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Decidual NK Cells Alter In Vitro First Trimester Extravillous Cytotrophoblast Migration: A Role for IFN-γ

Yuxiang Hu,†‡ Jan P. Dutz,†‡ Colin D. MacCalman,*¶ Paul Yong,†‡¶ Rusung Tan,†‡¶ and Peter von Dadelszen2*¶

Abnormal placentation results in either inadequate (consequences: recurrent miscarriage, intrauterine growth restriction, and preeclampsia) or overzealous (consequences: placenta accreta, increta, and percreta) placenta. NK cells dominate in first trimester decidua and probably control extravillous cytotrophoblast (EVT) invasion. We examined this interaction in a novel way, using NK cells and villous explants from concordant first trimester pregnancies cocultured using a new collagen (two-dimensional) model of placenta. Decidual NK (dNK) cells exerted contact-independent inhibition of normal cytotrophoblast migration, associated with changes in the cytotrophoblast expression of metalloproteinase-2 and -9, and plasminogen activator inhibitor-1. dNK cells did not affect EVT proliferation and apoptosis, and cell column formation. dNK cell effects were partially reversed by neutralizing Abs against IFN-γ. We provide ex vivo human evidence of a direct role for dNK in modulating EVT differentiation as they form columns and then migrate from anchoring villi. The Journal of Immunology, 2006, 177: 8522–8530.

The successful and tightly regulated interaction between the maternal immune system and fetal tissue is a prerequisite for normal pregnancy outcomes. Insufficient acquisition by the maternal immune system may lead to the inadequate placenta that supports normal pregnancy, and preeclampsia (1). Overzealous fetal invasion may lead to morbid adherence of the placenta, placenta accreta, increta, and percreta (2).

It has been postulated that maternal decidual NK (dNK)3 cells play an important role in the immune regulation of trophoblast invasion. There is considerable evidence to support that view (3). NK cells dominate during the early phase of gestation when placental trophoblast cells invade into decidua (4), and are found particularly around infiltrating trophoblast. This activity suggests a role in regulating the invasion and differentiation of the trophoblast (5). Preeclampsia, and the related fetal growth restriction, are interpreted as consequences of impaired trophoblast invasion (1, 6). Severe reduction of trophoblast cells, in combination with a strong increase of maternal lymphocytes and dNK cells, is found in intrauterine growth restriction (7).

Extravillous cytotrophoblast (EVT) express novel HLA-Ib proteins, HLA-G, and HLA-E (8, 9). These molecules have special roles in protecting trophoblast cells from maternal immune cells, especially dNK cells. In vitro studies have shown that the cytotoxic reactivity of dNK cells against HLA-G expression target cells of various origins was inhibited (10). HLA-E is a major ligand for the NK inhibitory receptor CD94/NKG2A (11), which mediates inhibition of killing of dNK cells (9). In the mouse, IL-15 was essential for the support of NK cell differentiation in the decidualizing uterus (12). dNK maintained with IL-15 in vitro have no cytotoxicity against trophoblast (13). This result suggests that, in normal pregnancy, dNK are not cytotoxic against cytotrophoblast.

During implantation, cytotrophoblast leave the tips of anchoring villi and become EVT. Initially, the EVT form cell columns, then invade and migrate from the columns into the maternal tissue, the decidua. The EVT at the villus tip are αβ, integrin positive, the EVT in the columns are αβ, integrin positive, and the invading EVT are αβ integrin positive (14, 15). A well-established experimental model of placentation involves the use of chorionic villi from first trimester terminations of pregnancy cultured as tissue explants on either collagen (two-dimensional model) (16, 17) or Matrigel (three-dimensional model) (18). In the two-dimensional model, the integrin α and α changes of invading EVT are not noted (16), whereas they are with the Matrigel model (18).

IFN-γ, a cytokine secreted by both activated T lymphocytes and NK, is best known for its immunological functions. It has been used to treat viral infections (19, 20). However, the use of IFN-γ in pregnancy might exert a deleterious effect, such as increased miscarriage (21). IFN-γ halts trophoblast migration in explant culture (22, 23), as does TNF-α. Others have reported that the following factors also contribute trophoblast migration inhibition: TGF-β1, TGF-β2, IL-10, and tissue inhibitor of metalloproteinases (TIMP)-1 (17, 22, 24–29). The role of dNK in controlling EVT differentiation and invasion has not been fully determined in a human model of placentation.

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In this study, we have tested the hypothesis that cotrailing first trimester villus explants with dNK will alter in vitro EVT migration. Using a new model in which dNK and villus explants were cocultured, we show that dNK cells limit trophoblast migration but not proliferation, apoptosis, and differentiation. This activity is mainly through the action of soluble mediators. Using Abs to neutralize IFN-γ partially reversed this effect.

