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Receptors for non-MHC ligands contribute to uterine natural killer cell activation during pregnancy in mice

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ABSTRACT

Introduction: Activated uterine natural killer (uNK) cells are abundant in early human and mouse decidual basalis. In mice, distinct uNK cell subsets support early endothelial tip cell induction, the pruning of new vessels and initiation of spiral arterial modification. While genetic studies indicate that NK/uNK cell activation via receptors recognizing Class I MHC-derived peptides promotes human pregnancy, roles for other activation receptors expressed by NK cells, such as the aryl hydrocarbon receptor (AHR) and natural cytotoxicity receptors (NCR) are undefined in human or mouse pregnancies.
Methods: Expression of AHR and NCR1 (ortholog of human NKp46) by gestation day (gd)10.5 mouse uNK cell subsets was measured by quantitative real-time RT-PCR. Early implantation sites from mice lacking expression of either receptor were examined histologically.
Results: Gd10.5 uNK cell subsets, separated by reactivity to *Dolichos biflorus* agglutinin lectin, differed in relative transcript abundance for *Ahr* and *Ncr1*. Quantitative histology revealed that, in comparison to C57BL/6 controls, implant sites from gd10.5 $Ahr^{-/-}$ and gd6.5–12.5 *UkCa:B6.Ncr1*$^{Gfp/Gfp}$ mice had normal uNK cell abundance but the uNK cells were smaller than normal and unable to trigger spiral arterial remodeling. Whole mount immunohistochemistry comparisons of viable, gd6.5–8.5 $Ncr1^{Gfp/Gfp}$ and C57BL/6 implant sites revealed deficits in implant site angiogenesis and conceptus growth in $Ncr1^{Gfp/Gfp}$.
Discussion: In mice, activation of AHR and of NCR1 by endogenous, as yet undefined ligands, contributes to uNK cell activation/maturation and angiogenic functions during early to mid-gestation pregnancy. MHC-independent activation of uNK cells also likely makes critical contributions to human pregnancy success.

1. Introduction

Endometrial decidualization is a hallmark of hemochorial placentation, a placental type found in many species including humans and rodents [1,2]. During the period of early pregnancy marked by decidualization, the endometrium experiences an influx of a large number of leukocytes. The most dominant cell population amongst these is a distinctive population of natural killer (NK) lymphocytes known as uterine (u)NK cells [3–5]. In mice, uNK cells first appear as small, agranular uNK cells around gestation day (gd) 5 and preferentially localize to the mesometrial decidua basalis [3,4,6,7]. UNK cells reach peak numbers at mid-pregnancy (gd10–12) then decline numerically as pregnancy progresses toward term [1,5,7]. Concurrent with mounting numbers toward mid-gestation, uNK cells enter a state of activation in which their size increases dramatically and they acquire numerous cytoplasmic granules [4–6]. The majority of uNK cells, but not peripheral NK cells, display surface N-acetylgalactosamine as a terminal sugar, which can be detected by *Dolichos biflorus* agglutinin (DBA) lectin [8–10]. Reactivity with this lectin, which also stains the membranes of uNK cell granules and some endothelial cells, divides uNK cells into DBA+ and DBA− subsets, each with distinct functions and intervals of dominance between early to mid-gestation [10–12]. DBA+ uNK cells are dominant at mid-gestation and angiogenic with heightened expression of vascular endothelial growth factor (*Vegf*), placenta growth factor (*Pgf*) [11], and delta-like ligand 1 (*Dll1*) [13]. The DBA− uNK cells are dominant immediately post implantation, declining to approximately 10% at mid-gestation [11,12]. DBA− uNK cells phenotypically resemble peripheral NK cells and are responsible for most interferon gamma (IFNG) production within early

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decidua [10,11]. IFNG is a key agent responsible for triggering spiral arterial (SA) modifications [10,14], which produces a more vein-like vessel [15].

Our recent studies suggest that uNK cells promote proper uterine lumen closure through promotion of very early decidual angiogenesis and thereby regulate the rate of pre-placental conceptus development [Hofmann AP, Gerber SA, and Croy BA, MS submitted]. Studies on major histocompatibility complex (MHC) class 1 molecules expressed by human extravillous trophoblasts (EVT) indicate that key interactions between uNK cells and trophoblasts regulate human pregnancy success. Class I human leukocyte antigen (HLA)-C is the only polymorphic MHC molecule expressed by trophoblasts. HLA-C is the major ligand for NK cell receptors of the killer immunoglobulin-like receptor (KIR) family [16,17]. KIR haplotypes are diverse, displaying both inhibitory and activation biases [18,19] and maternal KIR activation by fetal HLA-C enhances depth of trophoblast invasion [17] and the success of implantation [19,20].

