Receptors for non-MHC ligands contribute to uterine Natural Killer cell activation during pregnancy in mice

A. M. Felker¹, Z. Chen¹, W. G. Foster², and B.A. Croy^{1,3}

¹Department of Biomedical and Molecular Sciences, Queen's University, Kingston, ON, Canada K7L3N6

²Department of Obstetrics and Gynecology, McMaster University, Hamilton, ON, Canada L8S4L8

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 (orthologue 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.

Keywords

Arylhydrocarbon receptor; Natural cytotoxicity receptor; decidua; histology; reproductive immunology; spiral artery remodeling

³Corresponding author: Dr. B. Anne Croy, Department of Biomedical and Molecular Sciences, Queen's University, Kingston ON Canada K7L 3N6, Phone: 1-613-533-2859; FAX: 1-613-533-2022; croya@queensu.ca.

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 (*DIII*) [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 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 I 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 orthologue 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

Ranbmbred 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. Q (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 20µL and conditions were initial incubation (2 min at 95°C) and 40 cycles of 3 sec at 95°C and 34 sec 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 midsagittally or transversely using a scalpel blade to expose the embryonic crypt. Implant site halves were incubated in 200µL 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 1mL 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 antimesometrial 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 histochemistry, 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 6µm. 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 400x 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 200x

magnification. A SA wall-lumen ratio was calculated using measurements of the largest arterial diameter and the largest lumen diameter in the following calculation: $(D_{\text{Total}} - D_{\text{Lumen}})/D_{\text{Lumen}}$. The DBA+/DBA- phenotype of uNK cells was scored in DB on 8 or more sections/implant site at 400x 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 $\pm$ 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 $\pm$ SD. For uNK cell data, Mann-Whitney tests were used to assess statistical significance between two samples. SA data are expressed as mean $\pm$ SEM. For SA data student T-test assessed statistical significance of the difference between two means. One-way ANOVA with Tukey's posttest assessed statistical significance across multiple means. Statistical analyses were performed using Prism5 Software (GraphPad Software, Inc.). $P < 0.05$ was considered significant.

3. Results

3.1. DBA+ and DBA- uNK cell subsets differ in expression of *Ahr* and *Ncr1*

DBA+ and DBA- uNK cells from gd10.5 randombred CD1 mice were flow sorted for RNA isolation. Use of this strain with large litter sizes was necessary to obtain sufficient RNA from the minor DBA- uNK cell subset for study. QRT-PCR analyses revealed distinct differences between the uNK cell subsets. *Ahr* expression was essentially undetectable in DBA+ uNK cells and appeared to be exclusive to DBA- uNK cells (~638x higher relative expression than by DBA+ cells; Fig. 1A). *Ncr1* transcripts were ~8 fold relatively more abundant in DBA+ than in DBA- uNK cells (Fig. 1B). Relative expression of the NCR1 downstream transcription factor *Ets1* was ~2 fold higher in DBA+ than in DBA- uNK cells (Fig. 1C).

3.2. *Ahr* expression is required for uNK cell maturation and spiral arterial remodeling

Difficulties in obtaining matings and implant sites from *Ahr*^{-/-} females limited our study to multiple implant sites from only 2 gd-matched samples collected over 3 months of constant pairing. Since loss of AHR function in mice was previously linked to aberrant follicular selection, blastocyst formation and implantation [33,34], *Ahr*^{-/-} x *Ahr*^{-/-} pairings are not normally made in our colony. Even *Ahr*^{+/-} dams mated by *Ahr*^{-/-} males are reproductively deficient. Over a 3-year period, our *Ahr*^{+/-} dams mated by *Ahr*^{-/-} males gave birth to 84 live and 41 dead litters; 12 pups died shortly after birth and the average litter size (4.7 pups/litter) was smaller than control. Within live litters, the sex ratio of male to female pups was normal (1.09:1).

Histological comparisons between gd10.5 *Ahr*^{-/-} and gd10.5 B6 controls (n=3) showed that mean numbers of total PAS+ uNK cells were similar ($p=0.213$) but the proportion of DBA

+PAS+ uNK cells amongst total uNK cells was significantly different. In *Ahr*^{-/-} mice, the proportion of DBA+PAS+ uNK cells was 86.5% of total uNK cells. This was greater than the 79.8% DBA+PAS+ uNK cells in B6 ($p=0.0180$) [12]. Thus, in the absence of AHR, there are fewer DBA-PAS+ uNK cells (the *Ahr*-expressing subset). DBA- uNK cells in DB were significantly shorter in diameter in gd10.5 *Ahr*^{-/-} (10.15 μm) versus B6 (11.51 μm) ($p<0.0001$) (Fig. 2A3). This suggests retarded maturation and/or activation in the DBA- *Ahr*^{-/-} uNK cells compared with controls. Diameters of DBA+ *Ahr*^{-/-} cells were also significantly shorter (11.33 μm in *Ahr*^{-/-} vs. 13.67 μm in B6; $p<0.0001$) (Fig. 2B3), suggesting retarded maturation and/or activation is also present in the non-*Ahr* expressing DBA+ uNK cell subset. To determine whether the normal numbers of seemingly less mature uNK cells could initiate SA remodeling, arterial morphometry was conducted. The SA wall-lumen ratio was significantly greater in *Ahr*^{-/-} (0.326) than in B6 (0.211); $p=0.0019$ (Fig. 2C3). This observation suggests that despite normal total uNK cell numbers, uNK cells of *Ahr*^{-/-} mice are deficient in function.

