBA).[38] DBA expression increases during mid-gestation (second week) and is confined to a limited fraction of uterine NK cells ($CD3^-CD122^+NK1.1^-$).[39] Previous studies in mice used DBA to identify uterine NK cells, thus ignoring the sizable fraction of $CD3^-CD122^+NK1.1^+$ cells during early and mid-gestation.[39] A recent report suggested that mouse uNK cells are heterogeneous and considering only DBA^+ uNK cells could bias information on the uNK cell population.[40] Since studies in humans clearly indicated that NK cells represent the most frequent lymphoid cell population during early pregnancy (first trimester), playing a fundamental role in the establishment and maintenance of pregnancy, it would be important to gain more information in mice.[12,18,41,42]

In the present study, for the first time, murine NK cells were analyzed separately in decidua and uterus. We found that high proportions of immature NK cells are present both in decidua and uterus during the first week of pregnancy. In addition, we identified NK-committed hematopoietic precursors (Lin^-CD122^+) in decidua and uterus of pregnant mice. Transfer experiments of peripheral $EGFP^+$-NK cells indicated that these cells are not recruited mainly into decidua and uterus, thus do not contribute to the accumulation of dNK and uNK cells during early pregnancy. In contrast, $EGFP^+$-NKP transferred into pregnant mice rapidly migrated into decidua and uterus where they underwent proliferation and differentiation towards mature NK cells. Immature dNK and uNK display phenotypic and functional features similar to those previously described in humans. These data provide important information regarding the biology of NK cells in pregnancy, and identify novel tissues (decidua and uterus) able to sustain peripheral NK cell differentiation *in vivo*.

Methods

C57BL/6 and RAG-$2^{-/-}$ mice were purchased from Charles River. Transplant donors were EGFP+ transgenic mice ((C57BL/6-Tg(ACTB-EGFP)1 Osb/J mice 5 (GFP-Tg)). Cells derived from spleen, decidua, uterus and BM were incubated with different mAbs and run on a flow cytometer. $EGFP^+$ NK cells and NKP were injected intravenously into unirradiated wild-type syngeneic mice at gestational day (gd) 0,5. The presence and the phenotype of the transferred $EGFP^+$ cells were analyzed in the different tissues at gd 3,5-5,5-7,5 by flow cytometry. Mice were given one i.p. injection of 1 mg of 5-bromo-2'-deoxyuridine (BrdU). After 18 h, mice were sacrificed and organs were analyzed. Frozen sections of pregnant uteri were stained with anti-NKp46 (R & D Systems, Minneapolis, MN, USA) and anti-Ki-67 (Abcam, Cambridge, UK) followed by Alexa 594 chicken anti-goat and Alexa 488 goat anti-rabbit (Life Technologies, CA, USA). The nuclei were counterstained with DAPI. Cells derived from decidua, uterus and spleen of pregnant mice at gd 5.5 were stimulated during 4 h-culture with the different antibodies, or with YAC-1 mouse lymphoma cells (effector/target ratio 1:1), or with a combination of phorbol myristate acetate (PMA) and ionomycin (IONO). Cells were analyzed by flow cytometry and supernatants were tested by ELISA (VEGF-A Platinum ELISA, eBioscience) or by MagPix (Luminex, The Netherlands).

Statistical analysis

The unpaired two-tailed Student's t-test and one-way ANOVA analysis of variance followed by post-test for linear trend were used.

Results

Natural killer cells are abundant both in decidua and uterus during early pregnancy

Pregnant mice were sacrificed at different gd and the uteri containing implants (embryo+decidua) were collected. At gd 5,5, the entire implants were separated from uterus and processed. Starting from gd 9,5 it was possible to separate decidual tissues from the embryo (*Online Supplementary Figure S1*).

During development of murine NK cells, NK1.1 is acquired at an early stage.[28,36] In order to identify NK cells during the early phase of pregnancy (first week in mice), we analyzed the NK1.1 expression in cells gated as $CD3^-CD122^+$ derived from the implant (referred to as decidua) or uterus, at different gd. Spleen was used as control. Decidua was first analyzed at gd 4,5 when NK1.1+ cells were 28±2% of the total lymphoid cells (Figure 1). At gd 5,5, dNK cells decreased (21±2%) and they were extremely rare at gd 14.5 (2±1%). In uterus of virgin mice, NK cells were 20±2% of the total lymphoid cells. In pregnant mice, at gd 3,5 the proportions of uNK cells increased to 34±3%, remained high until gd 5,5 (32±2%) and after gd 7,5 (23±3%) progressively decreased to reach 12±2% at gd 14,5. The percentages of spleen NK (sNK) cells from pregnant and virgin mice displayed no significant differences (Figure 1). We also analyzed the proportions of T ($CD3^+NK1.1^-$) and NKT ($CD3^+NK1.1^+$) cells from the same mice. No significant variations were detected during pregnancy in any tissues (*Online Supplementary Figure S2*). Taken together, these data provide clear evidence that NK cells are present in high proportions in murine decidua and uterus at the early phase of gestation and that subsequently they progressively decrease.

dNK and uNK do not derive from peripheral natural killer cells but from hematopoietic precursors recruited in situ

The origin of dNK and uNK cells is still poorly defined: they could be recruited from the periphery or arise from progenitors that develop *in situ*. In order to assess the possible recruitment of peripheral NK cells into decidua and uterus, we isolated NK cells from spleens of $EGFP^+$ virgin mice. These cells were injected into syngeneic WT mice at gd 0,5 of pregnancy. The recruitment of $EGFP^+$-NK cells in

different tissues was analyzed at different gd. Both in pregnant and virgin mice, EGFP+-NK cells were mainly localized in the spleen and lymph nodes rather than in decidua and uterus (Figure 2A). Thus, no substantial homing of peripheral NK cells to uterus was detectable in pregnant mice. However, the presence of transferred EGFP+-NK cells in uterus-draining lymph nodes suggested that peripheral NK cells may traffic, but do not stay, in uterine tissues. Results also show that the homing capacity of NK cells to spleen and lymph nodes does not differ between pregnant and virgin mice. Similar results were obtained when recipient mice were depleted with anti-NK1.1 mAb (*data not shown*), suggesting that the presence of resident NK cells in decidua and uterus does not affect the recruitment of peripheral NK cells. These data indicate that dNK and uNK cells that accumulate in decidua and uterus during early pregnancy do not derive from peripheral NK cells.

