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Conversion of Peripheral Blood NK Cells to a Decidual NK-like Phenotype by a Cocktail of Defined Factors

Ana Sofia Cerdeira,∗,†,‡ Augustine Rajakumar,∗,§ Caroline M. Royle,∗ Agnes Lo,∗ Zaheed Husain,∗ Ravi I. Thadhani,‡ Vikas P. Sukhatme,* S. Ananth Karumanchi,∗,§ and Hernan D. Kopcow∗,∗∗

NK cells that populate the decidua are important regulators of normal placentation. In contrast to peripheral blood NK cells, decidual NK (dNK) cells lack cytotoxicity, secrete proangiogenic factors, and regulate trophoblast invasion. In this study we show that exposure to a combination of hypoxia, TGF-β1, and a demethylating agent results in NK cells that express killer cell Ig-like receptors, the dNK cell markers CD9 and CD49a, and a dNK pattern of chemokine receptors. These cells secrete vascular endothelial growth factor (a potent proangiogenic molecule), display reduced cytotoxicity, and promote invasion of human trophoblast cell lines. These findings have potential therapeutic applications for placental disorders associated with altered NK cell biology.

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Natural killer cells are lymphocytes of the innate immune system characterized by high cytolytic potential against virus-infected and tumor-transformed cells (1–3). In addition to their role in host defense, NK cells are proposed to play an important role in reproduction. NK cells are present in the human cycling endometrium. Their numbers augment after ovulation and during early pregnancy, becoming a prominent population of the decidua (4). By the end of the first trimester of pregnancy, decidual NK (dNK) cells account for 70% of the local lymphocytes and 30–40% of all decidual cells (5–7).

dNK cells are functionally and phenotypically different from peripheral blood NK (pNK) cells (8–10). pNK cells are commonly divided into two main subsets. CD56dimCD16+ pNK cells form the major subset that represents 90% of pNK cells. They are granular and express killer cell Ig-like receptors (KIRs), which regulate NK cell activity upon engagement with MHC class I molecules (1, 11, 12). The second and minor subset (~10% of pNK cells) is composed of CD56brightCD16− pNK cells, which are nongranular, do not express KIRs, and secrete cytokines in response to nonspecific stimuli (12, 13). In general, cytotoxic activity is associated with CD56dim pNK cells and cytokine production with CD56bright pNK cells (12, 13). However, CD56dim pNK cells are also capable of cytokine production, and CD56bright pNK cells may acquire cytotoxic capacity upon cytokine-induced activation (14–16).

Human dNK cells are a distinct NK cell subset (9). They are CD56brightCD16+, express KIRs, are granular, and have severely reduced cytotoxicity (17–19). They express markers that are not expressed by pNK cells such as CD9 (9) and CD49a (20) and they produce proangiogenic factors and trophoblast migratory factors (8, 21–25).

The abundance of NK cells in the decidua has suggested that these cells might play a role in pregnancy support and maintenance (10). Initial evidence in this direction has come from NK cell–deficient mice. NK-null mice display placental vascular anomalies, including thickening of the walls of spiral arteries with luminal narrowing (26, 27), a phenotype that is reverted upon restoration of uterine NK cell populations (26–28). In rats, NK cell depletion leads to altered trophoblast invasiveness, delayed spiral artery development, and reduced placental oxygen tensions (29). In humans, spiral artery narrowing is associated with preeclampsia and intrauterine growth restriction. Certain combinations of maternal KIR haplotypes (KIR AA) and paternal HLA-C alleles (HLA-C2) have been associated with preeclampsia, intrauterine growth restriction, and recurrent miscarriage, suggesting that dNK cells may play a role in the pathogenesis of these disorders (30, 31). Most importantly, a recent report associates impaired decidual NK cell function with high uterine artery resistance in pregnancy (25).

The induction of a dNK-like phenotype on pNK cells would be of utmost importance. It may open new venues for research on NK cell–based therapeutic approaches for preeclampsia and related disorders. In this study, we show that exposure of pNK cells to a combination of hypoxia, TGF-β1, and 5-aza-2′-deoxycytidine (Aza), a demethylating agent, yields NK cells that secrete vascular endothelial growth factor (VEGF)–A (a potent proangiogenic molecule), display reduced cytotoxicity, and promote invasion of human trophoblasts. CD56brightCD16− NK cells of these cultures express CD9, CD49a, and KIRs and display a chemokine receptor profile similar to dNK cells.

*Department of Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02215; †Gulbenkian Program for Advanced Medical Education, 1067-001 Lisbon, Portugal; ‡Department of Obstetrics and Gynecology, Centro Hospitalar do Porto, Porto University, 4050-001 Porto, Portugal; §Howard Hughes Medical Institute, Chevy Chase, MD 20815; ‡Division of Nephrology, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114; and †Harvard Stem Cell Institute, Cambridge, MA 02138

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Address correspondence and reprint requests to Dr. Hernan D. Kopcow and Dr. S. Ananth Karumanchi, Department of Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, 330 Brookline Avenue, RN359 (H.D.K.) and RN370D (S.A.K.), Boston, MA 02215. E-mail addresses: hkopcow@bidmc.harvard.edu (H.D.K.) and sananth@bidmc.harvard.edu (S.A.K.).

