Uterine natural killer cells pace early development of mouse decidua basalis

A.P. Hofmann1, S.A. Gerber2, and B.A. Croy1,*

1Department of Biomedical and Molecular Sciences, Queen’s University, Kingston, ON, Canada K7L3N6 2Department of Microbiology and Immunology, University of Rochester Medical Center, Rochester, NY 14642, USA

*Correspondence address. Tel: +1-613-533-2859; Fax: +1-613-533-2022; E-mail: croya@queensu.ca

Submitted on June 24, 2013; resubmitted on July 31, 2013; accepted on August 22, 2013

ABSTRACT: Pregnancy involves progressive relationship changes between conceptus-derived trophoblasts and maternal decidual vessels and leukocytes. Uterine natural killer (uNK) cells, the dominant leukocytes in early human and mouse decidua, have late gestational cardio-protective roles through mid-gestational initiation of decidual spiral arterial modification. The earlier gestational functions of uNK cells are unknown. Comparisons of gestation days (GD) 6.5–9.5 implant sites from allogeneically mated alymphoid or normal BALB/c mice (Rag2−/−Il2rg−/−; NK-T-B-versus +/+ ) by whole mount immunohistochemistry revealed delays in Rag2−/−Il2rg−/− uterine lumen closure, trophoblast invasion and conceptus development. Also delayed were onset of mesometrial angiogenesis and pruning of neo-vascular networks in decidua basalis. This phenotype was fully reversed in BALB/c-Rag2−/−Il2rg−/− pregnancies that followed adoptive Rag2−/−(NK+B-T-) marrow transfer. These data suggest that uNK cells coordinate GD-appropriate phases of decidual angiogenesis, which in turn paces progressive changes in early implant sites that support normal fetal growth. Similar roles for human CD56bright decidual NK cells could explain the importance of CD56bright decidual NK cell activation to pregnancy success.

Key words: UNK / embryo development / angiogenesis / early pregnancy

Introduction

Blastocyst implantation triggers radical changes to the uterus that sustain pregnancy. In many species, including humans and mice, stromal cells decidualize and recruit maternal immune cells, blood vessels undergo molecular and structural changes and fetal trophoblasts invade as the placenta differentiates. Many human gestational complications arise from abnormal progression of these processes (Germain et al., 2007; Hibi et al., 2008; Wang et al., 2009; Brosens et al., 2011). Decidually recruited uterine natural killer (uNK) cells are the dominant immune cells found in early decidua basalis (Hibi et al., 2008; Croy et al., 2012). uNK cells are essential for initiation of mid-pregnancy spiral arterial remodeling, a process that enlarges and stabilizes the implant site blood supply that comes from mesometrial vessels (Ashkar and Croy, 1999; Hazan et al., 2010). uNK cell functions earlier in pregnancy are poorly defined (Greenwood et al., 2000).

Healthy human pregnancy is genetically linked with activation of uNK cells via killer Ig-like receptors (KIRs) that provide allogeneic recognition of the restricted HLA Class I molecules expressed by trophoblast (Hibi et al., 2008; Sharkey et al., 2008). Co-engraftment of human primary first trimester trophoblast cells and NK cells in immune deficient mice indicated that decidual, but not blood CD56+ NK cells, promote trophoblast invasion and angiogenesis functions supportive of pregnancy (Hanna et al., 2006). In normal mice, uNK cells are rare just after implantation (gestation day (GD) 4.5) but numerous and highly proliferative in mesometrial decidua basalis by GD 6.5 (Peel, 1989; Croy et al., 2012). As in the human, murine uNK cells are activated cells that express interferon gamma (Ashkar et al., 2000), perforin (PRF1) (Parre et al., 1991; Lima et al., 2012), KIR-equivalent LY49 receptors (Yadi et al., 2008; Zhang et al., 2009; Chen et al., 2012), VEGFA (Wang et al., 2000; Lima et al., 2012), placental growth factor (PGF) (Tayade et al., 2007; Chen et al., 2012) and other hydrolytic enzymes and glycoproteins (Croy et al., 2006).

Endometrial stromal cell decidualization (Lee et al., 2007), decidual neoangiogenesis (Matsumoto et al., 2002) and trophoblast invasion (Das et al., 1997) are early post-implantation events (GD 4.5–7.5) associated with murine uNK cell localization to decidua basalis (Croy et al., 2012). If early uNK cells contribute to these processes in vivo, as suggested from our previous ultrastructural studies and from the xenogeneic engrafting studies of others (Hanna et al., 2006), we postulated that early vascular development within decidua basalis would be aberrant in mice lacking lymphocytes (NK−, T−, B−; alymphoid). We further predicted that preconception engraftment of NK+, T−, B− marrow in alymphoid mice would correct the anomalies. Early pregnancy implant sites of mice co-deficient in NK/uNK and T+B cells have been described from paraffin and resin-embedded histological materials (Croy et al., 1997; Guimond et al., 1998; Ashkar et al., 2000; Greenwood et al., 2000; Ashkar et al., 2003). From GD 7.5 onwards, hypomorphic decidual cells, swollen endothelial cells (Croy et al., 2000) and large intercellular areas in decidua basalis (interpreted as edema) were reported. From
GD 10.5, lack of spiral arterial modification was apparent (Guimond et al., 1998). Despite these features, litters of normal size are born apparently due to a series of maternal cardiovascular and placental growth adjustments described elsewhere (Burke et al., 2010; Croy et al., 2011; Zhang et al., 2011). Differences between normal and NK/uNK cell deficient mice at GD 10.5–14.5 were normalized by pre-conception engraftment of marrow from mice genetically deficient in T and B cells using a protocol that sustains fertility and fetal survival (Guimond et al., 1998).

