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Uterine natural killer cells as modulators of the maternal-fetal vasculature

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ABSTRACT Precise and local control of the innate immune system within the placenta is an essential component for achieving a normal and healthy pregnancy. One of the most abundant immune cells of the placenta is a subpopulation of natural killer (NK) cells that profusely populates the uterine decidua during early pregnancy. Uterine NK (uNK) cells and trophoblast cells of the placenta communicate both directly and indirectly to contribute to the critical process of spiral artery remodeling. Here, we discuss recent findings that expand our knowledge of uNK cell-trophoblast cell crosstalk and the important role it plays in the maternal vascular adaptation to pregnancy.

KEY WORDS: *NK cell, trophoblast cell, placenta, spiral artery remodeling, preeclampsia*

Introduction

Natural killer (NK) cells are lymphocytes belonging to the innate immune system that attack virally-infected cells and tumor cells via exocytosis of perforin- and granzyme-containing granules. In females, there are two populations of NK cells: peripheral blood (pb) NK cells and decidual (d) or uterine (u) NK cells. Both NK cell populations are capable of cytotoxicity and cytokine secretion. However, pbNK cells are primarily lytic cells, while uNK cells are primarily cytokine and chemokine producers.

A dramatic expansion of uNK cells occurs during early pregnancy, populating two adjacent areas of the implantation site, the decidua basalis (DB) and the mesometrial lymphoid aggregate of pregnancy (MLAp). Proliferation continues until mid-pregnancy, at which point uNK cells comprise up to 70% of immune cells present in the decidua, the progesterone-altered endometrium of the uterus that supports the conceptus (Bulmer *et al.*, 2010). uNK cell population size then declines until the end of pregnancy.

Molecular characterization of uNK cells has led to the identification of subpopulations of uNK cells in the decidua. Mouse uNK cells, previously identified by periodic acid Schiff (PAS) staining, are also currently identified by *Dolichos biflorus* agglutinin (DBA) lectin staining (Paffaro *et al.*, 2003). PAS and DBA lectin staining defines two subpopulations of uNK cells: PAS^+DBA^- and PAS^+DBA^+ cells, which exhibit different gene expression profiles (Chen *et al.*, 2012, Zhang *et al.*, 2011). While PAS is a pan-uNK cell marker, DBA lectin detects the subpopulation of cells that expands during pregnancy, as 90% of uNK cells at mid-gestation are DBA^+ (Zhang *et al.*, 2011).

In contrast to the mouse, human uNK cells are identified by their CD56 CD16 signature. The vast majority of uNK cells are $CD56^{bright}$ $CD16^-$, and pbNK cells are typically $CD56^{dim}$ $CD16^+$. Interestingly, while both subpopulations are present in the decidua, the proportion of these two subsets can shift to favor cytotoxic $CD56^{dim}CD16^+$ cells in the presence of infectious agents like cytomegalovirus (Siewiera *et al.*, 2013) and *Toxoplasma gondii* (Xu *et al.*, 2013a), both common intrauterine infections that cause severe birth defects.

Despite belonging to the immune system, uNK cells' primary contributions to the developing pregnancy are not immune in nature. Rather, uNK cell-secreted cytokines and chemokines communicate with fetal trophoblast cells of the placenta (Hanna *et al.*, 2006); these two cell types act in concert to remodel spiral arteries, conduits of blood from the uterus to the placental bed and growing fetus (Smith *et al.*, 2009). The importance of this process is stressed by the association of insufficient spiral artery remodeling with several diseases of pregnancy, such as fetal growth restriction (FGR) and preeclampsia. Here, we briefly review aspects of uNK cell-trophoblast cell crosstalk and their role in spiral artery remodeling and the maintenance of pregnancy, as summarized in Fig. 1.

Signals promoting differentiation of uNK cells

Little is known about uNK cell precursors and the source of the expanded uNK cell population during pregnancy. However, there

Abbreviations used in this paper: AM, adrenomedullin; DBA, *Dolichos biflorus* agglutinin; FGR, fetal growth restriction; HLA, human leukocyte antigen; KIR, killer cell Ig-like receptor; MMP, matrix metalloproteinase; uNK, uterine natural killer.

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is evidence to suggest that the majority of uNK cell precursors originate from outside the uterus. In the early 2000s, Croy and colleagues were unable to detect differentiated uNK cells in NK cell-deficient animals engrafted with parts of wild type uteri, suggesting that uNK cell precursors are extra-uterine (Chantakru *et al.*, 2002). More recently, analysis of changes in uNK cell surface markers during pregnancy suggest that uNK cells endogenous to the uterus decline between gestation day (gd) 0 and gd6, perhaps because their cytotoxicity would be lethal to the foreign conceptus (Takashima *et al.*, 2013). Therefore, the endogenous uNK cell population may be less critical for the maintenance of pregnancy than uNK cells that differentiated from extra-uterine precursors.