Another possible explanation for the origin of dNK and uNK cells is that they undergo *in situ* differentiation from precursors. Thus, we transferred Lin-CD122+ NKP isolated from the BM of EGFP+ mice into syngeneic WT mice at gd 0.5. The EGFP+ cells recovered in all organs were CD3-NK1.1+ (at gd 7.5) (Figure 2B). No EGFP+ cells were detected in the uterus of virgin mice, used as control (*data not shown*). In addition, we analyzed the presence of Lin-CD122+ NKP in decidua and uterus. High amounts of NKP were present in decidua and uterus of pregnant mice, thus suggesting that dNK and uNK cells derived from hematopoietic progenitors recruited when pregnancy was established (*Online Supplementary Figure S3*).

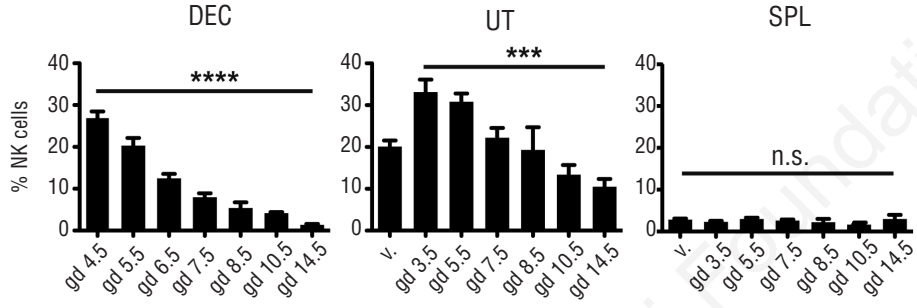

Figure 1. Identification and kinetics of murine NK cells in decidua and uterus during early pregnancy. Percentages of NK cells identified as CD3-CD122+NK1.1+ cells in decidua, uterus and spleen from virgin (v.) and pregnant mice at the indicated gd. A one-way analysis of variance test with post-test of linear trend was used. Results show the mean ± SEM. N=10 mice per group. n.s.: not significant; ***$P<0.001$; ****$P<0.0001$. DEC: decidua; UT: uterus; SPL: spleen.

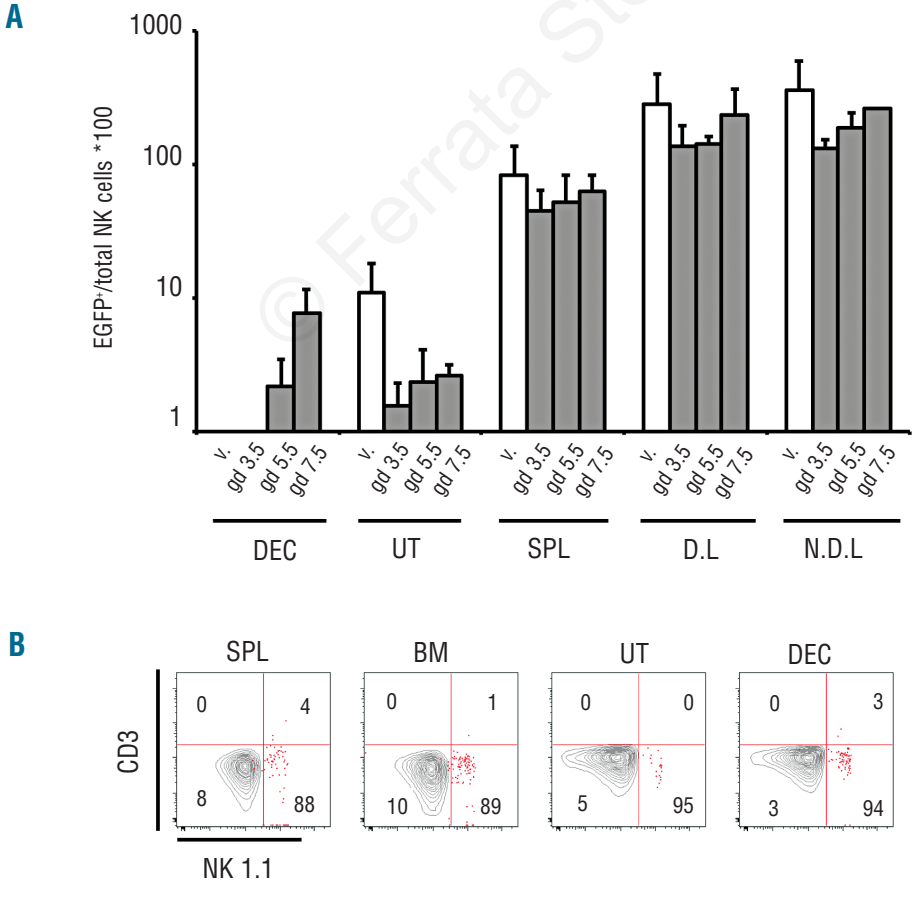

Figure 2. Trafficking of peripheral NK cells and NKP during early pregnancy. (A) Peripheral EGFP+-NK cells (CD3-CD122+ NK1.1+) were isolated from the spleen of virgin mice and injected in syngeneic WT mice at gd 0.5. Histogram represents percentages of EGFP+-NK cells recovered in decidua, uterus, spleen, uterus-draining lymph node (DR. LN.) and not draining lymph node (NOT DR. LN.) of pregnant mice at the indicated gd, normalized with the percentage of the total NK cells per organ (EGFP+/total NK cells *100). Virgin (v.) mice were used as control (white bars). Results show the mean±SEM. N= 4 mice per group. (B) NKP (Lin-CD122+) were isolated from the BM of EGFP+ mice and injected in syngeneic WT mice at gd 0.5. At gd 7.5 the presence and the phenotype of EGFP+-cells were analyzed in the different organs. Red dot plots represent the phenotype of the transferred EGFP+ cells, overlayed with the negative control (contour plot, gray). Numbers refer to dot plots and indicate the percentages of transferred EGFP+ cells in the different quadrants. One representative experiment out of 3.

