EVALUATION OF UNK CELL CAPACITY TO INITIATE
PREGNANCY-ASSOCIATED SPIRAL ARTERY REMODELLING

By: Michael Joseph Bilinski

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Abstract

Transient uterine Natural Killer (uNK) cells are the predominant leukocytes of early gestational human and murine uteri. Murine uNK cells promote changes in endometrial structure including initiation of perivascular smooth muscle reduction in spiral arteries. Less is known about human uNK cell functions due to sampling constraints. Xenogeneic engraftment of human lymphocyte progenitors to alymphoid mice has been useful in understanding human lymphocyte functions in vivo. Irradiation of recipients is required to create a niche for successful humanization of the mice but renders recipient mice sterile. The goal of my thesis was to develop a protocol enabling engraftment of human hematopoietic stem cells in alymphoid mice that would permit differentiation of functional human uNK cells. I then planned to evaluate human uNK cell functions and their regulation in vivo. Neonatal Rag2\(^{-/-}\) Il2rg\(^{-/-}\) mice, which lack T cells, B cells and NK cells were preconditioned with 5-fluorouracil and inoculated with syngeneic mouse bone marrow cells. As adults, inoculated female mice conceived and differentiated functional mouse uNK cells. In contrast, neonatally-preconditioned Rag2\(^{-/-}\) Il2rg\(^{-/-}\) mice inoculated with human cord blood hematopoietic stem cells conceived but differentiated non-lymphoid cells in sites normally occupied by uNK cells. Weekly injections of human IL-15, which is required for NK cell differentiation, proliferation and survival, did not promote uNK cell differentiation. Rather, treatment with IL-15 altered gestational uteri, even in mice receiving neither preconditioning nor hematopoietic stem cells. I was successful in developing a protocol that enables hematopoietic stem cell
engraftment in neonatal mice without compromising mouse fertility. However, this model is apparently not suitable for *in vivo* studies of human uNK cell functions.
Acknowledgements

Dr. B. Anne Croy (Department of Anatomy and Cell Biology, Queen’s University) supervised this work. Drs. Graeme Smith and Sam Basta supervised this work as Advisory Committee members. Dr. Marianne van den Heuvel performed 5-FU injections and C57BL/6 bone marrow inoculations of C57BL/6 Rag2−/−/Il2rg−/− mice at the University of Guelph. Dr. Zhilin Chen performed PCR analyses and human immunoglobulin ELISAs at Queen’s University. Graduate study funding was provided to Michael Joseph Bilinski by Queen’s Graduate Scholarships, the Ellen O’Rourke Merrin Award, the Robert John Wilson Fellowship and Ontario Graduate Scholarships in Science and Technology. Additional funding was provided by National Science and Engineering Research Council (NSERC) and the Canada Research Chairs Program funding awarded to Dr. B. Anne Croy.

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Table of Contents

Abstract ................................................................................................................................. ii
Acknowledgements ............................................................................................................. iv
Table of Contents .................................................................................................................. vi
List of Figures ....................................................................................................................... viii
List of Tables ......................................................................................................................... x
List of Abbreviations ........................................................................................................... xi

Chapter 1: Introduction and Review of Literature ............................................................... 1
  Mouse uNK cell life cycle ...................................................................................................... 5
  UNK cell activation ............................................................................................................. 7
  NK cell receptors and their ligands ..................................................................................... 8
    Killer immunoglobulin-like receptors ............................................................................. 9
    NKG2 receptors ............................................................................................................... 9
    Ly49 receptors ............................................................................................................... 10
    Natural cytotoxicity receptors ...................................................................................... 11
  UNK cell functions ............................................................................................................ 11
    I. Trophoblast invasion .................................................................................................... 11
    II. Vascular remodelling ................................................................................................. 13
    III. Cytokine production ................................................................................................. 13
    IV. Angiogenesis ............................................................................................................ 14
  UNK cell localization is a highly ordered process ............................................................... 14
  Humanized mouse models ............................................................................................... 16
    Nude mouse .................................................................................................................... 17
    SCID mouse .................................................................................................................. 17
    Non-obese diabetic SCID (NOD-SCID) mouse ............................................................ 18
    Recombinase activating gene (Rag) knockout mouse .................................................... 18
    Interleukin-2 receptor gamma chain knockout (Il2rg<sup>-/-</sup>) mouse ......................... 19
    Rag2 gamma chain double knockout (Rag2<sup>−/−</sup>/γc<sup>−/−</sup>) mouse .......................... 20
  Preconditioning of mice for HSC engraftment ................................................................. 20