Macrophage-derived interleukin-15 (IL-15) is a critical regulator of NK and uNK cell differentiation. It was first observed that the time course of IL-15 expression during pregnancy parallels that of uNK cell granule contents (Ye *et al.*, 1996). Soon thereafter, IL-15$^{-/-}$ animals were generated (Kennedy *et al.*, 2000). IL-15$^{-/-}$ females lack uNK cells, MLAps, and spiral artery remodeling despite birthing litters of average size but slightly below-average weight (Ashkar *et al.*, 2003, Barber and Pollard, 2003). Recent microarray analysis comparing IL-15$^{-/-}$ and IL-15$^{+/+}$ animals did not detect differences in expression levels of genes involved in decidualization (Bany *et al.*, 2012). Therefore, the finding of below-average weight pups born to IL-15$^{-/-}$ dams cannot be explained by differences in decidualization and is likely due to the effect of IL-15 on uteroplacental circulation via stimulation of uNK cell differentiation.

Recent studies offer candidate regulators of IL-15 expression. For example, there is evidence that the transcription factor Runx3 acts together with other transcription factors to promote IL-15 expression (Levanon *et al.*, 2014). However, there are also potentially indirect regulators of IL-15 expression. For example, a conditional knockout (cKO) of bone morphogenic protein receptor 2 (BMPR2) in the female reproductive system demonstrated decreased IL-15 expression (Nagashima *et al.*, 2013). As may be expected, BMPR2 cKOs lacked uNK cells at implantation sites and exhibited defects in placentation.

In another animal model of placental underperfusion, heme oxygenase-1 (HO-1) heterozygotes and knockouts also downregulated IL-15 expression and exhibited fewer uNK cells in the DB and MLAp (Linzke *et al.*, 2013). Similar to BMRP2 cKOs, Hmox1$^{+/-}$ dams demonstrated intrauterine growth restriction (IUGR), suggesting poor uteroplacental circulation. Interestingly, treatment with CO, a byproduct of HO's heme metabolism, elevated uNK cell numbers, promoted spiral artery remodeling, and decreased the incidence of fetal death. However, CO treatment did not elevate IL-15 levels. In an attempt to explain why IL-15 is downregulated in Hmox1$^{+/-}$ and Hmox1$^{-/-}$ animals, the authors suggest that CO may affect the activity of macrophages, a demonstrated source of IL-15 in the pregnant uterus (Ye *et al.*, 1996). If true, this hypothesis could address the well-established, counterintuitive protection that smoking confers against preeclampsia. Altogether, these observations suggest potential indirect mechanisms of uNK cell differentiation via regulation of IL-15 expression.

Direct interactions between uNK and trophoblast cells: KIRs and HLAs

Differentiated uNK cells express activating and inhibitory cell surface receptors. Stimulation of these subtypes by trophoblast-expressed ligands, for example, determines the degree of uNK cell activity. Interestingly, the proportions of activating and inhibitory receptors may shift in the presence of a foreign pathogen to modify uNK cell activity and promote cytotoxicity (Xu *et al.*, 2013b). Many of these uNK cell surface receptors belong to the killer cell Ig-like receptor (KIR) family. KIR A and B haplotypes preferentially express inhibitory and activating receptors, respectively, and bind to fetal trophoblast-expressed human leukocyte antigen C (HLA-C), a major histocompatibility complex (MHC) type I molecule.

Importantly, Moffett and colleagues demonstrated associations of certain KIR-HLA combinations, specifically KIR AA and HLA-C2, with diseases of pregnancy like preeclampsia and miscarriage (Hiby *et al.*, 2008, Hiby *et al.*, 2004). Binding of fetal HLA-C2 to the inhibitory receptor KIR2DL1 in these women may predispose to preeclampsia and other placental disorders via insufficient uNK cell activation (Hiby *et al.*, 2004). In contrast, KIR B women preferentially express uNK cell activating receptors. In these women, HLA-C2 likely binds to the activating receptor KIR2DS1, protecting women against these diseases by activating uNK cells and stimulating trophoblast invasion (Hiby *et al.*, 2010, Hiby *et al.*, 2004). Recent evidence that KIR2DS1 stimulates secretion of cytokines like granulocyte macrophage colony-stimulating factor (GM-CSF) by uNK cells and trophoblast migration supports this paradigm (Xiong *et al.*, 2013).