3.3. Ncr1 is required for uNK cell maturation, early decidual angiogenesis, and spiral arterial remodeling

Despite the loss of NCR1 function, *Ncr1*^{Gfp/Gfp} mice had breeding performances similar to B6 controls. Our *Ncr1*^{Gfp/Gfp} breeding pairs produced 9 live litters with an average of 6.8 pups/litter. The sex ratio of male to female pups at birth was 1.2:1 ($n=9$ litters). For *Ncr1*^{Gfp/Gfp} euthanized during pregnancy ($n=11$), the average litter size was 7.2 pups/litter and implantation sites were more common in the right than left uterine horn (1.8:1; $n=11$) while resorbed fetuses were twice as common in the right as in the left horn. The overall rate of resorption was 18% in *Ncr1*^{Gfp/Gfp} mice ($n=11$ litters), appears higher compared to a resorption rate of <10% in control matings [9].

Gestation day 6.5—H&E stained, gd6.5 *Ncr1*^{Gfp/Gfp} implantation sites sections were similar in overall appearance to gd-matched B6 controls. The proportion of DBA+PAS+ uNK cells among the total population of PAS+ uNK cells was similar between gd6.5 *Ncr1*^{Gfp/Gfp} (42.7%) and B6 (47.3%) mice ($p=0.0511$) [12], which indicates that loss of NCR1 function has no effect on either total uNK cell or DBA+PAS+ uNK cell numbers in implantation sites. *Ncr1*^{Gfp/Gfp} DBA+ uNK cell diameter, however, was significantly shorter at gd6.5 (10.6 μm in *Ncr1*^{Gfp/Gfp} vs. 12.45 μm in B6; $p=0.0005$; Table 1). DBA- uNK cell diameter was also significantly shorter at gd6.5 between *Ncr1*^{Gfp/Gfp} (8.13 μm) and B6 controls (10.22 μm ; $p<0.0001$; Table 1).

Reported results from B6 whole mount staining provide benchmarks for comparisons of the mutant mice [31; Fig. 3]. In gd6.5 B6,

- i. leukocytes, including many expressing CD31 (Fig. 3C), are localized to the mesometrial side of implants sites,
- ii. invasion of individual trophoblast cells has commenced from the ectoplacental cone (Fig. 3B),
- iii. CD31+ superbright staining appears surrounding the embryonic crypt (Fig. 3A),

- iv. active decidual angiogenesis is widespread, as evidenced by wide, fuzzy vessels indicative of endothelial tip cell formation (Fig. 3C).

Whole mount staining at gd6.5 in *Ncr1^{Gfp/Gfp}* mice revealed that

- i. CD31+ leukocytes were abundant and had localized to DB (Fig. 3F)
- ii. trophoblast invasion had begun (Fig. 3E), as in B6 controls.

In contrast to B6, gd6.5 *Ncr1^{Gfp/Gfp}* implant sites and fetuses appeared smaller. There was no elevation in CD31+ reactivity around the gd6.5 *Ncr1^{Gfp/Gfp}* embryonic crypt (Fig. 3D) and decidual vessels were narrow and well defined (Fig. 3F). This appearance suggested delayed endothelial tip cell induction and was quite unlike the angiogenic vessels in B6.

Gestation days 8.5 and 9.5—Scoring of total PAS+ uNK cells in paraffin sections revealed no differences in uNK cell numbers between *Ncr1^{Gfp/Gfp}* and B6 at gd8.5 ($p=0.285$). DBA+ gd8.5 *Ncr1^{Gfp/Gfp}* and B6 uNK cell numbers were also not significantly different ($p=0.274$) but the proportion of DBA+PAS+ cells among total PAS+ uNK cells was significantly increased in *Ncr1^{Gfp/Gfp}* (85.0%) compared to B6 (76.7%; $p=0.0003$). DBA+ *Ncr1^{Gfp/Gfp}* uNK cell diameters were shorter than B6 diameters (11.77 μ m in *Ncr1^{Gfp/Gfp}* vs. 15.11 μ m in B6; $p<0.0001$; Table 1). In both *Ncr1^{Gfp/Gfp}* and B6, there was a significant increase in the size of DBA+ uNK cells between gd6.5 to gd8.5. This indicates that uNK cell maturation is occurring in *Ncr1^{Gfp/Gfp}* as gestation proceeds but not at the rate expected in normal pregnancy. Similarly, DBA- uNK cell diameters increased between gd6.5 to gd8.5 but were shorter in *Ncr1^{Gfp/Gfp}* mice than in B6 (9.29 μ m vs. 11.24 μ m respectively; $p<0.0001$; Table 1).