Chapter 2: Materials and Methods .................................................................................... 22
  Animals ............................................................................................................................. 22
  Animal procedures .......................................................................................................... 23
  Tissue fixation and processing ......................................................................................... 23
  Preconditioning of graft recipients .................................................................................. 24
  Preparation of bone marrow suspensions ....................................................................... 25
  Preparation of enriched human CD34<sup>+</sup> cell suspensions ........................................ 25
  Inoculation procedures .................................................................................................... 26
    Syngeneic mouse bone marrow inoculation of Rag2<sup>−/−</sup>/Il2rg<sup>−/−</sup> mice .................. 26
    Human cord blood CD34<sup>+</sup> cell inoculation ........................................................... 27
  Histological evaluation of syngeneic bone marrow-inoculated
  C57BL/6 Rag2<sup>−/−</sup>/Il2rg<sup>−/−</sup> and BALB/c Rag2<sup>−/−</sup>/Il2rg<sup>−/−</sup> mice ................................ 28
  DNA isolation and polymerase chain reaction (PCR) ...................................................... 30
  Immunohistochemistry ..................................................................................................... 30
  Human Ig ELISA ................................................................................................................ 32
  Statistics ............................................................................................................................ 32
Chapter 3: Results........................................................................................................33
Implantation sites of untreated C57BL/6 mice.........................................................33
Implantation sites of unmanipulated C57BL/6 Rag2⁻/⁻/Il2rg⁻/⁻ mice..................35
Selection of 5-FU dosage for treatment of neonatal C57BL/6
Rag2⁻/⁻/Il2rg⁻/⁻ mice ......................................................................................................35
Differentiation of uNK cells from C57BL/6 syngeneic bone marrow transplantation..........................................................................................................................37
Assessment of spiral arterial modification in C57BL/6 Rag2⁻/⁻/Il2rg⁻/⁻
mice inoculated with C57BL/6 bone marrow..........................................................44
Implantation sites of unmanipulated BALB/c mice..................................................45
Implantation sites of unmanipulated BALB/c Rag2⁻/⁻/Il2rg⁻/⁻ mice..................45
Differentiation of uNK cells in BALB/c Rag2⁻/⁻/Il2rg⁻/⁻ mice inoculated
with syngeneic BALB/c bone marrow.........................................................................48
Assessment of spiral arterial modification in BALB/c Rag2⁻/⁻/Il2rg⁻/⁻
mice inoculated with syngeneic BALB/c bone marrow..............................................48
Growth trajectory of mice inoculated as neonates with enriched human
cord blood CD34⁺ cells..................................................................................................50
Gestation day 7–12 implantation sites of BALB/c Rag2⁻/⁻/Il2rg⁻/⁻
mice inoculated as neonates with enriched human cord blood
CD34⁺ cells.....................................................................................................................51
Implantation sites of mice inoculated as neonates with enriched human
cord blood CD34⁺ cells and supplemented with IL-15/RA complex....................56
Effects of IL-15/RA complex alone..........................................................................60
Growth trajectory of mice born to dams inoculated as neonates with
human CD34⁺ cells.......................................................................................................60
Detection of Chimerism............................................................................................62
Chapter 4: Discussion.............................................................................................67
Reference List.............................................................................................................80
## List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1</td>
<td>Implantation sites of C57BL/6 mice.</td>
<td>34</td>
</tr>
<tr>
<td>Figure 2</td>
<td>Implantation sites of gestation days 7, 9 and 12 C57BL/6 Rag2(^{-/-})/Il2rg(^{-/-}) mice.</td>
<td>36</td>
</tr>
<tr>
<td>Figure 3</td>
<td>Implantation site of gestation day 8 C57BL/6 Rag2(^{-/-})/Il2rg(^{-/-}) mouse treated with 25 mg/kg 5-FU at 24 hr of age followed by 5 x 10(^5) C57BL/6 bone marrow cells at 48 hr.</td>
<td>39</td>
</tr>
<tr>
<td>Figure 4</td>
<td>Implantation site of gestation day 10 C57BL/6 Rag2(^{-/-})/Il2rg(^{-/-}) mouse treated with 25 mg/kg 5-FU at 24 hr of age followed by 5 x 10(^5) C57BL/6 bone marrow cells at 48 hr.</td>
<td>40</td>
</tr>
<tr>
<td>Figure 5</td>
<td>Implantation site of gestation day 12 C57BL/6 Rag2(^{-/-})/Il2rg(^{-/-}) mouse treated with 25 mg/kg 5-FU at 24 hr of age followed by inoculation with 5 x 10(^5) BALB/c bone marrow cells at 48 hr.</td>
<td>42</td>
</tr>
<tr>
<td>Figure 6</td>
<td>Comparison of mean uNK cells/mm(^2) in MLAp and decidua basalis of C57BL/6 Rag2(^{-/-})/Il2rg(^{-/-}) mice inoculated with syngeneic bone marrow and wild type C57BL/6 mice.</td>
<td>43</td>
</tr>
<tr>
<td>Figure 7</td>
<td>Implantation sites of unmanipulated BALB/c mice at gestation days 7, 9 and 12.</td>
<td>46</td>
</tr>
<tr>
<td>Figure 8</td>
<td>Implantation sites of unmanipulated gestation day 12 BALB/c and BALB/c Rag2(^{-/-})/Il2rg(^{-/-}) treated with 25 mg/kg 5-FU at 24 hr of age followed by inoculation with 5 x 10(^5) BALB/c bone marrow cells at 48 hr.</td>
<td>47</td>
</tr>
<tr>
<td>Figure 9</td>
<td>Stages of uNK cell maturation in unmanipulated BALB/c and BALB/c Rag2(^{-/-})/Il2rg(^{-/-}) mice treated with 25 mg/kg 5-FU at 24 hr of age followed by inoculation with 5 x 10(^5) BALB/c bone marrow cells at 48 hr.</td>
<td>49</td>
</tr>
<tr>
<td>Figure 10</td>
<td>Gestation day 7 implantation site of Rag2(^{-/-})/Il2rg(^{-/-}) mice inoculated with 10(^6) human CD34(^+) cells.</td>
<td>52</td>
</tr>
<tr>
<td>Figure 11</td>
<td>Gestation day 9 implantation site of Rag2(^{-/-})/Il2rg(^{-/-}) mouse inoculated with 10(^6) human CD34(^+) cells.</td>
<td>54</td>
</tr>
<tr>
<td>Figure 12</td>
<td>Gestation day 12 heterogeneous implantation sites of Rag2(^{-/-})/Il2rg(^{-/-}) mice inoculated with 10(^6) human CD34(^+) cells.</td>
<td>55</td>
</tr>
<tr>
<td>Figure 13</td>
<td>Gestation day 10 implantation site of human CD34(^+) cell-engrafted BALB/c Rag2(^{-/-})/Il2rg(^{-/-}) mouse treated with IL-15/RA complex.</td>
<td>57</td>
</tr>
<tr>
<td>Figure 14</td>
<td>Gestation day 12 implantation site of BALB/c Rag2(^{-/-})/Il2rg(^{-/-}) mouse inoculated with human CD34(^+) cells and treated with IL-15/RA complex.</td>
<td>58</td>
</tr>
<tr>
<td>Figure 15</td>
<td>Gestation day 12 implantation site of BALB/c Rag2(^{-/-})/Il2rg(^{-/-}) mouse treated with IL-15/RA complex without preconditioning or hematopoietic stem cell inoculation.</td>
<td>61</td>
</tr>
<tr>
<td>Figure 16</td>
<td>Growth trajectories of mice born to female BALB/c Rag2(^{-/-})/Il2rg(^{-/-}) mice inoculated with human CD34(^+) cells.</td>
<td>63</td>
</tr>
<tr>
<td>Figure 17</td>
<td>Gel electrophoresis of PCR product following amplification of DNA using a primer specific to the alpha satellite region of human chromosome 17.</td>
<td>64</td>
</tr>
</tbody>
</table>
Figure 18: Immunohistochemistry to detect human cells
List of Tables