Presence of immature natural killer cells in decidua and uterus

In order to verify the NK cell differentiation in decidua and uterus, different markers acquired during NK cell maturation were analyzed.[36,43] The expression of NK1.1 and DX5 allows the identification of 4 subsets: NK1.1$^-$DX5$^-$, NK1.1$^+$DX5$^-$, NK1.1$^+$DX5$^+$ and NK1.1$^-$DX5$^+$ (Figure 3A). A previous study reported that NK1.1$^-$DX5$^-$ uterine NK cells derived from pregnant mice selectively express the DBA lectin.[39] Thus, we analyzed the expression of DBA by NK1.1$^-$DX5$^-$ NK cells at different gd (5.5–9.5–14.5) in decidua and uterus separately (*Online Supplementary Figure S4*). It should be noted that the NK1.1$^-$DX5$^-$ NK cells represent only a minor fraction of the total CD3$^-$CD122$^+$ NK cells in all tissues analyzed (Figure 3A). Therefore, DBA expression does not comprehensively identify the NK cell pool present in decidua and uterus during early pregnancy. On the other hand, the majority of CD3$^-$CD122$^+$ NK cells expressed NK1.1. Notably, although not shown, most of NK1.1$^+$ cells also expressed NKp46.[36] A large population of NK1.1$^+$ cells (expressing or not expressing DX5) was already present as early as gd 5.5 in decidua and uterus (Figure 3A). Therefore, we focused on NK1.1$^+$DX5$^-$ and NK1.1$^+$DX5$^+$ subsets. We first analyzed their percentages during gestation (Figure 3B). The results show that the more mature NK1.1$^+$DX5$^+$ NK cells are abundant in all organs analyzed (decidua, uterus, BM and spleen) and displayed minor variations during pregnancy. On the other hand, the more immature NK1.1$^+$DX5$^-$ NK cells were mainly present in decidua and uterus. The percentage of these cells in decidua was 28±6% at gd 5.5 and decreased during the second week of pregnancy. In uterus, the NK1.1$^+$DX5$^-$ NK cell subset was already present in virgin mice and did not significantly change during pregnancy. These data indicate that during early pregnancy decidua and uterus contain large proportions of immature NK cells.

Natural killer cells undergo maturation in decidua and uterus during early pregnancy

In order to better characterize CD3$^-$CD122$^+$NK1.1$^+$ NK cells in decidua and uterus, we analyzed the expression of CD27 and CD11b, two informative markers of NK cell maturation, during pregnancy (Figure 4A). BM and spleen were included as controls. dNK cells were characterized by an immature phenotype (with prevalence of CD27^{high}CD11b^{low}, stage II) in early pregnancy (gd 4.5–gd 5.5). Notably, at gd 4.5–gd 5.5 stage II was more represented in decidua than in BM-NK cells. After gd 5.5, a rapid NK cell maturation occurred in decidua, as CD27^{high}CD11b^{high} (stage III) and CD27^{low}CD11b^{high} (stage IV) had substantially increased. This process continued until gd 8,5 when no further changes were observed. uNK cells, similar to sNK cells, were mostly represented by stage IV. There were no differences between pregnant and virgin mice, and there was no variation in the maturation level of uNK and sNK during gestation. Also in the BM,

