Uterine natural killer cells: insights into their cellular and molecular biology from mouse modelling

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In primates, including women, and in rodents, natural killer lymphocytes (NK cells) have a unique relationship with the decidualizing uterus. Implantation sites from genetically modified and transplanted mice have proven useful models for understanding potential mechanisms involved in the recruitment, activation and functions of human CD56^{bright} uterine (u)NK cells. Key findings are reviewed in this article. In mice, uNK precursor cells are recruited from secondary lymphoid tissues and are activated coincident with their uterine arrival. uNK cells proliferate, produce cytokines (interferon gamma (IFN- γ) and interleukin 18 (IL-18) and IL-27), and terminally differentiate into granulated lymphocytes. Many uNK cells proliferate within the myometrium at each implantation site forming a structure, the mesometrial lymphoid aggregate of pregnancy (MLAp) that surrounds blood vessels servicing each placenta. Post-mitotic uNK cells are abundant within decidua basalis; frequently (> 25%) associating with spiral arteries, intramurally and intraluminally. From mid-gestation, numbers of uNK cells decline. Studies of implantation sites in mice lacking uNK cells, IFN- γ , components of IFN- γ -induction and -signalling pathways or IFN- γ -regulated genes indicate that uNK cell-derived IFN- γ is essential in triggering pregnancy-induced spiral artery modification. Decidual maintenance and uNK cell death are additional effects of uNK cell-derived IFN- γ . Thus, during the first half of gestation, uNK cells contribute to and sustain important changes in the maternal placental bed.

During uterine decidualization in women, non-human primates and rodents, lymphocytes of the natural killer (NK) lineage appear and become abundant as large, granulated cells (Fig. 1; Parr *et al.*, 1987; Peel, 1989; Moffett-King, 2002). NK cells differ from T and B lymphocytes because they lack somatically rearranged antigen-sensing receptors (Natarajan *et al.*, 2002). NK

cells contribute to innate immunity, participating in early immune protection, before clonal expansion of B and T lymphocytes. Functions of NK cells are lysis and cytokine production, with individual cells having single or dual capacity. Lysis is directed against virally infected cells and tumour cells. Interferon-gamma (IFN- γ), which restricts viral infection, is a major cytokine product (Trinchieri, 1995). Uterine (u)NK cells are predominantly activated, cytokine-producing NK cells (Moffett-King, 2002). Fifteen years lapsed between NK cell identification in marrow, blood and spleen and recognition of endometrial granulocytes (human designation) and

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Fig. 1. (a,c,e) Photomicrographs and (b,d,f) line drawings of the (a,b) virgin, (c,d) day 6 of gestation and (e,f) day 10 of gestation mouse uteri. In all images, the uterine artery access through the broad ligament (mesometrium) is at the top. Implantation triggers transformation of endometrial stroma into decidua. This process begins anti-mesometrially and progresses mesometrially (compare (a) with (c)), resulting in formation of the decidua basalis (DB) and loss of the uterine lumen. Uterine natural killer (uNK) cells are first found in the DB. As additional structures differentiate, uNK cells also localize between the layers of the uterine wall, in a transient structure called the mesometrial lymphoid aggregate of pregnancy (MLAp), marked with an arrowhead in (e), and are found at lower frequency within the placenta (P). (a) and (e) Stained with haematoxylin and eosin; (c) stained with the lectin *Dolichos biflorus* agglutinin (DBA) and haematoxylin. uNK cell granules are distinctive in sections stained with periodic acid–Schiff (PAS) reagent or DBA lectin (Peel, 1989; Paffaro *et al.*, 2003). DBA lectin also stains uNK cell plasma membranes but PAS does not (Paffaro *et al.*, 2003). uNK cells are somewhat difficult to recognize by haematoxylin and eosin staining as their granules, which are histochemically eosinophilic, are relatively indistinct. Virgin uterus is DBA lectin-negative. Scale bars represent (a) 400 μm and (c,e) 200 μm .

granulated metrial gland cells (murine designation) as NK cells.

uNK cells share many, but not all, of their features with peripheral NK cells. For example in humans, most blood NK cells analysed by flow cytometry express the surface marker CD16, an immunoglobulin domain receptor, and have dim expression of CD56, an adhesion molecule. About 1% of circulating lymphocytes are CD16⁺, CD56^{bright} NK cells and these co-express high amounts of the vascular addressin L-selectin (Campbell *et al.*, 2001). In contrast, most human uNK cells express CD56 brightly but lack CD16 and L-selectin (Searle *et al.*, 1999). Lymphotoxin β receptor (LT β R) is a murine example. LT β R is essential for peripheral NK cell differentiation and secondary lymphoid tissue formation (Fu and Chaplin, 1999). However, uNK cells differentiate and an architecturally correct mesometrial lymphoid aggregate of pregnancy (MLAp) forms in pregnant LT β R^{0/0} mice (Kather *et al.*, 2003). Such findings limit the usefulness of studying NK cells not isolated from or functioning within the uterus and make the unique features of

uNK cell recruitment, activation and differentiation key questions. It is of critical importance to recognize that these processes occur without pregnancy or fetal trophoblast tissue. In women, uNK cells differentiate in every menstrual cycle, 3–5 days after the LH surge (Bulmer *et al.*, 1987; King, 2000). In rodents, induction of artificial decidualomata induces fully mature uNK cells (Peel, 1989).