Materials and Methods
Preparation and culture of dNK cells
We obtained decidual tissue from healthy women undergoing elective pregnancy termination of a normal pregnancy at 6- to 12-wk gestation, after informed consent. dNK cells were isolated as described previously (26), with minor modifications. Specimens were extensively washed with PBS, minced thoroughly into fragments of ~1 mm³, and digested for 1 h at 37°C in RPMI 1640 medium with 1.5 mg/ml collagenase and 2 mg/ml hyaluronidase (Sigma-Aldrich). Cell suspensions were washed and plated in dishes with 2 mg/ml IL-15 (R&D Systems), and, after an overnight incubation at 37°C, nonadherent were harvested from the culture dishes, leaving behind adherent stromal cells and macrophages. The cell suspension obtained was then separated from dead cells and RBC by Ficoll-Hypaque gradient 1077 (Amersham Biosciences). NK cells were then enriched with human NK cell enrichment mixture following the manufacturer’s instructions (StemCell Technologies). On average, 6 × 10⁵ enriched dNK per gram of decidual tissue were isolated.

We performed flow cytometer analysis (FACScan flow cytometer; BD Biosciences) with enriched dNK cells prepared in this way. Aliquots of ~1 × 10⁵ cells were incubated with fluorescence-labeled Abs (BD Biosciences) for 30 min on ice. In each sample 10,000 cells were evaluated. To increase the purity of dNK cells, and to confirm highly purified dNK cells have the same effect on trophoblast, allophycocyanin-conjugated anti–CD56 Ab-labeled NK cells were sorted by FACS. The purity of CD56+ cells was 96.3% after sorting.

Enriched dNK were cultured in 200 μl of DMEM/F12 supplemented with 10% FBS (Invitrogen Life Technologies) and incubated for 3 days at 37°C with 5% CO₂. IL-15 was added at 0–50 ng/ml concentrations. dNK proliferation was measured by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) (Promega) staining.

Explant culture
We prepared 30 first trimester placental explants as described previously (30). All dNK cells and villi were from concordant pregnancies; dNK from different individuals were not pooled. Small pieces of tissue (2–3 mm³) from the periphery of the placental debris were dissected (usually 4–8 villus tips per sample). For preparation of rat-tail collagen 1-coated plates (BD Biosciences), 0.8 ml of collagen I was mixed with 0.1 ml of 10× DMEM (1:10) and 0.1 ml of 7.5% NaHCO₃ (1:10). For each explant, 80 μl of collagen was placed in the center of a 12-well culture dish. After formation of gels (30 min at 37°C), the dissected tissue pieces were carefully put on the top of each gel drop, covered with 20 μl of medium, and incubated for 2–4 h to allow anchorage. Subsequently, explants were flooded with 1 ml of DMEM/F12 supplemented with 10% FBS (DMEM/F12/FBS) and incubated overnight at 37°C with 5% CO₂. After an initial period of 24 h, during which cytotrophoblast proliferation occurs at the villous tips to produce anchoring cell columns, the explant cultures were exposed to a variety of experimental conditions, including Jurkat cells (a human T cell leukemia; ATCC), Type Cultured Stromal Cells (R&D Systems) (described in Results). The villi that did not show good attachment were discarded. The day when cultures were exposed to various conditions was time 0.

In preliminary experiments, we investigated the attachment of villus explants to collagen-coated surfaces, and found that the explants did not attach efficiently; EVT outgrowth rate was much lower (6 vs 50% with collagen-coated surface). Also, EVT differentiation (including column formation, then migration) was poor. This result is consistent with previous findings (31).

To assess potential involvement of TNF-α, TGF-β₁, TGF-β₂, IL-10, TIMP-1, plasminogen activator inhibitor (PAI)-1, and IFN-γ secreted by dNK, explants were incubated with dNK in the presence of neutralizing Abs (10 μg/ml; R&D Systems). Abs or TGFs were added individually, or in combination, added daily. dNK-derived conditioned medium (CM) were preincubated with 10 μg/ml (TIMP-1, 2 μg/ml) Abs for 1 h on ice, then exposed to explant cultures for a total of 96 h. Neutralizing Ab to PAI-1 (clone MA-56A7C10; University of Leuven, Leuven, Belgium) was also tested at 10 μg/ml, both individually and in combination.