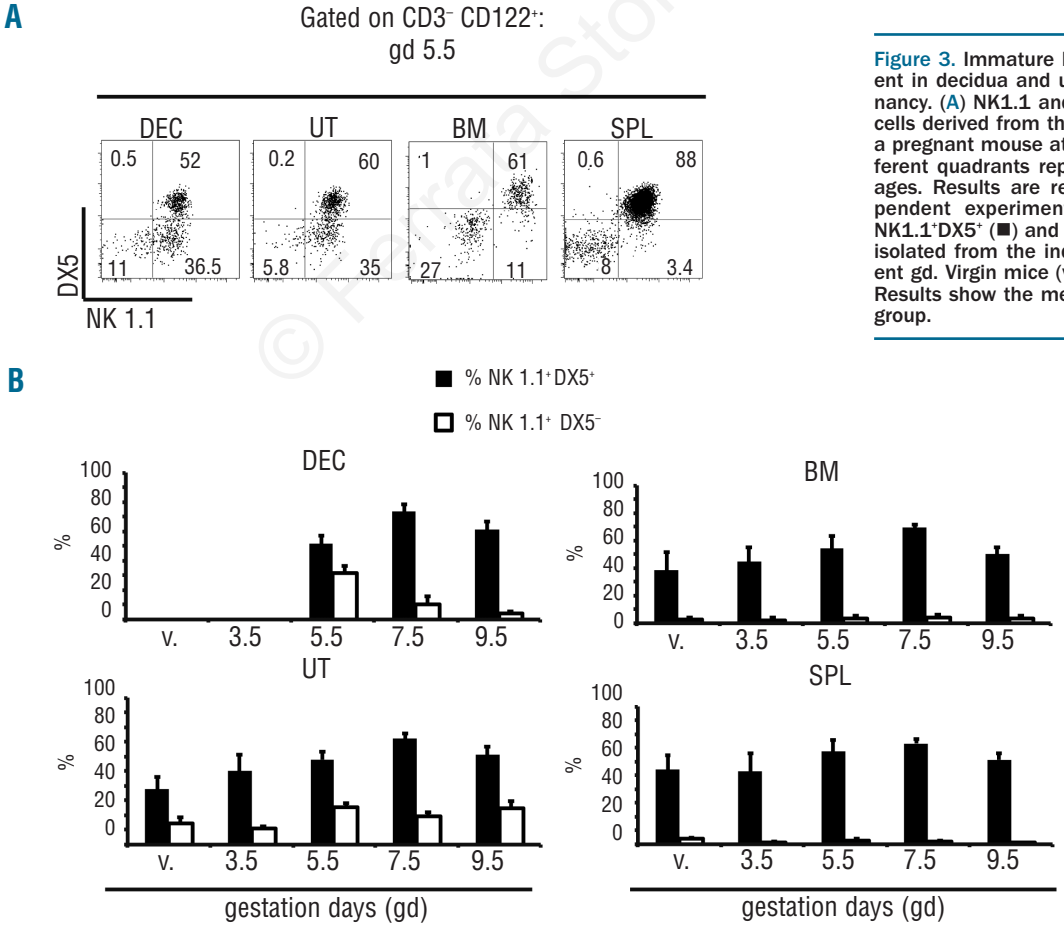

Figure 3. Immature NK cells are highly present in decidua and uterus during early pregnancy. (A) NK1.1 and DX5 expression by NK cells derived from the indicated organs from a pregnant mouse at gd 5.5. Numbers in different quadrants represent the cell percentages. Results are representative of 5 independent experiments. (B) Percentages of NK1.1$^+$DX5$^+$ (■) and NK1.1$^+$DX5$^-$ (□) NK cells isolated from the indicated organs at different gd. Virgin mice (v.) were used as control. Results show the mean±SEM. N=5 mice per group.

NK cell maturation level did not change during pregnancy. Representative cytofluorimetric analysis at gd 5.5 is shown in *Online Supplementary Figure S5*. Taken together, these data suggest that NK cells undergo differentiation in decidua during early pregnancy. In order to further document the occurrence of *in situ* differentiation of dNK and uNK cells, we also analyzed the stage of maturation of adoptively transferred EGFP$^+$-peripheral NK cells (Figure 2) that had been recruited in the different organs during gestation (Figure 4B). Analysis of CD27 and CD11b expression revealed that the few EGFP$^+$-NK cells recruited into uterus at gd 3.5 displayed an immature phenotype (high percentages of CD27^{high}CD11b^{low} and CD27^{high}CD11b^{high}). These cells underwent rapid maturation since, at gd 5.5 and 7.5, EGFP$^+$-NK cells in uterus were mostly stage IV. Remarkably, also EGFP$^+$-NK cells present in the uterus of virgin mice displayed a mature phenotype. In lymph nodes, EGFP$^+$-NK cells were mostly stage III, with no differences between pregnant and virgin mice. Taken together, these results clearly indicate that NK cells can undergo rapid *in situ* maturation both in decidua and uterus during early pregnancy.

Natural killer cells undergo intensive proliferation in decidua and uterus in early pregnancy

The presence of high proportions of immature CD3$^-$CD122$^+$NK1.1$^+$ NK cells in decidua and uterus during the early phase of gestation may reflect an intensive NK cell proliferation. Thus, we comparatively analyzed the spontaneous NK cell proliferation in different tissues during pregnancy. To this end, mice at different gd were injected with BrdU. After 18 h, BrdU incorporation was measured in NK cells isolated from decidua, uterus, BM and spleen. Virgin mice were used as controls. dNK cells displayed the highest proliferation rate (Figure 5A). Indeed, they showed a peak of proliferation at gd 5.5 (13±5%), then the proportion of proliferating dNK cells rapidly decreased (4±1% at gd 7.5). uNK cells from virgin mice displayed a low proliferation rate (2.5±1%) that increased after pregnancy was established to reach maximal levels at gd 5.5 (9±3%). Similar to dNK cells, proliferation of uNK cells at gd 7.5 had already decreased (2±1%) and remained low during the second week of gestation. In the BM, the percentages of proliferating NK cells increased after pregnancy was established (from 3±2% in virgin to

