This information is current as of March 5, 2021.

Vascular Endothelial Growth Factor C Facilitates Immune Tolerance and Endovascular Activity of Human Uterine NK Cells at the Maternal-Fetal Interface

Satyan S. Kalkunte, Teddy F. Mselle, Wendy E. Norris, Charles R. Wira, Charles L. Sentman and Surendra Sharma

J Immunol 2009; 182:4085-4092; ;
doi: 10.4049/jimmunol.0803769
http://www.jimmunol.org/content/182/7/4085

References This article **cites 52 articles**, 15 of which you can access for free at:
http://www.jimmunol.org/content/182/7/4085.full#ref-list-1

Why *The JI*? Submit online.

- **Rapid Reviews! 30 days*** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Fast Publication!** 4 weeks from acceptance to publication

**average*

Subscription Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
Copyright © 2009 by The American Association of Immunologists, Inc. All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Downloaded from http://www.jimmunol.org/ by guest on March 5, 2021

Vascular Endothelial Growth Factor C Facilitates Immune Tolerance and Endovascular Activity of Human Uterine NK Cells at the Maternal-Fetal Interface[1]

Satyan S. Kalkunte,* Teddy F. Mselle,† Wendy E. Norris,* Charles R. Wira,‡ Charles L. Sentman,† and Surendra Sharma[2]*

Although replete with cytotoxic machinery, uterine NK (uNK) cells remain tolerant at the maternal-fetal interface. The mechanisms that facilitate the uNK cell tolerance are largely unknown. In this study, we demonstrate that vascular endothelial growth factor (VEGF) C, a proangiogenic factor produced by uNK cells, is responsible for their noncytotoxic activity. VEGF C-producing uNK cells support endovascular processes as demonstrated in a three-dimensional coculture model of capillary tube formation on Matrigel. Peripheral blood NK cells fail to produce VEGF C and remain cytotoxic. This response can be reversed by exogenous VEGF C. We show that cytoprotection by VEGF C can be related to induction of the TAP-1 expression and MHC class I assembly in target cells. Small interfering RNA-mediated silencing of TAP-1 expression abolished the VEGF C-imparted protection. Overall, these results demonstrate that empowerment of uNK cells with angiogenic factors keeps them noncytotoxic. This phenotype is critical to their pregnancy-compatible immunovascular role during placentation and fetal development. *The Journal of Immunology,* **2009, 182: 4085–4092.**

Maternal immune tolerance of the semiallograft fetus is a subject of intense debate. Although the decidualized uterus is replete with immune cells, fetal development ensues unhindered and results in normal term delivery. An important aspect of successful pregnancy outcome is that they invoke immune tolerance and de novo angiogenesis, two highly intriguing, indispensable, and well-choreographed processes (1–3). These processes are central to optimal placentation and involve interactions between immune cells, endothelial cells, and invading trophoblasts. With respect to immune cells, T cell-mediated rejection of the fetus has been shown in mice, but not in humans (4). Moreover, uterine T cells or macrophages may not promote immunovascular cross-talk with the invading trophoblast lacking expression of HLA (HLA-A, HLA-B, or HLA-D) Ags (1, 5). Thus, it is reasonable to propose that the predominant immune tolerance cross-talk is between uterine NK (uNK)[3] cells and the trophoblast. Interestingly, uNK cells have been uniquely detected in the proximity of endothelial cells and invading trophoblasts associated with spiral arteries undergoing physiological transformation (6, 7).

How uNK cells induce tolerance to the fetal tissue is an important unresolved question, with implications for our understanding of the role of not only uNK cells but peripheral blood NK (pNK) cells in adverse pregnancy outcomes, tumor surveillance, and autoimmunity and immunity against infections. In humans, unlike peripheral blood NK cells of the cytotoxic $CD56^{dim}CD16^{+}$ phenotype, the pregnant uterus (decidua) is populated with specialized uNK cells that are of the noncytotoxic $CD56^{bright}CD16^{-}$ phenotype (8–10). These cells are present in the endometrium during the secretory phase of the menstrual cycle and reach peak numbers (60–70%) during first trimester of pregnancy. Thereafter, their numbers decline rapidly (11–13). Although the physiological role of uNK cells remains poorly understood, several groundbreaking observations and views have implicated these cells in decidualization, invasion of the trophoblast, and production of angiogenic factors and chemokines (14–18). Indeed, vascular endothelial growth factor (VEGF) mRNA and protein have previously been shown to be expressed in NK cells from endometrium and decidua (19, 20). Major mechanistic findings on uNK cells have come from animal studies suggesting their role in spiral artery remodeling. NK cell-deficient mice display abnormalities in decidual artery remodeling and trophoblast invasion, possibly due to lack of uNK cell-derived IFN-γ (3, 14). In humans, lack of "physiological transformation" of decidual spiral arteries is thought to be associated with preeclampsia, preterm birth, and intrauterine growth restriction (7, 21, 22). In this regard, evidence has been presented for defective interactions between uNK cells and the invading trophoblast in a cohort of preeclampsia patients (7, 23).

Despite their pregnancy-compatible role, uNK cells at the maternal-fetal interface harbor a repertoire of natural cytotoxicity receptors (NCRs), cytolytic granules, and activating and inhibitory killer IgG receptors (16, 24–26). This suggests that these innate cells retain their primordial role as sentinels with an ability to attack target cells in response to "danger signals" in their proximate milieu. Indeed, recent studies from our laboratory and others

*Department of Pediatrics, Women and Infants Hospital-Warren Alpert Medical School of Brown University, Providence, RI 02905; †Department of Microbiology & Immunology and ‡Department of Physiology, Dartmouth Medical School, Hanover, NH 03755

Received for publication November 12, 2008. Accepted for publication January 27, 2009.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported in part by grants from the National Institutes of Health and National Institute on Environmental Sciences, P20RR018728, and Superfund Basic Research Program Award (P42ES013660), National Institutes of Health Grant AI51877, and Fogarty International Center Grant FIC 2D43TW006807.

[2] Address correspondence and reprint requests to Dr. Surendra Sharma, Department of Pediatrics, Women and Infants Hospital, 101 Dudley Street, Providence, RI 02905. E-mail address: ssharma@wihri.org

[3] Abbreviations used in this paper: uNK, uterine NK; HUtEC, human uterine endothelial cell; pNK, peripheral blood NK; dNK, decidual NK; VEGF, vascular endothelial growth factor; PlGF, placenta growth factor; NCR, natural cytotoxicity receptor; NPS, normal pregnancy serum; siRNA, small interfering RNA.

Copyright © 2009 by The American Association of Immunologists, Inc. 0022-1767/09/$2.00

www.jimmunol.org/cgi/doi/10.4049/jimmunol.0803769

Downloaded from http://www.jimmunol.org/ by guest on March 5, 2021

have shown that uNK cells "switch on" their cytolytic machinery, leading to fetal resorption or preterm birth in mice exposed to LPS (27, 28) or dsRNA (29). In addition, increased cytolytic CD16$^+$ NK cells have been reported in the endometrium of patients with a history of recurrent spontaneous abortion and implantation failure (30–32). Other studies have shown that uNK cells cultured in vitro can be readily activated with IL-2 to lyse target cells, including trophoblasts (33, 34). Thus, the mechanisms that facilitate pregnancy-compatible, noncytotoxic characteristics of uNK cells need to be delineated. We hypothesize that the signature angiogenic machinery of uNK cells is responsible for their noncytotoxic behavior during normal pregnancy. We show that the noncytotoxic phenotype of uNK cells is accomplished through uNK cell-produced VEGF C, not VEGF A, which enhances resistance to lysis of trophoblasts and endothelial cells through induction of the TAP-1 protein. TAP-1 is associated with transport of processed peptides to the endoplasmic reticulum to ascertain functional MHC class I assembly (35).

