

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 Otavia Barbieri,^{2,3} Cristina Bottino,^{1,2} Lorenzo Moretta,¹ and Maria Cristina Mingari^{2,3}

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

**LC and PV equally contributed to this work*

©2014 Ferrata Storti Foundation. This is an open-access paper. doi:10.3324/haematol.2013.091421

The online version of this article has a Supplementary Appendix.

Manuscript received on May 10, 2013. Manuscript accepted on October 29, 2013.

Correspondence: lorenzomoretta@ospedale-gaslini.ge.it

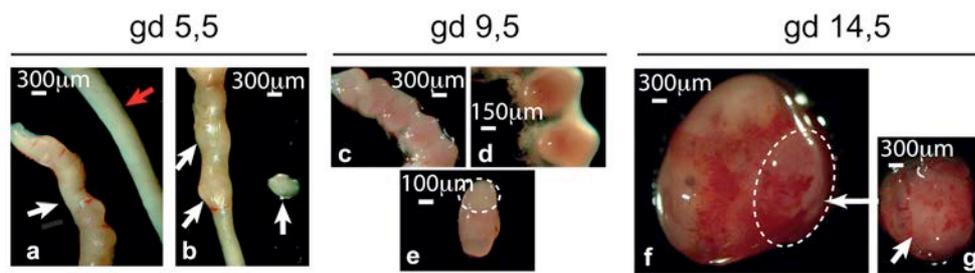


Figure 1S

Supplemental Figure 1. Images of pregnant uteri and decidua

(a): A representative image of uteri isolated at gd 5,5 from either pregnant (white arrow) or virgin mice (red arrow). **(b):** implants inside the uterus or isolated (white arrows). **(c-d):** images of pregnant uterus with implants from pregnant mice at gd 9,5. **(e):** an implant isolated from the uterus; dotted circle indicates decidua in the mesometrial pole **(f):** image of the amniotic sac containing fetus and placenta/decidua (dotted circle) at gd 14,5. **(g):** placenta/decidua area isolated from the embryo; the white layer corresponds to decidua (white arrow).

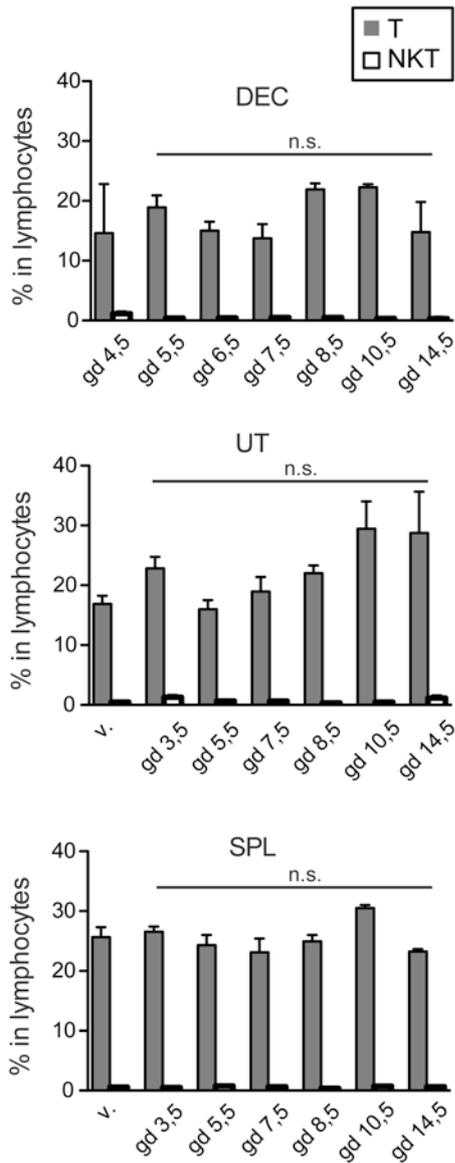


Figure 2S

Supplemental Figure 2. Kinetics of T and NKT cells during pregnancy.

Percentages of T cells (CD3⁺NK1.1⁻) and NKT cells (CD3⁺NK1.1⁺) 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. Not significant: n.s. Results show the mean±SEM. N=10 mice per group.