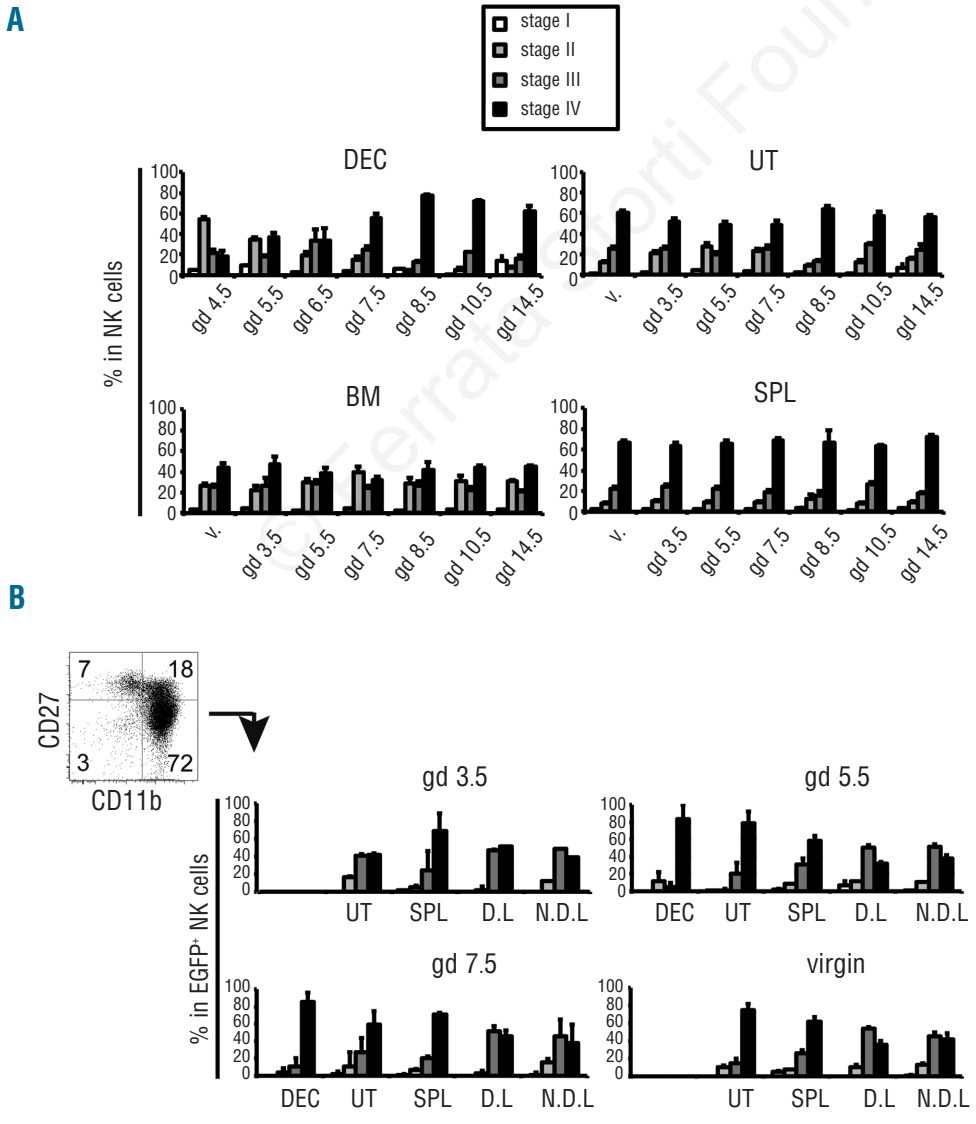

Figure 4. dNK and uNK cells undergo *in situ* maturation during early pregnancy. (A) Percentages of CD27^{low}CD11b^{low} (stage I), CD27^{high}CD11b^{low} (stage II), CD27^{high}CD11b^{high} (stage III) and CD27^{low}CD11b^{high} (stage IV) NK cells from the indicated organs from virgin (v.) and pregnant mice at different gd. Results show the mean ± SEM. N=10 mice per group. (B) Dot plot shows CD27 and CD11b expression in the peripheral EGFP$^+$-NK cells (CD3$^-$CD122$^+$ NK1.1$^+$) injected in syngeneic WT mice at gd 0.5. Percentages of CD27^{low}CD11b^{low} (stage I), CD27^{high}CD11b^{low} (stage II), CD27^{high}CD11b^{high} (stage III) and CD27^{low}CD11b^{high} (stage IV) in EGFP$^+$-NK cells recruited in the indicated organs from virgin (v.) and pregnant mice at different gd. DR. LN.: uterus-draining lymph node; NOT DR. LN.: not draining lymph node. Results show the mean±SEM. N=3 mice per group.

9±4% at gd 3.5) and remained stable through gd 14.5. On the other hand, in the spleen of virgin mice, the percentage of proliferating NK cells was low (3±1%) and did not vary during pregnancy (Figure 5A). In addition, we comparatively analyzed the proliferation rate of T and NKT cells derived from decidua, uterus, BM and spleen. Both cell types displayed a low proliferation rate, which was not significantly modified during pregnancy (*Online Supplementary Figure S6*). Proliferation of NK cells was confirmed by Ki-67 staining on frozen sections derived from pregnant *uteri* containing implants (Figure 5B).

IL-15 is the main cytokine involved in the NK differentiation/proliferation, thus we analyzed the expression of membrane-bound IL-15 (mIL-15) on decidual stromal cells (DSC). DSC expressed high levels of mIL-15, while stromal cells derived from BM were negative (Figure 5C).

Immature natural killer cells from decidua and uterus express high levels of Ly49D receptor

We further analyzed CD3-CD122^{+}NK1.1^{+} NK cells derived from decidua and uterus at gd 5.5 for the expression of informative markers (Figure 6A). The expression of NKp46, NKG2D, CD69, granzyme B and NKG2A/C/E in dNK and uNK was similar to that of NK cells isolated from BM and spleen at gd 5.5. We also analyzed the MHC-specific Ly49 receptors, which are acquired during NK cell maturation. sNK cells expressed high levels of Ly49C/I and Ly49G2, in agreement with their advanced maturation stage. BM-NK cells expressed low percentages of Ly49C/I and Ly49G2, in line with their partially immature phenotype. An intermediate level of expression of these receptors was detected in dNK and uNK cells. Notably, the activating receptor Ly49D was expressed in higher percentages in dNK cells as compared to NK cells isolated from the other tissues.

In addition, the expression of Ly49C/I, Ly49G2 and Ly49D was analyzed in the 4 NK cell subsets defined by CD27 and CD11b expression (Figure 6B). In all tissues, the percentages of Ly49C/I were low in CD27^{low}CD11b^{low} (stage I) and progressively increased in the other maturation stages. A similar increase was observed for Ly49G2 in uterus-, BM- and spleen-NK cells. In contrast, in decidua, the expression of Ly49G2 was similar in all subsets. Ly49D, in BM-NK and sNK cells, was more expressed at stage III and IV, while in dNK and uNK cells it was more expressed in the immature subsets (stages I and II). Ly49 receptor expression was also assessed in NK1.1^{+}DX5^{-} and NK1.1^{+}DX5^{+} NK cell populations derived from the different tissues of pregnant mice at gd 5.5 (*Online Supplementary Figure S7*). This analysis confirmed that dNK and uNK expressed higher levels of Ly49D receptor, as compared to BM-NK and sNK cells. Notably, Ly49D was highly present even on the more immature NK1.1^{+}DX5^{-} NK cell population. These data provide evidence that Ly49D is already