While KIR-associated uNK cell activation is strongly linked to pregnancy success, roles for non MHC-related NK cell activation receptors have not been well addressed. Since syngeneic matings in mice stimulate uNK cell activation and trigger SA remodeling, receptors other than MHC-binding KIR orthologues must participate in mouse uNK cell activation and function. The aryl hydrocarbon receptor (AHR) [21] and natural cytotoxicity receptors (NCR)-1 (human ortholog NKp46) [7,22,23] are known MHC-independent, activation receptors found on mouse NK cells. AHR is a transcription factor that responds to xenobiotics and endogenous metabolites [24]. It is found on the surfaces of T_H17 T cells and of interleukin (IL)-22 producing human stage 3 immature NK and uNK cells [21,24]. IL22 is a leukocyte-derived cytokine with key roles in intestinal defense and mucosal wound healing; some DBA+ mouse uNK cells transcribe and translate *Il22* [11]. NCR1 is a stress-activated receptor found on NK cells and some T cell subsets [25,26] that signals downstream via the transcription factor ETS1 [27]. Studies in mice with heterozygous loss of NCR1 function showed this receptor is essential in NK cell-mediated gastrointestinal mucosal immunity [26]. Studies using mice homozygous for NCR1 loss revealed NCR1's role in the recognition and destruction of tumors, clearance of influenza virus infection [25] and in type 1 diabetes development [28,29].

Here we address the roles of AHR and NCR1 in promotion of uNK cell differentiation, function and implantation site development by determining *Ahr* and *Ncr1* gene expression levels in DBA+ and DBA− uNK cells of normal mice using quantitative real-time RT-PCR (qRT-PCR) and by histological examination of implantation sites from mice lacking receptor expression. Each receptor was characterized as making an independent contribution to uNK cell maturation, activation and angiogenic function.

2. Materials and methods

2.1. Mice

Randombred CD1 males and females (Charles River, St. Constant, QU) at 8–10 wk age were mated for uNK cell subset isolation by flow cytometry. $Ahr^{-/-}$ mice on a B6 background [30] were mated by $Ahr^{-/-}$ males at McMaster University, Hamilton, ON for histological study. C57BL/6 (B6)-Tg(UBCGFP)/30SchaJ(*Gfp*+/−) mice having ubiquitous GFP expression, were purchased from The Jackson Laboratory (Bar Harbor, ME), bred to be homozygous *Gfp/Gfp* at Queen's University, and used as stud males to breed C57BL/6 (B6) and *Ncr1* loss of function females. Heterozygous *Ncr1* loss of function breeding pairs (*UkCa:B6.Ncr1*$^{+/Gfp}$ (*Ncr1*$^{+/Gfp}$)) [25] were provided by Dr. O. Mandelboim (Hebrew University – Hadassah Medical School, Jerusalem, Israel, through the laboratory of Dr. F. Colucci (University of Cambridge, Cambridge UK)) and bred to homozygosity (*Ncr1*$^{Gfp/Gfp}$) at Queen's University. Matings were timed from copulation plug detection (gd0.5). Mice were euthanized and uteri were removed and dissected as described below. All mouse handling was in accordance with approved animal care procedures at the respective institutions.

2.2. Cell subset separation and quantitative PCR

Cell separation and qRT-PCR were performed on gestation day (gd)10.5 as previously described and validated [11]. Briefly, CD1 decidua basalis (DB) and mesometrial lymphoid aggregate of pregnancy (MLAp) were mechanically separated into cell suspensions that were incubated in 1% BSA, stained with conjugated antibodies (PE-IL2RB, FITC-DBA, CY5-CD3E), and separated by flow sorting (EPICS Altra Flow Hy-PerSort Cytometer (Beckman Coulter, Mississauga, ON)). The first gate was set for CD3E−CD122+ cells. These cells were then sorted as DBA+ or DBA− NK cells. Cells were lysed and RNA was isolated, reverse transcribed, and amplified to template cDNA. QRT-PCR used the ABI Prism 7500 system and Bio-Rad iTaq™Fast Supermix with ROX. PrimeTime® qPCR Assays containing probes and primers were purchased from Integrated DNA Technologies, Inc. as follows: Mm.PT.53a.11116644 (*Ahr*), Mm.PT.53a.32103644 (*Ncr1*), Mm.PT.53a.16270650.g (*Ets1*), and Mm.PT.39a.22214828 (hypoxanthine guanine phosphoribosyl transferase; *Hprt*). RT-PCR volume was 20 μL and conditions were initial incubation (2 min at 95 °C) and 40 cycles of 3 s at 95 °C and 34 s at 60 °C. Relative expression of target transcripts was normalized to *Hprt* transcripts.