Table 1: Immunohistochemistry protocol..........................................................31
Table 2: Distribution of 5-FU-preconditioned (25 mg/kg) female neonatal
bone marrow recipients studied.................................................................38
### List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>α</td>
<td>Alpha</td>
</tr>
<tr>
<td>β</td>
<td>Beta</td>
</tr>
<tr>
<td>γ</td>
<td>Gamma</td>
</tr>
<tr>
<td>γc</td>
<td>Common cytokine receptor chain gamma</td>
</tr>
<tr>
<td>5-FU</td>
<td>5-fluorouracil</td>
</tr>
<tr>
<td>A2M</td>
<td>Alpha 2 macroglobulin</td>
</tr>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>ACD</td>
<td>Acid citrate dextrose</td>
</tr>
<tr>
<td>B cell</td>
<td>Bursa of Fabricius-derived cell</td>
</tr>
<tr>
<td>BLT</td>
<td>Bone marrow-liver-thymus</td>
</tr>
<tr>
<td>BM</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CCL</td>
<td>Chemokine (CC motif) ligand</td>
</tr>
<tr>
<td>cGy</td>
<td>Centigray; unit of radiation equivalent to 1 rad</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
</tr>
<tr>
<td>CX3CL</td>
<td>Chemokine (CX3C motif) ligand</td>
</tr>
<tr>
<td>CXCL</td>
<td>Chemokine (CXC motif) ligand</td>
</tr>
<tr>
<td>D</td>
<td>Diameter</td>
</tr>
<tr>
<td>DAB</td>
<td>Diaminobenzidine</td>
</tr>
<tr>
<td>DB</td>
<td>Decidua basalis</td>
</tr>
<tr>
<td>DBA</td>
<td><em>Dolichos biflorus agglutinin</em></td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>Fc</td>
<td>Fragment crystallizable antibody region</td>
</tr>
<tr>
<td>Foxp3</td>
<td>Forkhead box p3</td>
</tr>
<tr>
<td>FSH</td>
<td>Follicle stimulating hormone</td>
</tr>
<tr>
<td>Gd</td>
<td>Gestation day</td>
</tr>
<tr>
<td>GnRH</td>
<td>Gonadotropin-releasing hormone</td>
</tr>
<tr>
<td>GvHD</td>
<td>Graft-versus-host disease</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>Hr</td>
<td>Hour(s)</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>HSC</td>
<td>Hematopoietic stem cell</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IFNG</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IL-15/RA</td>
<td>Interleukin 15 receptor alpha chain</td>
</tr>
<tr>
<td>IP</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>------------</td>
<td>----------</td>
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<tr>
<td>IV</td>
<td>Intravenous</td>
</tr>
<tr>
<td>IP-10</td>
<td>Interferon gamma-induced protein 10</td>
</tr>
<tr>
<td>ITAM</td>
<td>Immunoreceptor tyrosine-based activating motif</td>
</tr>
<tr>
<td>ITIM</td>
<td>Immunoreceptor tyrosine-based inhibitory motif</td>
</tr>
<tr>
<td>KIR</td>
<td>Killer immunoglobulin-like receptor</td>
</tr>
<tr>
<td>LH</td>
<td>Luteinizing hormone</td>
</tr>
<tr>
<td>LIF</td>
<td>Leukemia inhibitory factor</td>
</tr>
<tr>
<td>Ly49</td>
<td>Mouse NK cells receptors analogous to human KIRs</td>
</tr>
<tr>
<td>MAdCAM</td>
<td>Mucosal addressin cell adhesion molecule</td>
</tr>
<tr>
<td>Map</td>
<td>Mesometrial aggregate of pregnancy</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MLAp</td>
<td>Mesometrial lymphoid aggregate of pregnancy</td>
</tr>
<tr>
<td>MULT1</td>
<td>Mouse UL16-binding protein-like transcript 1</td>
</tr>
<tr>
<td>Ncr</td>
<td>Natural cytotoxicity receptor</td>
</tr>
<tr>
<td>NK</td>
<td>Natural Killer cell</td>
</tr>
<tr>
<td>NK/T</td>
<td>Natural Killer T</td>
</tr>
<tr>
<td>NKG2</td>
<td>Natural Killer cell lectin-like receptor gene 2</td>
</tr>
<tr>
<td>NOD</td>
<td>Non-obese diabetic</td>
</tr>
<tr>
<td>NOG</td>
<td>Non-obese diabetic gamma chain null</td>
</tr>
<tr>
<td>OPD</td>
<td>o-phenylenediamine dihydrochloride</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PE</td>
<td>Preeclampsia</td>
</tr>
<tr>
<td>PLGF</td>
<td>Placental growth factor</td>
</tr>
<tr>
<td>RAE-1</td>
<td>Retinoic acid early inducible gene-1</td>
</tr>
<tr>
<td>Rag</td>
<td>Recombinase activating gene</td>
</tr>
<tr>
<td>RAS</td>
<td>Renin-angiotensin system</td>
</tr>
<tr>
<td>rh</td>
<td>Recombinant human</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>SCID</td>
<td>Severe combined immunodeficient</td>
</tr>
<tr>
<td>SDF-1</td>
<td>Stromal cell-derived factor 1</td>
</tr>
<tr>
<td>T cell</td>
<td>Thymus-derived cell</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>Th</td>
<td>Helper T</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>ULBP</td>
<td>UL16-binding protein</td>
</tr>
<tr>
<td>uNK</td>
<td>Uterine Natural Killer</td>
</tr>
<tr>
<td>VCAM</td>
<td>Vascular cell adhesion molecule</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>wk</td>
<td>Week(s)</td>
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</tbody>
</table>
Chapter 1: Introduction and Review of Literature