The benchmark features for whole mount stained gd8.5 B6 implant sites [31] are

- i. many wide vessels with a webbed appear in central DB, indicative of angiogenesis involving intussusception and endothelial progenitor cell recruitment,
- ii. long, wide arching vessels appearing in lateral decidua,
- iii. only a small, narrow residual uterine lumen,
- iv. frequent leukocyte-leukocyte conjugated pairs and changes in leukocyte shape (loss of roundness; gain in irregularity suggestive of activation and/or migration (Fig. 4A)).

Ncr1^{Gfp/Gfp} gd8.5 implant sites were smaller than controls though angiogenesis in the central DB and lateral decidua qualitatively resembled B6 angiogenesis. The residual uterine lumen appeared similar in size to B6 controls. CD45+ leukocytes were abundant in *Ncr1^{Gfp/Gfp}* DB but were smaller than in B6 (Fig. 4C), consistent with the morphometric data obtained from paraffin sections (Table 1). *Ncr1^{Gfp/Gfp}* leukocytes remained round and had not acquired the irregular shapes seen in B6 (Fig. 4C). As in B6 however, conjugated pairs of CD45+ leukocytes were present in gd8.5 *Ncr1^{Gfp/Gfp}* implant sites, suggestive of immune synapse formation, an event that leads to cell activation. Conjugation occurred exclusively between CD45+CD31+ cell pairs in both *Ncr1^{Gfp/Gfp}* and B6 (Fig. 4A,C).

An unexpected difference between gd8.5 B6 and *Ncr1^{Gfp/Gfp}* was present in the antimesometrial decidua, a region that physiologically lacks uNK cells. Here a honeycomb-like vasculature (Fig. 4B,D) was present only in *Ncr1^{Gfp/Gfp}*. Antimesometrial vessels in gd8.5 B6 implant sites were more than twice as wide as in *Ncr1^{Gfp/Gfp}* (41.3 μ m vs. 15.8 μ m respectively; $p < 0.0001$). There was also a decreased area of space between antimesometrial vessels in *Ncr1^{Gfp/Gfp}* (1134.9 μ m²) compared to B6 controls (2346.6 μ m²; $p = 0.0134$) that appeared to represent disorganization of vascular branching in the *Ncr1^{Gfp/Gfp}* antimesometrial vasculature (Fig. 4B,D).

Whole mount studies in B6 mice identified gd9.5 as the earliest time of trophoblast transit across decidual vascular endothelium and entry into vessel lumens [31]. Since uNK cells are hypothesized to promote trophoblast invasion, whole mount staining of gd9.5 *Ncr1^{Gfp/Gfp}* mice was undertaken. In *Ncr1^{Gfp/Gfp}* implant sites, trophoblast cells were co-localized with vessel endothelium and many trophoblasts were apparent in the lumens of decidual vessels. Thus, NCR1 does not appear to be utilized for regulation of intravascular trophoblast invasion.

Gestation day 10.5 and 12.5—Scoring of total PAS+ uNK cells in paraffin sections revealed that total cell numbers in *Ncr1^{Gfp/Gfp}* and B6 were similar at gd10.5 ($p = 0.287$) and gd12.5 ($p = 0.316$). At both gd10.5 and 12.5 DBA+PAS+ uNK cell numbers were also similar in *Ncr1^{Gfp/Gfp}* and B6 (gd10.5 ($p = 0.352$); gd12.5 ($p = 0.247$)). The proportion of DBA+PAS+ uNK cells among total PAS+ cells was now equivalent for *Ncr1^{Gfp/Gfp}* and B6 mice (gd10.5, 78.9% in *Ncr1^{Gfp/Gfp}* vs. 79.8% in B6; $p = 0.391$; gd12.5, 83.3% in *Ncr1^{Gfp/Gfp}* vs. 85.3% in B6; $p = 0.217$) [12]. Thus, the gain in proportion of DBA+ uNK cells with progress of early pregnancy, as previously reported for B6, is an NCR1 independent process. DBA+ uNK cell diameters, however, continued to differ between the strains at both gd10.5 (12.31 μ m in *Ncr1^{Gfp/Gfp}* vs. 13.67 μ m in B6; $p = 0.0001$) and gd12.5 (14.94 μ m in *Ncr1^{Gfp/Gfp}* vs. 18.73 μ m in B6; $p < 0.0001$; Table 1). This suggests about a 48 hr delay in maturation of *Ncr1^{Gfp/Gfp}* versus B6 DBA+ uNK cells. DBA- uNK cell diameter was also shorter in *Ncr1^{Gfp/Gfp}* mice compared to B6 controls at gd10.5 (10.80 μ m in *Ncr1^{Gfp/Gfp}* vs. 11.51 μ m in B6; $p = 0.0041$) and gd12.5 (11.26 μ m in *Ncr1^{Gfp/Gfp}* vs. 13.19 μ m in B6; $p < 0.0001$; Table 1). These data are the first to indicate that B6 DBA- uNK cells in normal mice are smaller throughout pregnancy than DBA+ uNK cells. The overall rate of change in uNK cell diameter per day for each subset in both *Ncr1^{Gfp/Gfp}* and B6 was approximately 5%, representing a 15% increase in cell volume per day.

