Sphingosine signalling regulates decidual NK cell angiogenic phenotype and trophoblast migration

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Submitted on April 12, 2013; resubmitted on July 4, 2013; accepted on July 30, 2013

STUDY QUESTION: Is sphingosine-1-phosphate (S1P) signalling involved in the regulation of the angiogenic function of decidual (d)NK cells during human pregnancy?

SUMMARY ANSWER: Human dNK cells, characterized by S1P receptor 5 (S1PR5) expression, are reactive to microenvironmental S1P to modify their VEGF expression and to regulate trophoblast migration and endothelial angiogenesis.

WHAT IS KNOWN ALREADY: S1P signalling can modulate peripheral (p)NK cells migration and function. As a unique NK population, human dNK can produce multiple cytokines and angiogenic growth factors to mediate extravillous trophoblast (EVT) invasion and spiral artery remodelling during pregnancy.

STUDY DESIGN, SIZE, DURATION: The study was designed to examine S1PR expression and function by freshly isolated human dNK cells in response to different S1P scenarios, created by FTY720, an S1P analogue and S1PR modulator. Ex vivo and in vitro experiments were performed to evaluate the functions of dNK cells. The study was performed between September 2011 and June 2013.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Human peripheral blood and decidual samples were collected and the S1PR expression by the decidual leukocytes population was examined. FTY720-induced dNK phenotypic and functional changes (including VEGF and IL-8 expression) were evaluated by multi-colour flow cytometric assays and transwell migration studies. Human placental explant culture and wound healing assays were performed to investigate whether S1P-activated dNK mediated trophoblast migration while angiogenesis was assessed by human umbilical vein endothelial cells (HUVEC) tube formation assays. Both first and second trimester dNK cells were studied to compare the difference in S1PR expression over time at the fetal—maternal interface.

MAIN RESULTS AND THE ROLE OF CHANCE: Freshly isolated NK cells (CD45+CD56+CD16−) from blood (pNK) and decidua (dNK) had low S1PR1 reactivity while S1PR5 was prominently expressed by dNK (40%) and, to a lesser extent, by pNK (18%; P<0.05) cells. S1PR5 expression by dNK was significantly down-regulated by FTY720 treatment, which also impaired decidual leukocyte mobility and cellular contact with invasive EVT. FTY720 significantly reduced VEGF expression by dNK, both in the numbers of VEGF+ cells and in fluorescence intensity (P<0.05). IL-8 expression by dNK was not changed by FTY720 and remained low at 8% positivity. Trophoblast migration and HUVEC tube formation were stimulated by control leukocytes, enriched CD56+ dNK or their conditioned medium, respectively, but this effect was markedly abrogated once they were pretreated with FTY720 (P<0.05). There was a significant decrease in S1PR5 expression in second trimester dNK cells, compared with those from first trimester (P<0.05). No significant differences in the levels of angiogenic factors (VEGF or IL-8) were detected between first and second trimester dNK cells.

LIMITATIONS, REASONS FOR CAUTION: Our ex vivo and in vitro experimental samples were from healthy women undergoing elective pregnancy termination. FTY720 is a chemical ligand for the S1PRs; little is known regarding the levels or actions of the naturally occurring ligand S1P in human gestational tissues. The in vivo function of S1PR5−/− dNK may be further investigated by using a genetically modified animal model.

WIDER IMPLICATIONS OF THE FINDINGS: This is the first study to investigate the role of S1PR and S1P interaction on dNK cell physiology and their downstream effects on trophoblast migration. We suggest that S1PR5 may represent a potential target for cellular targeted treatments for gestational diseases such as pre-eclampsia and intrauterine growth restriction that are characterized by inadequate dNK/trophoblast-coordinated uterine spiral artery transformation.
**Introduction**