While HLA-C has attracted attention for its disease associations, trophoblast cells express HLAs other than HLA-C. Specifically, they also express HLA-E and HLA-G. HLA-E binds to CD94/NKG2A, an inhibitory receptor on uNK cells (King *et al.*, 2000), and HLA-G binds to leukocyte immunoglobulin-like receptors (LILRs) on uNK cells (Apps *et al.*, 2007). However, HLA-G also binds to a KIR, CD158d/KIR2DL4, which is expressed in endosomes, not on the uNK cell surface like other KIRs (Rajagopalan, 2010). Binding of HLA-G to KIR2DL4 activates downstream pathways that confer a senescent phenotype on the uNK cell (Rajagopalan and Long, 2012). Supernatants from KIR2DL4-stimulated uNK cells enhance the permeability and angiogenic capacity of human ubilical vein endothelial cells (HUVECs). It is easy, therefore, to imagine a role for uNK cells in placental vascular remodeling via HLA-G stimulation of KIR2DL4.

Despite the attention paid to the consequences of HLA-KIR interactions, there is likely a role for non-KIR uNK cell receptors that bind to non-HLA ligands. Specifically, the aryl hydrocarbon receptor (AHR) is expressed by DBA^{-} uNK cells and may be important for the proliferation of this oft-ignored uNK cell subset (Felker *et al.*, 2013). While Ahr$^{-/-}$ implantation sites demonstrated wild type levels of total uNK cells, DBA^{+} cells were smaller, and DBA^{-} cells were fewer in number (Felker *et al.*, 2013). Ahr$^{-/-}$ animals also demonstrated insufficient spiral artery remodeling. Similarly, loss of natural cytotoxicity receptors (NCR), expressed by DBA^{+} cells, didn't affect total uNK cell numbers but impaired uNK cell maturation and spiral artery remodeling (Felker *et al.*, 2013). While the ligands for these receptors are unknown, they are clearly playing an important role in uNK cell maturation and activity, highlighting the importance of interactions outside the KIR-HLA axis.

Trophoblast-derived factors affect uNK cell recruitment

uNK cell-trophoblast cell crosstalk extends beyond contacts between cell surface proteins. For example, trophoblast-derived

peptides of the calcitonin (CT)/calcitonin gene-related peptide (CGRP) family are involved in maintaining proper placental perfusion, possibly by communicating with uNK cells. The clearest link between a CGRP family member, uNK cells, and spiral artery remodeling is adrenomedullin (AM).

Levels of AM, an anti-inflammatory vasodilator, are physiologically elevated in normal pregnancy (Gibbons *et al.*, 2007) but altered in adverse pregnancy outcomes (Lenhart and Caron, 2012). Polymorphisms in the AM gene are associated with birth weight, glycemic regulation, and preeclampsia (Lenhart *et al.*, 2013). AM localizes to implantation sites and is expressed by the uterine epithelium and fetal trophoblast cells (Li *et al.*, 2006). Fetal trophoblast cells also express the AM receptor, calcitonin receptor-like receptor (CLR) (Tsatsaris *et al.*, 2002). Interestingly, $AM^{+/-}$ females are less fertile due to diminished uterine receptivity (Li *et al.*, 2008), and pups born to $AM^{+/-}$ females are more likely to demonstrate FGR (Li *et al.*, 2006). Altogether, these data suggest a role for maternal AM in implantation and placentation.

However, $AM^{-/-}$ embryos are more likely to exhibit FGR than $AM^{+/-}$ or $AM^{+/+}$ embryos, suggesting a role for *fetal*-derived AM in placentation. $AM^{-/-}$ placentas demonstrate fewer uNK cells and retention of vascular smooth muscle cells lining spiral arteries compared to $AM^{+/+}$ placentas (Li *et al.*, 2013). Concordantly, placentas from $AM^{hi/hi}$ pregnant females, a gene-targeted animal model of AM overexpression, exhibit 30% more uNK cells than $AM^{+/+}$ placentas and upregulate cytokine, chemokine, and matrix metalloproteinase (MMP) expression. *In vitro*, uNK cell-conditioned media supplemented with AM promotes apoptosis of vascular smooth muscle cells, supporting the emerging role for uNK cells in spiral artery remodeling (Li *et al.*, 2013). It remains to be seen whether AM-mediated uNK cell recruitment and activity is dosage-dependent.