The question of uNK cell function during pregnancy is also of tremendous interest. The cells are highly mobile and their high content of lytic molecules plus partnering antigen receptor display makes them potentially dangerous to trophoblast cells in implanting primate blastocysts and in developing placentae (primates and rodents). uNK cells could play essential physiological roles limiting normal trophoblast invasion or could destroy trophoblast, leading to pregnancy loss. Full definition of uNK cell antigen recognition on trophoblast and the intracellular signalling involved in uNK cell activation, differentiation and senescence would resolve such functional debates and provide clinically relevant

Table 1. Summary of histological findings in uterine natural killer (uNK) cell-deficient mice with or without bone marrow transplantation

Strain		Manipulations	Histological findings			
Phenotype	Name		uNK	DB	SA	MLAp
Normal	C57Bl/6J 129J Balb/cJ CD1	—	+	Cellular	Modified	Present
T ± B deficient	Nude SCID	—	+	Cellular	Modified	Present
NK/uNK, T ± B deficient	IL-2/15Rβ ^{o/o} IL-2/15Rγ ^{o/o} (γc) IL-15 ^{o/o} Tg ε 26 RAG-2 ^{o/o} /γc ^{o/o}	—	—	Hypocellular	Unmodified	Absent
Bone marrow graft into uNK deficient	RAG-2 ^{o/o} /γc ^{o/o}	+ SCID BM + C57Bl/6J BM + IL-15 ^{o/o} BM + βERKO BM + αERKO BM	+	Cellular	Modified	Present

BM: bone marrow; DB: decidua basalis; ERKO: oestrogen receptor knockout mouse; MLAp: mesometrial lymphoid aggregate of mouse pregnancy; RAG-2^{o/o}/γc^{o/o}: double knockout mouse for recombinaase activating gene-2/common cytokine chain gamma; SA: spiral artery of the decidua basalis; SCID: severe combined immunodeficient.

information. Mice have proven excellent models for characterization of uNK cell biology by allowing manipulative studies that are not possible in pregnant women (Table 1). Strains of genetically mutant mice are available to address uNK cell regulation and studies are relatively short in duration (mouse pregnancy is 19–20 days). Furthermore, murine pregnancies can be interrupted and entire implantation sites studied. Inbred strains permit investigations of replicate pregnancies in time-course analyses that precisely define the changing cell and tissue relationships during gestation (Fig. 1). Murine uNK cell analyses are usually histological or molecular because no successful culture conditions or long-term cell lines are known. In the present study, at least six implantation sites derived from two or three pregnant females on at least two different days of gestation were serially sectioned. From each implant site eleven central sections were selected for computer-based image analysis in a manner ensuring that no duplicate counting of individual cells occurred. Control pregnancies were matched by genetic background as closely as possible, being sibling matings or matings of a congenic inbred partner strain matched for day of gestation. Study of human uNK cells occurs at more restricted times, largely those associated with biopsy or elective termination, and is genetically heterogeneous. In addition to histopathology, flow cytometry is widely used to study lymphocytes dissociated from human specimens and primary cloning has been achieved (Christmas *et al.*, 1990). Application of expression microarrays and laser capture microdissection combined with quantitative analyses will provide information unique to human uNK

cells and their environment, an environment distinct from that of the rodent anatomically, endocrinologically and in duration of gestation. The mouse will remain a key tool in critical assessment of new data from women because this model can move studies from correlative findings to genetically defined *in vivo* gestational data with potential for experimental interventions. uNK cells are infrequent or absent at term in both mice and women (Delgado *et al.*, 1996; King, 2000), making term tissue irrelevant for defining uNK cell functions.

NK cell differentiation and establishing the uNK cell lineage

Origins

NK cells are generated from pluripotent bone marrow stem cells. Their differentiation in mice is highly dependent upon stromal factors including stem cell factor, interleukin 7 (IL-7) and IL-15 (Kennedy *et al.*, 2000; Rosmaraki *et al.*, 2001). Lineage-committed NK-precursor cells have limited self-renewal ability and are present in secondary lymphoid tissues (blood, spleen, lymph nodes (LN)) but not the uterus (Chantakru *et al.*, 2002 and references therein). Transplants from NK cell-sufficient mice to NK cell-deficient mice established that uNK precursors are present in fetal liver; fetal, neonatal and adult thymus; marrow, LN and spleen (Chantakru *et al.*, 2002). Only spleen showed enhanced ability to generate uNK cells if harvested from pregnant donors (days 3–7 of gestation), whereas LN draining