Here we employ the technique of whole mount in situ immunohistochemical staining newly adapted to studies of viable, intact, early decidua to implant sites from alymphoid BALB/c mice (Guimond et al., 1998) and to implant sites from GD-matched syngeneically BALB/c colony and compared with the Jackson Laboratory and SAGE Labs report for BALB/c colony data show an average of 5.13 pups per litter (n = 15). All animal usage was approved by Queen’s University and compliant with guidelines of the Canadian Council on Animal Care for Care and Use of Laboratory Animals.

Histological techniques

Paraffin embedded sections
Implant sites were dissected and placed in 4% paraformaldehyde, 0.1 M sucrose in PBS, pH 7.4 (PFA) for 6 h then transferred to 70% ethanol. Standard automated processing was used to embed the tissue. Sections were cut at 5 μm, mounted onto glass slides, stained by hematoxylin and eosin (H&E) and photographed using AxioVision SE64 Rel. 4.8 (Carl Zeiss, Oberkochen, Germany).

Whole mount staining
The uterus was transected between implant sites. Then, the uterine wall of each site was incised along the anti-mesometrial border using watchmaker’s forceps, and peeled back over the mesometrial side where the wall attached to the decidua. Excess uterine wall tissue was trimmed away leaving a small amount of mesometrial myometrium at the decidual attachment site as a landmark. Decidual capsules and the residual mesometrium were then halved mid-sagittally using a scalpel (Croy et al., 2012), while viewed under a stereomicroscope. Sample tissues were placed into chilled PBS containing 1% bovine serum albumen and 0.1% sodium azide (PBA).

Dissected tissues were incubated in 200 μl PBA in a 5 ml tube, along with 10 μg/ml of Fc blocking antibody (anti-CD16/32; supernatant of clone 2.4G2 ATCC, Manassas, VA, USA), and 5–10 μg/ml of fluorescently conjugated primary antibodies; PE (phycoerythrin) and APC (allophycocyanin) tagged CD31 (EC13.3 #553373; BD Pharmingen, Mississauga, ON, Canada) and CD45 (50-F11 #557235, BD Pharmingen), respectively. Incubating samples were placed on an orbital shaker in 4°C, and agitated at medium intensity for 1 h. Samples were then mounted onto glass slides with PBA and examined and photographed under epifluorescence microscopy using AxioVision SE64 Rel. 4.8. At the time of dissection, unsectioned tissue was examined under epifluorescence microscopy to identify whether the cut had revealed the GFP conceptus and to assess autofluorescence background which was negligible. PE-tagged IgG was used to stain some implant sites as an isotype control. Only indistinct staining was present.

Bone marrow transplants

Rag2−/−Il2rg−/− (n = 6) served as the recipients for tail vein injections of bone marrow from donor (n = 6) Rag2del mice (NK+, T−, B−). Rag2−/−Il2rg−/− mice were primed with 150 mg/kg of 5-FU (5-fluorouracil) 48 h prior to bone marrow injection. Marrow cells were obtained by flushing the femurs and tibias of each donor mouse with sterile PBS. The suspended marrow was centrifuged at 233 g for 10 min and the pellet was resuspended at 107 cells in 0.5 ml. Recipients were given 1% bovine serum albumen and 0.1% sodium azide (PBA).

Recipients were halved mid-sagittally using a scalpel (Croy et al., 2012) but distinctly different from the amount of mesometrial myometrium at the decidual attachment site as a landmark. Decidual capsules and the residual mesometrium were then halved mid-sagittally using a scalpel (Croy et al., 2012), while viewed under a stereomicroscope. Sample tissues were placed into chilled PBS containing 1% bovine serum albumen and 0.1% sodium azide (PBA).

Dissected tissues were incubated in 200 μl PBA in a 5 ml tube, along with 10 μg/ml of Fc blocking antibody (anti-CD16/CD32; supernatant of clone 2.4G2 ATCC, Manassas, VA, USA), and 5–10 μg/ml of fluorescently conjugated primary antibodies; PE (phycoerythrin) and APC (allophycocyanin) tagged CD31 (EC13.3 #553373; BD Pharmingen, Mississauga, ON, Canada) and CD45 (50-F11 #557235, BD Pharmingen), respectively. Incubating samples were placed on an orbital shaker in 4°C, and agitated at medium intensity for 1 h. Samples were then mounted onto glass slides with PBA and examined and photographed under epifluorescence microscopy using AxioVision SE64 Rel. 4.8. At the time of dissection, unsectioned tissue was examined under epifluorescence microscopy to identify whether the cut had revealed the GFP conceptus and to assess autofluorescence background which was negligible. PE-tagged IgG was used to stain some implant sites as an isotype control. Only indistinct staining was present.