Like AM, adrenomedullin 2 (AM2), also known as intermedin, is physiologically elevated during pregnancy (Chauhan *et al.*, 2007). Administration of an AM2 antagonist causes FGR, highlighting the importance of AM2 in a healthy pregnancy (Chauhan *et al.*, 2006). AM2 is expressed by trophoblast cells and stimulates their invasion via the mitogen-activated protein kinase (MAPK) signaling pathway (Chauhan *et al.*, 2011, Havemann *et al.*, 2013). AM2 also dose-dependently stimulates HLA-G expression in trophoblasts cells, possibly stimulating KIR2DL4 signaling on uNK cells that could then acquire a senescent phenotype and increase vascular permeability and angiogenesis (Chauhan *et al.*, 2011, Rajagopalan and Long, 2012). It is appealing to test whether AM2 dosage correlates with uNK cell recruitment to the decidua and consequent spiral artery remodeling.

Other CGRP family members also appear to modulate the uteroplacental circulation. For example, CGRP affects blood pressure regulation at the maternal-fetal interface, and its levels are altered in pregnancy-induced hypertension and preeclampsia (Dong *et al.*, 2005, Dong *et al.*, 2004, Fei *et al.*, 2012, Gangula *et al.*, 2003, Knerr *et al.*, 2002). However, CGRPs' effects on uNK cell recruitment and activity remain to be elucidated.

As may be expected, there are trophoblast-derived factors outside the CGRP family that communicate with uNK cells. For example, thrombopoietin (TPO) and its receptor c-Mpl are expressed by uNK cells and trophoblast cells and act in concert to stimulate the proliferation and migration of these cell types via the JAK/STAT pathway (Segerer *et al.*, 2013). As we come to understand more about uNK cell-trophoblast cell crosstalk, we imagine that other trophoblast-derived factors will come to light as important effectors of uNK cell recruitment and activation.

uNK cell-derived factors promote placental vascular remodeling

uNK cells generate an array of angiogenic growth factors, cytokines, and chemokines in different proportions at different times of pregnancy, suggesting a continuous and evolving role for uNK cells as pregnancy progresses. Here, we briefly discuss several examples of uNK cell-derived factors, acknowledging that there

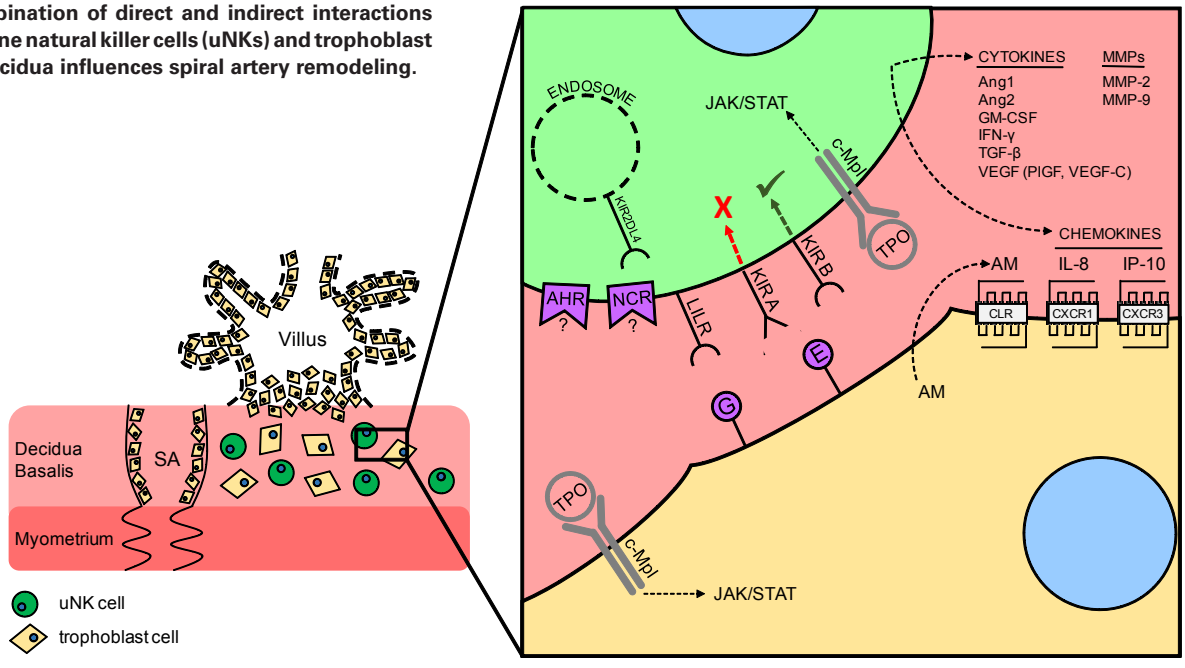

Fig. 1. A combination of direct and indirect interactions between uterine natural killer cells (uNKs) and trophoblast cells in the decidua influences spiral artery remodeling.

are many others we do not address.