Fig. 2. The *in vivo* assay system, used to assess uterine natural killer (uNK) cell precursor differentiation, homing and regulation, and to define uNK cell functions, depends on pregnancy in mice genetically deficient in the NK/uNK cell lineages. At present, the most reliable mouse used for these studies and manipulations is the RAG-2^{o/o}/γC^{o/o} double knockout. The RAG-2^{o/o}/γC^{o/o} is alymphoid but fertile under appropriate housing. Cell suspensions or tissues are grafted into virgin or mated uNK cell-deficient recipients. Donors can be normal or altered in the gene(s) of interest. At different times after transplantation, implantation sites are analysed for graft-derived uNK cells. Any and all uNK cells will be graft-derived in RAG-2^{o/o}/γC^{o/o} recipients. Independent microdomains (usually the decidua basalis (DB) and the mural, mesometrial lymphoid aggregate of pregnancy (MLAp)) are assessed within each implantation site. The procedure is summarized in (a). (b,c) Graft-derived uNK cells in the DB and MLAp (white and black arrowheads, respectively), 10 days after inoculation of peripheral lymph node cell suspensions from C57Bl/6J mice at day 5 of gestation to mated RAG-2^{o/o}/γC^{o/o} recipients at day 0 of gestation. uNK cells in the MLAp are smaller, have fewer cytoplasmic granules and more mitotic figures than those in the DB (compare (c) with (b)), findings replicating implantation sites in pregnant, genetically normal mice. (b) The common intravascular (*) position of uNK cells (black arrowhead) in the DB is replicated in transplant recipients. (d) The DB in unmanipulated RAG-2^{o/o}/γC^{o/o} (KO) and C57Bl/6J (N) females at day 10 of gestation. (e) The MLAp in unmanipulated RAG-2^{o/o}/γC^{o/o} (KO) and C57Bl/6J (N) females at day 10 of gestation. No uNK cells are found in unmanipulated RAG-2^{o/o}/γC^{o/o} but numerous uNK cells (arrows) are present in these tissues in normal mice. Scale bars represent 20 μm; all sections are stained with periodic acid–Schiff.

pregnant uteri were devoid of uNK precursors (Fig. 2). These observations strongly indicate that, before implantation, endocrine signals co-ordinately mobilize uNK precursors from spleen and induce mechanisms for trapping and retaining these circulating cells within the uterus (Chantakru *et al.*, 2002).

Ovarian hormone effects

The present authors addressed functionally the postulated steroid hormone-regulated interactions between lymphocytes and endothelium by assaying human blood lymphocyte adhesion to high endothelial venules in cryostat sections of mouse subcutaneous LN pools (PLN). Lymphocytes from anonymous blood-bank donors were evaluated using PLN from virgin and pregnant mice. Endothelium from pregnant donors attracted more cells (Chantakru *et al.*, 2002). Endothelium in PLN from ovariectomized mice given oestradiol or progesterone or both also gained adhesive function equalling pregnancy-induced gains. Antibody-blocking studies established that L-selectin and α4-integrin ligands mediated the functional changes. Adhesion assays were extended using mouse uterine tissues as substrate (virgin, pregnant or steroid hormone-treated) instead of PLN. Binding again occurred via L-selectin and α4-integrin-dependent mechanisms and numbers of cells adhering per mm² of uterus were increased if tissue came from pregnant or steroid hormone-treated (oestrogen, progesterone, or both) ovariectomized mice. Prelabelling the human lymphocytes with anti-CD56 revealed that gains in functional adhesion to decidua basalis (DB) occurred in both CD56⁺ and CD56[−] cells but greatest enrichment occurred in CD56⁺ cells (>70-fold compared with the starting blood-cell suspension; Chantakru *et al.*, 2002). Gain in ability of endothelium to attract lymphocytes was not systemic, as no pregnancy- or steroid hormone-induced changes were observed in pancreatic endothelium (Chantakru, 2002; S. Chantakru, W-C Wang, B. A. Croy and S. S. Evans, unpublished).

Two studies were performed to determine whether hormones regulate lymphocytes independently but co-ordinately with endothelium. First, splenic lymphocyte suspensions from virgin, pregnant or ovariectomized mice treated with placebo, or oestrogen in the presence or absence of progesterone, were tested for adhesion to PLN from a single donor. Adhesion rates for splenocytes from virgin and ovariectomized, placebo-treated mice were similar and statistically lower than adhesion of splenocytes from pregnant or steroid hormone-treated donors, indicating that pregnancy, oestrogen and progesterone enhance the ability of murine lymphocytes to interact with endothelium (Chantakru, 2002; S. Chantakru, W-C Wang, B. A. Croy and S. S. Evans, unpublished). For the second study, blood samples were collected from women three times per week over their