Impairments in SA modification became more defined in mid-pregnancy *Ncr1^{Gfp/Gfp}* mice. In comparison to controls, *Ncr1^{Gfp/Gfp}* mice had smaller vessel diameters, thicker arterial walls (Fig. 4E) and significantly greater wall to lumen ratios at gd10.5 (0.394 in *Ncr1^{Gfp/Gfp}* vs. 0.211 in B6; $p < 0.0001$) and gd12.5 (0.195 in *Ncr1^{Gfp/Gfp}* vs. 0.113 in B6; $p < 0.0001$; Fig. 4F). At gd10.5, *Ncr1^{Gfp/Gfp}* SA wall to lumen ratio did not differ significantly from *Ahr^{-/-}* ($p = 0.0742$), however there were significant differences in DBA+ ($p = 0.0089$) and DBA- ($p = 0.0255$) uNK cell diameters between the strains with smaller uNK cells in *Ahr^{-/-}* mice.

4. Discussion

In humans, genetic relationships between mother and fetus that activate NK cell KIR receptors, promote pregnancy. Syngeneic pregnancies in inbred mice also activate uNK cells, suggesting that endogenous ligands are present at the maternal-fetal interface that bind NK cell activation receptors. Mouse uNK cell activation is reflected by gestational gains in cell size and in numbers of cytoplasmic granules, expression of activation markers such as CD69 [31] and production of IFNG and VEGF [11,35]. Consequences of uNK cell activation are induction of endothelial tip cells in vessels of early decidua basalis [Hofmann AP, Gerber SA, and Croy BA, MS submitted], pruning of neoangiogenic vessels [36] and initiation of spiral arterial remodeling. It is probable that uNK cell-promoted angiogenesis underlies the beneficial effect of KIR activation in human pregnancy because human uNK cells express molecules associated with angiogenesis such as Tie-2, VEGF, and PGF [37,38].

To address the roles of MHC-independent NK cell receptors in pregnancy, inbred pregnancies are required. The MHC-independent NK cell activation receptors AHR and NCR1 have been identified on the surface of stage 3 and stage 4 NK cells in human uterine mucosa [21,39] and were therefore chosen for study. Since mouse uNK cells have been divided, based on reactivity with DBA lectin, into a blood-like subset (DBA-) and a specialized decidual mucosal population (DBA+), that appears to be more angiogenic [11], data were collected by subset based on DBA lectin expression. Adoptive transfer studies have shown that DBA+ uNK cells arise from bone marrow precursors that homed to the uterus; DBA- uNK cells arise from as yet undefined sources [11]. *Ahr* and *Ncr1* had differential expression on uNK cell subsets separated by DBA lectin expression; *Ahr* expression was restricted to DBA- uNK cells while *Ncr1* expression was heightened in DBA+ uNK cells. The availability of mice lacking functional AHR and NCR1 permitted further studies that addressed whether absence of AHR or NCR1 altered uNK cells in number, size, or DBA- to DBA+ cell ratios. Additionally, implantation sites were assessed to mid-pregnancy for evidence of changes in uNK cell-promoted events that might indicate altered functions due to a deficit in either receptor. Absence of either receptor had damaging effects on uNK cell maturation/activation and on the progression of normal gestational changes in the decidual vasculature.

Mice with the functional lack of either AHR or NCR1 have the same total numbers of PAS+ uNK cells compared to gd-matched B6 controls at each time point studied. Loss of AHR resulted in a greater proportion of DBA+PAS+ uNK cells present in gd10.5 DB [12] and indicates decreased numbers of DBA-PAS+ uNK cells. Since *Ahr* is expressed by the DBA- uNK cell subset, these data suggest that AHR has roles in recruitment and proliferation of DBA- uNK cells and that AHR may be the first useful marker defined for this subset since IFNG [10]. While the overall number of uNK cells in *Ahr*^{-/-} was unchanged from B6, *Ahr*^{-/-} implant sites were distinct with uNK cells being smaller and SA remodeling being deficient. Endogenous ligands for uNK cell-expressed AHRs are not known. The modulation of AHR during pregnancy, however, has been linked to maternal steroid hormones, particularly 17 β estradiol (E2) [34,40–42]. E2 has been shown to regulate *Ahr* mRNA and AHR protein levels in rats [40,41]. There is also evidence of crosstalk between AHR and the estrogen receptor (ER α) in the reproductive tract [41,42]. AHR is a particularly important

receptor for further study since it identifies a link between the environment and pregnancy disease pathogenesis that could be caused by disturbances to lymphocyte-promoted angiogenesis.

Histological time course studies were possible in *Ncr1^{Gfp/Gfp}* due to their fertility. DBA+ uNK cells were the minority uNK cell subset at gd6.5 (42.7%) but the dominant uNK cell subset at mid-gestation (gd10.5 (78.9%) and gd12.5 (83.3%)), the pattern typical of normal mice [12]. Preliminary flow cytometric analyses of decidual cells from gd10.5 B6 and *Ncr1^{Gfp/Gfp}* decidua, revealed that approximately half of all CD45+ cells in each strain expressed DBA lectin (data not shown). Thus, both histological and flow cytometric approaches suggest that homing of DBA+ uNK cells to the uterus is not dependent on NK cell receptor activation; homing more likely results from changes to uterine stromal environments [23]. Thus, even in the absence of *Ncr1*, which is heavily expressed by DBA+ uNK cells, recruitment, localization and proliferation of the DBA+ uNK cell population remained unchanged. This finding is supported by studies of intestinal mucosa that show NCR1 is not essential for maintaining cell numbers or tissue distribution of Nkp46+ cell subsets [26].