Materials and Methods

Human subjects

All human studies were done with the approval of the Institutional Review Board of Dartmouth Medical School (Hanover, NH) and Women and Infants Hospital (Providence, RI). Informed consent was obtained from all tissue donors.

Isolation of primary pNK cells

Mononuclear cells from blood were collected from nonpregnant healthy women 35 ± 5 years of age separated from heparinized blood samples by Ficoll gradient centrifugation as previously described (36). NK cells were further purified using a human NK cell isolation kit (Miltenyi Biotec) based on depletion of non-NK cells (negative selection) as per the manufacturer's instructions.

Isolation of decidual NK (dNK) cells

First-trimester decidua were obtained from pregnant women who underwent first-trimester elective termination. All subjects were between 7 and 11 wk of gestation. Gestational age was calculated based on last menstrual period or ultrasound at the time of procedure. Decidual tissue (~6 g) was processed for NK cell isolation immediately after extraction as previously described (36). The cells were suspended in RPMI 1640 supplemented with 10% FCS and antibiotics and adjusted to a concentration of 1×10^6 cells/ml. dNK cells were further separated using a human NK cell isolation kit (Miltenyi Biotec) as described above. Cell viability was confirmed to be >90% by trypan blue staining. The yield of dNK cells was ~400,000 cells/g of decidual tissue. The purity of the preparation (>95%) was confirmed by FACS by gating on forward scatter and side scatter dot plots of CD45$^+$ cells to exclude noncellular debris (36).

Generation of uNK cell clones

CD56^{bright}CD3$^-$ uNK cells were isolated from endometrial tissue specimens obtained from women undergoing hysterectomies as previously described (37). We usually received/digested 1 g of tissue after dissecting the muscular tissue out. After processing and magnetic selection, we obtained ~500,000 uNK cells/g of tissue. Tissue samples used were distal to any pathological changes. To generate uNK cell clones, enriched NK cells were cultured in 500 U/ml IL-2 for 2–3 days to allow cell expansion in complete medium (RPMI 1640 supplemented with 2-ME (50 μM), penicillin (100 U/ml), streptomycin (100 μg/ml), sodium pyruvate (1 mM), nonessential amino acids (0.1 mM), and 5% human serum). After 2–3 days in culture, uNK cells were cloned using a limiting dilution method as previously described (36). Actively growing cells from wells were analyzed to confirm their NK cell phenotype (CD56$^+$CD3$^-$), and these uNK clones were expanded and maintained in complete medium containing 500 U/ml IL-2 and 5 U/ml IL-15 for the remainder of their culture. It is important to note that uNK cells were made free of cytokines by washing them three times with PBS before their use in tube formation and cytotoxicity assays.

Trophoblast cells and endothelial cells

Immortalized first-trimester trophoblast cell line HTR8 with properties of invasive extravillous cytotrophoblasts was established and provided by Dr. C. Graham (38). HTR8 cells were grown to ~80% confluence in RPMI 1640 standard growth medium and used only during eight passages. HUVEC and human uterine endothelial cells (HUtEC) were obtained from Cambrex and cultured in EBM-2 medium (Cambrex). All cells were maintained in standard culture conditions of 5% CO_2 at 37°C. It is important to note that cells were thoroughly washed free of growth medium before their use in tube formation and cytotoxicity assays.

In vitro three-dimensional tube formation assay

We have recently established a three-dimensional dual culture system to study endovascular activity involving trophoblasts and endothelial cells (39). This method has been now modified to examine the role of uNK cells in angiogenic processes. Briefly, growth factor-reduced Matrigel (BD Biosciences) was thawed overnight at 4°C and mixed to homogeneity. Culture plates (48-well) were coated with 0.1 ml of Matrigel and allowed to gelatinize at 37°C for 30 min. Trophoblasts or endothelial cells (2.5×10^4) were labeled with cell tracker green CMFDA or cell tracker red CMTMR (Molecular Probes) respectively, in serum-free medium for 1 h. Cells were washed, trypsinized, and mixed with pNK or uNK cells (1:1:1) on Matrigel-coated plates in serum-free medium. The tube assay was initiated using 10% v/v normal pregnancy serum (NPS; 35- to 40-wk gestation). NPS is used for mimicking pregnancy-related physiological milieu. The spontaneous interaction and endothelial cell-directed tube formation by trophoblasts was monitored and recorded 12–14 h after incubation under standard culture conditions using florescence microscopy (Nikon Eclipse TS 100 coupled with a charge-coupled device camera). The average number of tubes/vacuoles formed was quantified by counting the number of tube-like structures formed by the connected capillary bridge in four different fields (×4 magnification) by two independent investigators (39). Based on the ability of NK cells to either maintain or disrupt the tube formation in this three-dimensional coculture model,, clones are henceforth termed as "nondisruptive" and "disruptive," respectively.

Calcein-AM retention assay for cytotoxicity measurement

We determined the cytotoxic ability of uNK cells and pNK cells on target endothelial (HUVEC, HUtEC) or trophoblast (HTR8) cells using a calcein-AM retention assay (40). Briefly, adherent confluent target cells (HUVEC, HUtEC, or HTR8; 2×10^3) were plated in a 96-well plate and labeled with 8 μM calcein-AM (Molecular Probes) in serum- and phenol red-free RPMI 1640 medium (Life Technologies) for 40 min at 37°C. Based on pilot experiments, effector cells (pNK or uNK cells) were added at a 25:1 ratio to calcein-AM-labeled target cells in quadruplicate. Maximal lysis was determined by solubilizing target cells in lysis buffer (0.1% Triton X-100 in PBS, pH 9.0). After the indicated time (2–3 h) of incubation at 37°C, the assays were terminated by washing the plates twice and the remaining fluorescence was read using a 96-well fluorescence plate reader (SpectraMax Gemini EM; Molecular Devices) with excitation and emission at 485 and 538 nm, respectively. Percent specific cytotoxicity was calculated as described elsewhere (40). To monitor the cytoprotective activity of VEGF C or VEGF A, the target cells were preincubated for 14 h with growth factors (100 ng/ml) and subjected to a cytotoxicity assay. The dose chosen was determined based on pilot experiments that were conducted to rescue three-dimensional tube formation between trophoblasts and endothelial cells.

Quantification of uNK cell-secreted factors

We cultured 1×10^6 uNK cell clones ($n = 22$) and pNK cells ($n = 5$) individually for 48 h in RPMI 1640 medium containing 500 U/ml IL-2 and 5 U/ml IL-15. Measurement of the concentrations of VEGF A, VEGF C, placenta growth factor (PlGF), IFN-γ, and TNF-α in supernatants was completed in triplicate using respective ELISA kits (Quantikine Kits; R&D Systems) according to the manufacturer's instructions.

Flow cytometry

We evaluated the surface expression by FACS analysis using fluorochrome-conjugated mAb. Cells were harvested and incubated in the dark at 4°C for 30 min with Abs. The Abs used were anti-class I HLA (clone W6/32; Abcam), anti-HLA E (MEM-E/08; Abcam), anti-HLA G (MEM-G/9; Abcam), anti-human CD16 (clone 3G8), anti-human CD56, CD45, CD3, NKP46 (BD Biosciences/BD Pharmingen), NKp30, NKp44, NKG2D (BioLegend), VEGF R1 (49560), VEGF R2 (89106), and VEGF R3 (54733) (R&D Systems). For HLA-E staining we used the anti-human HLA-E (MEM-E/08) as primary Ab, followed by FITC-labeled goat anti-mouse secondary Ab. TAP-1 expression in response to VEGF C (100 ng/ml), IFN-γ (100 ng/ml), VEGF A (100 ng/ml), PlGF (100 ng/ml), or the combination of VEGF C and IFN-γ (100 ng/ml each) was conducted by