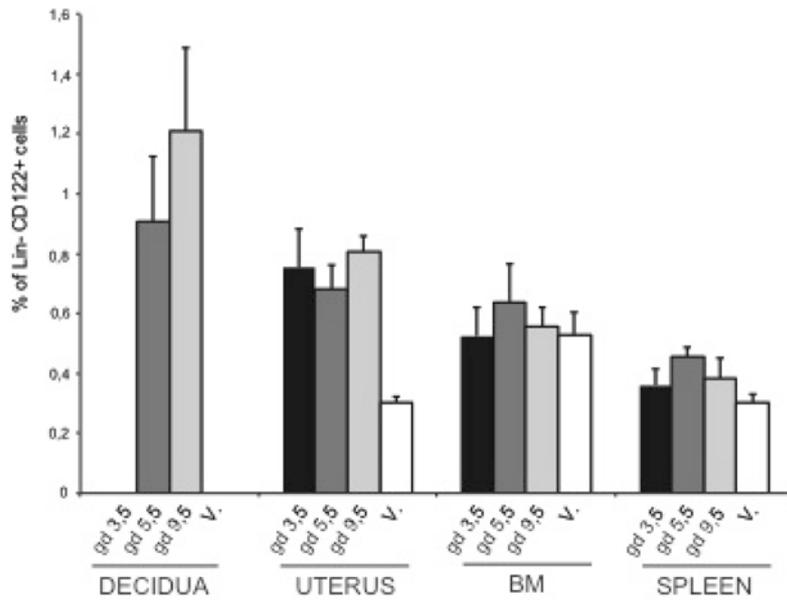


Figure 3S

Supplemental Figure 3. Presence of NK cell committed precursors in decidua and uterus of pregnant mice. Percentages of Lin⁻CD122⁺ were measured in the indicated organs at different gd.

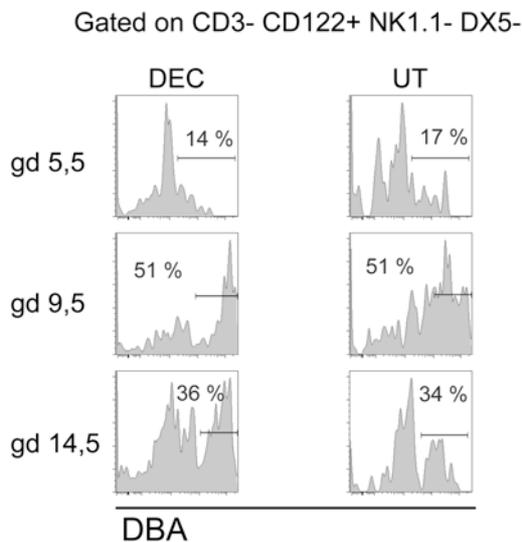


Figure 4S

Supplemental Figure 4. Expression of DBA in decidua and uterus during pregnancy.

DBA expression on CD3⁻CD122⁺NK1.1⁻DX5⁻ NK cells isolated from decidua and uterus at gd 5,5-gd 9,5-gd 14,5. Results are representative of 3 independent experiments.

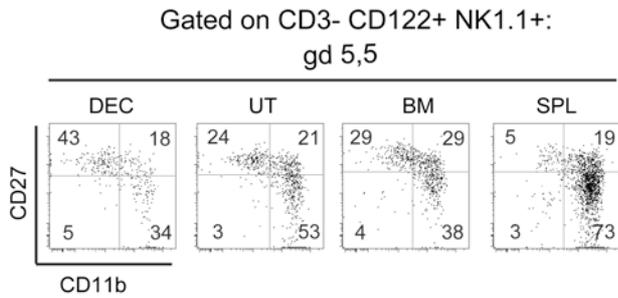


Figure 5S

Supplemental Figure 5. Comparative expression of CD27 and CD11b expression on NK cells derived from decidua, uterus, BM and spleen.

CD27 and CD11b expression by NK cells derived from decidua, uterus, BM and spleen at gd 5,5. Numbers in different quadrants represent the cell percentages. Results are representative of 10 independent experiments.