2.3. Whole mount immunohistochemistry

For whole-mount *in situ* immunofluorescent staining [31], at least 3 *Ncr1*$^{Gfp/Gfp}$ females syngeneically mated by either *Ncr1*$^{Gfp/Gfp}$ or *Gfp/Gfp* males (for visualization of GFP+ conceptus-derived cells) were studied on each of gd6.5, 8.5 and 9.5. Uterine dissections were performed under microscopic magnification as previously reported [31]. Uteri were transected into individual implantation sites. The myometrium was incised along the anti-mesometrial side using fine forceps, and gently retracted, leaving the decidua basalis attached. Excess myometrium was trimmed away, leaving only a small landmark remnant at the decidual attachment site. Implantation sites were then halved midsagitally or transversely using a scalpel blade to expose the embryonic crypt. Implant site halves were incubated in 200 μL PBS-1% BSA-0.1% sodium azide (PBA) for 1 h with 10ug/mL of blocking antibody to the IgG Fc receptor (anti-CD16/CD32; supernatant of hybridoma 2.4G2, ATTC, Manassas, VA) and 4ug/mL of fluorescently conjugated primary antibodies (CD45-FITC, CD31-PE; BD Pharmingen, Mississauga ON). After incubation and addition of 1 mL PBA, tissues were placed onto microscope slides with myometrium towards the slide, coverslipped, viewed by fluorescence microscopy, and photographed using Axiovision 4.8 software on an AxioCam-equipped Zeiss M1 imager (Zeiss; Toronto, ON, Canada). Using ImageJ software, three randomly selected whole mount images from each *Ncr1*$^{Gfp/Gfp}$ and B6 pregnancy studied were selected for quantification of anti-mesometrial vessel width and of the area between vessels. Vessel width was measured across the thickest point between two branch points. The area between vessels was measured by outlining the dark spaces created in the image due to vessel branching and calculating the surface area of the inscribed space.

2.4. Histopathology and morphometry

For routine histochemisty, implantation sites were collected from two $Ahr^{-/-}$ females at gd10.5 and *Ncr1*$^{Gfp/Gfp}$ mice on gd6.5, 8.5, 10.5, and 12.5 with three pregnancies per time point. Three implant sites were studied per litter when available. For smaller litters, all implant sites were examined. Implant sites were immersion fixed with 4% paraformaldehyde (PFA), processed, embedded in paraffin and cut as serial sections of 6 μm. Slides were stained using published protocols for hematoxylin and eosin (H&E) [32] for arterial morphometric measurements, and DBA lectin or DBA lectin/Periodic Acid Schiff's (PAS) double staining for uNK cell quantification, measurement, and subset quantification [8,12]. Using ImageJ software, the diameter of 20 randomly selected DBA+ or DBA− uNK cells (subsets I or II from each of 3 implant sites from the DB only) [8] were measured at 400× magnification for each specimen across their longest and shortest axes. An average of both values was used for statistical analysis. Multiple measurements of 3 round SA in each section (2–3 sections per specimen; $n = 3$ implant sites) were also taken at 200× magnification. A SA wall-lumen ratio was calculated using measurements of the largest arterial diameter and the largest lumen diameter in the following calculation: $(D_{Total} - D_{Lumen})/D_{Lumen}$. The DBA+/DBA− phenotype of uNK cells was scored in DB on 8 or more sections/implant site at 400× magnification as previously described [12]. Cells included in the count had visible nuclei and were scored as PAS+DBA− (purple) or PAS+DBA+ (orange to rust) after careful examination. Archived, identically prepared paraffin block samples from gd6.5–12.5 B6 females mated by B6 males were also sectioned and stained as above as control tissue. All scoring was conducted by a single individual (AMF).

2.5. Statistical analysis

QRT-PCR data are expressed as mean ± SEM. Statistical significance for qRT-PCR was assessed by Student *T*-test; $p < 0.05$ was considered significant. UNK cell data are expressed as mean ± SD. For uNK cell data, Mann–Whitney tests were used to