Downloaded from http://www.jimmunol.org/ by guest on March 5, 2021

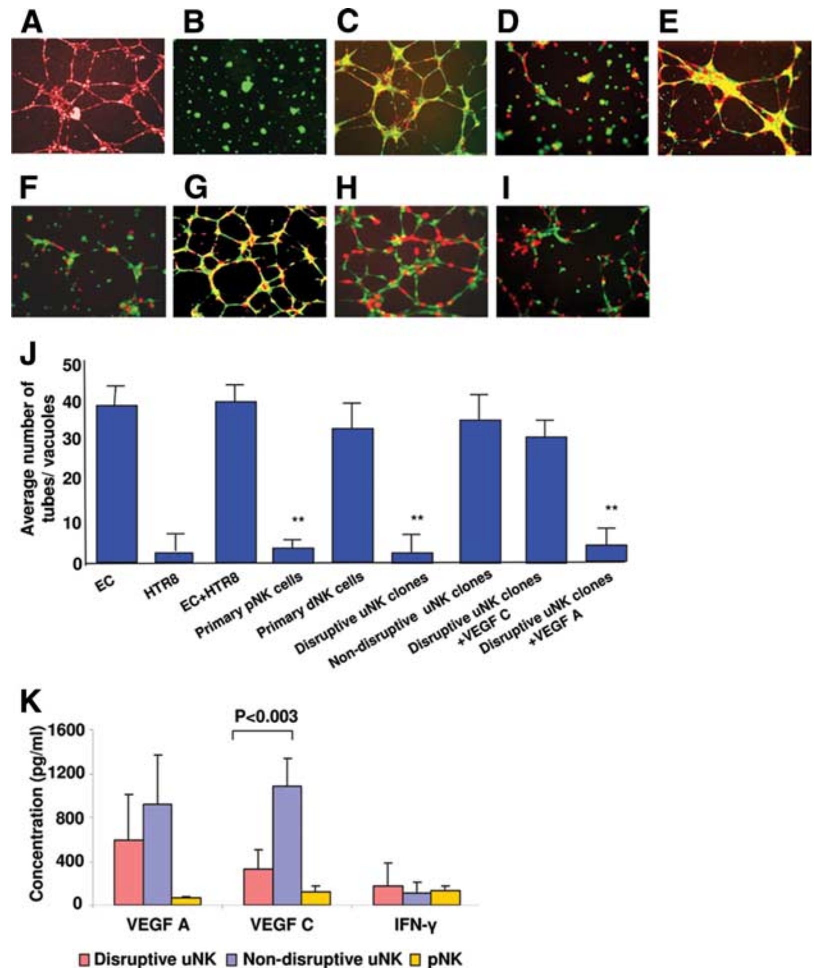

FIGURE 1. Effect of uNK cell clones and VEGF C on tube formation between endothelial cell and trophoblasts. HUVEC (EC, labeled red) and first-trimester extravillous trophoblasts (HTR8, labeled green) were cultured overnight on Matrigel in the presence or absence of primary pNK cells, dNK cells, or uNK cell clones (unlabeled). The capillary tube formation was recorded as described in *Materials and Methods*. *A* and *B* show tube formation with HUVEC or HTR8 cells alone, respectively. *C–I*, EC-directed tube formation by HTR8 cells involving: (*C*) no NK cells, (*D*) primary pNK cells ($n = 4$), (*E*) primary dNK cells ($n = 3$), (*F*) a disruptive uNK cell clone ($n = 18$), and (*G*) a nondisruptive uNK cell clone ($n = 4$; original magnification, ×4). Exogenous addition of VEGF C (100 ng/ml; *H*) but not VEGF A (100 ng/ml; *I*) rescued the EC-directed tube formation by HTR8 cells in the presence of disruptive uNK clones. *J*, Average number of tubes/vacuoles formed was quantified in four different fields (original magnification, ×4). The numbers (bars, ± SD) are the average of multiple experiments in triplicate assessed by two independent investigators. *K*, Average levels of VEGF A, VEGF C, and IFN-γ secreted by disruptive uNK, pNK, and nondisruptive uNK cell clones are shown. Values are mean ± SD of all of the samples. **, $p < 0.05$ (Student's *t* test).

intracellular staining using specific Ab (clone TAP1.28; MBL Laboratory) after fixing and permeabilizing the cells as recommended by the manufacturer. Negative controls were performed for each cell type by incubating the cells with isotype-matched Abs. The stained cells were acquired (10,000 cells) and analyzed by FACSCalibur (BD Biosciences).

Small interfering RNA (siRNA)-mediated silencing of TAP-1 in trophoblasts

We transfected HTR8 trophoblast cells in a 6-well plate with control siRNA (scrambled) or siRNA against TAP-1 (Santa Cruz Biotechnology) using Lipofectamine 2000 (Invitrogen) for 12 h in OptiMEM I-reduced serum medium (Invitrogen). After the transfection period, the cells were allowed to recover in complete RPMI 1640 medium for another 12 h. Cells were then harvested for FACS analysis and immunoblotting or used as targets for cytotoxicity assay.

Western blotting

We determined the protein expression of TAP-1 in HTR8 trophoblast cells treated with recombinant human IFN-γ (100 ng/ml), VEGF C (100 ng/ml; R&D Systems), or the combination of both for 24 h. The cell lysates were prepared in lysis buffer (10 mM Tris-HCl (pH 7.6), 50 mM NaCl, 5 mM EDTA, 50 mM NaF, 0.1 mM sodium orthovanadate, 30 mM sodium pyrophosphate, 1% Triton X-100, 10 μg/ml leupeptin, and 10 μg/ml aprotinin) on ice for 30 min. The cell lysates were separated on 12% SDS-polyacrylamide gels and blotted onto polyvinylidene difluoride membranes and probed with anti-TAP-1 Ab (clone TAP1.28; MBL Laboratory) (1/200 dilution) in 1% BSA in PBST. Actin was probed using anti-actin Abs (Chemicon International) and used as an internal loading control. The bands were visualized using HRP-conjugated secondary Abs followed by ECL (Amersham Biosciences). The protein bands were recorded using a Konica SRX 101A developer (Konica Minolta Medical Imaging).

Statistical analysis

All values are expressed as arithmetic mean of the number from triplicates ± SEM. Significance was determined by a two-tailed Student's *t* test. Values of $p < 0.05$ were considered statistically significant.

Results

VEGF C-producing uNK cells facilitate endovascular processes

Recent observations have suggested that human dNK cells chemoattract trophoblasts and regulate their invasion and participation in vascular remodeling (16). A central question that still remains to be addressed is what factors regulate the immune and vascular activities of noncytotoxic uNK cells. To address this, we have taken advantage of primary uNK and pNK cells and short-term cultured clones of these cells to assess their ability to influence vascular remodeling. NK cell clones from peripheral blood or endometrial tissue were established and propagated as described in *Materials and Methods*. We used a coculture model of vascular remodeling that involves trophoblast-endothelial cell-NK cell interactions on Matrigel and mimics trophoblast invasion of the spiral arteries during pregnancy (39). Using this approach, we demonstrate that in the absence of NK cells, the endothelial HUVEC inherently formed tube-like structures in response to NPS (Fig. 1*A*). However, HTR8 cells representing first-trimester extravillous trophoblasts did not exhibit tube formation under identical conditions (Fig. 1*B*), but footprinted the endothelial cell-guided capillary-like tube structures (Fig. 1*C*). Primary pNK cells disrupted the interaction (tube structure formation) between HTR8 cells and