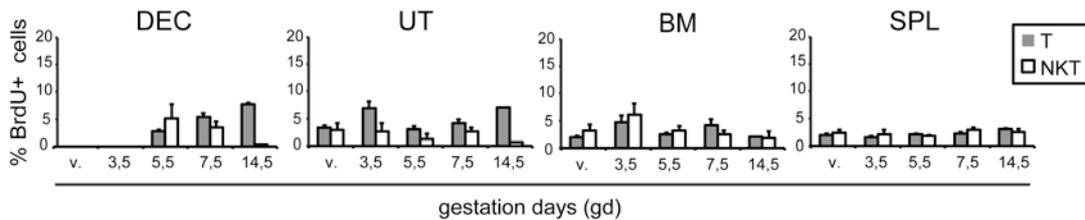


Figure 6S

Supplemental Figure 6. Proliferation of T and NKT cells during pregnancy

Percentages of proliferating (BrdU⁺) T cells (CD3⁺NK1.1⁻) and NKT cells (CD3⁺NK1.1⁺) from virgin (v.) and pregnant mice at different gd. Results show the mean±SEM of BrdU⁺ cells. N=9 mice per group.

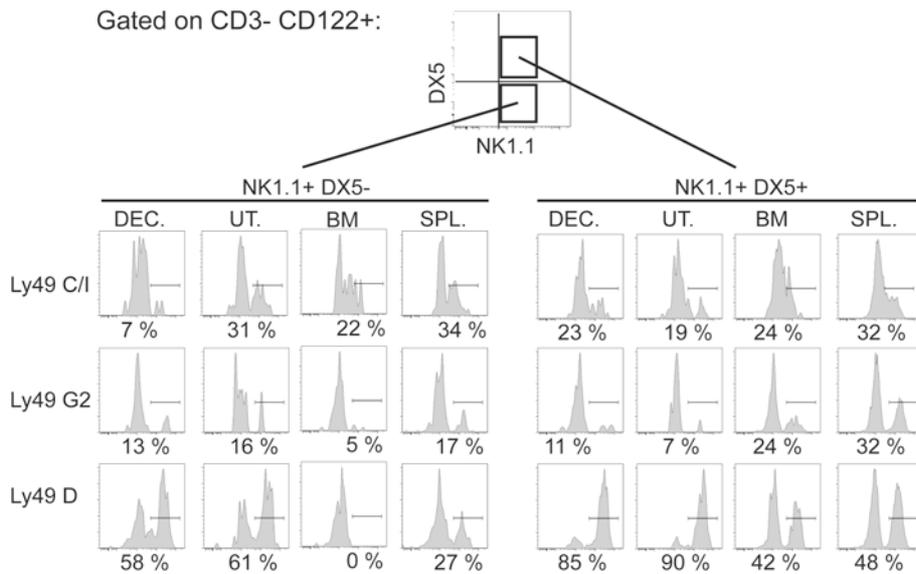


Figure 7S

Supplemental Figure 7. Expression of Ly49 receptors by DX5⁻ and DX5⁺ NK cell subsets during early pregnancy.

Representative flow cytometric analysis of NK cells derived from decidua (DEC), uterus (UT), bone marrow (BM) and spleen (SPL) of pregnant mice at gd 5,5. Numbers indicate the percentages of positive cells (%). As shown in the scheme, CD3⁻ CD122⁺ NK cells were stained with anti-NK1.1 and anti-DX5 monoclonal antibodies (mAbs) and gated into NK1.1⁺ DX5⁺ and NK1.1⁺ DX5⁻. Gated cell populations were analyzed for the expression of different Ly49 receptors. Results are representative of 5 independent experiments.