During human pregnancy, a specialized subset of natural killer (NK) cells becomes abundant at the fetal–maternal interface. These CD56-positive decidual (d)NK cells, either homed from peripheral organs or differentiated in situ, are accommodated within the decidual niche and are thought to play a critical role in uterine spiral artery remodelling and the increasing placental perfusion in normal pregnancy (Moffett *et al.*, 2004). Abnormal human gestation is associated with disorganization of immune cell populations in the placental bed and a failure in uterine spiral artery remodelling (Romero *et al.*, 2010). Increasing evidence has suggested an active role for dNK in the initiation of spiral artery remodelling by facilitating vascular smooth muscle cell separation and regulating trophoblast invasion (Pijnenborg *et al.*, 2006; Smith *et al.*, 2009; Hazan *et al.*, 2010; Robson *et al.*, 2012). Several studies have indicated that dNK cells may control extravillous trophoblast (EVT) invasion during early pregnancy via the secretion of various cytokines and chemokines (Chakraborty *et al.*, 2011; Wallace *et al.*, 2012). Human dNK cells are capable of producing various soluble factors such as VEGF, IL-8, IFNG, IL-1B and CCL2/MCP-1 (Hanna *et al.*, 2006; Hu *et al.*, 2006; Lash *et al.*, 2011; Bulmer *et al.*, 2012). Among these, the VEGF family plays a prominent role in promoting trophoblast motility, proliferation and spiral artery remodelling. The recent confirmation of a significant decrease in placental VEGF mRNA in severe early onset pre eclamptic women (Andraweera *et al.*, 2012) has further raised the possibility that the dysregulation of angiogenic dNK may be a contributing factor to the development of such gestational disease. Different approaches have also indicated that the functional capability of dNK cells may be greatly influenced by their microenvironment (Vacca *et al.*, 2011). Thus, a detailed investigation of the mechanisms governing dNK cell function, as both regulators and effectors of immunity and tissue remodelling during the establishment of the uteroplacental circulation, will clarify their role in human pregnancy.

Sphingosine-1-phosphate (SIP) is a bioactive phospholipid that transmits signals through the G-protein-coupled receptors (GPCRs), such as S1PR1-5, to control differentiation, survival and function of immune cells (Volker 2007; Allende *et al.*, 2011; Maceyka *et al.*, 2012). The distribution of different S1PRs varies across cell types. S1PR1 controls the trafficking and migration of numerous immune cells (Maceyka *et al.*, 2012), while S1PR5 is preferentially expressed in murine and human peripheral (p)NK cells and is required for their mobilization to inflammatory sites (Valkzer *et al.*, 2007). More recently, it was identified that S1PR5 expression by NK cell can be promoted by multiple transcription factors (Jenne *et al.*, 2009). S1PR5 can also coordinate with another GPCR, CXCR4, to control pNK trafficking (Mayol *et al.*, 2011). Although S1PR5 knockdown has been shown to reduce circulating NK cells in mouse liver, spleen and lungs (Walzer *et al.*, 2007), the effect of such a challenge on the regulation or recruitment of specialized human dNK cells is still unknown. However, a study in mice has shown that the disturbance of SIP signalling by knockdown of the sphingosine kinase (Sphk) gene can cause defective decidualization, and was associated with severely compromised uterine blood vessel development and early pregnancy loss (Mizugishi *et al.*, 2007). The S1PK1/SIP pathway has also been identified as a new modulator of the transcription factor hypoxia-inducible factor 1 alpha (HIF1A) to control hypoxia-mediated angiogenesis in tumours (Adler *et al.*, 2009). Therefore, the sphingolipid signalling pathway may play an important role in the coordination of decidual angiogenesis during early gestation (Kaneko-Tarui *et al.*, 2007). In human pregnancy, decidual S1PK1 expression is increased on Vimentin+ decidual cells during gestation (Yamamoto *et al.*, 2010) and may cause an increase in decidual S1P levels. Together these studies led us to hypothesize that SIP signalling, in particular via S1P R1/5, may be linked to both dNK cell migration and the stimulation of their angiogenic functions to mediate uterine spiral remodelling during early pregnancy. In this study, we investigate whether in vitro SIP-S1PR stimulation of human dNK cells can alter their phenotype and function and their subsequent effects on EVT migration.

**Materials and Methods**

**Primary tissues**

Decidual and placental tissues were obtained following informed consent from healthy women undergoing elective pregnancy termination during early pregnancy (6–20 weeks of gestation). In some experiments, peripheral blood from healthy pregnant women (6–14 weeks) was included.

**Ethical approval**

All sample collections were approved by the Morgantaler Clinic and the Research Ethics board of Mount Sinai Hospital (Toronto, Canada).