Assessment of cell columns, migration, and trophoblast differentiation
EVT outgrowth and migration occurs largely across the surface of the gel as described previously (32, 33). Outgrowth was examined qualitatively. For example, in assay 1, the migration distances for the four control tips were 35, 30, 30, and 35 μl, the mean was 32.5 μl. For the five hollow fiber (HF)–containing HF–exposed tips, the migration distance were 12, 20, 10, 13, and 20 μl. Therefore, for those five tips, the distance was corrected and calculated to be 0.37, 0.62, 0.62, 0.4, and 0.62, respectively, using the control tip mean as unity. For sample 5, the control tip distances were 70, 50, 50, and 60 (mean 57.5) μl, and the four HF dNK-exposed tip distances were 35 (0.61), 60 (1.04), 20 (0.35), and 45 (0.78) μl. The magnitude and direction of effect were similar between samples; using the observed/expected approach facilitated interpretation and analysis of the data.

Cell outgrowth patterns were examined by two individuals blinded to the experimental group, and described on an arbitrary scale of 1 (tightly packed cells through the outgrowth especially at the edge of the column, no gaps visible between EVT) to 3 (majority of cells radially oriented and migrating beyond the column, large gaps visible between EVT). The score 2 was assigned when cell outgrowth patterns were intermediate between 1 and 3.

The purity of EVT was assessed by fluorescence microscopy to determine the level of fibroblast contamination of EVT columns and migrating cells. Explant cultures fixed by 70% methanol were double-stained with rabbit anti-human cytokeratin (1/500 dilution; DakoCyto) and mouse anti-human vimentin (1/250 dilution; DakoCyto) for 1 h, washed, and then incubated with Alexa Fluor 594 goat anti-rabbit IgG (H+L) or Alexa Fluor 488 goat anti-mouse IgG (H+L) (Invitrogen Life Technologies). Stromal cells harvested from decidua were used as a positive control for vimentin Ab.

Immunohistochemistry staining for integrins
Immunocytochemistry staining was used to assess whether NK cells affected integrin expression in EVT. Briefly, 70% methanol-fixed cultured explants were incubated with mouse anti-human integrin α₂ or anti-integrin α₅ (Chemicon International), overnight at 4°C, and washed extensively with PBS. HRP-labeled secondary Abs (Bio-Rad) were added, and the plates were incubated for 1 h at room temperature for integrin α₂ staining; or biotinylated link universal secondary Ab and streptavidin–HRP (DakoCyto) were used for integrin α₅. After washing with PBS, 3-amino-9-ethylcarbazole substrate was applied. Images were taken at ×4 magnification.

Total RNA preparation and first-strand cDNA synthesis
Villi were carefully dissected from EVT. Total RNA was prepared from EVT after EVT were lysed and pooled from same assay groups according to the manufacturer’s protocol (RNAeasy mini kit; Qiagen). For this type of assay, EVT were always from the HF contained NK-treated group when in coculturing system was involved. cDNA synthesis was followed as described by the manufacturer (ThermoScript RT-PCR System; Invitrogen Life Technologies).

Real-time quantitative PCR
The SYBR Green real-time quantitative PCR was performed to quantify mRNA expression using the ABI Prism 7300 Sequence Detection System (Applied Biosystems). After 2 min at 50°C and 10 min at 95°C, the samples were cycled 40 times at 95°C for 15 s and 60°C for 60 s. The relative quantification of gene expression was calculated using 18S gene
expression as endogenous control, as described previously (34), and presented as fold changes in EVT in treated groups compared with the medium control group. The following proteins were evaluated: matrix metalloprotease (MMP)-2 and -9, urokinase-type protease activator (uPA), PAI-1, caspase-3, and E-cadherin. (Applied Biosystems). The primer sequences were listed as described below. MMP-2 and -9, uPA, and PAI-1 were a gift from Dr. P. Leung’s (Vancouver, British Columbia, Canada) laboratory.

The primers used were as follows: MMP-2, (sense) CCGCAGTGACGGAAAGATGT and (antisense) CACTTGCGGTCGTCATCG-TA; MMP-9, (sense) GGACGATGCCTGCAACGT and (antisense) CAAATACAGCTGG-TTCCCAATCT; uPA, (sense) CGCTTTCTTGCTGGTTGT and (antisense) CCCAGTCTCTTCTTACAGCTGAT; PAI-1, (sense) CCGCCGCC-TCTTCCA and (antisense) GCCATCATGGGCACAGAGA; caspase-3, (sense) GCCTACAGCCCATTTCTCCAT and (antisense) GCCGCTCTTCTTACAGGATGAT; and E-cadherin, (sense) AGGTGACAGAGCCTCTGGATAGA and (antisense) GGATGACACAGCGTGAGAGAAG.