Results

Whole mount studies of early BALB/c × B6-Gfp and Rag2−/−Il2rg−/− × B6-Gfp implant sites

To address the early contributions of lymphocytes to trophoblast invasion and to decidual angiogenesis, implant sites from GD 6.5, 8.5 and 9.5 BALB/c and Rag2−/−Il2rg−/− females mated by B6-Gfp males were studied by whole mount immunohistochemistry. GFP expression identified conceptus-derived cells including trophoblasts, PE-anti-CD31 was used to identify endothelium and APC-tagged CD45 to identify leukocytes. Implant sites in allogeneically mated BALB/c controls were similar to those previously reported in GD-matched syngeneically mated B6 females (Croy et al., 2012) but distinctly different from the implant sites in allogeneically mated Rag2−/−Il2rg−/− females, as detailed below.

Materials and Methods

Animals and ethical approval

B6-Tg(UBC-GFP)/30ScJa (B6-Gfp) and BALB/c+/+ (BALB/c) mice were from the Jackson Laboratory, Bar Harbor, ME, the former were bred to homozygosity. BALB/c-Rag2−/−Il2rg−/− (Rag2−/−Il2rg−/− or alymphoid) were bred in house (Queen’s University). C.12956 (B6)-Rag2−tm1Fwa->N12 (Rag2del) mice were purchased from Taconic, Germantown, NY. GD-matched BALB/c control and Rag2−/−Il2rg−/− females mated by B6-Gfp males were examined on GD 6.5, 8.5 and 9.5 using four pregnancies with three to five implant sites examined per gestational day. Mice were euthanized by cervical dislocation and uteri were removed and dissected as described below. Breeding data were collected for our Rag2−/−Il2rg−/− colony and compared with the Jackson Laboratory and SAGE Labs report for BALB/c+/+ litter size; between 5 and 7 pups, an intermediate level of breeding performance that was confirmed as 5.1 pups per litter by others (Nagasawa et al., 1973). Our Rag2−/−Il2rg−/− colony data show an average of 5.13 pups per litter (n = 15). All animal usage was covered by animal utilization protocols approved by Queen’s University and compliant with guidelines of the Canadian Council on Animal Care for Care and Use of Laboratory Animals.

Paraffin embedded sections

Implant sites were dissected and placed in 4% paraformaldehyde, 0.1 M sucrose in PBS, pH 7.4 (PFA) for 6 h then transferred to 70% ethanol. Standard automated processing was used to embed the tissue. Sections were cut at 5 μm, mounted onto glass slides, stained by hematoxylin and eosin (H&E) and photographed using AxioVision SE64 Rel. 4.8 (Carl Zeiss, Oberkochen, Germany).

Whole mount staining

The uterus was transected between implant sites. Then, the uterine wall of each site was incised along the anti-mesometrial border using watchmaker’s forceps, and peeled back over the mesometrial side where the wall attached to the decidua. Excess uterine wall tissue was trimmed away leaving a small amount of mesometrial myometrium at the decidual attachment site as a landmark. Decidual capsules and the residual mesometrium were then halved mid-sagittally using a scalpel (Croy et al., 2012), while viewed under a stereomicroscope. Sample tissues were placed into chilled PBS containing 1% bovine serum albumen and 0.1% sodium azide (PBA).
Gestation day 6.5
At GD 6.5, BALB/c implant sites contained cylindrical, green-fluorescing, primitive streak embryos with individual, round trophoblast cells (arrow in Fig. 1A) that appeared to have advanced mesosomally from the ectoplacental cone (Fig. 1A). These embryos were estimated as Theiler stage (TS9) (Kaufmann, 1992). Vessels in the central decidua basalis were narrow, highly branched and more abundant than in other decidual regions. These vessels were associated with a dense infiltrate of CD45+ cells (Fig. 1B above the yellow line). The vessels more proximal to myometrium had a webbed vascular appearance noted particularly at vessel branch points (arrows in Fig. 2A). Lateral and anti-mesometrial decidua had lower vessel abundance and were relatively deficient in CD45+ leukocytes (Fig. 1B below the yellow line). Leukocytes of normal GD 6.5 decidua basalis were cells of mixed sizes and were predominantly round. The larger leukocytes occasionally occurred as conjugated pairs. No conjugates were present between CD45+ cells. Many of the larger leukocytes co-expressed CD31+ (Fig. 2A, small circles not integrated into vessels, shown on PE channel to illustrate CD31 expression but co-stained with anti-CD45-APC to identify leukocytes and viewed to estimate CD45+CD31+ cell co-staining at ~50% (not shown)). Leukocytes in the other decidual regions were smaller CD45+ cells that never expressed CD31 (Fig. 1B). These smaller cells were most noticeable anti-mesometrially but were present in all regions of decidua, including decidua basalis where their presence was usually obscured by the abundance of larger CD45+ cells.