UNK cells in *Ncr1^{Gfp/Gfp}* mice were consistently smaller than in B6 between early to mid-gestation. DBA+ and DBA- uNK cell diameters increased throughout gestation at a rate of approximately 5%/gd in both strains. Over this interval, DBA+ and DBA- *Ncr1^{Gfp/Gfp}* uNK cells were consistently 80% of the size of B6 uNK cells, even when the DBA-/DBA+ uNK cell ratios became normalized at gd10.5 and 12.5. The smaller size suggests that terminal maturation/activation of both uNK cell subsets is impaired but not blocked in *Ncr1^{Gfp/Gfp}* mice. The absence of SA remodeling in *Ncr1^{Gfp/Gfp}* mice supports the conclusion that uNK cells in this strain are not fully functional. However, leukocyte-leukocyte conjugates were identified in gd8.5 *Ncr1^{Gfp/Gfp}* and B6 implant sites. These conjugations never involved trophoblasts and only rarely (~1/10) included DBA+ uNK cells (unpublished observation). In both *Ncr1^{Gfp/Gfp}* and B6 implant sites, conjugations were exclusively between CD31+ leukocytes. CD31 expression in B6 decidual leukocytes is first detected in whole mounts at gd6.5 [31] and the same time course was seen for *Ncr1^{Gfp/Gfp}* leukocytes. GD8.5 *Ncr1^{Gfp/Gfp}* leukocytes, although able to form conjugates, appeared less activated than control leukocytes due not only to their small size but because of their retention of a round shape. Several leukocyte populations, including NK cells and T cells, express CD31. Expression of CD31 promotes transmigration of CD31+ leukocytes through endothelial cells and endothelial cell basement membranes [43–44] and designates circulating angiogenic and vasculogenic cells [45]. CD31+, but not CD31-, T cells are known to contribute to angiogenic functions including endothelial repair and the secretion of angiogenic cytokines [43]

Whole mount studies indicated that lumen closure and trophoblast invasion appeared normal in gd6.5 *Ncr1^{Gfp/Gfp}*, suggesting NCR1 is not mediating these events. However, a deficiency in onset of decidual angiogenesis was observed, suggesting the endogenous ligands for NCR1 promote early steps in angiogenesis, including tip cell induction, within the DB. The striking difference between *Ncr1^{Gfp/Gfp}* and B6 antimesometrial vessels was a novel finding. Endothelial cells, vascular smooth muscle cells and endometrial fibroblasts are not known to

express NCR1 [26] and uNK cells are not present in this area of change. If this difference is truly mediated by ligation of NCR1 in the uNK cells of normal mice, an interpretation would be that angiogenic events involving or resulting from uNK cell activation in the DB have inductive consequences on antimesometrial angiogenesis that may decrease tip cell induction and promote pruning. Although erythrocytes are seen in the antimesometrial vessels during dissection and study, it will be important to determine whether these vessels support flow and may represent a compensatory mechanism for impaired angiogenic functions of uNK cells. At this time, we can only conclude that normal maturation and function of uNK cells in implantation site requires engagement of NK cell activating receptors that have ligands other than MHC molecules.

Acknowledgments

We thank Dr. Scott Gerber, University of Rochester, Rochester NY for advice and manuscript discussions and Dr. Anne Gannon, McMaster University, Dr. Patricia Lima and Mr. Alexander Hofmann at Queen's University, for technical assistance.

Supported by awards from the R. Samuel McLaughlin Fellowship and QGA, Queen's University (AMF); CIHR (WGF) and NSERC, CIHR, CFI and the Canada Research Chairs Program (BAC).