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Abbreviations used in this article: Aza, 5-aza-2′-deoxycytidine; dNK, decidual NK; HIF, hypoxia-inducible factor; i-dNK, induced decidual NK; KIR, killer cell Ig-like receptor; pNK, peripheral blood NK; VEGF, vascular endothelial growth factor.

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Materials and Methods

Ethical approval

Human specimens were collected according to protocols approved by the Institutional Review Board at the Beth Israel Deaconess Medical Center.

Human dNK cells and pNK cells

dNK cells were isolated from decidua basalis tissue from elective first trimester pregnancy terminations as previously described (9, 19). Specimens were washed with PBS, minced with scissors, and digested 30 min at 37˚C in 0.1% collagenase type IV and 0.01% DNAse I (both from Sigma-Aldrich, St. Louis, MO) in plain RPMI 1640 media (Invitrogen, Carlsbad CA). The digestion was stopped by the addition of an excess of RPMI 1640 media containing heat-inactivated 10% FCS. Cell suspensions were then sequentially passed through 100-, 70-, and 40-μm cell strainers (BD Falcon, San Diego, CA) washing three times with RPMI 1640 media containing 10% FCS. Decidua stromal cells and macrophages were adhered to the surface of tissue culture plates by incubation at 37˚C for 2 h in humidified incubators. Decidual lymphocyte suspensions were prepared from the overlying cell suspension by density gradient (Ficoll-Hypaque Plus from GE Healthcare, Pittsburgh, PA) and stained for analytical flow cytometry, preparative pNK cell FACs sorting, or dNK cell isolation with magnetic beads. For the generation of conditioned media, dNK cells were FACS sorted as CD56brightCD16+ cells. For cytotoxicity assays, dNK cells were resuspended with magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) by depletion of CD3+ cells and CD16+ cells and subsequent isolation of CD56+ cells.

pNK cells were enriched from discarded leukopacks using a positive enrichment Ab mixture (NK RosetteSep, Stem Cell Technologies, Vancouver, BC, Canada) following the manufacturer’s instructions. Enriched pNK cell preparations were used for establishment of NK cell cultures or stained with mAbs for analytical flow cytometry, for FACS sorting of CD3+CD56bright CD16− and CD3+CD56dimCD16+ pNK cells, or incubated with magnetic beads (Miltenyi Biotec) for NK cell isolation by depletion of CD3+ cells and subsequent isolation of CD56+ cells used in cytotoxicity assays.

Cell culture studies

All cell cultures in this study were done using IL-15 complete media (RPMI 1640 media containing 10% FCS, 1 U/ml penicillin, 100 μg/ml streptomycin, 1 mM l-glutamine, 1 mM sodium pyruvate, nonessential amino acids, and 55 mM 2-ME [all from Invitrogen]) with the addition of 10 ng/ml recombinant human IL-15 (PeproTech, Rocky Hill, NJ).

Conversion of pNK cells to dNK-like cells

Enriched pNK cells were seeded at 1 × 10^6 cells/ml in IL-15 complete media in the presence or absence of recombinant human TGF-β1 (PeproTech) at 2 ng/ml or at the concentrations indicated in the figures, and in the presence or absence of 1 μM Aza (Sigma-Aldrich) or at the concentrations indicated in the figures. Duplicate cultures were established and incubated at 37˚C in humidified incubators in an atmosphere containing 5% CO2. One of the NK cell cultures was maintained under 2% O2, and the other culture was maintained under hypoxia (1% O2). Cell culture supernatants were harvested after 7 d and used to determine VEGF-A content by ELISA or used in HUVEC tube formation assays. Cells were stained to evaluate CD9 and KIR, CD49a, CD94, CD158a (clone 1B5), anti-CD158b (clone CHL-1), anti-NKAT2 (clone DX27), anti-NK1B1 (clone DX9), anti-CD151 (clone 1A2H1), anti-CD49d (clone SR84), anti-CD94 (clone HP-3D9), anti-granzyme B (clone GB11), anti-granzyme A (clone CB9), anti-perforin (clone 6G9), and isotype controls were all from BD Biosciences (San Diego, CA). Anti-2B4 mAb (clone PP35) was from eBioscience. Cell surface molecules were stained with fluorescent dye–conjugated Abs in PBS supplemented with 2% FCS for 30 min on ice and washed three times with PBS containing 2% FCS. For chemokine receptor expression profiling, cells were first stained with anti-CD3, anti-CD16, and anti-CD56 mAbs, washed, blocked with 10% human serum (Sigma-Aldrich) in PBS 1 h on ice, and then stained with the following PE-conjugated mAbs or corresponding isotype controls: anti-CXCR1 (clone 42705), anti-CXCR3 (clone 49801), anti-CXCR4 (clone 120S), anti-CCR5 (clone 53103), anti-CCR7 (clone 150503) (all from R&D Systems), and anti-CX3CR1 (clone 2A9-1; BML International, San Diego, CA). For intracytoplasmic staining, cells were first stained with anti-CD3, anti-CD16, and anti-CD56 mAbs, then fixed and permeabilized with a Cytofix/Cytoperm Plus fixation and permeabilization kit (BD Biosciences) following the manufacturer’s instructions and stained with anti–granzyme A, anti–granzyme B, anti-perforin, or the corresponding isotype control Abs. Cell sorting was performed on a FACSaria II sorter (Becton Dickinson, San Jose, CA). Analytical measurements were done with a BD FACsCan flow cytometer (Becton Dickinson). NIR expression evaluation was done using a mix of PE-conjugated anti-CD158a, anti-CD158b, anti-NKAT2, and anti-NK1B1 mAbs.