Downloaded from http://www.jimmunol.org/ by guest on March 5, 2021

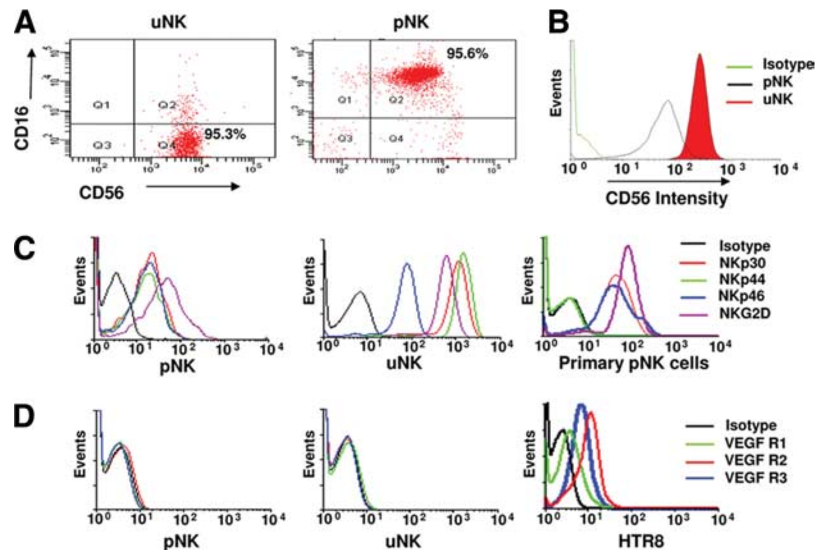

FIGURE 2. Characterization of uNK and pNK cells. Representative FACS analysis is shown for the expression of CD56 and CD16 Ags in (*A*) uNK cell clone and pNK cell clone with $CD56^{dim}$ and $CD56^{bright}$ characteristics (*B*). *C*, Presents phenotypic characterization of NCRs in pNK cell clones, uNK cell clones, and primary pNK cells. *D*, A representative phenotypic profile of VEGF receptors in pNK and uNK cell clones as compared with HTR8 trophoblast cells. One representative data set is shown of four different experiments.

HUVEC (Fig. 1*D*). In contrast, dNK cells were nondisruptive and supported formation of definitive tube structures (Fig. 1*E*). NK cells are not seen in these panels because they were not marked with a cell tracker.

To further delineate the mechanism(s) for differences between pNK and uNK cells, we used NK cell clones maintained in the presence of low doses of IL-2. uNK and pNK cell clones were established from four different individuals and further characterized as described in *Materials and Methods*. We tested a total of 22 uNK cell clones, 18 of which mimicked primary pNK cells with the tube disruption phenotype (data with a representative clone are shown in Fig. 1*F*), whereas four clones were nondisruptive similar to dNK cells (data with a representative clone are shown in Fig. 1*G*). A total of five pNK cell clones was also tested (data not shown) and the observations were similar to their primary counterparts (Fig. 1*D*). The average numbers of tubes/vacuoles formed from multiple experiments in the presence or absence of disruptive or nondisruptive uNK clones were quantified as described in *Materials and Methods* and are presented in Fig. 1*J*.

Next, we examined production of growth factors (VEGF A, VEGF C, PlGF) and cytokines (IFN-γ, TNF-α) secreted by disruptive and nondisruptive uNK cell clones and compared them with pNK cell clones. For simplicity, data are shown only for VEGF A, VEGF C, and IFN-γ since PlGF and TNF-α were minimally produced as detected by ELISA (Fig. 1*K*). Although uNK cell clones produced variable amounts of VEGF A and VEGF C, a striking observation was that nondisruptive uNK cell clones produced 3- to 4-fold higher levels of VEGF C compared with disruptive uNK or pNK clones. VEGF C mRNA and protein have previously been shown to be expressed in NK cells from endometrium and decidua (19, 20); however, its immunoregulatory role has not been described. On the other hand, no VEGF C mRNA was detected in the human placenta (41), suggesting a unique role of uNK cell-produced VEGF C at the maternal-fetal interface. This prompted us to evaluate exogenous VEGF C and VEGF A for their ability to rescue tube formation in the presence of disruptive uNK cell clones. Interestingly, VEGF C (Fig. 1*H*), not VEGF A (Fig. 1*I*), was partially able to rescue tube formation even in the presence of disruptive uNK cell clones. Quantification of VEGF C-mediated rescue of formation of tube structures is shown in Fig. 1*J*.

Disruptive and nondisruptive uNK clones do not differ in their phenotypic features

Cytolytic functions are generally associated with $CD56^{dim}CD16^{+}$ NK cells (8). To rule out disparate behavior of uNK cell clones due to heterogeneity in phenotypic characteristics, we performed FACS analysis for the surface expression of CD16 and CD56 on all uNK cell clones and compared them with pNK cell clones. CD56 expression on uNK and pNK cell clones was found to be bright and dim, respectively (Fig. 2*B*), in agreement with published observations (37). All of the uNK cell clones ($n = 22$) used in the study were found predominantly to be CD16 null ($CD56^{+}$ $CD16^{-}$), whereas pNK cell clones ($n = 5$) were mostly $CD56^{+}$ $CD16^{+}$ (Fig. 2*A*). In both groups, NK cells were $CD45^{+}$ and $CD3^{-}$ (data not shown). Since the expression of NCRs is a distinguishing feature of NK cells, we monitored the surface expression of NKp30, NKp44, NKp46, and NKG2D. uNK cell clones not only expressed all NCRs irrespective of their ability to disrupt or support tube formation, but the intensity of their surface expression was significantly higher compared with pNK cell clones (Fig. 2*C*). In addition, freshly isolated pNK cells lacked expression of NKp44. Thus, it is possible that NKp44 expression is regulated by activation stimuli. Since addition of VEGF C-producing uNK cells or exogenous VEGF C was able to restore capillary tube formation (Fig. 1*H*), we evaluated the expression of VEGF receptors R1, R2, and R3 on uNK cells. As shown in Fig. 2*D*, none of the VEGF receptors were expressed on either pNK cells or uNK cell clones. It is thus possible that VEGFs secreted by uNK cells may not have a direct effect on producer cells but may act in a paracrine manner on target cells expressing VEGF receptors like HTR8 trophoblast cells (Fig. 2*D*) and vascular endothelial cells (39).

VEGF C rescues target cells from NK cell-mediated cytotoxicity

Disruption of tube formation by uNK cell clones could result from the cytolytic activity of these cells. This prompted us to assess the cytolytic activity of uNK cell clones. Using the calcein-AM retention assay (40), we evaluated the cytotoxic effects of uNK and pNK cell clones on HUVEC, HUtEC, and HTR8 cells. Although nonpregnant endometrium does not contain trophoblasts, the use of extravillous HTR8 cells is justified because the same endometrial NK cells are further amplified in the decidua where they encounter invading trophoblasts. Furthermore, since the addition of VEGF C