menstrual cycle and adhesion to constant mouse tissue was assessed. A dynamic, predictable pattern was found with significant gains in cells adhering to PLN and to decidualized uterus at the LH surge (M. van den Heuvel, S. Bashar, J. E. Lewis, X. Xie, J. Horrocks and B. A. Croy, unpublished). These data are consistent with a hypothesis of periovulatory mobilization of human uNK cell precursors to blood and their uterine appearance at LH+3–5 (King, 2000). Limited duration and importance of the luteal phase in mice may explain why different hormones appear to mediate the pregnancy-associated gains in lymphocyte–endothelial interactions in women and mice. Whether these dynamic changes reflect important steps *in vivo* in recruitment of uNK precursors to human uteri remains to be addressed experimentally. In an *in vivo* model of fever-range hyperthermia, doubling *in vitro* adhesion correlated with quadrupled lymphocyte trafficking to the targeted site (Evans *et al.*, 2001).

IL-15

IL-15, a stromal cell and macrophage-derived cytokine, is critical for NK cell differentiation in human and murine lymphoid tissue (Carson *et al.*, 1994; Kennedy *et al.*, 2000). IL-15 acts on precursor and immature NK cells but not during NK progenitor cell differentiation. Human and murine endometria express IL-15 (Ye *et al.*, 1996; Kitaya *et al.*, 2000; Okada *et al.*, 2000; Dunn *et al.*, 2002). In mice, IL-15 mRNA is transcribed between day 6 and day 11 of gestation (Ye *et al.*, 1996; A. A. Ashkar, G. P. Black, Q. Wei, J. R. Head and B. A. Croy, unpublished). In humans, IL-15 transcription is more abundant during the secretory than during the proliferative cycle phase and is sustained in early pregnancy with localization to endothelium and perivascular stromal cells of decidual spiral arteries (SA; Kitaya *et al.*, 2000). These correlative time-course data strongly indicate that IL-15 participates in uNK cell differentiation, whereas the localization data indicate that IL-15 may contribute to chemotactic localization of uNK cells within the lumina and walls of the spiral arteries.

IL-15 shares two of its three receptor chains (β and γ) with IL-2, a cytokine not normally found in gestational uteri. Mice genetically ablated for either shared receptor or for IL-15 do not differentiate uNK cells (Guimond *et al.*, 1998; Croy *et al.*, 2003). Reconstitution and blocking bone marrow transplantation experiments were conducted to establish that IL-15 contributes to terminal uNK cell differentiation. The reconstitution experiment involved transplants between two strains that do not differentiate uNK cells. A lymphoid mice (RAG-2^{o/o}/common cytokine receptor chain γ (γ c)^{o/o}, a double knockout strain that makes IL-15) were grafted with bone marrow from IL-15^{o/o} mice, then mated and studied histologically. uNK cell differentiation occurred

at frequencies identical to those observed in normal congenic C57Bl/6J mice. For the blocking experiment, C57Bl/6J bone marrow was given to IL-15^{o/o} mice. This bone marrow produces normal uNK cells when grafted into alymphoid mice (Ashkar *et al.*, 2000), but was totally blocked from differentiation in uteri of IL-15^{o/o} recipients (Croy *et al.*, 2003; A. A. Ashkar, G. P. Black, Q. Wei, J. R. Head and B. A. Croy, unpublished). These experiments indicate that IL-15 is the crucial factor regulating uNK cell differentiation and provoke questions concerning the regulation of uterine IL-15 and whether its deficiency or over-abundance has clinical consequences in women, as suggested by Chegini *et al.* (2002) for women who experience recurrent spontaneous abortions. Interferon regulatory factor (IRF)-1, a key regulator of IL-15 in marrow, is expressed in the uterus in humans and mice (Jabbour *et al.*, 1999; Kitaya *et al.*, 2001; A. A. Ashkar, G. P. Black, Q. Wei, J. R. Head and B. A. Croy, unpublished) but does not appear to regulate murine uterine IL-15 (Ashkar and Croy, 1999; A. A. Ashkar, G. P. Black, Q. Wei, J. R. Head and B. A. Croy, unpublished). In humans, uterine IRF-1 appears to be regulated by prolactin (Jabbour *et al.*, 1999), whereas uterine IL-15 expression appears to be regulated by progesterone (Okada *et al.*, 2000; Kitaya *et al.*, 2000) and prostaglandins (Dunn *et al.*, 2002). Additional information on uterine specific regulation of IL-15 is needed.