**Flow cytometry**

Flow cytometry assays of peripheral blood cells were performed after density gradient centrifugation on Ficoll-Paque (GE Healthcare Biosciences, PA, USA). For decidual tissues, samples were first macroscopically identified and rinsed with Hank’s Buffered Salt Solution (HBSS). Then they were cut into small pieces (~ 1 mm3) and shaken for 30 min at 37 °C in orbital incubator (140/ min) in pre-warmed Ca, Mg-free HBSS (25 mM HEPES, 1 mM DTT, 1 mM EDTA). The dissociated cell suspension was filtered and incubated in a tissue culture dish for 20 min (37 °C, 5% CO2) to enrich decidual mononuclear cells. Red blood cells were lysed by BD Pharm LyseTM buffer and LIVE/DEAD® Fixable Dead Cell Stain Kit (violet; Invitrogen, NY, USA) was followed to eliminate staining artefacts from analysis. After non-specific blocking with serum-free protein block (Dako, Denmark), cells were stained for surface markers then subsequently stained with following antibodies: mouse anti-human CD45-APC/Cy7, CD3-FITC, CD56-PE/Cy7 (BD Pharmingen, CA, USA) and CD16-Krome Orange, CD335 (NKp46)-PE/Cy5, CD244 (2B4)-PE/Cy5 (Beckman Coulter, CA, USA). Monoclonal anti-human S1PR1-APC and S1PR5-PE were purchased from R&D Systems (MN, USA).

When intracellular staining was performed, cells were stimulated with Cell Stimulation Cocktail (plus protein transport inhibitors; eBioscience, CA, USA) for 4 h at 37 °C and stained for surface markers then subsequently fixed and permeabilized (BD Cytofix/CytopermTM Plus Kit; BD Biosciences,
CA, USA) and stained with cytoplasmic cytokines with anti-human VEGF-APC, IL-8-PE (R&D Systems) and IFNG-FITC (BD Pharmingen).

Flow cytometric data were acquired by Gallios flow cytometer (Beckman Coulter). Offline data analyses were performed on the Fixable viability dye negative population (live cells). The viable CD45+CD56+CD16+ cells were further investigated for dNK surface marker and functional analysis. Data were analysed by FlowJo Version 7.6 software (TreeStar, OR) or Kaluza 1.2 (Beckman Coulter).

Decidual leukocytes treatment with FTY720

The spingosine analogue, FTY720 (fingolimod; 2-amino-2-(2-[4-octylphenethyl]yl)-1,3-propanediol), an immunosuppressant that can selectively block S1PR1 and S1PR5 (Gräler and Goetzi, 2004), was purchased from Cayman Chemical (MI, USA). Isolated decidual leukocytes/dNK were incubated with 5 μM FTY720 at 37°C for 1.5 h, then pelleted cells were washed and prepared for the subsequent in vitro assays.

CD56+ dNK cell sorting

Direct positive magnetic sorting of dNK cells were performed using CD56 microbeads and MACS technology (Miltenyi Biotech, CA, USA). Cell separation was performed according to the manufacturer’s instruction. The cell sorting buffer was PBS with 0.5% (vol) BSA and 2 mM EDTA. Decidual leukocytes were prepared as described in the flow cytometry section. Cell separations were performed with a manual system using LS MACS cell separation columns. Briefly, cells were loaded on the column while attached to the magnet, and after three washes, non-bound cells were collected in buffer as the flow-through fraction. Then the column was removed from the magnet and the cells remaining on the column were eluted as the bound fraction. The enriched CD56+ cell fractions were used for wound healing or human umbilical vein endothelial cells (HUVEC) tube formation assays. Flow cytometric analysis confirmed that the purity of enriched CD56+ dNK cells was >95% (Supplementary Fig. S1).

Wound-healing assays

Wound healing experiments were performed as previously described (Nadeem et al., 2011). Briefly, an immortalized human trophoblast cell line, HTR-8/SVneo (Graham et al., 1993) (obtained from Dr Charles Graham, Queen’s University, Canada), was cultured in RPMI 1640 medium supplemented with 10% FBS, 100 IU/ml of penicillin and 100 μg/ml of streptomycin (Invitrogen) at 37°C with 5% CO₂. A confluent monolayer of cells was serum starved (Opti-MEM; Invitrogen) overnight and a scratch was made with a 200 μl plastic pipette tip. Then freshly prepared or FTY720 pretreated deciduial leukocytes, or MACS-purified CD56+ dNK cells (1.5×10⁵ cell per well) were added to the HTR-8-coated culture plate (24 well). After 16 h of culture, photographs were taken at 10 randomly marked locations along the wound edge by Leica DMIL LED microscope. The distance migrated by the HTR-8 cells was measured using ImagePro-plus software (Media Cybernetics). In some experiments, recombinant human (rh)VEGF (Sigma; 100 ng/ml) or IL-8 (R&D, 50 ng/ml) was added to stimulate HTR-8 cell migration.

HUVEC angiogenesis

MACS-enriched CD56+ dNK cells were stimulated with or without FTY720 for 1.5 h, then washed and cultured in RPMI1640 for 16 h. Collected conditioned media were applied on 24-well plates, coated with Matrigel and 3.0×10⁶ HUVEC cells (ATCC). After 18 h, HUVEC branching was examined by light contrast microscopy and images were captured. In a positive control group, 50 ng/ml rhVEGF was added to stimulate tube formation of HUVEC cells. Tube formation was assessed using Wimtube program (Wimasis Image).