Downloaded from http://www.jimmunol.org/ by guest on March 5, 2021

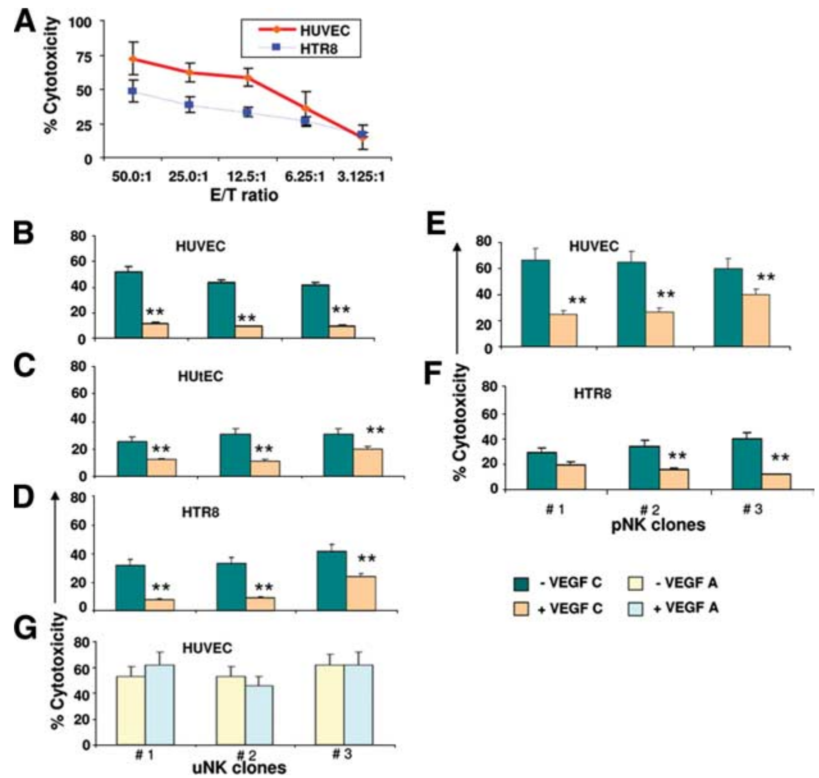

FIGURE 3. VEGF C rescues target cells from NK cell cytotoxicity. *A*, Percent cytotoxicity of pNK cells on HUVEC, HUtEC, and HTR8 cells at different E:T ratios. Values are mean + SD for triplicate reaction. *B–G*, Cytotoxicity of three representative disruptive uNK cell clones with an E:T ratio of 25:1 with HUVEC (*B*), HUtEC (*C*), and HTR8 (*D*) as target cells in the presence (tan bars) or absence (teal bars) of VEGF C. *E* and *F*, Cytotoxicity of three pNK cell clones against HUVEC and HTR8 cells, respectively, in presence (tan bars) or absence (teal bars) of VEGF C. *G*, Cytotoxicity of three uNK cell clones on HUVEC in the presence (light blue) or absence (light yellow) of VEGF A. **, $p < 0.05$ (Student's *t* test).

was able to restore tube formation (Fig. 1*H*), we investigated whether VEGF C or VEGF A would be able to rescue target cells from NK cell-mediated killing. To establish a suitable E:T cell ratio, we performed the assay using a wide range of E:T ratios involving a disruptive NK cell clone and target HUVEC or HTR8 cells (Fig. 3*A*). Based on these data, we used a 25:1 E:T ratio for all subsequent experiments involving all disruptive uNK cell clones ($n = 18$) and pNK cell clones ($n = 5$). Fig. 3, *B–G*, shows data for representative uNK or pNK cell clones. Disruptive uNK cell clones demonstrated robust cytolytic activity toward all target cells, HUVEC (Fig. 3*B*), HUtEC (Fig. 3*C*), or HTR8 (Fig. 3*D*), albeit at varying levels. Similarly, pNK cells were able to robustly lyse HUVEC (Fig. 3*E*) and, to a lesser extent, HTR8 cells (Fig. 3*F*). Importantly, preincubation of target cells overnight with VEGF C (Fig. 3, *B–F*) but not VEGF A (Fig. 3*G*) inhibited NK cell-mediated lysis.

Cytoprotection by VEGF C is mediated by up-regulation of TAP-1

Since both cytotoxic and noncytotoxic uNK cell clones express a similar profile of cytotoxic machinery (Fig. 2*C*), it is plausible that VEGF C modifies target cells to escape NK cell-mediated killing. In this regard, induction of TAP-1 expression has been shown to be associated with cytoprotection of microvascular endothelial cells from activated NK cells (40, 42). We found that VEGF C up-regulated the expression of TAP-1 in HTR8 cells and HUVEC cells as confirmed by FACS analysis (Fig. 4, *A* and *B*) and by Western blotting (Fig. 5*B*). This induction of TAP-1 was found to be synergistic with IFN-γ on HTR8 cells (Figs. 4*A* and 5*B*). Quantification of the percent TAP-1 positive HTR8 or HUVEC in response to VEGF C, VEGF A, PlGF, and IFN-γ by flow cytometry is presented in Fig. 4*B*. Both VEGF C and IFN-γ induced TAP-1

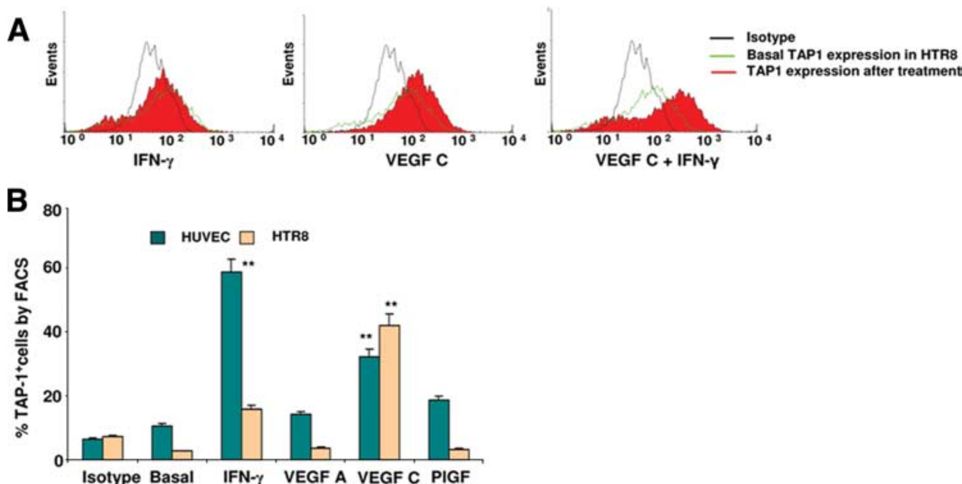

FIGURE 4. VEGF C induces TAP-1 expression. *A*, Intracellular staining and FACS analysis of TAP-1 expression (solid red) in HTR8 cells in response to IFN-γ (100 ng/ml), VEGF C (100 ng/ml), or VEGF C + IFN-γ (100 ng/ml each) as compared with untreated (green line) and isotype control (black line). One representative experiment is shown of three performed. *B*, Represents the percentage of TAP-1 expressing endothelial (teal bars) and HTR8 cells (tan bars) in response to different treatments. Values are mean ± SD of three experiments. Note that VEGF C but not VEGF A or PlGF were able to significantly increase the expression of TAP-1.

Downloaded from http://www.jimmunol.org/ by guest on March 5, 2021

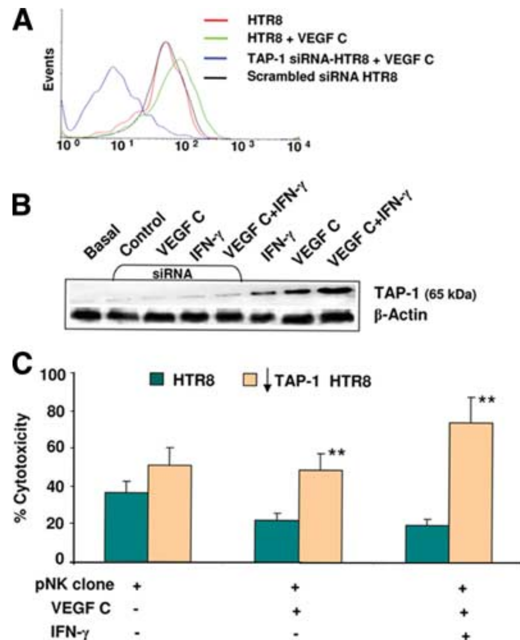

FIGURE 5. Silencing TAP-1 abrogates VEGF C-induced cytoprotection in HTR8 trophoblasts. *A*, A representative histogram of TAP-1 knockdown by siRNA as compared with scrambled siRNA, VEGF C-treated and untreated HTR8 cells. *B*, A representative Western blot analysis indicating TAP-1 induction in response to VEGF C, IFN-γ, or their combination. TAP-1 induction is abrogated in TAP-1 siRNA-treated HTR8 cells in response to VEGF C, IFN-γ, or a combination of both. *C*, Cytotoxicity of a representative pNK cell clone on TAP-1 siRNA-treated HTR8 cells (tan bars) as compared with TAP-1- expressing HTR8 cells (teal bars) in the presence or absence of VEGF C or VEGF C plus IFN-γ. Values are mean ± SD of triplicate reactions. **, $p < 0.05$ (Student's *t* test) as compared with the respective HTR8 treatment group. One representative data set is shown of three experiments performed.