Clearly, ovarian steroid hormones act on uterine stroma, including endothelium, as discussed above, in ways that promote differentiation of uNK cells, but whether the hormones act directly on the lymphocytes or their precursors is less clear. Availability of mice ablated for the oestrogen receptor (ER) α or β (α ERKO and β ERKO) (Couse and Korach, 1999) permitted functional assessment of ER utilization by uNK cells. Marrow transplanted from α ERKO and β ERKO to alymphoid mice fully reconstituted uNK cells, indicating no direct functional action in mouse uNK cells through oestrogen receptors. This conclusion was supported by RT-PCR analysis of highly purified (99%) uNK cells at days 12 and 13 of gestation, isolated rapidly from perfused, normal (C57Bl/6J) mice using a newly reported protocol involving magnetic beads coated with the lectin *Dolichos biflorus* agglutinin (DBA) (Borzychowski *et al.*, 2003; Paffaro *et al.*, 2003). mRNA for ER α and ER β were absent, whereas mRNA for other genes was detected (Borzychowski *et al.*, 2003). Human uNK cells have recently been reported to be ER α negative, as in earlier studies, but to express ER β (Henderson *et al.*, 2003), indicating species difference or transcripts from rare contaminating cells. In both humans and mice, studies of the complex interactions between the endocrine and stromal environments on lymphocyte differentiation within the uterus will be profitable and of clinical importance for endometrial as well as reproductive health.

NK cell and uNK cell activation

Lymphocyte activation requires signals additional to those involved in lymphocyte differentiation. IL-12 is a cytokine important for induction of IFN- γ synthesis in NK and T cells (Trinchieri, 1995). The action of IL-12 is enhanced by IL-18 (Nakanishi *et al.*, 2001). IL-12 and IL-18 are found in normal human and mouse implantation sites (Devergne *et al.*, 2001; Yoshino *et al.*, 2001; Zourbas *et al.*, 2001; Chaouat *et al.*, 2002; Zhang *et al.*, 2003). IL-18 shows a dynamic protein expression pattern by immunohistology, appearing first in decidual stromal cells at days 4–6 of gestation, then exclusively from day 8 to day 14 of gestation in uNK cells (Chaouat *et al.*, 2002; Zhang *et al.*, 2003). Pregnant mice deficient in both IL-12 and IL-18 (Takeda *et al.*, 1998) have been compared with littermates deficient in only one or neither cytokine (Zhang *et al.*, 2003). Females and males matched in genotype were mated. uNK cell differentiation was morphologically and numerically similar in the four strains. In the three cytokine-deficient strains, mid-gestation IFN- γ (after day 8 of gestation) was somewhat reduced and SA dilation was impaired but not as severely as in IFN- $\gamma^{o/o}$ implantation sites. This finding indicated that additional cytokines contribute to uNK cell activation. Two recently described cytokines, IL-23 and IL-27, are related to IL-12 and contribute to IFN- γ induction (Parham *et al.*, 2002; Pflanz *et al.*, 2002 and references therein). IL-23 and IL-27 expression is absent from virgin mesometrial uterus and mesometrial uterus at day 3 of gestation, but present in mesometrial decidua from day 4 of gestation (Zhang *et al.*, 2003). Analyses of mRNA, from DBA lectin-purified uNK cells, indicate that stromal cells produce IL-23, whereas uNK cells produce IL-27. The time-course patterns for induction of these cytokines match well with putative roles for these cytokines in uterine IFN- γ regulation in normal mice because IFN- γ protein is absent mesometrially from virgin and pre-implantation uteri at day 3 of gestation but is detected from day 6 of gestation by ELISA. Peak mesometrial IFN- γ occurs at day 10 of gestation and then declines (Ashkar *et al.*, 2000).

NK cells interact with other cells via surface receptors that are classified as activating or inhibitory, depending on whether lytic activity is displayed when the receptor is engaged in *in vitro* assays. Individual cells display multiple receptors to give target recognition specificity. The ligands initially defined for NK cell receptors were classic major histocompatibility complex (MHC) class I molecules or their structural relatives (Cerwenka and Lanier, 2001; Natarajan *et al.*, 2002). Because the pathway resulting in terminal uNK cell differentiation in primates and rodents is triggered in the absence of conception, the role of non-self antigen recognition during uNK cell activation is unclear. Trophoblast has restricted transplantation antigen expression that, in outbred matings, will include paternally derived, non-self

antigens (Moffett-King, 2002). The human uNK cell population expresses a full repertoire of immunoglobulin-like NK cell receptors, although in different proportions than expressed by blood NK cells (Hiby *et al.*, 1997). The major NK cell receptors in mice are lectin-like LY49 gene family members. uNK cells from C57Bl/6 mice express all of the LY49 NK receptors typical of the strain (V. A. Paffaro, Jr, H. He and B. A. Croy, unpublished). This sharing of receptors between blood NK and uNK cells indicates that uNK cell activation will display many features in common with NK cell activation in other tissues and predicts that the unique properties of uNK cells are not defined by these receptors.