Transwell migration study

Freshly prepared decidual leukocytes were incubated without (RPMI 10% FBS; control group) or with FTY720 (5 μM; FTY720 group) prior to the establishment of transwell migration assays (Ofer 2006), which were carried out using 8 μm cell culture inserts (no Matrigel coating; BD Biosciences) in 24-well plates. RPMI 10% FBS culture medium was added to the bottom wells, and the leukocyte cell suspension (5×10⁵) was added to the top wells and incubated at 37°C for 3 h. Non-invading cells were carefully swabbed off from the upper surface of the membrane. Then the membranes were stained using crystal violet (Sigma, MO) and mounted onto glass slides. Cell invasion was determined by counting the number of stained cells on the membrane under Leica DMIL microscope. The migration index was defined as the number of FTY720-treated cells divided by that of the control group. The assay was carried out in duplicate in at least three independent experiments.

Culture of human placental explants with decidual leukocytes

To examine the biological effect of FTY720 on decidual leukocyte-facilitated trophoblast invasion, 1×10⁵ decidual leukocytes (with or without FTY720 pretreatment) were mixed with phenol red-free matrigel (BD Biosciences) and the leukocyte matrigel mixture was applied to coat culture inserts (4 μm; Millipore, MA, USA) in a 24-well plate. Patient-matched placental villous explants were then microdissected and placed on the top of polymerized matrigel, as previously described (Baczyn et al., 2009). Explants were allowed to attach to the matrigel surface overnight and then were supplied with 200 μl serum-free DMEM/F12 (Invitrogen) supplemented with 0.1% Normocin (Invitrogen). Culture medium was changed every 2 days. Explants were photographed after 3 and 6 days of incubation at 37°C, 3% O₂ to assess EVT outgrowth.

Immunohistochemistry

Human placental explants were fixed in 4% paraformaldehyde for 1 h at room temperature, processed and embedded in paraffin. The explant blocks were serial sectioned at 5 μm and deparaffinized in xylene and rehydrated through a gradient series of ethanol. Endogenous peroxidase activity was blocked by incubation of the sections in 3% hydrogen peroxide (Fisher Scientific, ON, Canada) for 30 min. After 30 min incubation with Dako protein blocking solution (Dako), primary mouse monoclonal anti-human CD45 (1:200; Dako), CD56 (1:100; Dako) or HLA-G antibody (1:500; Exbio, Czech) were loaded on the sections and incubated at 4°C, overnight. After washing with PBS, biotinylated rabbit anti-mouse IgG (1:200; Dako) was applied and followed with Universal LSAB®-HRP Kit (Dako). Slides were developed with Liquid DAB® Substrate Chromogen System (Dako) and counterstained with Gills Haematoxylin (Sigma). Photomicrographs were obtained using a Leica DMIL LED microscope with image Quality software.

Statistical analysis

Statistical significant differences between experimental treatments/groups were determined by independent t-test using SPSS13 software (IBM SPSS). Data are presented as mean ± SD. P < 0.05 was considered significant.

Results

S1P receptor expression levels on peripheral and decidual NK cells

To investigate whether different NK populations expressed different S1P receptors, we examined the expression levels of S1PR1 and S1PR5 on...
freshly isolated pNK and dNK cells. For flow cytometric data analysis, dead cells were excluded through the use of the LIVE/DEAD staining kit and viable CD45⁺CD56⁺CD16⁻ NK cells were investigated (Fig. 1A). We found only a few S1PR1⁺ NK cells in the blood but this population doubled in decidual samples (2.5 versus 5.5%). In both pNK and dNK, S1PR5⁺ populations were present in greater numbers than S1PR1⁺ cells (Fig. 1B), suggesting that the NK cells are preferentially reactive to S1PR5. Interestingly, the dNK cells showed a higher proportion of S1PR5 positive (40%) in comparison with the pNK subset where only 18% expressed S1PR5. In the dNK cells, the majority of the S1PR1⁺ population was also S1PR5 reactive (Supplementary Fig. S2). To further characterize the phenotype of S1PR1⁺ and S1PR5⁺ dNK cells, we also examined the surface expression of the classical NK receptor CD335 and CD244. As shown in Fig. 1C, there was no difference in CD335 or CD244 expression between the S1PR1⁺ and S1PR5⁺ dNK subsets.