References

1. Croy BA, van den Heuvel MJ, Borzychowski AM, Tayade C. Uterine natural killer cells: A specialized differentiation regulated by ovarian hormones. *Immunol Rev.* 2006; 1(4):161–85.
2. Benirschke K. Placentation. *Journal of Experimental Zoology.* 1983; 228:385–9. [PubMed: 6363611]
3. Zhang J, Adams MA, Croy BA. Alterations in maternal and fetal heart functions accompany failed spiral arterial remodeling in pregnant mice. *Am J Obstet Gynecol.* 2011; 205(5):485e1–485e16.
4. Chantakru S, Kuziel WA, Maeda N, Croy BA. A study on the density and distribution of uterine natural killer cells at mid pregnancy in mice genetically-ablated for CCR2, CCR 5 and the CCR5 receptor ligand, MIP-1a. *J Reprod Immunol.* 2001; 49:33–47. [PubMed: 11137111]
5. Peel S. Granulated metrial gland cells. *Adv Anat Embryol Cell Biol.* 1989; 115:1–112. [PubMed: 2658485]
6. Chantakru S, Miller C, Roach LE, Kuziel WA, Maeda N, Wang WC, et al. Contributions from self-renewal and trafficking to the uterine NK cell population of early pregnancy. *J Immunol.* 2002; 168:22–8. [PubMed: 11751942]
7. Manaster I, Mandelboim O. The unique properties of uterine NK cells. *Am J Reprod Immunol.* 2010; 63(6):434–44. [PubMed: 20055791]
8. Paffaro A, Bizinotto C, Joazeiro P, Yamada T. Subset classification of mouse uterine natural killer cells by DBA lectin reactivity. *Placenta.* 2003; 24(5):479–88. [PubMed: 12744924]
9. Murphy SP, Tayade C, Ashkar AA, Hatta K, Zhang J, Croy BA. Interferon gamma in successful pregnancies. *Biol Reprod.* 2009; 80:848–59. [PubMed: 19164174]
10. Yadi H, Burke S, Madeja Z, Hemberger M, Moffett A, Colucci F. Unique receptor repertoire in mouse uterine NK cells. *J Immunol.* 2008; 181:6140–7. [PubMed: 18941204]
11. Chen Z, Zhang J, Hatta K, Lima PD, Yadi H, Colucci F, et al. DBA-Lectin reactivity defines mouse uterine natural killer cell subsets with biased gene expression. *Biol Reprod.* 2012; 87(4):81. [PubMed: 22875907]
12. Zhang J, Yamada AT, Croy BA. DBA-lectin reactivity defines natural killer cells that have homed to mouse decidua. *Placenta.* 2009; 30:968–73. [PubMed: 19765824]
13. Degaki KY, Chen Z, Yamada AT, Croy BA. Delta-Like ligand (DLL)1 expression in early mouse decidua and its localization to uterine natural killer cells. *PLoS One.* 2012; 7(12):e52037. [PubMed: 23284862]

14. Ashkar AA, Di Santo JP, Croy BA. Interferon gamma contributes to initiation of uterine vascular modification, decidual integrity, and uterine natural killer cell maturation during normal murine pregnancy. *J Exp Med*. 2000; 192(2):259–69. [PubMed: 10899912]
15. Zhang J, Dong H, Wang B, Zhu S, Croy BA. Dynamic changes occur in patterns of endometrial EFNB2/EPHB4 expression during the period of spiral arterial modification in mice. *Biol Reprod*. 2008; 79(3):450–8. [PubMed: 18463357]
16. Lash GE, Bulmer JN. Do uterine natural killer (uNK) cells contribute to female reproductive disorders? *J Reprod Immunol*. 2011; 88(2):156–64. [PubMed: 21334072]
17. Male V, Sharkey A, Masters L, Kennedy PR, Farrell LE, Moffett A. The effect of pregnancy on the uterine NK cell KIR repertoire. *Eur J Immunol*. 2011; 41(10):3017–27. [PubMed: 21739430]
18. Chazara O, Xiong S, Moffett A. Maternal KIR and fetal HLA-C: A fine balance. *J Leukoc Biol*. 2011; 90(4):703–16. [PubMed: 21873457]
19. Moffett-King A. Natural killer cells and pregnancy. *Nature Reviews Immunology*. 2002; 2(9):656–63.
20. Hiby SE, Apps R, Sharkey AM, Farrell LE, Gardner L, Mulder A, et al. Maternal activating KIRs protect against human reproductive failure mediated by fetal HLA-C2. *J Clin Invest*. 2010 Nov; 120(11):4102–10. [PubMed: 20972337]
21. Male V, Hughes T, McClory S, Colucci F, Caligiuri MA, Moffett A. Immature NK cells, capable of producing IL-22, are present in human uterine mucosa. *The Journal of Immunology*. 2010; 185:3913–8. [PubMed: 20802153]
22. El Costa H, Casemayou A, Aguerre-Girr M, Rabot M, Berrebi A, Parant O, et al. Critical and differential roles of Nkp46- and Nkp30-activating receptors expressed by uterine NK cells in early pregnancy. *J Immunol*. 2008; 181(5):3009–17. [PubMed: 18713971]
23. Huntington ND, Vosshehrich CA, Di Santo JP. Developmental pathways that generate natural-killer-cell diversity in mice and humans. *Nat Rev Immunol*. 2007; 7(9):703–14. [PubMed: 17717540]
24. Colonna M, Jonjic S, Watzl C. Natural killer cells: Fighting viruses and much more. *Nat Immunol*. 2011; 12(2):107–10. [PubMed: 21245897]
25. Gazit R, Gruda R, Elboim M, Arnon TI, Katz G, Achdout H, et al. Lethal influenza infection in the absence of the natural killer cell receptor gene *Ncr1*. *Nat Immunol*. 2006; 7(5):517–23. [PubMed: 16565719]
26. Satoh-Takayama N, Vosshehrich CAJ, Lesjean-Pottier S, Sawa S, Lochner M, Rattis F, et al. Microbial flora drives interleukin 22 production in intestinal Nkp46+ cells that provide innate mucosal immune defense. *Immunity*. 2008; 29:958–70. [PubMed: 19084435]
27. Di Santo JP. An IL-1b-dependent switch in innate mucosal immunity? *Immunity*. 2010; 32:734–6. [PubMed: 20620939]
28. Seidel E, Glasner A, Mandelboim O. Virus-mediated inhibition of natural cytotoxicity receptor recognition. *Cell Mol Life Sci*. 2012; 69:3911–3920. [PubMed: 22547090]
29. Gur C, Porgador A, Elboim M, Gazit R, Mizrahi S, Stern-Ginossar N, et al. The activating receptor Nkp46 is essential for the development of type 1 diabetes. *Nat Immunol*. 2010 Feb; 11(2):121–8. [PubMed: 20023661]
30. Gonzalez FJ, Fernandez-Salgureo F-S, Lee SST, Pineau T, Ward JM. Xenobiotic receptor knockout mice. *Toxicology Letters*. 1995; 82/83:117–21.
31. Croy BA, Chen Z, Hofmann AP, Lord EM, Sedlacek AL, Gerber SA. Imaging of vascular development in early mouse decidua and its association with leukocytes and trophoblasts. *Biol Reprod*. 2012; 87(5):125, 1–11. [PubMed: 22954796]
32. Prophet, EB. Mills, B. Arrington, JB., Sobin, LH., editors. *Laboratory Methods in Histotechnology*. Washington, D.C: American Registry of Pathology; 1992.
33. Bussmann UA, Bussmann LE, Barañao JL. An aryl hydrocarbon receptor agonist amplifies the mitogenic actions of estradiol in granulosa cells: Evidence of involvement of the cognate receptors. *Biol Reprod*. 2006; 74(2):417–26. [PubMed: 16237154]
34. Hernández-Ochoa I, Karman BN, Flaws JA. The role of the aryl hydrocarbon receptor in the female reproductive system. *Biochem Pharmacol*. 2009; 77(4):547–59. [PubMed: 18977336]