The measurement of protein activity and level in explant culture supernatants

The supernatants of explant cultures were pooled from the same group and used to measure the protein activity or level. The total and active MMP-2 and -9 activity were measured using MMP-2 and -9 Biotrak Activity Assay System (Amersham Biosciences); TIMP-1 was detected by TIMP-1, human, Biotrak ELISA System (Amersham Biosciences); uPA activity was tested by uPA Activity Assay Kit (Chemicon International); and PAI-1 was detected with IMUBIND Tissue PAI-1 ELISA Kit (American Diagnostics). The expression of MMP-2 and -9, uPA, and PAI-1 are presented as fold changes in the treated groups compared with the medium control group.

The determination of EVT apoptosis by caspase-3 assay and annexin-V staining, E-cadherin expression by flow cytometry, and proliferation assay by MTS staining

Caspase-3 and annexin-V were used to determine whether EVT went apoptosis after exposure to dNK or CM. Caspase-3 activity was tested by Caspase 3/CPP32 Colorimetric assay kit (BioVision). Fold increase in CPP activity was determined by comparing EVT activity in HF dNK or CM-treated groups with the level in medium-treated control. Annexin V expression was measured by flow cytometry following the manufacturer’s suggestions (BD Pharmingen). In these two assays, EVT from the same group were pooled after villi were dissected and then assayed. Staurosporine was added to EVT in medium control group for an additional 4 h at the end of explant culture and then used as positive EVT apoptosis inducer.

E-cadherin expression was used to determine whether dNK and CM changed E-cadherin expression in EVT. After removing villi, EVT from the same assay group were pooled and labeled with rabbit Ab against E-cadherin (Santa Cruz Biotechnology) followed by FITC-conjugated goat anti-rabbit Ig. The E-cadherin expression was measured by flow cytometry.

MTS staining was applied to measure whether HF dNK or CM affected the EVT proliferation. After removing the villi, collagen gels attached with EVT were carefully transferred to 96-well plates individually. Medium
was added back to each well containing the gel. Then, EVT were exposed to 25 µl of MTS and incubated for 5 h. OD at 490 nm was measured.

Statistics

Groups were compared using either parametric one-way ANOVA, with Student’s t tests as posttests, or nonparametric Kruskal-Wallis ANOVA, with Mann-Whitney $U$ posttest, as appropriate, using GraphPad Prism 4.0 software. For posttests, significance was set at 0.01, to adjust for multiple comparisons, because Bonferroni corrections were deemed too strict.

Results

Purity and maintenance of dNK cell preparations, and purity of cell column and migrating villous outgrowth cells

We observed that >94% of decidual lymphocytes were CD45$. Of the CD45$ cells, 87.3% were dNK (CD56$/CD16$), CD3$ and CD14$ cells were all <0.4%, and CD16$ cells were <2.1% (BD Biosciences) (Fig. 1a). At IL-15 concentrations, >2 ng/ml dNK viability in culture was well maintained for 72 h (Fig. 1b). For all experiments, 10 ng/ml IL-15 was used. We found that cells forming cell columns and migrating on collagen were cytokeratin positive and vimentin negative (Fig. 1c); cytokeratin stains for trophoblast, an epithelial cell line, whereas vimentin stains for cells of fibroblast origin. Isolated stromal cells from decidua were vimentin positive (Fig. 1d).

dNK inhibit in vitro first trimester EVT migration

We examined the effect of dNK on EVT column formation and migration under the following experimental conditions: 1) control medium; 2) 10 ng/ml IL-15 in medium; and 3) Free dNK (enriched dNK with 10 ng/ml IL-15 suspended at 5 × 10$^3$ – 6 × 10$^5$ cells/explant in culture medium).

Upon seeding on collagen I (i.e., two-dimensional culture), we observed attachment and flattening at the distal parts of the tips and outgrowth of EVT in explant cultures, as described previously (32, 33). For explant cultures in medium or IL-15 control groups, EVT formed columns and multilayered sheets of cells in 24–48 h, and migrated out in a radially oriented direction especially from the column edge, usually in 48–96 h (Fig. 2 and Table I). In the presence of free dNK, proliferation and outgrowth occurred and increased in extent over several days, but migration of EVT away from column was not observed. For one experiment in which flow
Table I. Decidual NK cells inhibit extravillous trophoblast migration, but not proliferation, in a contact-independent manner