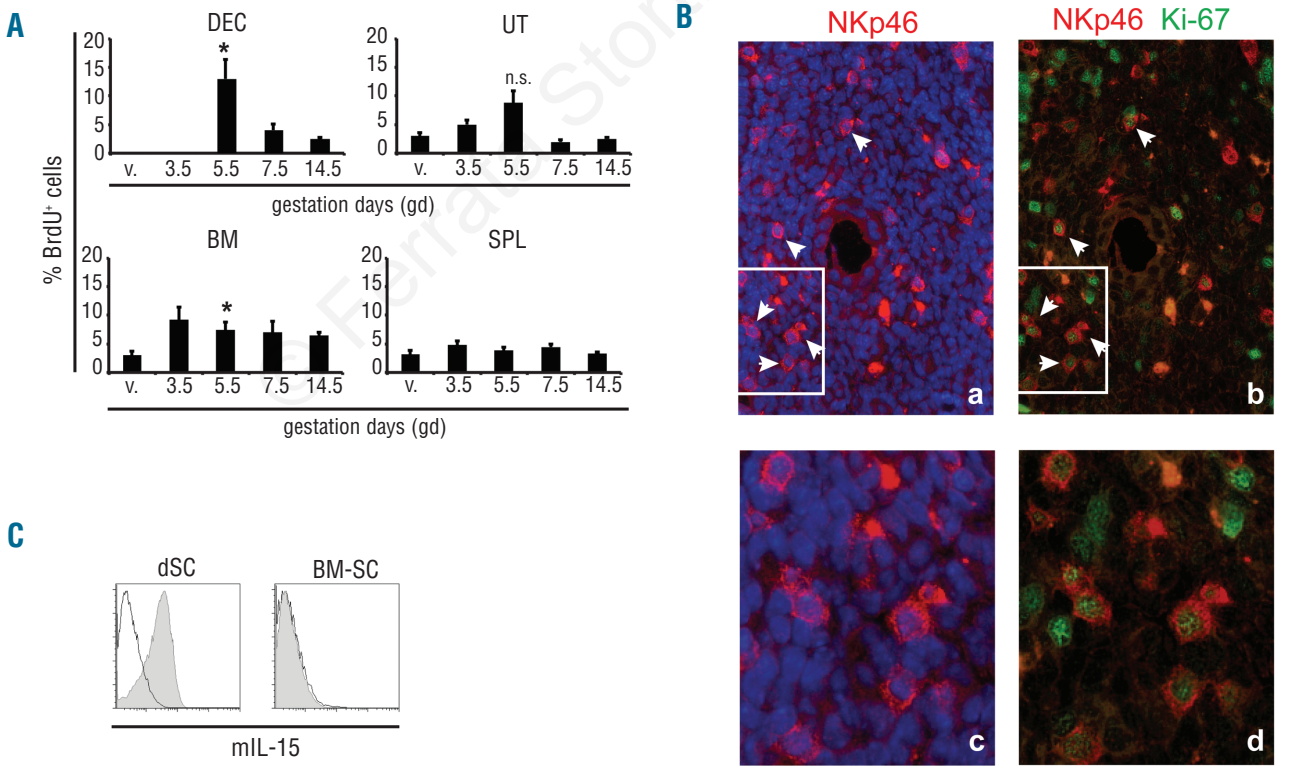

Figure 5. High rate of NK cells proliferation during early pregnancy. (A) Percentages of proliferating (BrdU^{+}) NK cells (CD3^{-}CD122^{+}NK1.1^{+}) from virgin (v.) and pregnant mice at different gd. Unpaired two-tailed Student's t-test was used to compare BrdU^{+} cells from decidua, uterus and BM *versus* BrdU^{+} cells from spleen at gd 5.5. Results show the mean±SEM of BrdU^{+} cells. N=9 mice per group. n.s.: not significant; *P<0.05. (B) Ki-67 (green) and NKp46 (red) staining on frozen sections of pregnant uterus containing implants. The white arrows indicate proliferating NK cells (magnification 40x). Panels c and d represent a magnification of white squares (C) mIL-15 expression (gray profiles) on stromal cells derived from decidua and BM. Empty profiles represent isotypic control.

expressed at the earliest stages of NK cell differentiation both in decidua and uterus.

dNK and uNK display regulatory rather than cytotoxic functions

$CD3^-CD122^+NK1.1^+$ NK cells derived from decidua, uterus and spleen at gd 5.5 were analyzed for their cytolytic potential using the degranulation assay based on CD107a surface expression. In parallel, these cells were tested for the IFN-γ production. In these assays, cells were stimulated either with monoclonal antibodies (mAbs) directed against NK1.1, NKG2D and Ly49D receptors or using the NK-sensitive target cell YAC-1 or PMA-IONO. dNK and uNK cells displayed much lower CD107a expression and intracytoplasmic IFN-γ than sNK cells upon triggering with all stimuli (Figure 7). In order to understand the function of dNK during early pregnancy, different cytokines/chemokines were analyzed in supernatants of $CD3^-CD122^+NK1.1^+$ NK cells isolated from RAG-$2^{-/-}$ mice at gd 5.5. Upon 18-h stimulation with the stimuli mentioned above, dNK cells isolated from pregnant mice at gd 5.5 were able to release VEGF, GM-CSF and IP10 (*Online Supplementary Figure S8*). Taken together, these results suggest that dNK and uNK cells are characterized by a low cytolytic potential and produced soluble factors involved in tissue remodeling and neoangiogenesis.