35. Lima PD, Croy BA, Degaki KY, Tayade C, Yamada AT. Heterogeneity in composition of mouse uterine natural killer cell granules. *J Leukoc Biol.* 2012; 92(1):195–204. [PubMed: 22566570]
36. Hatta K, MacLeod RJ, Gerber SA, Croy BA. Emerging themes in uterine natural killer cell heterogeneity and function. *Am J Reprod Immunol.* 2012 Oct; 68(4):282–9. [PubMed: 22626252]
37. Li XF, Charnock-Jones DS, Zhang E, Hiby S, Malik S, Day K, et al. Angiogenic growth factor messenger ribonucleic acids in uterine natural killer cells. *J Clin Endocrinol Metab.* 2001 Apr; 86(4):1823–34. [PubMed: 11297624]
38. Hanna J, Goldman-Wohl D, Hamani Y, Avraham I, Greenfield C, Natanson-Yaron S, et al. Decidual NK cells regulate key developmental processes at the human fetal-maternal interface. *Nat Med.* 2006; 12(9):1065–74. [PubMed: 16892062]
39. Hughes T, Becknell B, Freud AG, McClory S, Briercheck E, Yu J, et al. Interleukin-1B selectively expands and sustains interleukin-22+ immature human natural killer cells in secondary lymphoid tissue. *Immunity.* 2010; 32:804–14.
40. Kretschmar G, Papke A, Zierau O, Möller FJ, Medjakovic S, Jungbauer A, Vollmer G. Estradiol regulates aryl hydrocarbon receptor expression in the rat uterus. *Mol Cell Endocrinol.* 2010 Jun 10; 321(2):253–7. [PubMed: 20176079]
41. Bussmann UA, Bussmann LE, Baraño JL. An aryl hydrocarbon receptor agonist amplifies the mitogenic actions of estradiol in granulosa cells: Evidence of involvement of the cognate receptors. *Biol Reprod.* 2006; 74(2):417–26. [PubMed: 16237154]
42. Swedenborg E, Pongratz I. AhR and ARNT modulate ER signaling. *Toxicology.* 2010 Feb 9; 268(3):132–8. [PubMed: 19778576]
43. Kushner EJ, MacEneaney OJ, Morgan RG, Van Engelenburg AM, Van Guilder GP, DeSouza CA. CD31+ T cells represent a functionally distinct vascular T cell phenotype. *Blood Cells Mol Dis.* 2010; 44(2):74–8. [PubMed: 19897387]
44. Ma L, Cheung KC, Kishore M, Nourshargh S, Mauro C, Marelli-Berg FM. CD31 exhibits multiple roles in regulating T lymphocyte trafficking in vivo. *J Immunol.* 2012 Oct 15; 189(8):4104–11. [PubMed: 22966083]
45. Kim SW, Kim H, Cho HJ, Lee JU, Levit R, Yoon YS. Human peripheral blood- derived CD31+ cells have robust angiogenic and vasculogenic properties and are effective for treating ischemic vascular disease. *J Am Coll Cardiol.* 2010 Aug 10; 56(7):593–607. [PubMed: 20688215]

Fig. 1.