Rag2−/−Il2rg−/− implant sites contained conceptuses that were developmentally delayed. The implanted Rag2−/−Il2rg−/− embryos were smaller than controls and resembled GD 5.5 pre-primitive streak (TS7) embryos (Kaufmann, 1992) of normal mice in size and shape. Importantly, no trophoblast migration was present from the ectoplacental cone or from any other region of the conceptus (Fig. 1C). Smaller CD45+ cells, which can now be described as non-lymphoid, were present mesometrially and anti-mesometrially but no enrichment of CD45+ cells was present in decidua basalis (Fig. 1D above the yellow line), suggesting that lymphocytes are the only CD45+ cells restricted to mesometrial decidua basalis. These alterations did not appear to alter the onset of angiogenesis in the central decidua basalis. Vessels in this region had indistinct vessel edges (hazy appearance) as shown in Fig. 2B. This is characteristic of induction of endothelial tip cells, which is reported to occur on GD 5.5 in normal B6 implant sites (Croy et al., 2012). These vessels were also poorly defined suggesting a deficit in pruning and vessel maturation compared with the same decidual region in the controls (compare Fig. 2B with A). CD31 labeled vascular cells but not leukocytes (i.e. myeloid cells) in Rag2−/−Il2rg−/− implant sites (Fig. 2B, shown on PE channel to illustrate CD31 expression but co-stained with anti-CD45-APC that was viewed to confirm numerous leukocytes were present (not shown)).

Gestation day 8.5
GD 8.5 BALB/c implant sites contained well-developed, globular-shaped, GD-appropriate conceptuses estimated as TS11d (Kaufmann, 1992) (Fig. 3A). Of note was the formation and migration of the allantoic bud (AB) within the conceptus, which is an essential step in the formation of the placenta. Trophoblasts had migrated radially and, in correlation, anti-mesometrial decidua had regressed (Fig. 3A). Residual uterine lumens (RL) appeared as small, narrow slits in the mesometrial-anti-mesometrial plane (Fig. 3B). Vessels in the leukocyte-enriched decidua basalis were now indistinct. The vessels of the central decidua basalis that had been narrow and sharply defined at GD 6.5 were less defined and wider suggesting intense remodeling (Fig. 3B and C). Lateral-, large vessels had become apparent and arched along the decidual margins from the myometrium—decidua boundary to the edge of the invasive trophoblasts (Fig. 3B arrows). A distinct boundary continued to occur between CD45+ lymphocyte-associated vessels and vessels of lateral decidua that were relatively deficient in CD45+ cells. A large proportion of CD45+ cells continued to express CD31 (Fig. 3C). Conjugated CD45+ large cell pairs were frequent (indicated by asterisk in Fig. 3C); trophoblast cells continued to be excluded from CD45+ cell conjugates. Many CD45+ cells had acquired irregular shape (plus sign in Fig. 3C) suggestive of activation and mobilization (Hauser et al., 2007).

GD 8.5 Rag2−/−Il2rg−/− implant sites continued to appear ~24 h delayed. Embryos had not assumed a full sized globular shape (Fig. 3D), lacked allantoic growth and appeared to be in a transition phase from the normal GD 7.5 shape to the shape seen in controls at GD 8.5. They were estimated to be at TS10c (Kaufmann, 1992). Rag2−/−Il2rg−/− anti-mesometrial decidua was thicker and more vascular than in controls, suggesting that anti-mesometrial decidua had not regressed and thinned appropriately. This may be due to the smaller size of the amniotic cavity and limited expansion of trophoblast. A broad, apparently open RL (Fig. 3) persisted (compare Fig. 3E and F with B) and vessels of decidua basalis lacked the intense angiogenic remodeling seen in control sites. The Rag2−/−Il2rg−/− vessels maintained a narrower, more, sharply defined morphology similar to the unmodified vessels seen in the control uterus at GD 6.5 and 7.5. The large, arched lateral decidual vessels had not developed (Fig. 3E). Small, CD45+ cells were located in all regions of decidua. These non-lymphoid cells continued to lack CD31 expression, remained round and were sometimes seen in pairs (Fig. 3F and 3Fi).

Gestation day 9.5
At GD 9.5, BALB/c implant sites maintained a normal growth trajectory. The fetus and implantation chamber were now relatively large compared with the overall size of the implant site (not shown). Fetal head fold, body and tail regions were clearly identifiable as were forelimb buds and the developing heart, placing the fetus at TS14 (Kaufmann, 1992) (Fig. 4A). The organization and orientation of decidual vessels towards the maternal—fetal interface persisted mesometrially and CD45+ cells expressing CD31 were abundant (Fig. 4B).