**FTY720 treatment down-regulates dNK S1PR expression**

FTY720 is a sphingosine analogue phosphorylated by SPHKs to produce a S1PR ligand with potent effects, including S1PR modulation and down-regulation of S1PR expression (Rivera et al., 2008). After incubation of primary first trimester decidual leukocytes with FTY720, we found that the proportion of CD56⁺ dNK in the CD45⁺ decidual lymphocytes was unaltered (Fig. 2A). S1PR1 expression of these dNK was slightly decreased but not statistically different from the control cells. However, the S1PR5 signal was significantly down-regulated by

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**Figure 1** S1P receptors expression on human NK cells. (A) Representative gating strategy of human NK cell from peripheral blood and decidual tissue. Viable CD45⁺ NK cells were defined according to their CD56 and CD16 expression. (B) S1PR1 and S1PR5 expression on decidual (d) or peripheral (p) NK cell (CD56⁺ CD16⁻). The experiment shown is a representative plots of n = 3 (PBMC) and n = 14 (decidua). Fluorescence-minus-one (FMO) controls of S1PR1 and S1PR5 were included. (C) S1PR1- and S1PR5-positive dNK cells expressing classical NK cell surface markers CD335 and CD244.
FTY720 treatment, both in the frequency of S1PR5+ cells and in mean fluorescent intensity value (MFI; Fig. 2B and C). This ligand-induced internalization of S1PR5 did not change the CD56+ dNK phenotype, as illustrated by the maintenance of CD335 and CD244 expression (Fig. 2D). Since S1P-S1PR is essential for immune cell trafficking (Jenne et al., 2009; Mayol et al., 2011), we examined the migratory ability of decidual leukocytes following FTY720 treatment. This analysis revealed that decidual leukocytes mobility is impaired by FTY720 pretreatment (Fig. 2E). The migration index was significantly decreased by 30% following FTY720 treatment when compared with the control group treated with normal culture medium (*P < 0.05; Fig. 2E).

### FTY720 treatment decreases VEGF, but not IL-8, expression by dNK cells

Both VEGF and IL-8 are important factors which can promote decidual angiogenesis during human placental development (Hanna et al., 2006). Thus, we performed flow cytometry to examine VEGF and IL-8 production by dNK at the single cell level. In freshly isolated dNK cells (CD45+CD56+CD16−), ~80% were found to be VEGF+, but only ~8% of them were IL-8+. Interestingly, FTY720 treatment of the leukocytes significantly decreased dNK VEGF expression both in the numbers of VEGF+ dNK cells and in fluorescence intensity (MFI, *P < 0.05; Fig. 3A–C). IL-8 expression of dNK was not changed by FTY720 treatment. Detailed subset analysis demonstrated that among the CD56+ dNK population, only the VEGF+IL-8− subset is greatly decreased by FTY720, the VEGF+IL-8+ or VEGF-IL-8+ subsets remain unchanged (Fig. 3D). Meanwhile in CD3+ T cells, neither VEGF nor IL-8 expression were affected by FTY720 treatment (Fig. 3D). Furthermore, it was revealed that a greater proportion of S1PR5+ dNK were VEGF (*P = 0.17) and IFNG (*P = 0.003) positive, while more of the S1PR5+ dNK cells expressed low levels of these angiogenic factors (for both % and MFI; Fig. 3E). These results suggest the selectivity of S1P-S1PRs pathway for dNK VEGF expression.

**Figure 2** S1PR5 expression on dNK cell was down-regulated by FTY720. (**A**) Freshly isolated first trimester decidual leukocytes were incubated with 5 μM FTY720 for 1.5 h at 37°C and their CD56/CD3 profile was examined. (**B** and **C**) S1PR5 expression of the CD45+CD56+CD16− dNK cell was decreased at both percentage and median fluorescent intensity (MFI). (**D**) NK cell receptors CD335 and CD244 were not changed by FTY720 treatment. (**E**) Migration of decidual leukocytes was inhibited by FTY720 treatment. Decidual leukocytes were incubated with 5 μM FTY720 for 1.5 h, then washed with fresh medium and loaded into transwell inserts for 3 h, 37°C. Non-invading cells were counted by Imagepro Plus and the migration index was calculated by normalizing to the control group (freshly isolated decidual cells). Bar = 100 μm, n = 5. *P < 0.05.
FTY720 treatment inhibits dNK cell-facilitated trophoblast outgrowth and HUVEC tube formation