HUVEC tube formation

Growth factor–reduced Matrigel (BD Biosciences) was placed in the wells (100 μl/well) of a prechilled 48-well cell culture plate and incubated at 37˚C for 30 min to allow polymerization. Twenty thousand HUVECs were plated onto the Matrigel-coated wells and incubated with 200 μl NK cell–conditioned or control media at 37˚C for 6–8 h. Tube formation was then assessed through an inverted phase-contrast microscope at ×40 and ×100 (Nikon TE 300). Images were acquired using a Leica DFC50FX camera controlled with Leica FireCam version 1.5 (Leica Microsystems Imaging Solutions). The percentage surface per field covered by HUVECs was calculated from the average of three fields per well using Image J software (National Institutes of Health).

Matrigel invasion assay

A Matrigel invasion assay was performed using extravascular cytophoblasts immortalized HTR-8/SvNeo cells (a gift of Dr. Charles Graham) and BD BioCoat Matrigel invasion chambers (BD Biosciences) following the manufacturer’s instructions. HTR cells (1 × 10^3 or 2 × 10^3 in 200 μl serum-free RPMI 1640 medium) were placed on the upper chamber. pNK cells grown either under standard tissue culture conditions in IL-15 complete media (control) or under 1% O2 in IL-15 complete media in the presence of 2 ng/ml TGF-β1 and 1 μM Aza (i-dNK cells) for a week were washed with PBS and seeded in the lower chamber (6.5 × 10^5 to 7.5 × 10^5 cells) in 750 μl BIO-MPM-1 serum-free media (Biological Industries, Beit Haemek, Israel) containing 10 ng/ml IL-15, 1 U/ml penicillin, 100 μg/ml streptomycin, 1 mM l-glutamine, 1 mM sodium pyruvate, nonessential amino acids, and 55 mM 2-ME (all from Invitrogen). Additionally, fibronectin (1 μg/ml) was added to the lower chamber to aid adherence of HTR cells to the membrane after migration. After 48 h the wells were removed and washed with Dulbecco’s Modified Eagle Medium (Thermo Fisher Scientific, Waltham, MA). The membranes were mounted on microscopic slides, images were captured, and cells were counted using ImageJ software (National Institutes of Health). Invasion was calculated normalizing the number of

IL-8 measurements

IL-8 content in cell culture supernatants of 1-wk induced dNK (i-dNK) and control cells was evaluated by ELISA using an IL-8 Quantikine ELISA kit (R&D Systems) following the manufacturer’s instructions. dNK cell media IL-8 content was measured from 3-d cultures of freshly isolated dNK cells or dNK cells previously grown for 7 d. Cells were seeded at 1 × 10^5 cells/ml.

Cytotoxicity assays

Determination of NK cell cytotoxic activity on K562 cells was done for 4-h Cr-release assays as previously described (32).

Flow cytometry

The following mouse anti-human mAbs conjugated with FITC, Alexa Fluor 488, PE, PE-Cy7, PerCP-Cy5.5, allophycocyanin, or allophycocyanin-Cy7 were used for FACS analysis or FACS sorting: anti-CD56 (clone B159), anti-CD3 (clone SK7), anti-CD16 (clone 3G8), anti-CD9 (clone ML-13), anti-CD158a (clone HP-3E4), anti-CD158b (clone CHL-1), anti-NKAT2 (clone DX27), anti-NK1B1 (clone DX9), anti-CD151 (clone 1A2H1), anti-CD49d (clone SR84), anti-CD94 (clone HP-3D9), anti-granzyme B (clone GB11), anti-granzyme A (clone CB9), anti-perforin (clone 6G9), and isotype controls were all from BD Biosciences (San Diego, CA). Anti-2B4 mAb (clone PP35) was from eBioscience. Cell surface molecules were stained with fluorescent dye–conjugated Abs in PBS supplemented with 2% FCS for 30 min on ice and washed three times with PBS containing 2% FCS. For chemokine receptor expression profiling, cells were first stained with anti-CD3, anti-CD16, and anti-CD56 mAbs, washed, blocked with 10% human serum (Sigma-Aldrich) in PBS 1 h on ice, and then stained with the following PE-conjugated mAbs or corresponding isotype controls: anti-CXCR1 (clone 42705), anti-CXCR3 (clone 49801), anti-CXCR4 (clone 120S), anti-CCR5 (clone 53103), anti-CCR7 (clone 150503) (all from R&D Systems), and anti-CX3CR1 (clone 2A9-1; BML International, San Diego, CA). For intracytoplasmic staining, cells were first stained with anti-CD3, anti-CD16, and anti-CD56 mAbs, then fixed and permeabilized with a Cytofix/Cytoperm Plus fixation and permeabilization kit (BD Biosciences) following the manufacturer’s instructions and stained with anti–granzyme A, anti–granzyme B, anti-perforin, or the corresponding isotype control Abs. Cell sorting was performed on a FACSaria II sorter (Becton Dickinson, San Jose, CA). Analytical measurements were done with a BD FACsCan flow cytometer (Becton Dickinson). NIR expression evaluation was done using a mix of PE-conjugated anti-CD158a, anti-CD158b, anti-NKAT2, and anti-NK1B1 mAbs.