At GD 9.5, Rag2−/−Il2rg−/− implant sites continued to show some delay and embryos appeared to be at TS13 (Kaufmann, 1992). Head fold, body and tail regions were identifiable but forelimb buds were not present and the heart was not distinct. Angiogenesis in decidua basalis resembled that in control mice prior to the extensive remodeling seen at GD 8.5. No development of large lateral vessels was yet present in alymphoid implant sites (Fig. 4D versus B). The absence of CD31+ reactivity on CD45+ leukocytes remained a distinct difference from controls (Fig. 4D).

Paraffin-embedded histology
Because delayed uterine lumen closure and delayed conceptus development had not been recognized in our previous studies of uNK cell deficient mice, routine paraffin-embedded sections from GD 8.5 BALB/c...
control and alymphoid females mated by B-Gfp males were studied (Fig. 5). The size, shape and developmental features seen in semi-allogeneic embryos gestating in BALB/c dams (Fig. 5A) were similar to those reported for normal development of embryos within C57BL females that had been mated to CBA males (Kaufmann, 1992), and differed from those of semi-allogeneic, immune competent embryos gestating in alymphoid dams (Fig. 5D). The latter appeared to be developmentally normal but with a delay of ≏24 h (at TS10c). In comparison with controls, the implant sites in alymphoid mice had a larger RL (Fig. 5E compared with 5B), wider vessels in the central mesometrial decidua basalis (arrows in Fig. 5E compared with 5B) and a less cellular decidua basalis. In alymphoid implant sites, the central decidua basalis contained arterioles, which were larger and less frequent (arrows in Fig. 5F) than in control decidua (arrows in Fig. 5C), suggesting a lack of fine vessel branching. Closer inspection of central decidual arterioles revealed swollen endothelial cells suggesting a compromised vascular lining (Fig. 5Fi) compared with the continuous endothelial cell lining in control decidual arterioles (Fig. 5Ci). The large arching vessels seen in control decidua basalis are shown with an arrow head in Fig. 5B but were absent from histological sections of alymphoid implant sites (Fig. 5E). These observations are consistent with and support the conclusions made from the whole mount studies.

Implant sites in B6-Gfp mated BALB/c-Rag2-/- Il2rg-/- recipients of Rag2del marrow

The abnormal features of the early implant sites in alymphoid mice could arise from deficits in functions of T, B and/or NK cells or from deficits in interactions between lymphocyte subsets. To assess which of the anomalous features could be attributed to uNK cell functions, whole mount analysis of implantation sites from Rag2-/- Il2rg-/- that had been reconstituted 3 weeks earlier with Rag2del (NK+T-B-) marrow was undertaken at GD 6.5 and 8.5. On both GD, large CD45+ cells were present, enriched and prominent in the decidua basalis of normal mice. Modified diagrams (Kaufmann, 1992) are for orientation of embryonic features on the gestational time points discussed and are not drawn to scale. Labels are as follows: EE, embryonic ectoderm; EP, ectoplacental cone; ExE, extra-embryonic ectoderm; PC, proamniotic cavity; T, trophoblast cells. Size bars represent 150 μm in (A–D).

Figure 1 Whole mount staining comparing implant sites at GD 6.5 in control BALB/c (A and B) with Rag2-/- il2rg-/- (C and D). Anti-CD31-PE reactivity is shown in red; anti-CD45-APC reactivity and conceptus-associated GFP expression are shown in distinct shades of green. The normal GD 6.5 implant site (A) has an embryo of cylindrical shape showing the initiation of trophoblast invasion (arrow) from the ectoplacental cone. (B) An upper region (‘Meso’ for Mesometrial region, ‘Lat’ for Lateral region) of leukocyte enrichment (boundary delineated by the yellow line) made up of larger CD45+ cells. This enrichment is restricted to decidua basalis. Smaller leukocytes are diffusely located throughout the decidua. Alymphoid implantation sites differed (C) The GD-matched implant site in an alymphoid mouse uterus. Here the embryo, which is genetically Rag2+/- Il2rg+/- and not immune deficient, is smaller than its GD-matched control and typical of a GD 5.5 embryo in a normal mouse. No trophoblast invasion is apparent. Leukocytes are not enriched mesometrially (above yellow line in D) suggesting only lymphocytes enrich in decidua basalis of normal mice. Modified diagrams (Kaufmann, 1992) are for orientation of embryonic features on the gestational time points discussed and are not drawn to scale. Labels are as follows: EE, embryonic ectoderm; EP, ectoplacental cone; ExE, extra-embryonic ectoderm; PC, proamniotic cavity; T, trophoblast cells. Size bars represent 150 μm in (A–D).
6.5 conceptuses (arrow in Fig. 6A). Vessels of the decidua basalis were similar to those of GD-matched BALB/c sites; that is, highly branched and sharply defined. Localization and enrichment of the larger CD45+ cells to decidua basalis was restored (Fig. 6B) and CD31 co-expression by these cells was prominent (Fig. 6Bi). At GD 8.5 (Fig. 6C, D and Di), implant sites in graft recipients contained GD-appropriate embryos (Fig. 6C) and displayed other expected normal features. The RL was small, large arching vessels had developed in lateral decidua basalis (Fig. 6D arrows) and vessels of the central, leukocyte-enriched decidua basalis had acquired a wide, undefined-edge morphology suggestive of angiogenesis and remodeling, as in GD-matched BALB/c (Fig. 6D). Many leukocytes co-expressed CD31. Leukocyte–leukocyte conjugates and irregularly shaped CD45+ cells were prominent (Fig. 6Di), suggestive of GD-appropriate onset of decidual leukocyte activation in the engrafted mice.