Onset of the menstrual cycle initiates the period of reproductive competence in women. Gonadotropin-releasing hormone (GnRH), produced in the hypothalamus, causes follicle stimulating hormone (FSH) to be released from the anterior pituitary into circulation. FSH acts on the ovary, inducing follicular maturation and estrogen production. During the hormonally regulated cycle of approximately 28 days, important changes occur in the uterus. In the uterine proliferative phase, ovarian estrogen induces development of blood vessels and uterine glands within the endometrial stroma. These preparatory changes continue until approximately mid-cycle. By ~day 12, estrogen concentration reaches a threshold above which it stimulates release of luteinizing hormone (LH) from the anterior pituitary. LH contributes to terminal follicular maturation leading to ovulation. Post-ovulation, LH stimulates progesterone production in the evacuated ovarian follicle (corpus luteum). Elevated levels of estrogen and progesterone inhibit GnRH production and the consequent release of FSH and LH from the anterior pituitary. Progesterone initiates the uterine secretory phase. By LH+3, decidualization of the uterine stroma transforms the initially thin-walled uterine lining into a thick vascular bed rich in glandular secretions that promote implantation. These secretions include cytokines leukemia inhibitory factor (LIF) (Laird et al. 1997), CX3CL1 and CCL14 (Hannan et al. 2006) and IL-11 (Makkar et al. 2006). The decidua is divided into two functional layers, the superficial decidua functionalis and the deep decidua basalis.
During the secretory phase, uterine Natural Killer (uNK) cells become enriched in the decidua and become the dominant leukocytes in the uterus. These are activated lymphocytes that localize to maternal spiral arteries and uterine glands in the decidua basalis (Bulmer et al. 1991). UNK cells represent a distinct NK cell subset phenotypically characterized using flow cytometry and fluorochrome-tagged antibodies as surface CD56^{Bright}, CD16^{Dim} and CD3 negative cells. Cells of this phenotype are found in blood and represents approximately 10% of the circulating NK cell population. Gene array comparison (Koopman et al. 2003) has determined that uNK cells are functionally distinct from their circulating, phenotypically identical counterparts. Most of the differential gene expression is due to uNK cell overexpression, indicating uNK cell activation. Upregulated genes include those for surface and secreted proteins. Most surface proteins are either adhesion molecules such as L-selectin and alpha integrins or cell surface receptors such as NKG2, KIR2DL4. Secreted proteins include numerous cytokines (eg. IFNG, TNF), chemokines (eg. IL-8, IP-10) and angiogenic factors including VEGF and PLGF (Hanna et al. 2006; Koopman et al. 2003).