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migrated HTR-8-svNeo cells to that of the control for each of the donors or media alone.

**Immunoblot analysis for hypoxia-inducible factor-1α protein expression**

Total proteins from NK cells were extracted and Western blot was performed using published protocols (33). For detection of hypoxia-inducible factor (HIF)–1α, mAb against HIF-1α (clone 54/HIF-α; BD Transduction Laboratories; 1:500 dilution) was used. The membranes were stripped and reprobed with a monoclonal anti-β-actin Ab (clone AC-15; Sigma-Aldrich; 1:1000 dilution) to assess for protein loading.

**Statistical analyses**

Data were analyzed by a Student t test and a p value <0.05 was considered significant. A Wilcoxon rank-sum test was used whenever data were not normally distributed.

**Results**

**Phenotype of dNK and pNK cells**

Supplemental Fig. 1 displays characteristics that distinguish pNK cells from dNK cells. In peripheral blood, the number of CD56dimCD16+ NK cells is 10- to 20-fold the number of CD56 brightCD16− NK cells, whereas in the human decidua NK cells are almost exclusively CD56 brightCD16− (10, 12). Representative CD56 and CD16 expression profiles of ex vivo CD3−pNK and dNK cells are shown in Supplemental Fig. 1A. CD9 and CD49a serve as markers for dNK cells, as they are expressed by dNK cells but not by CD56 brightCD16− or CD56 dimCD16+ pNK cells (9, 20) (Supplemental Fig. 1B, 1D). Decidual NK cells and CD56 dimCD16+ pNK cells express KIRs, contrasting with CD56 brightCD16− pNK cells, the vast majority of which are devoid of this type of receptor (9, 12) (Supplemental Fig. 1C). dNK cells are functionally distinct from pNK cells. Ex vivo dNK cells have reduced cytotoxicity on K562 target cells when compared with pNK cells (19) (Supplemental Fig. 1E). Uterine and dNK cells have been reported to secrete proangiogenic molecules (8, 21, 22, 25).

**Reversibility of the dNK cell phenotype**

Is the phenotype of dNK cells conditioned by the decidual microenvironment? FACS-sorted CD3−CD56 brightCD16− dNK cells maintained CD9 expression after 7–10 d in culture (Fig. 1A, left panel). Surprisingly, the percentage of KIR+ dNK cells was substantially reduced (Fig. 1A, right panel, 1B) in these cultures. The expression of other molecules such as CD49a, which is expressed by dNK cells but not by pNK cells (20), CD62L, which is only expressed by CD56 brightCD16− pNK cells (9), CD94, which is differentially expressed by CD56 bright and CD56 dim NK cells (34), and 2B4 did not change (Fig. 1C, top panel, Supplemental Fig. 2).

Similar results were obtained when CD3−CD56 brightCD16− dNK cells were maintained in culture on total decidual cells (Fig. 1C, bottom panel). Thus, factors present in the decidual microenvironment, but not necessarily secreted by decidual stromal cells, may be necessary to either induce or maintain KIR expression on dNK cells. IL-8 was secreted by freshly isolated dNK cells (55–739 pg/ml, n = 4) but not by dNK cells previously grown for 7 d (below the ELISA detection level, n = 4). Magnetic bead–sorted dNK cells, cultured in IL-15 complete media for a week,

**FIGURE 1.** Reversibility of the dNK cell phenotype. (A) CD9 (left panel), KIR (right panel) expression by ex vivo CD3−CD56 brightCD16− dNK cells (dark gray histograms), and CD3−CD56 brightCD16− NK cells from 7- to 9-d dNK cell cultures in IL-15 complete media (light gray histograms). Filled histograms show isotype control. (B) Percentage of KIR+ cells among ex vivo FACS-sorted dNK cells and FACS-sorted dNK cells cultured for 7–9 d in IL-15 complete media evaluated by flow cytometry. Each line represents an independent sample. *p = 0.02. (C) KIR, CD49a, CD62L, and CD94 expression by ex vivo CD3−CD56 brightCD16− dNK cells (blue histograms) and CD3−CD56 brightCD16− NK cells from 7- to 9-d dNK cell cultures in which cells were seeded in IL-15 complete media (green histograms in top panels) or on total decidua cells (red histograms in bottom panels). Gray histograms show isotype control. (D) Cytotoxic activity of ex vivo dNK cells (○) and dNK cells cultured for a week in IL-15 complete media (●) on K562 cells evaluated by 51Cr-release assays. Error bars represent SD. Average of three experiments is shown.
were highly cytotoxic as opposed to freshly isolated dNK cells, which displayed reduced cytotoxicity (Fig. 1D). Thus, the reduction in the percentage of KIR+ cells and the acquisition of a cytotoxic phenotype by dNK cells in vitro are indicators of the importance of the decidual microenvironment in maintaining the phenotype of dNK cells.