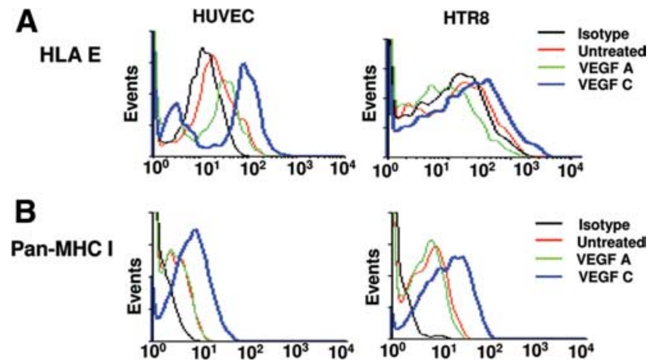

FIGURE 6. VEGF C induces HLA-E expression. *A*, Representative histograms for the surface expression of HLA-E in HUVEC and HTR8 in response to VEGF C and VEGF A. *B*, Representative histograms of MHC class I expression in HUVEC and HTR8 cells in response to VEGF C or VEGF A treatments. One representative data is shown of three experiments performed.

expression in HUVEC and HTR8, although IFN-γ was found to be more potent in HUVEC. On the other hand, IFN-γ elicited less activity in trophoblast HTR8 cells (Figs. 4*A* and 5*B*). Importantly, VEGF A and PlGF failed to induce any significant amount of TAP-1 in either cell type (Fig. 4*B*).

siRNA-mediated knockdown of TAP-1 expression reverses cytotoxicity of uNK cells

To further confirm the role of TAP-1 in the VEGF C-induced noncytotoxicity profile of uNK cells, we used gene-specific siRNA to knockdown TAP-1 expression in HTR8 cells induced by IFN-γ, VEGF C, or a combination of both. As demonstrated by FACS analysis (Fig. 5*A*) and Western blotting (Fig. 5*B*), siRNA treatment of cells significantly blocked induction of TAP-1 by VEGF C, IFN-γ, or a combination of both. Next, we tested whether siRNA-mediated inhibition of TAP-1 expression would reverse the protection profile of target cells. siRNA treatment abolished the protection imparted by VEGF C or VEGF C plus IFN-γ in HTR8 cells against NK cell-mediated lysis as indicated by the calcein-AM cytotoxicity assay (Fig. 5*C*). This clearly confirms both the mechanistic and functional importance of VEGF C in priming targets that otherwise would be susceptible to killing by cytotoxic NK cells.

Induction of TAP-1 by VEGF C leads to an increase in MHC class I presentation on target cells

Activation or inhibition of the cytolytic machinery in NK cells is determined by the extent of the MHC class I complex on target cells. Because we observed up-regulated TAP-1 expression in HUVEC and HTR8 cells in response to VEGF C, we propose that TAP-1 induction results in greater amounts of MHC class I on treated cells. In this regard, we analyzed HLA-E expression which engages the CD94-NKG2A inhibitory or CD94-NKG2C-activating receptors on NK cells (43, 44). Basal expression of HLA-E was low in both HUVEC and HTR8 cells. Interestingly, VEGF C but not VEGF A induced HLA-E expression on HUVEC and HTR8 trophoblast cells (Fig. 6*A*), which was consistent with TAP-1 induction (Fig. 4*B*). Next, we evaluated the surface expression of MHC class I molecules using a pan-class I Ab on HUVEC and HTR8 cells (Fig. 6*B*). Both cell types exhibited significant MHC class I expression in response to VEGF C but not VEGF A (Fig. 6*B*). Because trophoblasts are not known to express classical MHC class I molecules, the observed expression with pan-Ab possibly reflects binding to induced HLA-C or HLA-G. Thus, VEGF C-induced TAP-1 expression and MHC class I assembly in trophoblasts and endothelial cells could facilitate survival by providing inhibitory signals to uNK cells.

Discussion

Host defense, self-tolerance, and tissue transplantation are major health issues that invoke both innate and adaptive immune responses. However, the immune defense mechanisms of the fetus have challenged the concepts embedded in the traditionally defined pathways of immunity. This is further complicated by the "immunologically tolerant" phenotype of the predominant NK cell population in the pregnant uterus and its perceived role in placentation-associated endovascular activity (16, 26). In this study, we describe novel mechanisms by which uNK cells maintain the noncytotoxic and proangiogenesis status.

Our results provide evidence for a central link between production of VEGF C, a proangiogenic factor, by uNK cells and their noncytotoxic phenotype. Using primary human peripheral blood or endometrial NK cells and a panel of clones derived from primary cells, we demonstrated that VEGF C production is a hallmark property of noncytotoxic uNK cells. We further demonstrated that VEGF C, not VEGF A or PlGF, can protect target endothelial and trophoblast cells from killing by cytotoxic pNK cells. Lack of killing is directly associated with VEGF C-mediated induction of TAP-1 in target cells, a key molecule in the process of MHC class I assembly. This is directly demonstrated by siRNA-mediated knockdown of VEGF C-mediated TAP-1 expression which results in reversal of noncytotoxic characteristics of uNK cells.

Downloaded from http://www.jimmunol.org/ by guest on March 5, 2021

Using a three-dimensional culture system on Matrigel to evaluate the endovascular activity of uNK cells, we demonstrate that there are differences in the ability of activated uNK cell clones to facilitate or disrupt the endothelial-trophoblast dual cell interactions mimicking vascular remodeling. Although CD56^{bright}CD16^{-} uNK cell clones consistently expressed NCRs, they differed in their cytotoxic profile. This is not surprising because uNK cell clones were propagated in vitro in the presence of activating stimuli. In vivo, most uNK cells are likely to be of the noncytotoxic phenotype. Despite in vitro conditions, several uNK cell clones maintained their noncytotoxic phenotype. In these clones, the noncytotoxic phenotype correlated with the production of high amounts of VEGF C. Importantly, exogenous VEGF C could rescue trophoblasts and endothelial cells from traditionally cytotoxic NK cells. Dysregulation in VEGF C production at the maternal-fetal interface could be a signal for poor angiogenesis and pregnancy complications. Reduced expression of VEGF C has been reported in pregnancies experiencing intrauterine growth restriction and preeclampsia (45). Involvement of VEGF C may thus explain the non-killer phenotype of uNK cells despite possessing toxic granules and expressing cytotoxicity receptors.

Since our results show that uNK cells do not express receptors for VEGFs, it is tempting to argue that angiogenic factors are produced for a paracrine action on uterine endothelial cells and invading trophoblasts. Our results point to this activity of uNK cell-derived VEGF C. VEGF C acts by binding to VEGF R2 and VEGF R3 and triggers intracellular survival signals in tumor cells and promotes their invasion (46). In addition to maintaining uNK cell noncytotoxicity, VEGF C may support survival of endothelial cells (47). Although during pregnancy, intrauterine hormones and factors that up-regulate secretions of VEGF C in uNK cells are not known, in vitro studies suggest involvement of cytokines like IL-15 (20).

The noncytotoxic capacity of NK cells is based on its ability to recognize surface MHC class I molecules on target cells that deliver signals to suppress NK cell functions. A lack of engagement of such MHC-specific receptors leads to NK cell-mediated killing (8, 18, 25). Interestingly, Bender and colleagues (40) have shown that endothelial cells exhibited sensitivity to activated peripheral blood NK cells in the absence of expression of TAP-1. IFN-γ was shown to induce TAP-1 in endothelial cells and protect them from peripheral blood NK cell-mediated killing (40). However, our data suggest that VEGF C is robustly produced by noncytotoxic uNK cells compared with IFN-γ. It is thus proposed that VEGF C is the predominant regulator of TAP-1 expression in the uterus, although it is likely that VEGF C and IFN-γ act in tandem as supported by our observations (Fig. 5*B*). Interestingly VEGF A or PlGF failed to induce TAP-1 expression (Fig. 4*B*). TAP-1 is a key factor essential for peptide loading for MHC class I assembly and Ag presentation (35, 48). Silencing TAP-1 expression using siRNA in trophoblasts abolished the cytoprotective activity of VEGF C, confirming the mechanism of action of VEGF C.