<table>
<thead>
<tr>
<th>Culture Conditions (number of tips examined)</th>
<th>Corrected Migration Distance (median; IQR)</th>
<th>MWu (*, p)</th>
<th>Migration/Cell Packing Scorea (median; IQR)</th>
<th>Bonferroni (*, p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (229)</td>
<td>1.01 (0.8, 1.2)</td>
<td>NS</td>
<td>3.0 (3, 3)</td>
<td>NS</td>
</tr>
<tr>
<td>Control + IL-15 (51)</td>
<td>0.92 (0.66, 1.1)</td>
<td>(vs control)</td>
<td>3.0 (3, 3)</td>
<td>(vs control)</td>
</tr>
<tr>
<td>Free dNK (59)</td>
<td>0.66 (0.42, 0.83)</td>
<td>&lt;0.0001</td>
<td>1.0 (1, 1)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Free dNK + anti-IFN-γ (19)</td>
<td>0.85 (0.82, 0.98)</td>
<td>0.0047</td>
<td>1.0 (1, 1)</td>
<td>NS</td>
</tr>
<tr>
<td>Free dNK + anti-TNF-α (7)</td>
<td>0.70 (0.37, 0.76)</td>
<td>NS</td>
<td>1.0 (1, 1)</td>
<td>NS</td>
</tr>
<tr>
<td>Empty hollow fibers (7)</td>
<td>0.98 (0.93, 1.1)</td>
<td>(vs free dNK)</td>
<td>3.0 (3, 3)</td>
<td>(vs free dNK)</td>
</tr>
<tr>
<td>HF dNK (106)</td>
<td>0.58 (0.47, 0.70)</td>
<td>&lt;0.0001</td>
<td>1.0 (1, 1)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>HF dNK + anti-IFN-γ (16)</td>
<td>0.84 (0.61, 0.93)</td>
<td>0.0015</td>
<td>1.0 (1, 1)</td>
<td>NS</td>
</tr>
<tr>
<td>HF dNK + anti-TNF-α (12)</td>
<td>0.58 (0.41, 0.63)</td>
<td>(vs HF dNK)</td>
<td>1.0 (1, 1)</td>
<td>(vs HF dNK)</td>
</tr>
<tr>
<td>HF dNK + anti-PAI-1 (22)</td>
<td>0.66 (0.48, 0.76)</td>
<td>(vs HF dNK)</td>
<td>1.0 (1, 1)</td>
<td>(vs HF dNK)</td>
</tr>
<tr>
<td>Conditioned medium (137)</td>
<td>0.63 (0.46, 0.85)</td>
<td>&lt;0.0001</td>
<td>1.0 (1, 1)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CM + anti-IFN-γ (49)</td>
<td>0.79 (0.52, 1.05)</td>
<td>0.0089</td>
<td>1.0 (1, 1)</td>
<td>NS</td>
</tr>
<tr>
<td>CM + anti-TNF-α (53)</td>
<td>0.74 (0.62, 0.92)</td>
<td>(vs CM)</td>
<td>1.0 (1, 1)</td>
<td>(vs CM)</td>
</tr>
<tr>
<td>CM + anti-PAI-1 (13)</td>
<td>0.73 (0.59, 0.82)</td>
<td>NS</td>
<td>1.0 (1, 1)</td>
<td>NS</td>
</tr>
<tr>
<td>CM + anti-TGF-β3 (11)</td>
<td>0.68 (0.56, 0.74)</td>
<td>(vs CM)</td>
<td>1.0 (1, 1)</td>
<td>(vs CM)</td>
</tr>
<tr>
<td>CM + anti-TIMP-1 (7)</td>
<td>0.51 (0.47, 0.57)</td>
<td>(vs CM)</td>
<td>1.3 (0.5)</td>
<td>NS</td>
</tr>
<tr>
<td>IFN-γ (81)</td>
<td>0.48 (0.36, 0.65)</td>
<td>&lt;0.0001</td>
<td>1.0 (1, 1)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>IFN-γ + anti-IFN-γ (46)</td>
<td>0.86 (0.66, 1.08)</td>
<td>&lt;0.0001</td>
<td>3.0 (2.5, 3)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>TNF-α (24)</td>
<td>0.68 (0.40, 0.79)</td>
<td>&lt;0.0001</td>
<td>1.0 (1, 1)</td>
<td>(vs IFN-γ)</td>
</tr>
<tr>
<td>TNF-α + anti-TNF-α (15)</td>
<td>1.03 (0.81, 1.24)</td>
<td>0.0033</td>
<td>2.5 (2.3)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Jurkat cells (6)</td>
<td>0.95 (0.63, 1.04)</td>
<td>(vs TNF-α)</td>
<td>3.0 (3, 3)</td>
<td>NS</td>
</tr>
</tbody>
</table>

a Semiquantitative evaluation of cytotrophoblast outgrowth from explants was carried out by analyzing individual sites at villous tips. Corrected migration distance: farthest cell outgrowth from villous tips using aperture grid; corrected against mean migration distance for all control tips for each sample. Migration/cell packing score: arbitrary scale of 1 (tightly packed cells through the outgrowth especially at the edge of the column) to 3 (majority of cells radially oriented and migrated beyond the column). anti-Neutralizing Ab; MWu, Mann-Whitney U test.