A second type of NK cell activation receptor in mice and humans is NKG2D. The Rae-1 family of five retinoic acid-induced oncodevelopmental antigens was recently found to engage murine NKG2D, whereas the homologous human ligands are products of the HLA-associated genes *MICA* and *MICB* (Cerwenka and Lanier, 2001). Interactions between murine NK cells and Rae-1 genes are normally studied *in vitro* using embryonic cell-line targets. *In vivo* expression of Rae-1 is reported only in mid-gestation developing mouse brain (Nomura *et al.*, 1996). The present authors addressed transcription of Rae-1- α - β - γ - δ - ϵ in uteri from C57Bl/6 and 129/J strains by RT-PCR. Non-pregnant uteri expressed no Rae-1 signal but strain appropriate Rae-1 expression was induced by day 6 of gestation in DB and sustained at day 10 of gestation in DB, MLAp and placenta. Immunohistochemistry using a pan Rae-1 α polyclonal antibody showed that extra-fetal expression was most strongly localized to trophoblast (B. A. Croy and T. Seya, unpublished). These and other developmental antigens expressed by trophoblast may be of major importance in sustaining uNK cell activation when trophoblasts and NK cells begin to intermingle (in mice from about day 8 of gestation). Roles for oncodevelopmental antigens are not yet explored in humans.

Functions of uNK cells

uNK-deficient and IFN- γ signal disrupted mice

Several mouse strains have major (> 99%) or absolute deficits in NK cells. Five strains, each having different gene deletions, are uNK cell-deficient and have common histopathology (Table 1; Guimond *et al.*, 1998; Ashkar *et al.*, 2000). Three anomalies accompany absence of uNK cells: absence of myometrial MLAp development (that is, a decidual bed deficit), hypocellularity and oedema of DB and persistence of vascular smooth muscle in the SA with limited lumen dilation and vessel lengthening at mid-gestation. There is no overgrowth of trophoblast and no consistent quantifiable impairment in fetal or postnatal health (Greenwood *et al.*, 2000). Issues such as maternal vascular or fetal gene expression, fetal hypoxia, brain development, ventricular hypertrophy,

maturity onset diabetes and others have not been addressed experimentally between uNK-deficient and uNK cell-sufficient gestations. These findings imply that the normal functions of uNK cells during pregnancy are to congregate lymphocytes myometrially at the portals of uterine arteries and veins, to interact with and provide growth support for stromal cells committed to decidual differentiation, and to trigger events that culminate in normal, pregnancy-induced SA modification. These interpretations were confirmed by normalizing implantation sites in NK/uNK cell-deficient mice by bone marrow transplants from severe combined immunodeficient (SCID) (T-B-) mice (Guimond *et al.*, 1998). Effector mechanisms were addressed by transplanting NK/uNK cell-deficient females with bone marrow deleted for IFN- γ , IFN- γ R α or their downstream signalling molecule Stat-1. Implantation sites in the recipients revealed that IFN- γ is not required to initiate uNK cell differentiation but low concentrations are required for full maturation and senescence of uNK cells. Terminal uNK cell maturation required about 1 iu per implantation site, which is adequately provided by other types of cell (Ashkar *et al.*, 2000). uNK cell-derived IFN- γ provides higher tissue contents (> 6 iu per implantation site) and these contents support decidual integrity and SA modification. Daily infusions of mrIFN- γ (100–3000 iu per treatment) into alymphoid females at days 6–11 of gestation were effective in induction of normal SA modification and decidual maintenance in the absence of uNK cells (Ashkar *et al.*, 2000). Thus, pro-inflammatory cytokines act physiologically in normal pregnancy.

The α_2 -macroglobulin gene family, possible targets of uNK-cell derived IFN- γ

As the present study indicated that IFN- γ acts on gene expression in a complex tissue that includes lymphocytes, stromal, vascular smooth muscle, endothelial and decidual cells, a cDNA microarray (GEM[©], Incyte, St Louis, MO) was undertaken. Maternal mesometria from C57Bl/6J mice at day 6 to day 10 of gestation were compared. Forty-three genes, documented among the hundreds regulated by IFN- γ , were present on the array and differentially expressed (B. A. Croy, unpublished). Among these IFN- γ -regulated genes were mouse α_2 -macroglobulin (MAM) and its receptor. A related EST777415, defined as α_2 -macroglobulin (α_2 M) precursor-like, was among the most differentially upregulated genes. The array results were extended by northern blot analyses. Non-decidualized mouse uterus (virgin and at day 3 of gestation) did not express EST777415 but expression was induced by day 6 of gestation, peaked at days 10 and 12 of gestation and then declined by day 14 of gestation (H. He, D. McCartney, Q. Wei, S. Esadeg, J. Zhang, A. Hayes, F. van Leuven and B. A. Croy, unpublished). Members of the α_2 M family are

abundant plasma proteins that regulate the bioavailability of proteases and cytokines in tissue (Borth, 1994). The genes map closely in mice to the NK cell LY49 receptor gene complex on chromosome 6 (Ensembl, Mouse Genome Browser *BLASTView*).