Trophoblasts cells do not express classical MHC class I molecules but express HLA-C and unconventional HLA-G and HLA-E molecules (5). By using HLA-E as a molecule of choice, we show that VEGF C enhanced surface expression of HLA-E on first-trimester HTR8 trophoblasts. Surface expression of HLA-E normally depends on the recruitment and binding of TAP-dependent classical nonameric peptides with anchoring residues derived from HLA class I signal sequence (49, 50). Interestingly, TAP-1 expression has been shown to be significantly higher in extravillous trophoblasts that are positive for nonpolymorphic HLA class I molecules (5, 51, 52). We also detected significant presence of pan-MHC class I on both HTR8 trophoblast cells and HUVECs in response to VEGF C, suggesting that this angiogenic factor is a potent immunoregulator in the uterine microenvironment.

Taken together, these findings support a dual role of VEGF C in immune tolerance and promoting active angiogenesis by uNK cells. Our findings for the first time provide evidence that noncytotoxicity of uNK cells is directly coupled to their angiogenic translational machinery. uNK cells apparently prepare trophoblasts and endothelial cells to express modest levels of MHC class I molecules to evade killing by a major cytotoxic cell population at the maternal-fetal interface.

Acknowledgments

We thank James Padbury for the thoughtful critique of this manuscript.

Disclosures

The authors have no financial conflict of interest.

References

1. Moffett-King, A. 2002. Natural killer cells and pregnancy. *Nat. Rev. Immunol.* 2: 656–663.
2. Norwitz, E. R. D., J. Schust, and S. J. Fisher. 2001. Implantation and the survival of early pregnancy. *N. Engl. J. Med.* 345: 1400–1408.
3. Croy, B. A., S. Esadeg, S. Chantakru, M. van den Heuvel, V. A. Paffaro, H. He, G. P. Black, A. A. Ashkar, Y. Kiso, and J. Zhang. 2003. Update on pathways regulating the activation of uterine natural killer cells, their interactions with decidual spiral arteries and homing of their precursors to the uterus. *J. Reprod. Immunol.* 59: 175–191.
4. Aluvihare, V. R., M. Kallikourdis, and A. G. Betz. 2004. Regulatory T cells mediate maternal tolerance to the fetus. *Nat. Immunol.* 5: 266–271.
5. Shorter, S. C., P. M. Starkey, B. L. Ferry, L. M. Clover, I. L. Sargent, and C. W. Redman. 1993. Antigenic heterogeneity of human cytotrophoblast and evidence for the transient expression of MHC class I antigens distinct from HLA-G. *Placenta* 14: 571–582.
6. Pijnenborg, R., L. Vercruysse, and M. Hanssens. 2006. The uterine spiral arteries in human pregnancy: facts and controversies. *Placenta* 27: 939–958.
7. Parham, P. 2004. NK cells and trophoblasts: partners in pregnancy. *J. Exp. Med.* 200: 951–955.
8. Cooper, M. A., T. A. Fehniger, and M. A. Caligiuri. 2001. The biology of human natural killer-cell subsets. *Trends Immunol.* 22: 633–640.
9. Koopman, L. A., H. D. Kopcow, B. Rybalov, J. E. Boyson, J. S. Orange, F. Schatz, R. Masch, C. J. Lockwood, A. D. Schachter, P. J. Park, and J. L. Strominger. 2003. Human decidual natural killer cells are a unique NK cell subset with immunomodulatory potential. *J. Exp. Med.* 198: 1201–1212.
10. Saito, S., K. Nishikawa, T. Morii, M. Enomoto, N. Narita, K. Motoyoshi, and M. Ichijo. 1993. Cytokine production by CD16^{-}CD56^{bright} natural killer cells in the human early pregnancy decidua. *Int. Immunol.* 5: 559–563.
11. Kitaya, K., T. Yamaguchi, T. Yasuo, T. Okubo, and H. Honjo. 2007. Post-ovulatory rise of endometrial CD16^{-} natural killer cells: in situ proliferation of residual cells or selective recruitment from circulating peripheral blood? *J. Reprod. Immunol.* 76: 45–53.
12. Bulmer, J. N., and G. E. Lash. 2005. Human uterine natural killer cells: a reappraisal. *Mol. Immunol.* 42: 511–521.
13. Kalkunte, S., C. O. Chichester, F. Gotsch, C. L. Sentman, R. Romero, and S. Sharma. 2008. Evolution of non-cytotoxic uterine natural killer cells. *Am. J. Reprod. Immunol.* 59: 425–432.
14. Ashkar, A. A., J. P. Di Santo, and B. A. Croy. 2000. Interferon γ contributes to initiation of uterine vascular modification, decidual integrity, and uterine natural killer cell maturation during normal murine pregnancy. *J. Exp. Med.* 192: 259–270.
15. Ain, R., L. N. Canham, and M. J. Soares. 2003. Gestation stage-dependent intrauterine trophoblast cell invasion in the rat and mouse: novel endocrine phenotype and regulation. *Dev. Biol.* 260: 176–190.
16. Hanna, J., D. Goldman-Wohl, Y. Hamani, I. Avraham, C. Greenfield, S. Natanson-Yaron, D. Prus, L. Cohen-Daniel, T. I. Arnon, I. Manaster, et al. 2006. Decidual NK cells regulate key developmental processes at the human fetal-maternal interface. *Nat. Med.* 12: 1065–1074.
17. Le Bouteiller, P., and J. Tabiasco. 2006. J. Killers become builders during pregnancy. *Nat. Med.* 12: 991–992.
18. Moffett, A., and C. Loke. 2006. Immunology of placentation in eutherian mammals. *Nat. Rev. Immunol.* 6: 584–594.
19. Lash, G. E., B. Schiessl, M. Kirkley, B. A. Innes, A. Cooper, R. F. Searle, S. C. Robson, and J. N. Bulmer. 2006. Expression of angiogenic growth factors by uterine natural killer cells during early pregnancy. *J. Leukocyte Biol.* 80: 572–580.
20. Li, X. F. 2001. Angiogenic growth factor messenger ribonucleic acids in uterine natural killer cells. *J. Clin. Endocrinol. Metab.* 86: 1823–1834.
21. Kim, Y. M., E. Bujold, T. Chaiworapongsa, R. Gomez, B. H. Yoon, H. T. Thaler, S. Rotmensch, and R. Romero. 2003. Failure of physiologic transformation of the spiral arteries in patients with preterm labor and intact membranes. *Am. J. Obstet. Gynecol.* 189: 1063–1069.
22. Sargent, I. L., A. M. Borzychowski, and C. W. Redman. 2007. NK cells and pre-eclampsia. *J. Reprod. Immunol.* 76: 40–44.