* p < 0.0001, Kruskal-Wallis; MWu tests performed (significance set at 0.01 (multiple comparisons)).

Cytotherapy-sorted dNK were used (purity 96.3%), the similar inhibitory effect on trophoblast migration was observed (data not shown). The outgrowths in these sheets were densely packed, especially at the anticipated limits of normal cell columns from control cultures, and outgrowth distance was limited (Fig. 2 and Table I).

The dNK-dependent migration inhibition was cell number dependent. When the concentration of dNK was ≥100,000 cells/explant, EVT migration was invariably limited; when the dNK concentration was ≤50,000 cells/explant, then EVT migration was not affected (data not shown).

Therefore, we focused on whether the observed effect was contact-dependent or contact-independent.

dNK inhibit EVT migration in a contact-independent manner

To address the question of whether dNK-dependent migration inhibition was due to the contact inhibition, we undertook the following studies: 1) dNK contained within sealed protein permeable, but cell impermeable, HFs (MWCO 500,000, used mainly in pre-clinical drug efficacy tests in oncology (35); 2) HF dNK were cocultured with villous explants (enriched dNK cells 3 × 10⁵ cells/explant) with 10 ng/ml IL-15 suspended in culture medium; and 3) The HFs were placed at the periphery of the culture plates, to avoid close proximity between dNK and EVT. However, empty HFs were also tested and did not influence EVT migration.

The results showed that cell outgrowth and column formation was not interrupted, but migration distance and pattern were affected similar to free dNK cells (Fig. 2 and Table I). Enriched dNK conditioned medium (enriched dNK cells were cultured for 48 h at 0.5 × 10⁵ cells/ml with 10 ng/ml IL-15) suspended in culture medium; conditioned medium was added to explant culture at 1:1 ratio with medium) caused similar migration inhibition as free or HF dNK (Fig. 2 and Table I). Instead of dNK, Jurkat cells, at 3 × 10⁵ cells/explant culture, were used to assess cell contact-dependent changes in EVT differentiation. Normal EVT migration was observed (Fig. 2 and Table I). The assay indicated that interaction between EVT and Jurkat cells did not reveal contact inhibition.
Therefore, we investigated whether cytokines could be responsible for the observed effects of dNK on EVT.

**Assessment of the potential involvement of the soluble mediators secreted by dNK**

dNK appeared to inhibit trophoblast migration through soluble mediators. TNF-α has been reported to restrict EVT migration (17). As a positive control, we determined whether or not TNF-α influenced the alterations in EVT migration noted above. Briefly, 4 ng/ml TNF-α was added into explant cultures causing the migration inhibition; neutralizing Ab to TNF-α reversed the effect (Fig. 2 and Table I). dNK produced 21.5 (±13.5 SD) pg/ml TNF-α in coculture medium after 96 h, as measured by ELISA. However, dNK-dependent migration inhibition could not be reversed by a neutralizing anti-TNF-α Ab (Table I).

dNK also produce IFN-γ, TGF-β1, TGF-β2, IL-10, and TIMP-1 (23–26, 36), and these cytokines may either reduce MMP expression (27), MMP autoconversion into active forms (37), or inhibit EVT migration and invasion (28, 29). We found that IL-15-main-tained dNK released TIMP-1 into conditioned medium after 96 h, as measured by ELISA. However, neutralizing Abs to TGF-β1, TGF-β2, IL-10, PAI-1, and TIMP-1 did not reverse migration inhibition (Table I). Because the PAI-1 level was increased after exposure to dNK (Fig. 3), neutralizing Ab to PAI-1 was also tested, and no reverse effect was observed (Table I).

Anti-IFN-γ was associated with reversal of the farthest distance of EVT spread from villous tips (Table I). There was no discernible effect of anti-IFN-γ on migration inhibition/cell packing scores (Table I). Therefore, we tested the influence of IFN-γ on EVT. Briefly, 5 ng/ml recombinant human IFN-γ was added into explant cultures causing migration inhibition; neutralizing Ab to IFN-γ reversed the effect (Fig. 2 and Table I). Recombinant human IFN-γ showed this effect when the dose was as low as 200 pg/ml. dNK produced 207 (±122 SD) pg/ml IFN-γ in coculture medium after 96 h, as measured by ELISA.