In the absence of pregnancy, ovarian production of estrogen and progesterone declines in the late secretory phase. The decidua functionalis and invested immune cells are shed via menstruation, ending the menstrual cycle. Deep decidua is retained as the source of regenerative cells for future cycles. Decreased levels of estrogen and progesterone permit GnRH release from the
hypothalamus and the initiation of the subsequent cycle. Cycles continue until interrupted by pregnancy and lactation, resuming thereafter, or by the onset of menopause. Uteri of menopausal women lack significant numbers of uNK cells (Hickey et al. 2005). If pregnancy is established, sustained progesterone production in the corpus luteum inhibits GnRH release from the hypothalamus, preventing ovarian follicular maturation and halting the menstrual cycle. UNK cells rapidly increase in number and remain elevated until approximately wk 20, a time coincident with completion of the chorioamniotic placenta (Bulmer and Lash 2005).

During the first trimester of human pregnancy, vascular changes occur in the endometrial branches of the uterine artery, particularly at branches known as spiral arteries. These changes in structure are thought to promote embryonic development. Trophoblast cells traverse the decidua and invade the arterial walls as the vascular smooth muscle becomes replaced by chemically unidentified amyloid/fibrinoid secretions from the trophoblast. Other trophoblasts invade the maternal spiral arteries, replacing endothelial cells and forming a lining within the vasculature. These processes change the spiral arteries into large, flaccid vessels. Modified spiral arteries are capable of high volume flow at low resistance and provide increased nutrient and gas exchange to the fetus via the placenta. In most women who develop preeclampsia and in some women who have fetal growth restriction, spiral arterial modification is incomplete. Timing of uNK cell accumulation in the decidua, their secretion of vascular endothelial
growth factor (VEGF) (Li et al. 2001) and their close association with maternal arteries suggest uNK cell involvement in the vascular changes that influence pregnancy success. Indeed, high numbers of uNK cells in the secretory phase have been associated with uterine receptivity to implantation regardless of the blastocyst’s genetic quality (Quenby et al. 2002).

Preeclampsia is a common emergency syndrome seen only in human pregnancy. It is characterized by proteinuria and elevated blood pressure after 20 wk gestation (Redman and Sargent 2005). Pathogenesis of this disease develops much earlier in pregnancy and is incompletely understood. A large recent study across six developing countries attributed 23.6% of perinatal deaths to maternal hypertensive disorders (WHO 2009). In the developed world, PE occurs in approximately 5% of pregnancies (Redman and Sargent 2005). In the uteri of women with PE, spiral arteries retain perivascular smooth muscle and thus their responsiveness to vasoconstrictive agents. This results in a diminished volume pulsatile placental flow that is associated with placental insufficiency. A preeclamptic episode also has postnatal health consequences for the mother and her offspring. These include elevated blood pressure which promotes development of cardiovascular disease (CVD) later in life (Himmelmann et al. 1993; Seidman et al. 1991; Smith et al. 2001; Smith et al. 2009a; Vatten et al. 2003; Williams 2003). At one year post-partum, CVD risk is elevated 2.5 times in the mother (Vasan et al. 2001) due to a 9 mm Hg gain in baseline blood pressure (Smith et al. 2009b). Cardiovascular risk continues to rise rapidly in following
years (Smith, GN personal communication). Offspring of women with PE are also at increased risk of developing PE (Skjaerven et al. 2005), hypertension (Tenhola et al. 2003; Vatten et al. 2003) and stroke (Kajantie et al. 2009) later in life. In 2007, human NK cells and T cells were found to express all components of the renin-angiotensin system (RAS), the major system controlling blood pressure (Jurewicz et al. 2007). More recently, women with preeclampsia were compared with normotensive women at delivery. These investigations found that peripheral blood T cells in the PE patients had high Th17 to regulatory T (Foxp3+) cell ratios (Santner-Nanan et al. 2009). Further study suggested there was no gain in Th17 cells but rather abnormally low regulatory T cells that allow expression of IL-17. This is interesting because IL-17 derived from T cells promotes hypertension in mice (Madhur et al. 2010). Studies of Th17 cells have not been conducted at the maternal-fetal interface in PE patients and there are no studies of RAS molecules in human uNK cells. Indeed, practical, legal and ethical reasons constrain studies of human immune cell functions in the uterus. Few endometrial samples are available even following early termination or at delivery. Much of what we understand about uNK cells comes from animal models, especially the mouse.