Discussion

Natural killer cells are important for the establishment and maintenance of pregnancy.[17-21] However, information

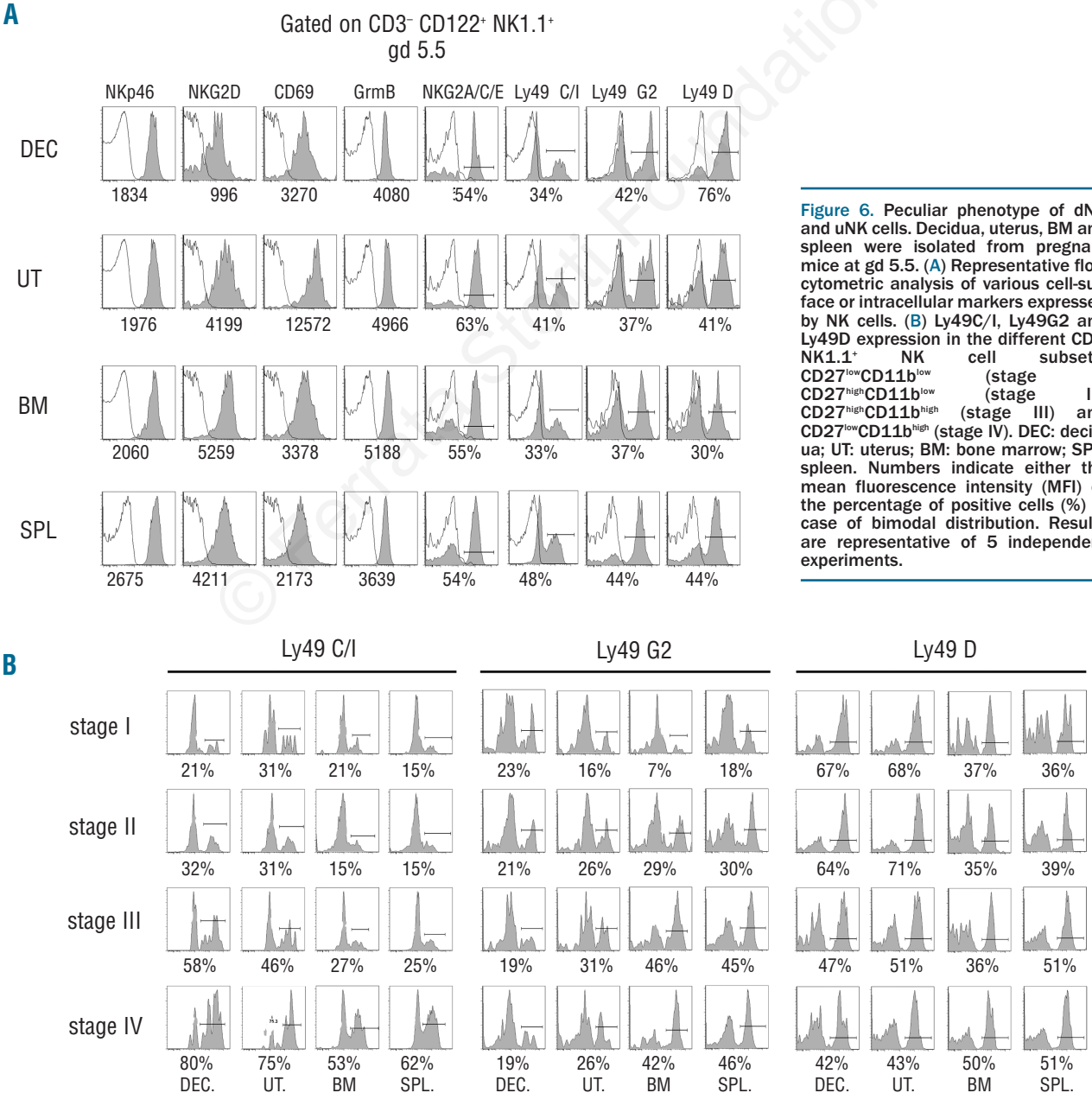

Figure 6. Peculiar phenotype of dNK and uNK cells. Decidua, uterus, BM and spleen were isolated from pregnant mice at gd 5.5. (A) Representative flow cytometric analysis of various cell-surface or intracellular markers expressed by NK cells. (B) Ly49C/I, Ly49G2 and Ly49D expression in the different $CD3^-NK1.1^+$ NK cell subsets: $CD27^{low}CD11b^{low}$ (stage I), $CD27^{high}CD11b^{low}$ (stage II), $CD27^{high}CD11b^{high}$ (stage III) and $CD27^{low}CD11b^{high}$ (stage IV). DEC: decidua; UT: uterus; BM: bone marrow; SPL: spleen. Numbers indicate either the mean fluorescence intensity (MFI) or the percentage of positive cells (%) in case of bimodal distribution. Results are representative of 5 independent experiments.

on the origin and phenotype of decidual and uterine NK cells is still limited.[39,41,42,44] In the present study, we analyzed murine dNK and uNK cells starting as early as gd 3.5 by using markers expressed since the initial stages of NK cell differentiation. We showed that, during early pregnancy, large proportions of NK cells were present in decidua and uterus. Adoptive transfer of peripheral NK cells revealed that only a minority of these cells was recruited in decidua and uterus, while NKP efficiently migrated in these organs where they underwent differentiation generating dNK and uNK. Moreover, NK-committed hematopoietic precursors were abundantly present in decidua and uterus of pregnant mice. These cells underwent rapid *in situ* proliferation and differentiation, thus generating dNK and uNK cells with poor cytotoxic function but able to produce soluble factors involved in the maintenance of pregnancy. Despite their immature phenotype, dNK and uNK cells expressed high levels of the activating Ly49D receptor, thus suggesting that a peculiar NK developmental pathway may occur in decidua and uterus.

Previous studies in mice have been focused on the analysis of NK cells present in pregnant uterus using DBA, a surface marker specifically expressed by uterine NK cells. However, DBA is acquired by a small percentage of uterine NK cells and its expression increases during pregnancy.[39,45,46] This led to the notion that mouse uterine NK cell numbers peak at mid-gestation, thus differing from what has been described in humans.[11] In our present study, we analyzed dNK and uNK cells by using NK1.1 and DX5 markers. NK1.1 is acquired very early during NK cell differentiation, while DX5 identifies the majority of mature NK cells. The use of NK1.1 and the possibility of analyzing decidua and uterus separately allowed us to identify and characterize NK cells isolated from these tissues since the early phase of gestation. We showed that NK cells were present in large proportions in both tissues immediately after implantation, while they subsequently decreased. Moreover, we provided evidence that the immature NK1.1^{+}DX5^{-} NK cells were present in high proportions both in decidua and uterus.