Angiopoietin (Ang) 1 and 2, transforming growth factor β (TGF-β), and vascular endothelial growth factor (VEGF) are several examples of uNK cell-secreted angiogenic factors (Lash *et al.*, 2006). Placental growth factor (PlGF) belongs to the VEGF family and has emerged as a potential biomarker for preeclampsia, emphasizing the significance of these angiogenic factors in proper uteroplacental circulation (Chappell *et al.*, 2013, Levine *et al.*, 2004). VEGF-C also belongs to the VEGF family and may assume an additional role in protecting trophoblast cells from uNK cell cytotoxicity (Kalkunte *et al.*, 2009). The concentration of these factors decreases in the decidua as pregnancy progresses, suggesting that they may be irrelevant after spiral artery remodeling concludes (Lash *et al.*, 2006).

Interferon-gamma (IFN-γ) is a uNK cell-secreted cytokine that has arguably attracted the most attention in the literature; it is necessary and sufficient for spiral artery remodeling (Ashkar and Croy, 2001). IFN-γ inhibits trophoblast invasion by promoting apoptosis of trophoblast cells and altering protease levels, keeping trophoblast invasion in check (Lash *et al.*, 2006). Perhaps counterintuitively, uNK cells from preeclamptic women secrete less IFN-γ than uNK cells from normotensive controls (Zhou *et al.*, 2013).

uNK cells also produce a variety of chemokines, including interleukin-8 (IL-8) and interferon-inducible protein-10 (IP-10). Receptors for these two chemokines, CXCR1 and CXCR3, respectively, are expressed on the trophoblast cell surface, substantiating uNK cells' candidacy as potent regulators of trophoblast invasion (Hanna *et al.*, 2006). However, IL-8 and IP-10 presence in uNK cell-conditioned media (CM) isn't different between pregnancies with normal and high uterine artery Doppler resistance indices (RIs) (Wallace *et al.*, 2013). To generate uNK cell CM, the authors plated equal densities of uNK cells from the different pregnancies, which could explain this finding. uNK cell chemokine secretion could very well be equivalent cell to cell, but the two types of pregnancies may have different population sizes of uNK cells. CM from these pregnancies did differ in extracellular signal-regulated kinase (ERK) and Akt pathway activation, which are critical for trophoblast invasion. Therefore, it is possible that uNK cells of these two types of pregnancies differentially express chemokines other than the ones examined in this study.

Finally, uNK cells are also sources of MMPs such as MMP-2 (Naruse *et al.*, 2009). The previously mentioned peptide hormone AM stimulates MMP-9 secretion from uNK cells, triggering spiral artery smooth muscle cell apoptosis (Li *et al.*, 2013). Altogether, this orchestra of signaling molecules coordinates the complex process of spiral artery remodeling to maintain a healthy pregnancy. Intriguingly, angiogenic growth factors and cytokines decrease in concentration when uNK cells and trophoblast cells are co-cultured, though the cell type from which these factors are derived in this co-culture is uncertain (Lash *et al.*, 2011). No doubt additional signals will be identified that will further our understanding of uNK cell-trophoblast interactions.

Conclusions

In summary, the overall immune milieu of the placenta is an important determinant of the health and success of a pregnancy (Arck and Hecher, 2013, Erlebacher, 2013). Perturbations in this complex environment by lipopolysaccharide (LPS)-induced inflammation, for example, can cause abnormal placental vascular remodeling and phenotypes resembling FGR and preeclampsia (Cotechini *et al.*, 2014). uNK cells dominate this immune landscape during early pregnancy and are important modulators of the maternal-fetal vasculature. Generalized inflammatory changes triggered by obesity, for example, can cause under-recruitment of uNK cells to the decidua, which could explain why obesity elevates a patient's risk of pregnancy complications (Parker *et al.*, 2013).

Certainly, there are other determinants of uNK cell density in the decidua during early pregnancy not discussed here, such as decidual cell-derived cytokines (Lockwood *et al.*, 2013). However, it is likely that there are important effectors of spiral artery remodeling other than uNK cells (Charalambous *et al.*, 2012); several studies suggest that uNK cells may be important only during a small time frame of this process. Ultimately, furthering our understanding of uNK-trophoblast cell interactions and their role in placental vascular remodeling will shed light on placentation disorders like FGR and preeclampsia and may potentially reveal new treatment modalities for these diseases.

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