**Mouse uNK cell life cycle**

Cells of the NK cell lineage first appear in the mouse uterus at 2 wk of age. These cells remain immature and only increase in number proportional to uterine growth (Kiso et al. 1992). In adult mice, successful blastocyst implantation on gestation day (gd) 4 induces decidualization. As stromal cells of
the endometrium become large, proliferative decidual cells (Lee and DeMayo 2004), uNK cells differentiate to large, granular cells (Peel 1989). These early (gd 3-6) uNK cells express inhibitory Ly49G2 and receptors for collagen, laminin, fibronectin and mucosal vascular addressin cellular adhesion molecule (MAdCAM)-1 (Kiso et al. 1994). Gd5 uNK cells acquire surface N-acetyl-galactosamine, detected using *Dolichos biflorus agglutinin* (DBA) lectin histochemistry (Paffaro, Jr. et al. 2003). DBA lectin staining reveals numerous cytoplasmic granules that contain mucins (Kiso et al. 1992), perforin (Parr et al. 1990), serine proteases (Zheng et al. 1991), phosphatases (Peel 1989), and FasL (Kusakabe et al. 2005). Cytoplasmic granules accumulate steadily and can be used to stage mouse uNK cell maturity (Paffaro, Jr. et al. 2003). The cytoplasmic granules are not normally released until senescence, when uNK cells are post-mitotic and vacuolated (Delgado et al. 1996a).

Gd6 uNK cells concentrate in the mesometrial decidua basalis (DB) where they continue to amass. By gd8, some uNK cells form a transient lymphoid structure between the smooth muscle layers of the uterine wall. This is the mesometrial lymphoid aggregate of pregnancy (MLAp). Each MLAp surrounds vessels that run from the uterine artery toward an implantation site (Croy et al. 2006). Although found at high numerical density within the MLAp, most uNK cells are located within DB (Paffaro, Jr. et al. 2003). UNK cells remain proliferative in the MLAp but are post-mitotic when widely distributed throughout DB and peak in number about midgestation (gd 10-12). From gd13, uNK cells decline in number
steadily toward term (gd19-20). Some uNK cells remain at term and are shed with the placenta (Delgado et al. 1996a; Peel 1989).

**UNK cell activation**

In humans, mice and other species, NK cell activation is governed by inhibitory and activating receptor ligation. Naive NK cells are inactive. During early mouse pregnancy, dramatic changes occur in uNK cell localization and function. These fluctuations appear to be responses to changes in estrogen and progesterone levels, although mouse uNK cells do not possess steroid receptors (Borzychowski et al. 2003; Oh and Croy 2008). Thus, important changes in other cell types in the uterus must occur that promote uNK cell activity.

A key progesterone-related response is induction of IL-15 synthesis in decidual stromal cells from gd6 to gd11 (Ye et al. 1996a). In humans and mice, IL-15 is essential for both systemic NK cell and decidual NK cell differentiation (Kennedy et al. 2000; Mrozek et al. 1996; Ohteki et al. 1997; Puzanov et al. 1996; Ye et al. 1996b), activation (Ashkar et al. 2000a; Carson et al. 1997) and survival (Barber and Pollard 2003; Carson et al. 1997). The IL-15 receptor is a heterotrimer composed of the IL-15RA chain and the shared IL-2Rβ and IL-2Rγ chains (Anderson et al. 1995; Leclercq et al. 1996). The IL-15RA chain is structurally similar to the IL-2Rα chain (Giri et al. 1995). The IL-2 receptor also forms a trimer of α, β and γ chains. The γ chain is a structural component of a
family of cytokine receptors including IL-2, -4, -7, -9, -15 and -21 (reviewed in (Rochman et al. 2009)).