To first examine the effect of S1P signalling in dNK-EVT interactions, we cultured primary EVT in Matrigel inserts seeded with patient-matched decidual leukocytes (mainly NK cells > 70%, Figs 1 and 2). As shown in Fig. 4A, decidual leukocyte-stimulated EVT migration over the gel surface was inhibited by pretreatment of the leukocytes with FTY720, apparent as decreased EVT outgrowth area and distance migrated from the villous tips. We performed immuno-histochemistry analysis of HLA-G, a marker of invasive EVT and CD45 to examine the positional relationship between EVT and decidual leukocytes (Fig. 4B). Compared with the control where CD45+ leukocytes (arrowheads; Fig. 4B) were frequently observed in close proximity to and in contact with the HLA-G+ EVT (arrows; Fig. 4B) in the extended outgrowth, only a few FTY720 pretreated decidual leukocytes were found close to HLA-G+ EVT (arrows; Fig. 4B). After applying CD56 staining to serial sections of the same explants, we further confirmed that FTY720 pretreatment affected the interaction between CD56+ dNK and HLA-G+ EVT (Fig. 4B, bottom panel).

We next utilized a wound healing assay to quantify the effect of decidual leukocyte-induced trophoblast migration. Serum-starved
trophoblast cells (HTR-8/SVneo) were wounded by scratching with a pipette tip and then freshly isolated decidual leukocytes, either with or without FTY720 pretreatment, were directly added into the wells (Fig. 5A). HTR-8 cell migration was significantly improved after 16 h incubation in the presence of control leukocytes, but this effect was markedly abrogated when the leukocytes were pretreated with FTY720 ($P < 0.05$; Fig. 5B). To further confirm the specific effect of dNK cells on trophoblasts, we repeated this experiment using purified...
Figure 5  Effect of S1P signal on dNK cell-associated HTR-8/SVneo trophoblast migration. The wound-healing assay was performed with HTR-8/SVneo cells. Once the wound was created, primary decidual leukocytes from first trimester pregnancy were added in the culture well and 16 h later migration of trophoblast cells into the wounded area was measured. (A) Representative images show HTR-8 cells migration alone (negative), or with fresh isolated (control) or FTY720 pretreated (FTY720) decidual leukocytes. Scale bar = 100 μm. (B) Summarized histogram of relative wound closure index of four repeated experiments using decidual leukocytes performed in duplicates. *P < 0.05. (C) MACS-enriched CD56+ dNK cells were applied in wound assay and same result was obtained. n = 5. *P < 0.05. (D) During the wound assay, rhVEGF (100 ng/ml) or rhIL-8 (50 ng/ml) was added in the HTR-8-coated well either alone or together with FTY720 pretreated decidual leukocytes. n = 6. *P < 0.05. (E) MACS-enriched CD56+ dNK were treated with or without FTY720 for 1.5 h, then they were cultured another 18 h to collect supernatant for an HUVEC tube formation assay. The negative group was cultured with medium from untreated cells. As a positive control, 50 ng/ml rhVEGF was added in the culture system. Representative images are from six experiments. Scale bar = 250 μm.
CD56+ dNK and similar results were observed. As shown in Fig. 5C, control dNK cells significantly improved HTR-8 cell migration, and FTY720 pretreatment of dNK prevented this effect ($P < 0.05$). Exogenous administration of rhVEGF or rhIL-8 to the co-culture system in the presence of FTY720-pretreated dNK was able to restore HTR-8 cell migration (Fig. 5D). We further investigated the effect of FTY720 on the angiogenic function of dNK using an HUVEC tube formation assay. Our results confirmed that FTY720 treatment not only decreased dNK VEGF expression, but downregulated the secreted VEGF in the conditioned media. As shown in Fig. 5E and Supplementary Fig. S3, HUVEC tube formation was inhibited when cultured with conditioned medium from FTY720-treated dNK, compared with that in the medium from the vehicle-treated normal group or the rhVEGF-positive control.

**Discussion**

Increasing evidence supports a critical role for the dNK cells in the establishment of the placenta and uteroplacental circulation. Human dNK cells have been shown by our group and others to stimulate trophoblast invasion and angiogenesis and to play an active role in the initiation of vascular remodelling in the first trimester (Hanna et al., 2006; Smith et al., 2009; Lash et al., 2010). However, little is known about the dNK cellular responses and angiogenic functions, which are regulated by the lipid signalling pathway. In the current study, we demonstrated that dNK cells exhibit an angiogenic phenotype, which is modulated by S1P signalling. Furthermore, such change is mediated by changes in dNK VEGF production and downstream effects of trophoblast migration and HUVEC angiogenesis.