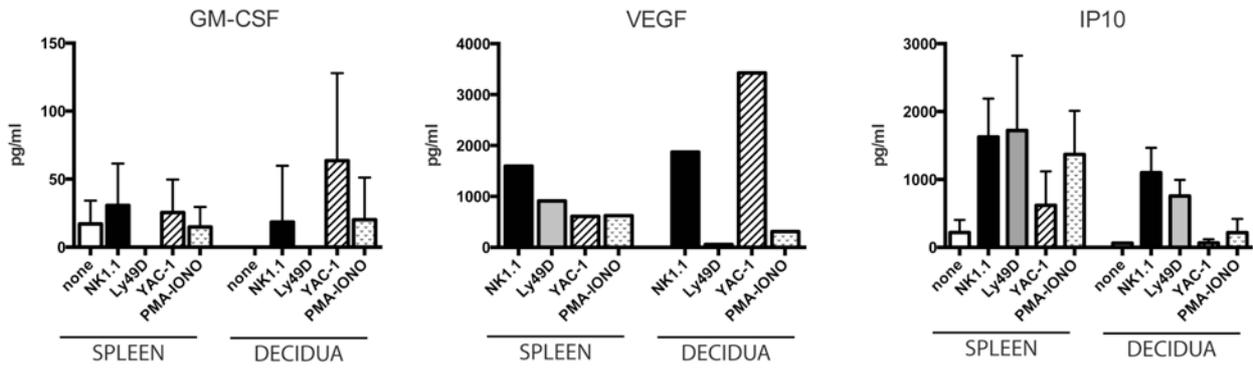


Figure 8S

Supplemental Figure 8. Cytokines/chemokines produced by NK cells during early pregnancy. GM-CSF, VEGF and IP-10 were analyzed in the supernatants derived from RAG-2^{-/-} NK cells isolated from spleen and decidua at gd 5,5 upon 18h triggering with the indicated stimuli. Results show the mean±SEM N=3 mice per group for GM-CSF and IP-10. VEGF was tested on the pool of 2 supernatant obtained from 2 different mice.

Supplemental Methods

Mice and collection of decidua and uterus tissues.

C57BL/6 and RAG-2^{-/-} mice were purchased from Charles River (Como, Italy), maintained and mated at the Animal Facility of the IRCCS San Martino-IST. Transplant donors were EGFP⁺ transgenic mice ((C57BL/6-Tg(ACTB-EGFP)1 Osb/J mice 5 (GFP-Tg)). All mice were used between 6 and 12 weeks of age. Housing and treatments of animals were in accordance with the Italian and European Community guidelines (D.L. 2711/92 No.116; 86/609/EEC Directive) and approved by the internal Ethic Committee. To time pregnant females, superovulation was induced by i.p. (intraperitoneal) injection of 5 IU of Pregnant Mares Serum (Folligon; Intervet, Italy), followed, 48 h later by 5 additional IU of hCG (Corulon; Intervet, Italy). Immediately following injection, each female was mated with a syngeneic male overnight. The morning after, females with copulation plug were separated and identified as gestation day (gd) 0,5. Mice were killed at the indicated gd by cervical dislocation. The uterus was first cleaned of surrounding fat and then explanted by cutting below the ovaries on each uterine horn and distal to the cervix. The isolated uterus containing implants was dissected using a stereomicroscope. The cervix was removed and the 2 horns were separated. At gd 3,5, the stage before blastocyst implantation, each horn was flushed with PBS by using a 1 ml syringe to let the blastocysts flow out. The presence and the quality of the blastocysts were assessed under a stereomicroscope. After flushing, only uteri containing blastocysts were analyzed as a source of uNK. At gd 4,5 when blastocysts start implanting, once verified their presence under a microscope, we got implants out using a sterile spatula to squeeze each horn. Then, we processed the entire implants as a source of dNK, since lymphoid cells present in the implant before gd 9 are of maternal origin and located in the decidual tissue. At gd 4,5 we did not analyze the uterus because it could retain implant residues and the purity of uterus-derived cells might be altered. Starting from gd 5,5 each horn of the uterus was open by a cut at the anti-mesometrial side and the implants were isolated and processed as a source of dNK. Starting from gd 9, the decidua was separated from the implant by collecting the

mesometrial pole and all deciduae derived from the same uterus were pooled together to collect dNK. Starting from gd 5,5 the whole uterine wall (once cleared out of the implants) was processed as a source of uNK. The uteri of virgin females were isolated as described above and processed entirely. Decidual and uterine tissues were mechanically disrupted.

Lymphocyte isolation and monoclonal antibodies (mAbs).