Studies of mice transfected with a deletion mutant of human IL-15RA chain revealed a lack of human IL-15 at the cell surface, establishing the necessity of the α chain but not the β chain for membrane-bound IL-15 (Dubois et al. 2002). IL-15 signalling is unusual in that the receptor α chain presents IL-15 to neighbouring cells in trans. Only trans-presented IL-15 is capable of stimulating T cell proliferation (Dubois et al. 2002) and differentiation of NK and NK/T cells (Ashkar et al. 2003; Koka et al. 2004; Schluns et al. 2004).

NK cell activation is incremental. During initial decidualization, endometrial stroma cells transiently produce IL-18, a known NK cell activating ligand that enhances IL-12-driven IFNG production by NK cells (Walker et al. 1999). IFNG is the hallmark cytokine of NK cells. From gd6 to gd14, uNK cells are the sole cells that produce and sustain endometrial IL-18 (Lash et al. 2006b; Zhang et al. 2003).

**NK cell receptors and their ligands**

In addition to activation by cytokines, antigen recognition further modulates NK cell activation. The antigen recognition receptors on human NK cells are known as killer immunoglobulin-like receptors (KIR). In mice, these include NKG2D and Ly49 receptors. It is widely speculated that the ligands for
these are found on trophoblast. For some receptors this has been demonstrated. For example, RAE-1 has been localized to spiral arteries, blastocysts and trophoblasts.

Killer Immunoglobulin-like Receptors (KIR)

Human NK cells possess unique Ig superfamily receptors (KIRs). These are highly polymorphic receptors that recognize human MHC-I antigens (HLA) expressed by all nucleated human cells. KIRs are functionally classified as activating or inhibitory and interaction between KIR and HLA regulates NK cell activation. Extensive polymorphism in classical HLA molecules and KIRs yields multiple possible genotype coupling combinations. Of particular interest is the interaction during pregnancy between maternal NK cell KIRs and fetal HLA-C. HLA-C is the only classical MHC antigen expressed by human trophoblast (King et al. 2000). In pregnancies complicated by PE or recurrent spontaneous abortion (RSA), increased frequencies of KIR-AA:HLA-C2 coupling is reported (Hiby et al. 2008; Hiby et al. 2004). This suggests that uNK cell recognition of fetal antigen is central to pregnancy success. The AA KIR genotype is unique in that it lacks activation motifs, further implicating uNK cell activation in pregnancy success.

NKG2 receptors

NKG2 proteins are C-type lectins found in both humans and mice. Most NKG2 family proteins form heterodimers with CD94 (Gunturi et al. 2004). Like
Ly49 receptors, both activating and inhibitory varieties are found. In humans, NKG2/CD94 receptors recognize human leukocyte antigen HLA-E. HLA are divided into classical MHC-Ia (HLA-A, -B, -C) and non-classical MHC-Ib (HLA-E, -F, -G) groups. Classical HLA genes are highly polymorphic (Davidson et al. 1985; Ploegh et al. 1981) while non-classical MHC-Ib genes are highly conserved (Shawar et al. 1994).

Notably, NKG2D, an activating receptor involved in recognition of virally infected cells, is unique in that it does not dimerize with CD94. Rather, it associates with adapter proteins DAP10 and DAP12 for NK cell activation (Gilfillan et al. 2002). NKG2D binds to a family of proteins structurally similar to MHC-I antigens. In humans, NKG2D ligands include MICA, MICB, ULBP1, ULBP2, ULBP3, and ULBP4. In mice, NKG2D ligands include RAE-1α, RAE-1β, RAE-1γ, RAE-1δ, RAE-1ε, H60, and MULT1 while humans ligands include (reviewed in (Lanier 2005)). In mice, RAE-1 is expressed by spiral arteries (Xie et al. 2005), providing an additional activation stimulus for uNK cells.

Ly49 receptors

Mouse NK cells recognize cell surface ligands via several receptors. The rodent Ly49 receptor family members recognize MHC class I antigens expressed by all nucleated cells. This forms the basis of the missing self hypothesis in the mouse. The number of Ly49 genes varies between mouse strains and there is evidence for extensive allelic polymorphism (Mehta et al. 2001). Most Ly49
genes encode inhibitory receptors with immunoreceptor tyrosine-based inhibitory motifs (ITIM) in their cytoplasmic domains. Others such as Ly49D and Ly49H in C57BL/6 mice encode activating receptors that associate with the adapter molecule DAP12 conjugated to immunoreceptor tyrosine-based activating motifs (ITAM) (Smith et al. 1998). Individual NK cells transcribe on average between 1 and 4 Ly49 genes in fetal and adult NK cell populations (Kubota et al. 1999).

**Natural cytotoxicity receptors**

NCRs are activating receptors that recognize non-MHC ligands and are central to the recognition and killing of tumour and virally-infected cells (Cantoni et al. 1999; Pende et al. 1999a; Sivori et al. 1997). Other NCRs have been identified, including Ig superfamily members NKp30, NKp44, NKp46 (Ncr-1 in mice) and 2B4 as well as the C-type lectin NKp80. NKp46 and NKp30 are selectively expressed by all NK cells (Pende et al. 1999b) while NKp44 is only expressed by activated cells (Vitale et al. 1998). NKp80 is expressed by nearly all naive and activated NK cells (Vitale et al. 2001). NKp80 and 2B4 appear to act as co-receptors, contributing to activation only when co-engaged with a triggering receptor (Sivori et al. 2000).