Discussion

Comparisons of intact, viable, pre-placental implantation sites from NK+ T+B+ (normal control), NK− T− B− and NK+ T− B− pregnancies by whole mount in situ immunohistochemistry support major roles for uNK cells in promotion of early decidual angiogenesis and in trophoblast invasion. The study approach revealed new anomalies in NK− T− B− implant sites that were normalized when NK/uNK cells were replaced, with delayed conceptus development of key importance. Attribution of this finding as primary or secondary to the absence of uNK cells is complicated by the novel observation of delayed uterine lumen closure. Because of this and the normal location of uNK cells on the mesometrial side of the RL, we suggest that the earliest actions of uNK cells are their angiogenesis-promoting effects on mesometrial maternal vessels at implant sites. Our observations would be consistent with the hypothesis that uNK cell-promoted neoangiogenesis enlarges mesometrial tissue and pushes it adlumenally to cause uterine lumen closure. Because of this and the normal location of uNK cells on the mesometrial side of the RL, we suggest that the earliest actions of uNK cells are their angiogenesis-promoting effects on mesometrial maternal vessels at implant sites. Our observations would be consistent with the hypothesis that uNK cell-promoted neoangiogenesis enlarges mesometrial tissue and pushes it adlumenally to cause uterine lumen closure.

Implantation site viability during early pregnancy

Between GD 6.5 and 8.5 viable Rag2−/− Il2rg−/− implant sites dropped from 9.4 ± 0.821 (n = 5 litters) to 7.2 ± 1.16 (n = 5 litters), with resorptions more common at GD 8.5 than in BALB/c +/+. In contrast, BALB/c +/+ had 10.7 ± 0.747 (n = 7 litters) viable implant sites on GD 6.5 and 10.3 ± 0.615 (n = 6 litters) on GD 8.5. At GD 8.5, the number of implant sites in Rag2−/− Il2rg−/− were significantly lower (P = 0.0331 < 0.05) than in BALB/c +/+ but there was no difference at term, suggesting loss in BALB/c +/+ but there was no difference at term, suggesting loss in BALB/c litters occurs after GD 8.5.
In the GD 6.5 NK−T−B− decidua basalis, widespread angiogenesis was present, but mature vessels were undefined. This appearance resembled that reported in B6 decidua (Croy et al., 2012) and seen in BALB/c at GD 5.5 (not shown) and suggested that a key action of early uNK cells is the promotion of vessel maturation following endothelial tip cell induction. This process would include pruning of nascent vessels to a mature functional state.

At GD 8.5, implant sites of the controls and of the NK cell engrafted recipients showed drastic vascular remodeling in decidua basalis, whereas vessels in NK−T−B− decidua basalis were thin and resembled controls at GD 6.5. This suggests that uNK-based mechanisms not only contribute to angiogenic factors such as VEGF (Matsumoto et al., 2002), PGF (Tayade et al., 2007) and delta-like ligand (DLL)1 (Degaki et al., 2012) but also to cytotoxic molecules that participate in neovessel

Figure 3 Whole mount staining at GD 8.5 visualizing the differences seen in implantation sites between control (A–C) and alymphoid (D–F) mice. In normal uteri (A), embryos have a globular shape typical for GD 8.5. This coincides with a rounded embryonic crypt (EC) and receding decidua due to the lateral and anti-mesometrial (AM) advance of trophoblast cells. (B) The morphological changes of mesometrial blood vessels that have occurred at this time point. Large, wide, arching vessels are now present in the lateral decidua basalis (arrows in B), whereas the vessels in the central region have become less defined. The RL appears as a slit in the central decidua basalis. Expression of CD31 continues on a large number of leukocytes in decidua basalis (C) within the region of blood vessel remodeling. Insert panel (Ci) shows the leukocyte conjugates (asterisk) and the activated irregular shape of leukocytes (plus sign), common at GD 8.5. In comparison with (A), implantation sites in alymphoid mice show an implantation crypt that remains more cylindrical. Embryos are smaller and still transitioning towards the globular phase and the anti-mesometrial decidua is less receded (D) indicating a delay in development. (E) The absence of the large lateral vessels in the decidua basalis as well as unmodified vessels in the central region. A larger RL persists. (F) The absence of leukocyte CD31 expression in Rag2−/−Il2rg−/− decidua basalis. The smaller, non-lymphoid cells remain distributed throughout the decidua basalis (insert panel Fi) and none co-express CD31. Modified diagrams (Kaufmann, 1992) are for orientation of embryonic features on the gestational time points discussed and are not drawn to scale. Labels are as follows: AB, allantoic bud; AC, amniotic cavity; EE, embryonic ectoderm; EPC, ectoplacental cavity; ExEC, extra-embryonic cavity; FG, foregut invagination. Size bars represent 200 μm (A, B, D, E), 150 μm in (C and F) and 50 μm in both insert images.
pruning and remodeling. A review of our previously published histologic-
al figures comparing GD 10–14 decidua basalis in uNK cell deficient and
control mice, and of the paraffin-embedded specimens used to prepare
Fig. 5 also showed that the development of fine vessels was markedly
reduced in the decidua basalis of uNK deficient mice. Thus, the large
vacant areas between decidual stromal cells previously interpreted as
edema from histological sections of NK/uNK cell deficient mice and
defined as GD 8.5 in Fig. 5F are unpruned, neovascular structures.