At early stages of pregnancy, decidualization enhances the recruitment of specialized immune cells and creates a temporary lymphoid niche at the fetal—maternal interface (Vacca et al., 2011). Such a well-controlled inflammatory stimulation (Sargent et al., 2006) may re-shape molecular and functional features of decidual leukocytes, particularly via the engagement of S1P-S1PR1/5 to orchestrate dNK cell adaptation (Shi et al., 2011; Walzer and Vivier, 2011). Interestingly, we have confirmed that in the first trimester, >70% CD45+ leukocytes express S1PR5 expression levels are decreased in second trimester dNK cells

Pregnancy is a dynamic process that requires decidual immune cells to adapt their phenotype and function to the surroundings. To investigate the potential change in the S1PR profile over time, we evaluated their expression on second trimester dNK cells and compared it with that found on the first trimester dNK (Fig. 6A). In normal pregnancy, there was no change in dNK S1PR1 expression between first and second trimester pregnancy. However, a significant decrease in S1PR5 expression on dNK cells was observed in the second trimester dNK cells, compared with that from the first trimester (20 versus 45%; $P < 0.05$). However, we did not find a significant difference in the proportions of dNK cells expressing VEGF or IL-8 between first and second trimester pregnancy (Fig. 6B).

**Figure 6** S1PR5 expression is decreased in second trimester dNK cells. (A) Gestational S1PR1 and S1PR5 expression on CD56+CD16− dNK cells from first ($n = 14$) or second ($n = 9$) trimester deciduas. (B) VEGF and IL-8 expression of dNK cells from first ($n = 8$) and second ($n = 5$) trimester pregnancy. Data were presented as mean ± SD.
CD56 and 40% of these dNK were S1PRS+. while only 18% of pNK expressed S1PRS. The CD56+ or CD16+ NK cells in the decidua are the main source of S1PRs signal as T cells which make up 15–25% of decidual leukocytes express very low levels of S1PRs (Supplementary Fig. S4). These findings correlate well with previous reports of pNK (Walzer et al., 2007) and also indicate that the enhanced dNK responsiveness to the S1P signal may facilitate their accumulation within the first trimester decidua. Moreover, the high numbers of S1PRS+ dNK cells presenting in the fetal–maternal interface suggests that they are reactivated in situ as effector cells, a mechanism that has been identified by Arnon et al. (2011).

In this study, we demonstrated the functionality of the S1P signalling by using FTY720 to affect dNK cell mobility and cytokine/chemokine expression profiles. It was reported that FTY720 application leads to sustained S1PR internalization and lymphocyte sequestration (Sensken and Gräler, 2010). However, more recent data indicated that FTY720 stimulation had a dual effect on external S1P activation and cellular function (Wu et al., 2013). In our experiments short-term exposure of dNK cells to FTY720 resulted in the down-regulation of both S1PR1 and S1PRS expression. The significant S1PRs decrease suggested that this receptor has a more important biological function than S1PR1 in regulating dNK reactivity. Interestingly, subset alterations and functional changes of pNK populations were also reported in patients with multiple sclerosis who received sustained FTY720 therapy (Johnson et al., 2011). We further confirmed that this modulation of the S1P signalling pathway can cause an impairment of decidual lymphocyte/dNK migration, mainly mediated by chemokine responsiveness (Mayol et al., 2011). These data agree with previous studies where S1P expression by lymphocytes correlates with their egress kinetics (Thangada et al., 2010). Our results indicate that investigating the S1P signalling pathway of NK cells in the early stages of gestation may provide valuable information regarding dNK homing to the uterus.

Human dNK cells are observed in close proximity to migrating HLA-G+ trophoblast cells in the decidua (Pijnenborg et al., 2006; Smith et al., 2009) and one of their most important functions is to regulate EVT invasion (Hanna et al., 2006; Lash et al., 2011; Wallace et al., 2012). Many angiogenic and chemokine factors have been reported to be expressed by dNK and herein we choose to investigate VEGF and IL-8 expression as both have been shown to mediate angiogenesis and trophoblast invasion (Hanna et al., 2006). In this study, we demonstrated that the majority of freshly isolated CD56+ dNK express a high level of VEGF while only a few of them are IL-8 reactive. This discrepancy with previous findings may be explained by the culture conditions, as the previous report of high IL-8 expression by dNK was found in cells cultured in the presence of IL-2 (Hanna et al., 2006) (personal communication with Dr O. Mandelboim). Alternatively, IL-8 production by NK cells may also depend upon their developmental status since this capability was reported to be confined to immature NK (CD56+CD117+CD94−) derived from tonsil (Montaldo et al., 2012), while the human dNK population is dominated by the mature subset (CD117+−/CD94+) (Male et al., 2010).