The experiments of adoptive transfer suggested that the NK cells present in decidua and uterus during the early phase of gestation did not derive from peripheral NK cells recruited when pregnancy was established. Notably, we showed that peripheral NK cells can traffic through the uterine tissues, as they were found in uterus-draining lymph nodes. However, only a minority remained in uterus and decidua. Moreover, the expression of CD27 and CD11b revealed that the few peripheral NK cells recruited into uterus at gd 3.5 displayed an immature phenotype, thus suggesting that the homing to uterine tissues is confined to immature NK cells. More strikingly, transferred-NKP efficiently migrated to decidua and uterus where they differentiated towards NK cells. Thus, it is conceivable that dNK and uNK cells may develop *in situ*. Previous studies in humans revealed the presence of CD34^{+} hematopoietic precursors in endometrial and decidual tissues that could give rise to mature NK cells when cultured *in vitro*.[15,16] On the contrary, graft experiments in mice suggested that uNK cells were derived from the recruitment of BM-precursors.[47] However, the formal evidence that precursors can differentiate into mature dNK and uNK cells *in vivo* was still lacking. In the present study, we showed that Lin^{-}CD122^{+} hematopoietic precursors were present in decidua and uterus during early pregnancy. These cells were able to give rise to immature CD27^{high}CD11b^{low} NK cells (stage II) that undergo intensive proliferation. Interestingly, at gd 5.5, proliferation of NK cells was higher in decidua than in the other organs. Subsequently, dNK cells undergo rapid maturation *in situ*. Taken together, these data indicate that the NK cell differentiation can occur in decidua, as previously described for other peripheral sites, such as inflamed lymph nodes, tonsils, thymus and liver.[8,28]

Studies in humans have been mostly focused on NK cells isolated from decidual tissues during the first trimester (i.e. early pregnancy). These studies revealed that dNK cells release a number of cytokines/chemokines involved in tissue remodeling and neoangiogenesis rather than pro-inflammatory cytokines (IFN-γ and TNF-α).[12,17-19,48] In addition, human dNK cells were poorly cytolytic, in spite of their relevant content of perforin and granzymes. They also displayed a peculiar surface phenotype, as they were CD56^{bright} (typical of immature NK cells), and KIRs^{+} (normally confined to mature NK cells). It has been shown that human dNK cells express both inhibitory and activating KIRs specific for HLA-C molecules that are present at the trophoblast cell surface during the first trimester. The

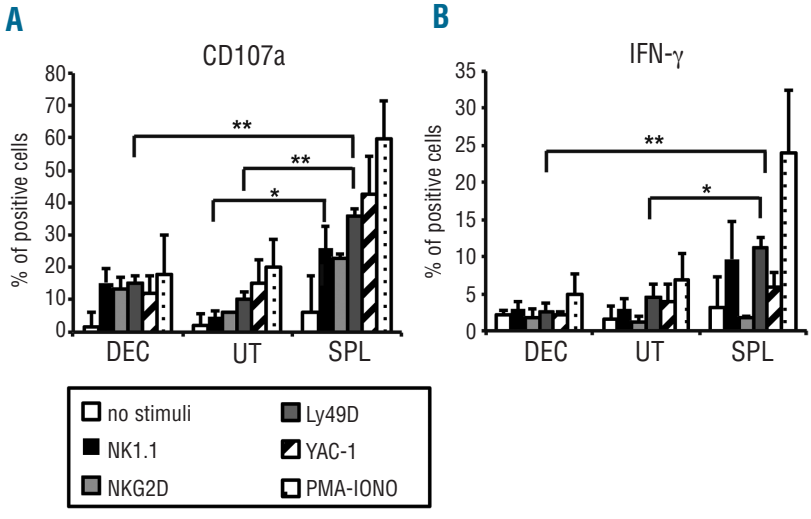

Figure 7. Cytotolytic activity and IFN-γ production by dNK and uNK cells in early pregnancy. (A) Percentages of CD107a^{+} NK cells upon 4h triggering with the indicated stimuli. (B) Percentages of IFN-γ^{+} NK cells upon 4h triggering with the same stimuli. Results show the mean±SEM. N=4 mice per group. Unpaired two-tailed Student's t-test was used to compare decidua and uterus *versus* spleen for each stimulus. When not indicated, results were not statistically significant. *P<0.05; **P<0.01.

interactions between KIRs and trophoblast HLA-C molecules appear to play a relevant role for a successful pregnancy.[12] Our present data showed that murine dNK and uNK cells display phenotypic features similar to those of human dNK cells since they express high levels of the MHC-specific Ly49 receptors (i.e. the mouse equivalent of human KIRs), in particular the Ly49D activating receptor. In addition, mouse dNK cells expressed high levels of CD27, a marker that is associated with a bright expression of CD56 in humans.[49,50] Similar to humans, murine dNK cells were poorly cytolytic and produced low amounts of IFN-γ despite their high expression of the activating receptors NK1.1, NKp46 and NKG2D and high levels of granzyme B. Moreover, we showed that murine dNK cells were capable of releasing cytokines and chemokines involved in tissue remodeling and neoangiogenesis, including GM-CSF, VEGF and IP10.

Taken together, our results suggest that decidua and uterus represent novel sites capable of sustaining NK cell differentiation. It is conceivable that this particular microenvironment may play a critical role in the acquisition of the unique dNK and uNK cell phenotypic and functional characteristics.[8,14] In this context, we showed that IL-15, a key cytokine for NK cell differentiation and functional maturation, is expressed by DSC, thus providing a suitable milieu for NK cell development and proliferation. Moreover, our present characterization of mouse dNK and uNK cells highlights important similarities with humans, thus providing a useful tool for a better understanding of the events occurring in the establishment/maintenance of pregnancy and of possible causes of recurrent miscarriages.

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Authorship and Disclosures

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