Because of the delay in uterine lumen closure in implant sites lacking
lymphocytes, the cause of delayed trophoblast migration in GD 6.5
NK-T−B− pregnancies is unclear. The absence of trophoblast out-
growth is compatible with the stage of development and size of the
embryos within alymphoid implant sites, which appeared to be at GD
5.5 (TS 7) rather than at GD 6.5 (TS 9). These are uterine effects
because the conceptus genotypes were identical. Since the same findings
were made in multiple implant sites from four GD 6.5 pregnancies and
delay persisted in the four later pregnancies examined, potential errors
in mating dates cannot explain these findings and an explanation for
the delay in conceptus development must be found. It may have a nutri-
tional explanation since maternal vascular supply to the conceptus would
be more abundant if lumen closure had been further advanced. Arterial
supply vessels would also have been more abundant had the initiation of
decidual angiogenesis been appropriately timed and vessel matur-
ation and pruning been effectively completed. It remains possible that
uNK cell-derived molecules promote conceptus growth and trophoblast
chemotaxis for normal embryonic development as postulated from

Figure 4  Comparison of implant sites in control (A and B) versus Rag2−/−Il2rg−/− (C and D) at GD 9.5. Both embryos have similar features of head,
trunk and tail sections. However, embryos in the normal uterus (A) are larger than embryos in the alymphoid uterus (C) (comparing size bars and magn-
ification, (A) is at 25 × and (C) at 50 × magnification). (B) The continued expression of CD31 on leukocytes in the normal decidua basalis and (D) the
absence of these cells from alymphoid decidua basalis. Blood vessel morphology also differs; vessels are wider and less branched in control decidua
basalis (B) versus thinner and sharply defined in alymphoid decidua basalis (D). Modified diagrams (Kaufmann, 1992) are for orientation of embryonic fea-
tures on the gestational time points discussed and are not drawn to scale. Labels are as follows: B, body; FB, forelimb buds; H, head; PH, primitive heart;
T, tail. Size bars represent 200 µm (A), 150 µm (C) and 100 µm (B, D).
human tissue xenografting studies (Hanna et al., 2006). However, this conclusion cannot be drawn from our study because of the developmental and anatomical changes identified. Thus, our study reveals a much more complex behavioral relationship between uNK cells and trophoblasts. This is reinforced by failure to observe conjugation between leukocytes and trophoblast cells between implantation and mid-pregnancy. Intrauterine growth restriction (IUGR) of the GD 8.5 conceptuses within NK− T− B− implant sites due to deficient maternal vascular supply likely also accounts for the delay in regression of the anti-mesometrial decidua.

**Figure 5** Hematoxylin and eosin histology of BALB/c (A, B, C and Ci) and Rag2−/−Il2rg−/− (D, E, F and Fi) implantation sites at GD 8.5. (A) and (D) The shape and anatomical features of a control embryo and an embryo within an alymphoid implantation site, respectively. Developmental delay in growth can be seen when comparing the embryo in (D) to (A). Importantly, the AB has developed and begun migrating towards the chorion in the control embryo (A), an essential step in the formation of the placenta. This has not yet happened in (D). Shape, size and anatomical structures differ. (B) and (E) The mesometrial decidua basalis of control and alymphoid implantation sites, respectively. Major vessels of the central decidua basalis are indicated with arrows. A notable size difference is present when comparing these vessels in alymphoid implant sites (E) with those seen in controls (B). The RL is larger in alymphoid implant sites (E) than in control implant sites (B). (C) The size and number of arterioles (arrows) within control central decidua basalis. (Ci) is a high magnification of a control implant site arteriole in the central decidua basalis. The endothelium is thin and continuous (arrow). In contrast, (F) The larger and less numerous arterioles present in the central decidua basalis of alymphoid implant sites. (Fi) is a high magnification of one of these arterioles which shows the swollen and discontinuous endothelium (arrow) characteristic of alymphoid implant site arterioles. Size bars represent 150 μm (B and E) and 100 μm (A and D), 50 μm (C and F) and 20 μm (Ci and Fi).
Human placental size, health and functionality are strongly correlated with size, health and post-natal development of offspring (Scifres and Nelson, 2009; Alwasel et al., 2012). Human IUGR is usually associated with a smaller and/or dysfunctional placenta leading to fetal growth deficiency (Proctor et al., 2009) and with increased perinatal morbidity and mortality. Induction of IUGR-like symptoms in animal models has been valuable for deciphering underlying causes of human IUGR. Here we present the BALB/c-\textit{Rag2}\textsuperscript{−/−}\textit{Il2rg}\textsuperscript{−/−} mouse as a model of naturally occurring (without surgical or dietary interventions) early onset IUGR. The very early delays in decidual vascularization, uterine lumen closure and in placental and fetal development in this mouse may offer insight into some of the initial causes of human IUGR and of early spontaneous pregnancy loss.