Single-cell suspensions were prepared from spleen, decidua and uterus by forcing the tissues through a 100- μ m cell strainer. Bone marrow cells were isolated by flushing femurs and tibias with PBS, using a 1ml syringe and a 25 G needle. White blood cells were isolated after lysis of red blood cells using ACK lysing buffer. For cell surface staining FcR blocking reagent (Miltenyi, Bergisch Gladbach, Germany) was added. Pacific Blue anti-mouse CD3 complex were purchased from Biolegend (San Diego, CA, USA). PE anti-mouse CD122, FITC anti-mouse Ly49C/I, Ly49G2, Ly49D and FITC anti-BrdU mAbs were from BD Pharmingen (San Diego, CA, USA). APC anti-mouse NK1.1, FITC anti-mouse CD49b (DX5), biotin anti-mouse NKG2D, biotin anti-mouse CD69, PE anti-mouse Granzyme B, biotin anti-mouse NKG2A/C/E, PE anti-mouse CD27, PE-Cy7 anti-mouse CD11b were from e-Bioscience (San Diego, CA, USA). Membrane-bound IL-15 was analyzed with biotinylated anti-mouse IL-15 (Peprotech, UK) before and after treatment with buffer pH4. Streptavidin PE and PercP-Cy5.5 from BD Pharmingen (San Diego, CA, USA) were used as a secondary reagent for biotinylated antibodies. Before intracellular staining, samples were treated with BD Cytofix/Cytoperm and Perm/Wash solutions (BD Biosciences, San Jose, USA). Samples were run on a FACS Canto II (Becton-Dickinson, San Jose, USA) or a Gallios (Beckman Coulter, Miami, FL) flow cytometers and analyzed using Flow Jo 9.4.10 software (TreeStar Inc., Ashland, OR).

Adoptive transfer of peripheral NK cells.

Lymphocytes were obtained from spleens and BM of EGFP⁺ transgenic mice. NK cells were isolated from the total splenocytes using the NK cell isolation kit and NKP from the BM-cell suspension using Lineage cell depletion kit (Miltenyi, Bergisch Gladbach, Germany) followed by sorting of CD122⁺ cells. Purity of isolated cells was more than 98% as checked by flow cytometry. For transfer experiments, 0,5-1x10⁶ cells were injected intravenously (i.v.) into unirradiated wild type (WT) syngeneic mice at 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.

Measurement of BrdU incorporation.

Mice were given one i.p. injection of 1 mg of 5-bromo-2'-deoxyuridine (BrdU) (BD Pharmingen, San Diego, CA, USA). 18 hours after, mice were sacrificed and organs harvested. Cells were stained with mAbs described above. After fixation and permeabilization, cells were treated with DNase and stained with FITC anti-BrdU mAb (BD Pharmingen, San Diego, CA, USA), according to manufacturer instructions. BrdU incorporation in the different cell populations was measured by flow cytometry.

Immunofluorescence.

Immunofluorescence was performed on 6- μ m-thick serial frozen sections. Sections were fixed with acetone before staining with goat anti-mouse NKp46, and rabbit anti-mouse Ki-67 polyclonal Abs followed by secondary chicken anti-goat IgG-alexa 594 and goat anti-rabbit IgG-alexa 488 Abs (Life Technologies). The nuclei were counterstained with DAPI. Images were captured using Apotome microscopy with AxioCam (Zeiss LSM 510, Welwyn Garden City, Hertfordshire, U.K.).

NK cell stimulation assay.

Purified mAbs directed against NK1.1, NKG2D (e-Bioscience, San Diego, CA, USA), Ly49D (BD Biosciences, San Diego, CA, USA) were allowed to bind to plastic 96-well plates (Thermo

Scientific) overnight in carbonate buffer. Cells derived from decidua, uterus and spleen of pregnant C57BL/6 and RAG-2^{-/-} mice at gd 5,5 were stimulated during 4 or 18 hours 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) (50 ng/ml; Sigma) and ionomycin (IONO) (500 ng/ml; Sigma). FITC anti-mouse CD107a and GolgiStop (BD-Biosciences) were added to cultures. At the end of the 4 hour-stimulation, C57BL/6 cells were stained with APC anti-NK1.1 (e-Bioscience) and Pacific Blue anti-mouse CD3 (Biolegend), then, following treatment with Cytofix/Cytoperm kit (BD Biosciences), PE anti-mouse IFN- γ (BD-Biosciences) was used to measure intracellular IFN- γ . Cells were then analyzed by flow cytometry. After 18 hour-stimulation supernatants derived from RAG-2^{-/-} mice were collected and tested by ELISA (VEGF-A Platinum ELISA, eBioscience) or by MagPix (Luminex, Netherlands).

Statistical analysis. Statistical analyses were performed using GraphPad Prism (GraphPad Software Inc, San Diego, CA). The unpaired two-tailed Student's t-test and one-way ANOVA analysis of variance followed by post-test for linear trend were used. n.s.: not significant. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$.