By MTS assay, HF dNK and CM did not alter EVT cell proliferation compared with controls (median (IQR): 0.39 (0.29, 0.358; n = 10), 0.44 (0.25, 0.78; n = 10), and 0.49 (0.35, 0.59), respectively), whereas IFN-γ (5 ng/ml) did (0.22 (0.15, 0.35; n = 13), Mann-Whitney U test, p = 0.0115 (vs control)). The effect of IFN-γ on proliferation was reversed by neutralizing anti-IFN-γ Ab (0.40 (0.29, 0.51; n = 6)).

**FIGURE 3.** dNK affect EVT protease gene expression and protein levels. a, HF dNK and CM reduced mRNA for total and active forms of MMP-2. b, Although HF dNK and CM increased mRNA for and total MMP-9, the active form was reduced. c and d, HF dNK and CM increased mRNA for and protein concentrations of uPA and PAI-1, respectively. Data are presented as fold increase or decrease compared with mean control tip mRNA or protein expression, as relevant, per pregnancy sample. For all graphs, ANOVA p < 0.01; Student’s t test **p < 0.01 and ***p < 0.001, compared with control (ctrl).
dNK and CM influenced MMP-2 and -9, uPA, and PAI-1 expression

We examined the effect of dNK exposure on EVT gene expression and protein levels of the following proteases: MMP-2 (total and active), MMP-9 (total and active), uPA, and PAI-1 (Fig. 3). These proteases have been shown by us and others to be involved in EVT migration and differentiation. For MMP-2, mRNA was reduced following exposure to HF dNK (reversed by anti-IFN-γ/H9253; data not shown), CM, and IFN-γ/H9253 (reversed by anti-IFN-γ/H9253); this was mirrored by both total and active MMP-2 protein, for free dNK and CM, but not HF dNK. Although mRNA message and total MMP-9 were increased following HF dNK and CM exposure, however, the active form was not increased or even decreased with exposure to free and HF dNK and CM. Both PAI-1 and uPA message RNA were increased following exposure to HF dNK and CM; however, HF dNK did not cause elevated levels of PAI-1, whereas free dNK and CM did. HF dNK caused elevated uPA expression.

Because the cell columns in the three dNK groups (free, contained, and conditioned medium) appeared tightly packed with EVT, we investigated whether or not either EVT adhesion molecule expression or apoptosis was influenced.

dNK and CM increased EVT E-cadherin expression, did not alter α1 and α5 integrin expression, and had no cytotoxicity against cytrophoblast

E-cadherin gene and protein expression were increased in EVT exposed to both HF dNK and CM (Fig. 4a). Cytrophoblast in explant cultures expressed α1 and α3 integrins in the collagen model as reported previously (16). In the absence of the biotin-streptavidin system, α5 integrin expression was strongly positive and α1 integrin expression was weakly positive in both column EVT and migrating EVT, in the medium and IL-15 control groups. But α5 integrin was also strongly positive following the introduction of the biotin-streptavidin system. α1 and α5 integrin expression was not affected by exposure to either HF dNK or CM, but was increased by staurosporine (positive control). ANOVA p < 0.001 for all comparisons; Student’s t test, **, p < 0.01 and ***, p < 0.001, compared with control. Scale bars, 250 μm.

Discussion

We have introduced a new model of the critical interaction between dNK and first trimester trophoblast that closely simulates the in vivo state. We found that dNK limit migration of EVT, through changes in protease activity and E-cadherin expression, and that the inhibition of EVT expansion was reversed by anti-IFN-γ. Also, we have introduced the use of HFs into the villus explant model, showing that dNK-dependent migration inhibition is contact independent.

dNK limit trophoblast migration in a dose-dependent manner. When dNK cells were ≥100,000 cells per explant culture, trophoblast migration was restricted, but no inhibition was observed.
when dNK cells were \(\leq 50,000\) cells per explant culture. HF dNK and CM, but not Jurkat cells, had the same inhibitory effects on EVT migration as free dNK. This indicated that dNK-dependent migration inhibition was contact independent and is mediated by soluble factors produced by dNK.

We have determined that IL-15 is required to maintain dNK cells in vitro, but does not affect EVT proliferation and migration. dNK activated by IL-15 do not have cytotoxicity against cytotrophoblast in vitro (13). We have found that trophoblast cocultured with dNK or CM are able to proliferate. The cell column expanded over several days of culture, with increased cellular packing within the columns, related to the observed up-regulation of E-cadherin gene expression. There was no evidence of apoptosis induction in EVT by dNK measured by caspase-3 and annexin V; indeed, CM seemed to reduce caspase-3 gene expression in EVT. However, because migration was limited, dNK alter EVT transition to a migrating phenotype, but not proliferation, and without cytotoxicity.