**Hypoxia induces proangiogenic activity on pNK cells**

Hypoxia is a regulator of angiogenesis (35, 36) and immunity (37, 38). To test whether hypoxia can induce a nontoxic and proangiogenic phenotype on pNK cells, pNK cells were cultured for 1 wk under normal tissue culture conditions (21% O2) or under hypoxia (1% O2). Cultures under hypoxia were enriched in CD56brightCD16− NK cells relative to cultures under 21% O2 (Fig. 2A). In all but one experiment the ratio of the percentage of CD56brightCD16− NK cells to the percentage of CD56−CD16+ NK cells was higher under 1% O2 than under 21% O2 (Fig. 2B).

dNK and uterine NK cells but not pNK cells have been reported to secrete VEGF when cultured under different conditions (8, 21, 24, 25). The levels of VEGF-A secretion by dNK cells were not detectable in 3-d cultures under 21% O2 in accordance with what others have reported (23). Independent dNK samples produced low levels of VEGF-A in 7- to 8-d cultures (Supplemental Fig. 3A). VEGF-A secretion was enhanced significantly when dNK cells were cultured under hypoxia (Supplemental Fig. 3A). To evaluate whether hypoxia could induce angiogenic function on pNK cells, pNK cells were cultured under hypoxia and cell culture supernatants were assayed for VEGF-A content by ELISA and for their capacity to induce tube formation by HUVECs on Matrigel. pNK cells did not secrete VEGF-A when cultured under 21% O2; however, they secreted considerable amounts of VEGF-A when cultured under hypoxia (Fig. 2C). pNK cells cultured under hypoxia but not under 21% O2 expressed HIF-1α, a transcription factor that mediates responses to hypoxia and induces VEGF expression (39) (Supplemental Fig. 3B). Furthermore, HUVECs formed more tubes when cultured with pNK supernatants generated under hypoxia than with supernatants generated under 21% O2 as evaluated by cell morphology and percentage area per field covered by HUVECs (Fig. 2D). pNK cell preparations enriched by negative selection Ab cocktails may contain contaminating cells that could be the source of VEGF-A. To confirm that indeed pNK cells secrete VEGF-A under hypoxia, 99% pure FACS-sorted CD56brightCD16− pNK and CD56dimCD16+ pNK cells were cultured under 1% O2 or 21% O2. VEGF-A was found in CD56bright CD16− pNK cultures under 1% O2 but not under 21% O2 (Fig. 2C). CD56dimCD16+ pNK cells secreted low amounts of VEGF-A (Fig. 2C).

pNK cells cultured under hypoxia or 21% O2 showed similar cytotoxic activity on K562 target cells (Fig. 2E). The percentage of target cells lysed by NK cells cultured under hypoxia or 21% O2 was significantly higher than the percentage of cells lysed by freshly isolated pNK cells owing to the presence of IL-15 in the

![FIGURE 2. Hypoxia enriches pNK cell cultures in CD56brightCD16− cells and turns pNKs into proangiogenic cells. (A) CD56 and CD16 expression profile of pNK cells cultured for 1 wk under hypoxia (1% O2) or normal tissue culture conditions (21% O2) in the presence of IL-15. Squares delineate CD56brightCD16− NK and CD56dimCD16− NK cell populations. Numbers indicate percentage of cells. (B) CD56bright CD16− NK cell/CD56−CD16+ NK cell ratio in hypoxia and 21% O2 cultures in experiments similar to the one shown in (A). Each line represents an independent experiment. **p = 6 × 10−5 by paired t test. (C) VEGF-A levels in the supernatants of pNK cells, FACS-sorted CD56brightCD16− pNK cells, and FACS-sorted CD56dimCD16− pNK cells cultured under 21 or 1% O2 in the presence of IL-15 for 1 wk. Cells were seeded at a density of 4 × 10^4 cells in 300 µl media. Error bars represent SD. Average of three experiments is shown. *p = 0.029. **p = 0.009. (D) Tube formation by HUVECs in the presence of supernatants of pNK cells seeded at 1 × 10^5 cells per ml and cultured 1 wk under 21% O2 or 1% O2. Representative fields of 540 phase contrast images (left) and tube formation quantitative evaluation (right) by the average percentage area per field covered by HUVECs. Results are average of six experiments. Error bars represent SD. **p = 0.002. (E) Cytotoxic activity of freshly isolated pNK cells and of pNK cells cultured in the presence of IL-15 under 21% O2 or 1% O2 on K562 target cells. Average of two ^51Cr-release assays is shown. (F) CD9 (top panel) and KIR (bottom panel) expression by CD3 CD56brightCD16− NK and CD3 CD56dimCD16− NK cells from pNK cell cultures maintained for a week under 1% O2 (black histogram) or 21% O2 (gray histogram) in the presence of 10 ng/ml IL-15. In the top panels, histograms are not visualized owing to overlap with isotype control. Filled histograms show isotype control. Histograms are representative of three experiments.
culture media in which they were maintained. The expression of cell surface markers such as CD9 or KIRs was not affected by hypoxia in 1-wk cultures (Fig. 2F). Thus, hypoxia alone does not affect pNK cell cytotoxicity nor does it induce the expression of CD9 or KIRs on CD56<sup>bright</sup>CD16<sup>−</sup> pNK cells, but it confers pNK cells proangiogenic properties and enriches pNK cell cultures in CD56<sup>bright</sup>CD16<sup>−</sup> cells.

**TGF-β1 treatment combined with hypoxia results in VEGF-A–secreting CD9<sup>+</sup> NK cells with reduced cytotoxicity**

TGF-β1 is a pleiotropic immunosuppressive molecule expressed by the decidual stroma (40) and trophoblasts (41) that is involved in NK cell differentiation and induces the expression of CD9 and to some extent of KIRs on NK cells (40, 42). TGF-β1 effects are often dependent on the environmental setting. We therefore tested whether the combined effects of hypoxia and TGF-β1 on pNK cells can generate noncytotoxic proangiogenic NK cells with similarities to dNK cells. As expected, TGF-β1 reduced the cytotoxic activity of NK cells on K562 targets, but interestingly the inhibition of cytotoxicity was augmented by hypoxia (Fig. 3A).