QRT-PCR data comparing the relative expression of *Ahr* (A), *Ncr1* (B), and *Ets1* (C) in uterine (u) DBA+ and DBA- uNK cell subsets from CD1 mice. (A) *Ahr* expression by DBA- uNK cells was 638x higher than by DBA+ uNK cells (*p<0.05). (B) *Ncr1* and (C) *Ets1* expression was predominantly by DBA+ uNK cells (**p<0.001). *Ncr1* transcripts were 8x and *Ets1* transcripts were 2x more abundant in DBA+ compared to DBA- uNK cells. Data is represented as mean±SEM. P<0.05 was considered significant.

Fig. 2.

Histological observations made at gd10.5 in B6 and *Ahr*^{-/-} mice. (A) DBA/PAS double stained implant sites from B6 (A1) and *Ahr*^{-/-} (A2) mice. White arrows indicate DBA-PAS + uNK cells. (A3) DBA+ uNK cells in *Ahr*^{-/-} mice are significantly shorter (*p<0.05) than in B6 indicating impaired maturation/activation of DBA+ uNK cells. (B) DBA lectin stained implant sites from B6 (B1) and *Ahr*^{-/-} (B2) mice. DBA+ uNK cells appear brown. (B3) DBA+ uNK cells in *Ahr*^{-/-} mice are significantly shorter (*p<0.05) than in B6 indicating impaired maturation/activation of DBA+ uNK cells. UNK cell data are expressed as mean ±SD. Scale bars represent 20μm (A1, A2, B1, B2). (C) H&E stained implant sites showing spiral arterials (SA) from B6 (C1) and *Ahr*^{-/-} (C2) mice. (C3) Spiral arterial wall to lumen ratio is increased (*p<0.05) in *Ahr*^{-/-} mice compared to B6 indicating impaired spiral arterial remodeling. Spiral arterial data are expressed as mean±SEM. Scale bars represent 50μm (C1, C2). P<0.05 was considered significant.

Fig. 3.

Whole mount staining comparing implant sites from gd6.5 B6 (A–C) and *Ncr1^{Gfp/Gfp}* (D–F) mice. CD31-PE expression is shown in red; GFP-conceptus expression is shown in green. (A,D) CD31-PE superbright expression is visible around the embryonic crypt (*) in B6 controls (A), but is absent in *Ncr1^{Gfp/Gfp}* implant sites (D). (B,E) Trophoblast invasion begins at gd6.5 in both B6 (B) and *Ncr1^{Gfp/Gfp}* (E) mice. (C,F) CD31+ leukocytes (arrows) are abundant in both B6 (C) and *Ncr1^{Gfp/Gfp}* (F) implant sites. (C) Active angiogenesis and tip cell formation is occurring in the mesometrial decidua of B6 mice. (F) *Ncr1^{Gfp/Gfp}* implant sites have thinner mesometrial vessels than B6 suggesting delayed decidual angiogenesis. Scale bars represent 500µm in A,B,D,E and 50µm in C and F.

Fig. 4.

Whole mount staining of gd8.5 implant sites and histological analysis of SA remodeling at gd10.5 and 12.5 in B6 and *Ncr1^{Gfp/Gfp}* mice. CD31-PE expression is shown in red; CD45-FITC expression is shown in green. (A) At gd8.5 in B6 mice, leukocytes increase in size, become activated, and acquire irregular shapes (circled). There is also abundant conjugation between CD31+CD45+ leukocytes (arrows). (C) Cell-cell conjugation is unchanged in *Ncr1^{Gfp/Gfp}* mice and occurs between CD31+CD45+ cells (arrows), but leukocytes are smaller in size and retain their round, inactivated appearance seen at earlier gd. Scale bars represent 100µm. (B) Antimesometrial vasculature in B6 implant sites shows wide vessels with large areas between branch points. (D) *Ncr1^{Gfp/Gfp}* antimesometrial vasculature appears to have a disorganized honeycomb structure with thinner vessels and decreased area between vessels. Scale bars represent 500µm. (E) Spiral arteries in B6 (E1) and *Ncr1^{Gfp/Gfp}* (E2) stained with H&E. The muscular wall of the artery is outlined in yellow. Scale bars represent 50µm. (F) Spiral arterial wall to lumen ratio was increased at gd10.5 and 12.5 in *Ncr1^{Gfp/Gfp}* compared to B6 controls (*p<0.05) indicating an impairment in mid-gestation spiral arterial remodeling in *Ncr1^{Gfp/Gfp}* mice. Data are expressed as mean±SEM. P<0.05 was considered significant.

Table 1

DBA+ and DBA- uNK cell diameters

Gd	C57BL/6	<i>Ncr1^{Gfp/Gfp}</i>	<i>p</i> -values	C57BL/6	<i>Ncr1^{Gfp/Gfp}</i>	<i>p</i> -values
	DBA+ (μm)	DBA+ (μm)		DBA- (μm)	DBA- (μm)	
6.5	12.45	10.60	0.0005	10.22	8.13	<0.0001
8.5	15.11	11.77	<0.0001	11.24	9.29	<0.0001
10.5	13.67	12.31	0.0001	11.51	10.80	0.0041
12.5	18.73	14.94	<0.0001	13.19	11.26	<0.0001