Potential roles of MAM and its related, expressed family member murinoglobulin-1 (MUG-1) in implantation sites were addressed by undertaking histological analyses using MAM^{o/o}/MUG-1^{o/o} mice (Umans *et al.*, 1999). Placental development was highly unusual; the labyrinth was reduced whereas giant cells were in excess (Fig. 3). In addition, intramural trophoblast invasion of the SA was aggressive and by mid-gestation reached the myometrial circular smooth muscle layer, a position normally achieved in very late gestation. uNK cells were numerous and SA, although cuffed by trophoblast, were dilated. This indicated that members of the α_2 M gene family regulate the rate at which trophoblast invades and led the authors to characterize EST777415, which remains expressed in decidua of MAM^{o/o}/MUG-1^{o/o} (Esadeg, 2002).

A full-length cDNA, now designated α_2 M of mouse pregnancy (A2Mp) was obtained from EST777415 (Genbank accession no. AY185125). The predicted amino acid sequence is typical of members of the α_2 M family with bait, thiol ester and receptor-binding domains. The bait region is most distinctive. The predicted A2Mp amino acid sequence is closest to rat α_2 M, an acute phase protein (90%), and 71% and 63% matched to the non-acute phase reactants human α_2 M and pregnancy zone protein, a molecule of undefined function, which is highly upregulated during human gestation. Homology to the four other α_2 M mouse family members is at 50% (H. He, D. McCartney, Q. Wei, S. Esadeg, J. Zhang, A. Hayes, F. van Leuven and B. A. Croy, unpublished). Unlike other members of the mouse gene family, A2Mp is not detected in liver by RT-PCR or by *in situ* hybridization. Rather, testes and ovaries constitutively transcribe A2Mp; uterus transcribes A2Mp only with decidualization and the gene is transcribed in lactating mammary gland. *In situ* hybridizations (Fig. 4) localized A2Mp to primary spermatocytes and Sertoli cells in testes and to granulosa cells of secondary and pre-ovulatory ovarian follicles, in patterns distinct from those reported for MAM (Zhu *et al.*, 1994; Dajee *et al.*, 1998). Expression in 24 h postpartum, lactating mammary tissue was localized to alveolar epithelium (H. He, D. McCartney, Q. Wei, S. Esadeg, J. Zhang, A. Hayes, F. van Leuven and B. A. Croy, unpublished). Uterine localization was dynamic. At day 6 of gestation, A2Mp was expressed by anti-mesometrial decidua. Signal then migrated mesometrially and by days 8–14 of gestation was in vascular smooth muscle of SA and unmodified fibroblasts interfaced between mesometrial myometrium and decidua (full description of these experiments will be published elsewhere). One interpretation of these findings is that A2Mp binds molecules needed for cells

Fig. 3. Photomicrographs of implantation sites at day 10 of gestation in mice with different genetic mutations. (a) An implantation site in IL-12^{o/o}/IL-18^{o/o}. These mice have normal uterine natural killer (uNK) cell differentiation and mesometrial lymphoid aggregate of pregnancy (MLAp) (I) and placental (P) development but lower than normal levels of interferon gamma (IFN-γ) at mid-gestation and only limited modification of the spiral arteries (SA). Intravascular (black arrowhead) and perivascular (white arrowhead) localization of uNK cells is seen in the decidua basalis of these mice (insert). (b) An implantation site in IFN-γ^{o/o} with over-development of uNK cells and the MLAp (I). uNK cells are unusually localized in decidua basalis close to the placenta (P), leaving a band of decidua relatively void of uNK cells (*). Upper insert shows this region at day 10 of gestation with healthy decidua cells (white arrow) clustered with darker, *Dolichos biflorus* agglutinin (DBA)-lectin stained uNK cells (arrowhead). Lower insert is of the same region at day 12 of gestation, when the decidua basalis has become necrotic (Ashkar *et al.*, 2000). Decidual cells have been destroyed and amorphous material fills the spaces between cells. Small mononuclear cells and fibroblasts (black arrow) replace the decidual cell population. uNK cells (arrowhead) appear less granulated at this time point (day 12 of gestation) than in normal mice. No spiral artery modification occurs in IFN-γ^{o/o} pregnancy. (c,d) Implantation sites in MAM^{o/o}/MUG-1^{o/o} with relative overgrowth of trophoblast particularly along the spiral artery (P: placenta); (d) is a higher magnification (×1000) of the boxed area in (c) on a serial section stained with anti-cytokeratin antibody and illustrates intramural trophoblast cells. Erythrocytes (RBC) are present in the spiral artery lumen and endothelial cells (E) were always present between trophoblasts (arrow) and circulating cells in this segment of the vessel. Scale bars represent (a,b) 200 μm, (c) 400 μm and (d) 20 μm. (a,b) Stained with DBA lectin and haematoxylin; (c) stained with haematoxylin and eosin; and (d) counter-stained with haematoxylin.

in reproductive tissues to transition from quiescence to active differentiation.