VEGF-A secretion by NK cells under hypoxia was only marginally inhibited by TGF-β1 in 1-wk cultures (Fig. 3B), whereas NK cells cultured under 21% O<sub>2</sub> did not secrete VEGF-A at any of the TGF-β1 concentrations tested. As expected, TGF-β1 induced CD9 expression on NK cells (Fig. 3C, top panel), but the level of CD9 expression on CD56<sup>bright</sup>CD16<sup>−</sup> NK cells was enhanced under hypoxia (Fig. 3D, Supplemental Fig. 4A). TGF-β1, however, failed to induce significant KIR expression on CD56<sup>bright</sup>CD16<sup>−</sup> NK cells present in 1-wk cultures under hypoxia (Fig. 4C, bottom panel).

Thus, the combination of hypoxia with TGF-β1 exposure resulted in NK cells that expressed CD9, had reduced cytotoxicity, and secreted proangiogenic VEGF-A but lacked KIR expression. We then focused our attention on other synergistic factors that could induce KIR expression to generate KIR<sup>−</sup>CD56<sup>bright</sup>CD16<sup>−</sup> NK cells with similarities to dNK cells.

**Induction of KIR expression on pNK cells by demethylating agents**

Epigenetic mechanisms are important regulators of KIR expression by NK cells, that is, promoter demethylation of CpG islands induces KIR expression (43, 44). Promoter demethylation may be one of the underlying mechanisms of KIR expression in dNK cells. Interestingly, T cells in the decidua have higher levels of KIR expression than do their peripheral blood counterparts (45). Although the transcriptional control of KIRs in T cells and NK cells may differ (46), DNA CpG demethylation was shown to increase KIR expression in T cells (47).

We therefore used Aza, a demethylating agent, to induce KIR expression on CD56<sup>bright</sup>CD16<sup>−</sup> pNK cells (Fig. 4A, top panel). Aza induced KIR expression by CD56<sup>bright</sup>CD16<sup>−</sup> pNK cells, with the percentage of KIR<sup>+</sup>CD56<sup>bright</sup>CD16<sup>−</sup> cells being higher under hypoxia than under 21% O<sub>2</sub> (Fig. 4A, 4B). TGF-β1 reduced to some extent the percentage of KIR<sup>+</sup> cells induced by Aza (Fig. 4B) under hypoxia, and Aza reduced to some extent the number of CD56<sup>bright</sup>CD16<sup>−</sup> NK cells in the cultures (data not shown). Nevertheless, the percentage of KIR<sup>+</sup> cells induced by 1 μM Aza under hypoxia in the presence of TGF-β1 was still high, ∼50–60% of CD56<sup>bright</sup>CD16<sup>−</sup> NK cells (Fig. 4B). Aza did not affect expression of CD9 on cells grown under hypoxia in the presence of TGF-β1 (Fig. 4A, bottom panel), and it only inhibited marginally the secretion of VEGF-A in 1-wk cultures (Fig. 4C). The cytotoxicity of pNK cells cultured under hypoxia with TGF-β1 was not affected by Aza and remained significantly reduced compared with cells grown under hypoxia alone (Fig. 4D). Thus, the exposure of pNK cells to a combination of hypoxia, TGF-β1, and Aza resulted in NK cells with reduced cytotoxicity that secrete proangiogenic VEGF-A, including CD56<sup>bright</sup>CD16<sup>−</sup> NK cells that similar to dNK cells express CD9 and KIRs. We refer to these cells as induced dNK-like cells (i-dNK).

**FIGURE 3.** NK cell incubation under hypoxia in the presence of TGF-β1 results in CD9<sup>+</sup> NK cells that secrete VEGF-A and have reduced cytotoxicity. (A) Cytotoxic activity of pNK cells cultured 1 wk under 21% O<sub>2</sub> or 1% O<sub>2</sub> in the presence or absence of 2 ng/ml TGF-β1 on K562 target cells. Average of two 51Cr-release assays is shown. (B) VEGF-A secretion by pNK cells cultured for a week under 21% O<sub>2</sub> (○) or 1% O<sub>2</sub> (□) with TGF-β1 (Fig. 3A). Results from one representative experiment are shown. Similar results were obtained in 7 of 10 experiments.

Induction of KIR expression on pNK cells by demethylating agents

Epigenetic mechanisms are important regulators of KIR expression by NK cells, that is, promoter demethylation of CpG islands induces KIR expression (43, 44). Promoter demethylation may be one of the underlying mechanisms of KIR expression in dNK cells. Interestingly, T cells in the decidua have higher levels of KIR expression than do their peripheral blood counterparts (45). Although the transcriptional control of KIRs in T cells and NK cells may differ (46), DNA CpG demethylation was shown to increase KIR expression in T cells (47).