VEGF expression by dNK cells is decreased by FTY720 treatment, this suggests that S1P signalling is involved in the regulation of VEGF production, which is essential for the functional integrity of angiogenic dNK cells. Indeed, we demonstrated that FTY720 can reduce the angiogenic ability of dNK cells in stimulating both trophoblast migration and HUVEC tube formation. Similarly, an ex vivo model has shown that the signalling and angiogenic properties of S1P (including basic fibroblast growth factor and VEGF) enhance vascular sprouting and neovascularization through the activation of S1P receptors (Spiegel and Milstien, 2011). Conversely, the non-classical class I HLA-G homodimer can induce secretion of IL-6 and IL-8 from dNK cells, but with no effect on VEGF production (Li et al., 2009). Combined with our data, these results suggest that dNK function is modulated by microenvironmental stimulation and that there are different signalling pathways governing the regulation of VEGF and IL-8 expression.

During pregnancy, there are significant changes in deciduial stromal and glandular cells as well as in immune cell phenotype and activation status at the fetal–maternal interface (Male et al., 2011; Marlin et al., 2012). Mature NK cells from peripheral organs are able to accommodate the changing cellular environment (Vivier et al., 2011). In contrast to decidual T cells, CD56+ or CD16+ NK are the main population reactive to S1PRs signal (Supplementary Fig. S4). Our current data provide additional evidence regarding the heterogeneity of dNK cells with increasing gestation as illustrated by their different S1PRS+ reactivity. There are more S1PRS+ dNK in the first trimester, a time characterized by increasing decidual angiogenesis, compared with that in the second trimester when the intervillous space circulation is established to support the rapidly growing fetus. Yamamoto et al. (2010) reported that decidual S1PHK, which can promote S1P synthesis from sphingosine, is increased during pregnancy. Although S1P is much less effective than FTY720 at inducing receptor internalization and degradation, high levels of S1P can induce S1PRS+ internalization in the dNK cells. Such a reduction in sensitivity of dNK to the S1P signal may affect their angiogenic function on trophoblast invasion. However, in our study no significant change was found in VEGF or IL-8 expression level by the second trimester dNK cells compared with those from the first trimester. Alternatively, the down-regulation of S1PRS may lead to retention and specialization of the dNK within the decidual niche, as such a mechanism has been observed in other lymphoid organs (Jenne et al., 2009; Mayol et al., 2011). Recent reports have emphasized the integrative and bidirectional effects between S1P and VEGF, as they have differential angiogenic effects that are dependant upon the S1P inhibitor and growth factor cocktail (Gough et al., 2013; Nguyen et al., 2013; Törnquist, 2013). Thus, a detailed investigation of the S1P signalling dynamic in the placental bed may yield significant insights into how dNK remain and function in situ.

In conclusion we have shown that: (i) modulation of the S1P pathway results in decreased S1PR5 expression by dNK cells; (ii) dNK VEGF but not IL-8 expression is significantly decreased by FTY720 treatment and (iii) dNK-mediated EVT migration and HUVEC angiogenesis are also impaired by FTY720 treatment. These results identify for the first time that the S1P pathway is an important player in the regulation of dNK cell physiology during normal pregnancy. We suggest that dNK homing and angiogenic function can be manipulated by modulating their S1PRS reactivity. Thus, S1PRS may represent a potential target in gestational diseases, such as pre-eclampsia and intrauterine growth restriction, which are characterized by the inadequate dNK/trophoblast coordinated uterine spiral artery transformation.

Acknowledgements
We thank the donors, the Research Centre for Women’s and Infants’ Health BioBank Program of Lunenfeld-Tanenbaum Research Institute (LTRI) and the Mount Sinai Hospital/University Health Network.
(Toronto, Canada) for providing human specimens. We appreciate the technical help of Dr Lubna Nadeem and Ms Melissa Kwan (LTRI). We thank Dr Andrea Jurisicova (Lunenfeld-Tanenbaum Research Institute, Toronto) for providing us with HUVEC. We thank Dr Oksana Shynlova (LTRI) for critical reviewing and Ms Annie Bang (LTRI, Mount Sinai Hospital) for technical support of flow cytometry.

**Authors’ roles**

J.H.Z. was responsible for the experiment design, data acquisition and analysis, and manuscript drafting. C.E.D. and S.J.L. were responsible for the data interpretation, critical discussion and manuscript revision.

**Funding**

This study was supported by Canadian Institutes of Health Research (CIHR), MOP82811 to S.J.L.

**Conflict of interest**

No conflicts of interest are declared.

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