BALB/c +/+ and \textit{Rag2}\textsuperscript{−/−}\textit{Il2rg}\textsuperscript{−/−} mice bear litters of similar size. However, ante-partum data collected in this small study suggest that implant site viability may drop earlier in \textit{Rag2}\textsuperscript{−/−}\textit{Il2rg}\textsuperscript{−/−} than in BALB/c +/+. In earlier studies, BALB/c-\textit{Rag2}\textsuperscript{−/−}\textit{Il2rg}\textsuperscript{−/−} placentas were shown to have a different growth trajectory than control placentas after GD 14.5. While size of the control placentas plateaued during later gestation, \textit{Rag2}\textsuperscript{−/−}\textit{Il2rg}\textsuperscript{−/−} placentae, which were significantly smaller than control placentae up to GD 16.5, continued to grow to term and were larger than those of the congenic controls at birth (Croy et al., 2011). By 10 weeks after birth, the \textit{Rag2}\textsuperscript{−/−}\textit{Il2rg}\textsuperscript{−/−} offspring had become heavier than their age-matched controls (Croy et al., 2011) and this statistical difference was maintained to study conclusion at Week 16. These data suggest that the earlier reduction in litter size in alymphoid mice may shunt rare nutritional resources to the remaining implant sites. Then, upon completion of placental development, less inter-litter competition is present for the enlarged nutrient pool and nutrients are able to sustain late placental growth. In contrast, in control litters, resources are more abundant before placental development and resorptions due to excessive conceptus demands occur.

**Figure 6** Whole mount analysis of implantation sites in NK cell reconstituted \textit{Rag2}\textsuperscript{−/−}\textit{Il2rg}\textsuperscript{−/−} at GD 6.5 (A, B, Bi) and GD 8.5 (C, D, Di). GD-appropriate embryonic developmental stages and trophoblast invasion (arrow in A) were restored (A, cylindrical; C, globular). Enrichment in CD45\textsuperscript{+} was present in decidua basalis (B) as was leukocyte expression of CD31 (B). The yellow line delineates the boundary of lymphocyte enrichment in the decidua basalis. Vessels of decidua basalis at GD 6.5 had a pruned, highly branched morphology (B and Bi) similar to control GD 6.5 implantation sites. (C) The large, globular heterozygous embryo and round embryonic crypt characteristic of normal GD 8.5 development. This crypt shape and embryo size were not present in embryos of the same genotype gestating in non-reconstituted females (compare with Fig. 2D). Vessel remodeling in the decidua basalis has resulted in the development of large lateral blood vessels (arrows in D) and the undefined morphology of vessels in the central decidua basalis. Large leukocyte numbers were restored and cells were forming conjugates (*) and were visibly active (+) (Di). Size bars represent 200 μm (C and D), 150 μm (A and B) and 50 μm (Bi and Di).
once rapid fetal growth is initiated following onset of placental function. However, studies of much larger numbers of mouse pregnancies are necessary to establish if reductions in litter size occur consistently in both strains, with different time courses and by what mechanisms.

Human and murine uNK cells are closely related in their developmental control and in their functional activities. The importance of the current study has been to define early functions of uNK cells following their implant site recruitment. This is a time interval largely refractory to experimental study during human pregnancy. The findings of uNK cell-based enhancement of early decidual angiogenic processes that are linked to appropriate timing of uterine lumen closure, and to conceptions development and growth, including onset of trophoblast invasion, are likely relevant to human pregnancies. They are processes that if impaired or inhibited could lead to the human reproductive complications that have been genetically linked with inhibition of uNK cell activation (Hilby et al., 2010).

Acknowledgements

We thank Dr Shawn P. Murphy, University of Rochester for helpful discussions and Dr Zhilin Chen and Ms Kimberly Laverty, Queen’s University for technical assistance.

Authors’ roles

A.P.H. performed the experimental procedures, image capture and analysis, and wrote and edited the manuscript. S.A.G. contributed to design of the experiments, assisted in image analysis and edited the manuscript. B.A.C. designed the experiment, assisted in image analysis and contributed to writing and editing of the manuscript.

Funding

Supported by awards from NSERC (RGPIN3219), CFI, and the Canada Research Chairs Program (to B.A.C.).

Conflict of interest

None declared.

References


Zhang JH, Yamada AT, Croy BA. DBA-lectin reactivity defines natural killer cells that have homed to mouse decidua. Placenta 2009;30:968–973.