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induction of a dNK-like phenotype on pNK cells

To assess the phenotypic similarity of i-dNK cells to dNK cells, i-dNK cells were more fully characterized by flow cytometry. CD56{bright}CD16{−} i-dNK cells presented a chemokine receptor expression profile similar to dNK cells and distinct from that of CD56{bright}CD16{+} pNK and CD56{dim}CD16{+} pNK cells as evaluated by the percentage of cells expressing CCR6, CCR7, CXCR1, CXCR3, CXCR4, and CXC3CR1 (Fig. 5A). Furthermore, i-dNK cells expressed a panel of molecules that are differentially expressed by the three NK cell subsets (dNK, CD56{bright} pNK, and CD56{dim} pNK) in a dNK-like pattern (Fig. 5B). These included CD49a, which is expressed by dNK but not by pNK cells (20); CCR7 and CD62L, overexpressed by CD56{bright}CD16{−} pNK cells (9) (Fig. 5A); CD151, overexpressed by dNK cells (9); and perforin, granzyme A, granzyme B, and CD94, all expressed by dNK cells. i-dNK cells, like dNK cells, expressed the receptor 2B4 (Supplemental Fig. 2) but did not secrete IL-8 as evaluated by ELISA (n = 3).

Reversibility of the i-dNK cell phenotype

To evaluate whether the phenotype induced in CD56{bright}CD16{−} pNK cells by the combined treatment with hypoxia, TGF-β1, and Aza reflected terminal differentiation of the cells or was reversible upon removal of the inducers, pNK cells were treated for a week with these stimuli and then were washed and put in culture for a second week either under i-dNK–inducing conditions or under basal conditions (21% O2). Cell culture supernatants were then tested for VEGF-A content and cells were stained to evaluate expression of CD9 and KIRs by CD56{bright}CD16{−} cells. VEGF-A secretion diminished dramatically when cells were not maintained under i-dNK cell–inducing conditions (Fig. 6A), indicating that hypoxia was necessary to maintain VEGF-A secretion. Under noninducing conditions cells still expressed CD9 (Fig. 6B, top panel). The percentage of KIR{+} cells among CD56{bright}CD16{−} cells was markedly reduced in cultures kept the second week under 21% O2 when compared with cultures maintained under i-dNK cell–inducing conditions (Fig. 6B, lower panel), or when compared with the first week of hypoxia, TGF-β1 and Aza culture from which the cells were derived (data not shown). Thus, i-dNK cell–inducing stimuli were necessary to maintain a high percentage of KIR{+}CD56{bright}CD16{−} cells, but not for maintenance of CD9 expression in 1-wk cultures. This pattern of phenotype “reversion” is similar to the one observed when dNK cells are cultured for a week under 21% O2, removed from the decidua microenvironment (Fig. 1A).

Regulation of trophoblast invasion by i-dNK cells

Trophoblast cells invade the maternal decidua and replace the endothelial lining, transforming spiral arteries into low-resistance, high-flow vessels capable of providing adequate placental perfusion (48). Decidual NK cells have been proposed to be involved in the regulation of trophoblast invasiveness and uterine spiral artery remodeling (8, 29, 49, 50).

NK cells treated under i-dNK cell–inducing conditions for 7 d were tested for their capacity to affect invasion of HTR-8/svNeo cells, an immortalized human extravillous trophoblast cell

The presence of TGF-β1 under hypoxia (O) or 21% O2 (D) at different concentrations of Aza. Average of two experiments is shown. (D) Cytotoxic activity of effector pNK cells cultured for a week under hypoxia in the presence (triangles) or absence (circles) of 1 μM Aza, in the presence (open symbols) or absence (filled symbols) of 2 ng/ml TGF-β1, on K562 target cells. Results are average of two independent experiments.
line that has been previously used in invasion studies (51), in Matrigel invasion assays. Treated NK cells promoted invasion of HTR-8-svNeo cells as compared with control NK cells from the same donor maintained in culture under 21% O2 (Fig. 7A, 7B).

Fig. 7C presents the HTR-8-svNeo migration-inducing capacity of dNK cells from three separate donors and NK cells treated under i-dNK cell–inducing conditions from four independent donors. Although definitive conclusions cannot be made, it appears that dNK cells obtained at later gestational age promote migration when compared with dNK cells obtained at earlier gestational age, in agreement with prior work by Lash et al. (49). The HTR invasion-inducing capacity of NK cells treated under i-dNK cell–inducing conditions was reduced when the cells were later maintained in culture under reversal conditions (21% O2 and IL-15) (Fig. 7C, open bars). In additional studies, reverted cells promoted less invasion than did control cells maintained in culture under i-dNK cell–inducing conditions for the same duration of the experiment (reverted cells, 0.84; control cells, 1.69; average of two experiments, normalized to media alone).

**Discussion**

Decidual NK cells as opposed to pNK cells are characterized by their reduced cytotoxicity and their capacity to secrete angiogenic molecules. In this study, human pNK cells cultured with a combination of hypoxia, TGF-β1, and the demethylating agent Aza displayed reduced cytotoxicity, secretion of a potent proangiogenic molecule (VEGF-A), and acquired capacity to promote trophoblast invasion in cell culture studies. Furthermore, CD56\textsuperscript{bright}CD16\textsuperscript{−} cells from these cultures express CD9 and KIRs, CD49a, chemokine receptors, and molecules such as CD151, CD62L, CD94, perforin, granzyme B, and granzyme A in a pattern that is unique to dNK cells (Fig. 5).