The present authors reasoned that if the A2Mp gene-derived product was the molecule of key importance induced by uNK cell-derived IFN-γ, administration of A2Mp to pregnant IFN-γ^{o/o} mice would induce SA modification. As no gene product is yet available but homology is significant to human α₂M, human plasma-derived α₂M or PBS was infused from days 6–11 of gestation into pregnant IFN-γ^{o/o} or alymphoid mice.

In both stains, native human α₂M induced full SA modification whereas PBS induced no modification (H. He, D. McCartney, Q. Wei, S. Esadeg, J. Zhang, A. Hayes, F. van Leuven and B. A. Croy, unpublished). Thus, human α₂M must have regulated proteases, cytokines or other molecules that signal vascular dilation and elongation during gestation. When human α₂M, activated by methylamine to destroy its protease binding sites, was infused into pregnant alymphoid mice, SA dilation was again achieved. This experiment indicates

Fig. 4. *In situ* hybridization for α_2 M of mouse pregnancy (A2Mp) in reproductive tissues. (a) Strong hybridization of an anti-sense DIG-labelled probe to anti-mesometrial decidual cells at day 6 of gestation. The embryonic crypt is labelled EC. (b) The sense strand hybridization negative control. (c) A secondary follicle in the ovary, showing hybridization to granulosa cells (arrowheads). Primary follicles (*) and corpora lutea (not shown) were unlabelled. (d) Localized expression of A2Mp in a seminiferous tubule from a mature C57Bl/6J mouse (arrowhead). Hybridization appeared restricted to spermatogonia, primary spermatocytes and Sertoli cells. Scale bars represent (a,b) 400 μ m and (c,d) 50 μ m.

that cytokines rather than proteases are the key molecules involved in SA destabilization. If A2Mp is the key molecule induced by uNK cell-derived IFN- γ and if A2Mp, through its bound molecules, effects SA dilation and elongation, it is not necessary to postulate additional vasoactive uNK cell products. However, these are known. In mice, inducible nitric oxide synthase, the enzyme generating the powerful vasodilator NO, is predominantly found in uNK cells of normal implantation sites (Hunt *et al.*, 1997). In contrast, implantation sites in uNK cell-deficient mice display expression of this enzyme in trophoblast (Hunt *et al.*, 1997). Vascular endothelial cell growth factor (VEGF), a molecule bound by α_2 M, is produced by murine and human uNK cells (Wang *et al.*, 2000; Li *et al.*, 2001). In humans, placenta growth factor, a molecule upregulating the bioavailability of VEGF, is reported to be expressed exclusively in uNK cells (Li *et al.*, 2001). Collectively these studies indicate that murine and human uNK cells contribute in various ways to the maternal vascular changes that occur in support of pregnancy.

Conclusions

Rodent studies strongly indicate that uNK cells are key contributors to maternal uterine changes induced by and in support of pregnancy. These studies indicate that many regulatory steps are involved, each of which could have a clinical correlate culminating in syndromes such as pre-eclampsia or intrauterine growth retardation. Although it is appealing to suggest that uNK cell-deficient mice may model pre-eclampsia, the present authors' opinion is that they model only vascular aspects. Serial urinalysis of mated NK/uNK cell-deficient IL-15 $^{o/o}$ and C57Bl/6 mice for protein (Lowry technique) failed to show proteinuria in uNK cell-deficient mice above that seen in pregnant normal mice (Fig. 5). Other strains, such as BMP5, which shows pregnancy-induced glomerulosclerosis, proteinuria and hypertension (Davisson *et al.*, 2002), or thrombomodulin $^{o/o}$, which displays trophoblast death and fibrinoid deposition (Isermann *et al.*, 2003), are important and investigations of pre-eclampsia may be best served by combining these or other strains as murine

Fig. 5. Analysis of mean urinary protein (Lowry assay) collected daily from individual uterine natural killer (uNK) cell-deficient IL-15^{0/0} mice (dashed line; $n = 6$) and C57Bl/6J mice (solid line; $n = 4$) from one oestrous cycle (-4-0) and pregnancy (0-18). Every day, individual mice were placed in clean cages having a base composed of sterile 96-well plates. The mice were allowed to roam freely and void. Wells containing urine but no faeces were collected and pooled when urine volumes of 50–200 μ l appeared to be available. The uNK cell-deficient mice were less proteinuric than their gestation-day matched normal congenic controls.

study models. Although there are many differences between implantation sites in rodents and women, this review illustrates the dynamic and detailed information that can be collected from serial time-course studies of murine pregnancy to provide valuable concepts in understanding the biology of human CD56^{bright} uterine cells. The resolving power of mouse genetics, combined with transplantation, in addressing questions of regulatory mechanisms in mammalian pregnancy *in vivo* are illustrated. The authors hope that others will develop and explore further variations of these approaches.

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