Downloaded from http://www.jimmunol.org/ by guest on March 5, 2021

23. Hiby, S. E., J. J. Walker, K. M. O'shaughnessy, C. W. Redman, M. Carrington, J. Trowsdale, and A. Moffett. 2004. Combinations of maternal KIR and fetal HLA-C genes influence the risk of preeclampsia and reproductive success. *J. Exp. Med.* 200: 957–965.
24. Jacobs, R., G. Hintzen, A. Kemper, K. Beul, S. Kempf, G. Behrens, K. W. Sykora, and R. E. Schmidt. 2001. $CD56^{bright}$ cells differ in their KIR repertoire and cytotoxic features from $CD56^{dim}$ NK cells. *Eur. J. Immunol.* 31: 3121–3127.
25. Riley, J. K., and W. M. Yokoyama. 2008. NK cell tolerance and the maternal-fetal interface. *Am. J. Reprod. Immunol.* 59: 371–387.
26. Kopcow, H. D., D. S. Allan, X. Chen, B. Rybalov, M. M. Andzelm, B. Ge, and J. L. Strominger. 2005. Human decidual NK cells form immature activating synapses and are not cytotoxic. *Proc. Natl. Acad. Sci. USA* 102: 15563–15568.
27. Murphy, S. P., L. D. Fast, N. N. Hanna, and S. Sharma,. 2005. Uterine NK cells mediate inflammation-induced fetal demise in IL-10-null mice. *J. Immunol.* 175: 4084–4090.
28. Murphy, S. P., N. N. Hanna, L. D. Fast, S. K. Shaw, G. Berg, J. F. Padbury, R. Romero, and S. Sharma. 2000. Evidence for participation of uterine natural killer cells in the mechanisms responsible for spontaneous preterm labor and delivery. *Am. J. Obstet. Gynecol.* 200: 308.e1 9. Epub 2008 Dec 27.
29. Kinsky, R., G. Delage, N. Rosin, M. N. Thang, M. Hoffmann, and G. Chaouat. 1990. A murine model of NK cell mediated resorption. *Am. J. Reprod. Immunol.* 23: 73–77.
30. Lachapelle, M. H., P. Miron, R. Hemmings, and D. C. Roy. 1996. Endometrial T, B, and NK cells in patients with recurrent spontaneous abortion: altered profile and pregnancy outcome. *J. Immunol.* 156: 4027–4034.
31. Quenby, S., M. Bates, T. Doig, J. Brewster, D. I. Lewis-Jones, P. M. Johnson, and G. Vince. 1999. Pre-implantation endometrial leukocytes in women with recurrent miscarriage. *Hum. Reprod.* 14: 2386–2391.
32. Kwak, J. Y., K. D. Beaman, A. Gilman-Sachs, J. E. Ruiz., D. Schewitz, and A. E Beer. 1995. Up-regulated expression of $CD56^+$, $CD56^+/CD16^+$, and $CD19^+$ cells in peripheral blood lymphocytes in pregnant women with recurrent pregnancy losses. *Am. J. Reprod. Immunol.* 34: 93–99.
33. King, A., and Y. W. Loke. 1990. Human trophoblast and JEG choriocarcinoma cells are sensitive to lysis by IL-2-stimulated decidual NK cells. *Cell. Immunol.* 129: 435–448.
34. Abadía-Molina, A. C., C. Ruiz, M. J. Montes, A. King, Y. W. Loke, and E. G. Olivares. 1996. Immune phenotype and cytotoxic activity of lymphocytes from human term decidua against trophoblast. *J. Reprod. Immunol.* 31: 109–123.
35. Cox, J. H., J. W. Yewdell, L. C. Eisenlohr, P. R. Johnson, and J. R. Bennink. 1990. Antigen presentation requires transport of MHC class I molecules from the endoplasmic reticulum. *Science* 247: 715–718.
36. Plevyak, M., N. Hanna, S. Mayer, S. Murphy, H. Pinar, L. Fast, C. Ekerfelt, J. Ernerudh, G. Berg, L. Matthiesen, and S. Sharma. 2002. Deficiency of decidual IL-10 in first trimester missed abortion: a lack of correlation with the decidual immune cell profile. *Am. J. Reprod. Immunol.* 47: 242–250.
37. Eriksson, M., S. K. Meadows., C. R. Wira, and C. L. Sentman. 2004. Unique phenotype of human uterine NK cells and their regulation by endogenous TGF-β. *J. Leukocyte Biol.* 76: 667–675.
38. Graham, C. H., T. S. Hawley, R. G. Hawley, J. R. MacDougall, R. S. Kerbel, N. Khoo, and P. K. Lala. 1993. Establishment and characterization of first trimester human trophoblast cells with extended lifespan. *Exp. Cell Res.* 206: 204–211.
39. Kalkunte, S., L. Zhongbin, N. Tewari, C. Chichester, R. Romero, J. Padbury, and S. Sharma. 2008. In vitro and in vivo evidence for lack of endovascular remodeling by third trimester trophoblasts. *Placenta* 29: 871–878.
40. Ayalon, O., E. A. Hughes, P. Cresswell, J. Lee, L. O'Donnell, R. Pardi, and J. R. Bender. 1998. Induction of transporter associated with antigen processing by interferon γ confers endothelial cell cytoprotection against natural killer-mediated lysis. *Proc. Natl. Acad. Sci. USA* 95: 2435–2440.
41. Clark, D. E., S. K. Smith, D. Licence, A. L. Evans, and D. S. Charnock-Jones. 1998. Comparison of expression patterns for placenta growth factor, vascular endothelial growth factor (VEGF), VEGF-B and VEGF-C in the human placenta throughout gestation. *J. Endocrinol.* 159: 459–467.
42. Ma, W., P. J. Lehner, P. Cresswell, J. S Pober, and D. R Johnson. 1997. Interferon-γ rapidly increases peptide transporter (TAP) subunit expression and peptide transport capacity in endothelial cells. *J. Biol. Chem.* 272: 16585–16590.
43. King, A., D. S. Allan, M. Bowen, S. J. Powis, S. Joseph, S. Verma, S. E. Hiby, A. J. McMichael, Y. W. Loke, and V. M. Braud. 2000. HLA-E is expressed on trophoblast and interacts with CD94/NKG2 receptors on decidual NK cells. *Eur. J. Immunol.* 30: 1623–1631.
44. Braud, V. M., D. S. Allan, C. A. O'Callaghan, K. Soderstrom, A. D'Andrea, G. S. Ogg, S. Lazetic, N. T. Young, J. I. Bell, J. H. Phillips, et al. 1998. HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C. *Nature* 391: 795–799.
45. Dunk, C., and A. Ahmed. 2001. Expression of VEGF-C and activation of its receptors VEGFR-2 and VEGFR-3 in trophoblast. *Histol. Histopathol.* 16: 359–375.
46. Ueda, M., Y. C. Hung, Y. Terai, K. Kanda, M. Kanemura, H. Futakuchi, H. Yamaguchi, D. Akise, M. Yasuda, and M. Ueki. 2005. Vascular endothelial growth factor-C expression and invasive phenotype in ovarian carcinomas. *Clin. Cancer Res.* 11: 3225–3232.
47. Zhou, Y., V. Bellingard, K. T. Feng, M. McMaster, and S. J. Fisher. 2003. Human cytotrophoblasts promote endothelial survival and vascular remodeling through secretion of Ang2, PlGF, and VEGF-C. *Dev. Biol.* 263: 114–125.
48. Goldszmid, R. S., A. Bafica, D. Jankovic, C. G. Feng, P. Caspar, R. Winkler-Pickett, G. Trinchieri, and A. Sher. 2007. TAP-1 indirectly regulates $CD4^+$ T cell priming in *Toxoplasma gondii* infection by controlling NK cell IFN-γ production. *J. Exp. Med.* 204: 2591–2602.
49. Lee, N., D. R Goodlett, A. Ishitani, H. Marquardt, and D. E. Geraghty. 1998. HLA-E surface expression depends on binding of TAP-dependent peptides derived from certain HLA class I signal sequences. *J. Immunol.* 160: 4951–4960.
50. Coupel, S., A. Moreau, M. Hamidou, V. Horejsi, J. P. Soulillou, and B. Charreau. 2007. Expression and release of soluble HLA-E is an immuno-regulatory feature of endothelial cell activation. *Blood* 109: 2806–2814.
51. Roby, K. F., D. Gershon, and J. S. Hunt. 1996. Expression of the transporter for antigen processing-1 (TAP-1) gene in subpopulations of human trophoblast cells. *Placenta* 17: 27–32.
52. Clover, L. M., I. L. Sargent, A. Townsend, R. Tampé, and C. W. Redman. 1995. Expression of TAP1 by human trophoblast. *Eur. J. Immunol.* 25: 543–553.

Downloaded from http://www.jimmunol.org/ by guest on March 5, 2021