Although different possible origins have been proposed for human dNK cells, namely being derived from pNK cells homing to...
the decidua or from hematopoietic precursors present in decidual tissue (40, 52, 53), it is evident that the decidual microenvironment is necessary to induce or maintain the dNK cell phenotype. When dNK cells, the vast majority of which express KIRs, are removed from the decidua and placed in culture with IL-15, they become cytotoxic and harbor a high proportion of KIR\(^\text{+}\)CD56\(^{\text{bright}}\)CD16\(^{\text{-}}\)dNK cells (Fig. 1). The reduction in the percentage of KIR\(^{+}\)dNK cells in the cultures may be due to loss of KIR expression by dNK cells, to a higher cell death rate of KIR\(^{+}\)cells, or to an increased proliferation of KIR\(^{+}\)cells that fail to acquire KIR expression in the absence of factors present in the decidua. The presence of other dNK markers such as CD94, CD49a, and absence of CD62L makes it less likely that these KIR\(^{+}\)cells are arising from contaminating KIR\(^{-}\)CD56\(^{\text{bright}}\)pNK cells. It should be noted, however, that exogenous IL-15 may have contributed to the cytotoxicity phenotype.

Hypoxia is a regulator of angiogenesis (35, 36) and immunity (37, 38). Hypoxia enriched pNK cell cultures in CD56\(^{\text{bright}}\)CD16\(^{-}\)cells and induced the secretion of VEGF by pNK cells (Fig. 2). Furthermore, hypoxia enhanced the effects of TGF-\(\beta\)1 on cytotoxicity inhibition and induction of CD9 expression (Fig. 3). It is possible that the enhancement of TGF-\(\beta\)1 effects on NK cells by hypoxia may be due to added TGF-\(\beta\)1 secreted by pNK cells when cultured under low oxygen tensions. It remains to be determined whether the effects of hypoxia on NK cells are regulated directly through HIF-1\(\alpha\) or through other downstream metabolites of hypoxia.

The i-dNK cells we have generated secrete the proangiogenic molecule VEGF-A. Previous reports have shown that dNK cells and uterine NK cells but not pNK cells secrete VEGF under different culture conditions (8, 21, 23, 24). Controversy exists as to the type of VEGF produced. Some studies support the secretion of VEGF-A but not VEGF-C (25), others VEGF-C but not VEGF-A (23), and others do not specify the VEGF type secreted (8, 24). This may reflect differences in culture conditions, cell isolation procedures, gestational age of the samples, or protein detection assays used. In our short-term (3 d) cultures under 21% O\(_2\), dNK cells did not secrete detectable levels of VEGF-A but they did produce it when exposed to low oxygen tensions similarly to pNK cells treated under i-dNK–inducing conditions. Interestingly, a recent study in mice has shown the presence of VEGF-A\(^*\) decidual NK derived from blood (54).

The combined effects of hypoxia and TGF-\(\beta\)1 yielded cells that express CD9, have reduced cytotoxicity, and secrete VEGF. However, CD56\(^{\text{bright}}\)CD16\(^{-}\)cells did not express KIRs like dNK cells. TGF-\(\beta\)1 has been reported to induce KIR expression on \(-20%\) of CD56\(^{\text{bright}}\)CD16\(^{-}\)cells in 2-wk or longer pNK cultures (40, 42). The absence of KIR induction by TGF-\(\beta\)1 on CD56\(^{\text{bright}}\)CD16\(^{-}\)cells in this study may be due to the shorter length of the cultures, to a confounding effect introduced by hypoxia, or to differences in media composition. The induction of KIR expression on a higher proportion of CD56\(^{\text{bright}}\)CD16\(^{-}\)pNK cells was achieved with the addition Aza to the culture media. Most interestingly, hypoxia augmented the percentage of KIR\(^{+}\)cells induced by Aza among CD56\(^{\text{bright}}\)CD16\(^{-}\)cells, suggesting that hypoxia may be a modulator of KIR expression or alternatively may favor the proliferation of KIR\(^{-}\)CD56\(^{\text{bright}}\)CD16\(^{-}\)NK cells. Note, however, that the addition of Aza reduced the number of CD56\(^{\text{bright}}\)CD16\(^{-}\)cells (data not shown).

Demethylating agents induce KIR expression on NK cells and to a lesser degree on T cells, but not on other tested cell types, which suggests a specific effect of demethylation on regulation of KIRs on NK cells and T cells (44). The endogenous factor responsible for KIR expression in dNK cells is unknown. It is
possible that the endocrine and cytokine milieu as well as cell–cell interactions may contribute to KIR induction. In this setting, hormones are particularly interesting because they are known to regulate epigenetic mechanisms (55).

Thus, in addition to the phenotypic similarities to dNK cells, NK cells exposed to i-dNK–inducing conditions secrete VEGF-A, a potent proangiogenic molecule, have reduced cytotoxicity and promote trophoblast invasion. Future research should establish whether i-dNK cells can mimic dNK cell function in vivo. Manipulation of ex vivo–expanded autologous peripheral NK cells to yield dNK–like cells may open the door to research on new therapeutic venues for the treatment of reproductive disorders associated with NK cell biology such as preeclampsia and intrauterine growth restriction.

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Disclosures
R.I.T., V.P.S., and S.A.K. are coinventors on patents related to angiogenic markers in preeclampsia and have financial interest in Aggamin LLC. The other authors have no financial conflicts of interest.

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