We have confirmed the findings of Bauer et al. (17) that TNF-\(\alpha\)-treated EVT remain as highly packed cell columns. This important cytokine, produced by dNK, may regulate at least some of the dNK effects on EVT differentiation. However, neutralizing Ab to TNF-\(\alpha\) added to dNK coculture did not reverse the effect, indicating that other soluble mediators must be involved.

Neutralizing the effect of IFN-\(\gamma\) reversed the EVT expansion associated with dNK exposure. IFN-\(\gamma\) is a major dNK cytokine (23), being secreted spontaneously by dNK isolated from human decidua and at higher concentrations than peripheral blood NK cells (38, 39). We found that IFN-\(\gamma\)-treated EVT remained as tight columns. However, unlike exposure to either HF dNK or CM, IFN-\(\gamma\)-reduced EVT proliferation, as measured by MTS assay. We believe that this due to the effect induced by dNK or CM results from multiple modulators, rather than a single inhibitory factor. In the mouse, dNK are recruited to the decidua, and dNK-derived IFN-\(\gamma\) plays a key role in the control of trophoblast migration and spiral artery modification (40). However, the subjective assessment of migration and cell packing remained significantly different between the anti-IFN-\(\gamma\)-treated group and controls, implying that the change in measured distance may have modulated through either a reduction in tight inter-EVT adhesion (imperceptible to the light microscope-aided eye) or an increase in the dimensions of the cell column due to EVT proliferation, without permitting change to the migrating phenotype.

dNK can produce other soluble molecules like TGF-\(\beta_1\), TGF-\(\beta_2\), and IL-10, all of which have been reported to limit trophoblast migration and invasion (24, 26–29, 41). However, in these studies, neutralizing Abs to these molecules did not reverse the effect. dNK produce a variety of cytokines and other immunomodulatory proteins (23, 24, 42). The observed effect of dNK on EVT almost certainly does not act through a single molecule, such as IFN-\(\gamma\). A protein array investigation of the CM would facilitate the identification of other cytokines or immunomodulatory proteins to be investigated.

Among other MMPs, MMP-9 is known to be self-activating, with the cleavage of the propeptide leading to the formation of an active enzyme (43). TIMP-1, the natural inhibitor of MMP-9 and MMP-2 (43), is secreted by dNK (25, 36), a finding that we have confirmed. TIMP-1 can block the activation of pro-MMP-9 in skin (37). Given our findings of decreased active MMP-9, despite increased MMP-9 mRNA and total MMP-9 protein, in association with dNK exposure, we support the proposed down-regulation of the proteolytic activation of MMP-9 in human tissue by TIMP-1 (37). However, we could not reverse the phenotypic effect on EVT migration with a blocking mAb to TIMP-1. Also, whereas free dNK and CM reduced EVT-free MMP-2 protein, HF dNK did not.

This implies that part of the dNK effect on MMP-2 activation is due to large solutes (molecular mass \(>500\) kDa). Such solutes might be complexed proteoglycans (e.g., serglycin or decorin).

The observed increase in uPA mRNA and protein probably represents a compensatory response by EVT, because increased uPA expression would be expected to increase EVT migration (44).

We have also confirmed the finding that EVT cultured on collagen do not undergo the integrin expression changes noted both in vivo and during culture on Matrigel, which permits true invasion, rather than migration (16, 18). dNK had no discernible effects on \(\alpha_5\) and \(\alpha_\text{vi}\) integrin expression in EVT in vitro.

In vivo, the interaction between dNK and EVT may be modulated by Ag-presenting dendritic cells, which are present in proximity to dNK in human first trimester decidua (45, 46), as well as other factors such as other cytokines, growth factors, and pregnancy hormones.

There are some limitations to this study. Primarily, as noted, the model that we chose for these experiments was two-dimensional, with explants being cultured on collagen, rather than three-dimensional, using Matrigel; we anticipate being able to replicate the findings with Matrigel. Second, the scale used to measure EVT migration was intrinsically arbitrary, and, therefore, prone to bias. However, the assessment of EVT migration was blinded in terms of experimental conditions and was averaged between two observers; the findings presented reflect this approach. As we refine the model to the use of Matrigel, we will apply objective invasion assays.

Despite the limitations, these data show ex vivo human evidence of a direct role for dNK in modulating EVT differentiation as they form columns and then migrate from anchoring villi. The control of this interaction, partially through IFN-\(\gamma\), is important in the processes that lead to either normal placentation, or deficient placentation and its sequelae (e.g., preeclampsia and intrauterine growth restriction), and morbidity and mortality of the placenta.

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**Disclosures**

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