**UNK cell functions**

I. Trophoblast invasion

NK cells were originally named for their ability to kill tumor cells without prior sensitization (Kiessling et al. 1975). Initial ideas about uNK cell function
focused on regulating trophoblast invasion via lytic mechanisms (Croy 1994). However, studies of mice with depleted or absent NK cells reveal normal trophoblast invasion during pregnancy (Guimond et al. 1998a). Also, despite abundant cytoplasmic granulation, ability to kill target cells through lytic mechanisms is reduced in human (Jones et al. 1997; Ritson and Bulmer 1989) and mouse (Linnemeyer and Pollack 1991) uNK cells. In mice, uNK cells do not appear to restrict trophoblast invasion. Rather, this role is attributed to decidua (Esadeg et al. 2003). In humans, where trophoblast invasion into maternal decidua and its vessels is more extensive than in mice, uNK cells have been shown to promote trophoblast invasion using an in vivo subcutaneous xenograft model in nude and Rag2\(^{-/-}\)/Il2rg\(^{-/-}\) mice (Grummer et al. 1999; Hanna et al. 2006).

Human uNK cells stimulated with IL-15 produce IL-8 and IP-10, cytokines that promote migration of primary trophoblast cells obtained from elective human pregnancy terminations at 8-10 wk gestation (Hanna et al. 2006), when trophoblast invasion is maximal (Lash et al. 2006a). Although 8-10 wk trophoblast was responsive to these cytokines, human uNK cells do not produce them before wk 12-14 (Lash et al. 2010). Therefore, human uNK cells may provide IL-8 and IP-10 required for trophoblast maintenance rather than establishment of trophoblast invasion. Information on functions of early human uNK cells is lacking.
II. Vascular remodeling

Mice that lack NK cells have significantly lower decidual IFNG (90% reduction) (Ashkar et al. 2003; Guimond et al. 1998a). In these mice (tgε26, Rag2\(^{-/-}\)/γc\(^{-/-}\)), decidual spiral arteries retain their pre-pregnancy perivascular smooth muscle coats (Greenwood et al. 2000), a distinguishing feature shared with PE in humans. From gd6 onward, decidua is also oedematous and hypomorphic. Exogenous mouse IFNG (Ashkar et al. 2000b) or a downstream IFNG target, human α2M (He et al. 2005), drives spiral arterial remodelling and normalizes decidua in the absence of systemic and uterine NK cells. Thus, in mice, uNK cell-derived IFNG is required for vascular smooth muscle reduction while in humans this process is normally described as a function of intravascular trophoblast. Recent studies of very early human termination samples suggest that initiation of vascular remodelling occurs prior to invasion of the vessel by trophoblast; uNK cells are candidate contributors (Bulmer and Lash 2005; Dunk et al. 2008; Hanna et al. 2006; Smith et al. 2009c).

III. Cytokine production

Cytokines are proteins produced by cells of the immune system that modulate immune cells in an autocrine, paracrine and/or endocrine manner (Cohen et al. 1974). The cytokine milieu of healthy men and non-pregnant women is described as type 1, a state that promotes cell-mediated immunity. UNK cells produce several pro-inflammatory type 1 cytokines (IFNG, IL-12, IL-18) that are thought to promote decidualization and blastocyst implantation in
humans (De et al. 1993; Gnainsky et al. 2010; van Mourik et al. 2009; von et al. 1999) and in mice (Ashkar et al. 2000b; Whiteside et al. 2003). Post-implantation, systemic immunity shifts to a type 2 anti-inflammatory immune bias that promotes humoral immunity (Dudley et al. 1993). This shift is reflected in the uterus, where type 2 cytokine levels (IL-4, IL-5, IL-10, TGFβ) increase in both women (Sargent et al. 2006) and mice (Krishnan et al. 1996; Lin et al. 1993). In women, uNK cells are the primary source of uterine type 2 cytokines (Borzychowski et al. 2005). Furthermore, women with PE have increased plasma levels of type 1 cytokines including TNFα and IL-1β (Kupferminc et al. 1996; Kupferminc et al. 1994), implicating uNK cells in the pathology of PE.

IV. Angiogenesis

In women and mice, uNK cells produce potent angiogenic molecules. VEGF produced by neurons is the primary guidance molecule in developmental angiogenesis. In decidualizing endometrium, VEGF is produced by uNK cells (Lash et al. 2006b; Xie et al. 2005) and trophoblast (Cooper et al. 1995; Shweiki et al. 1993). Distributed through DB, uNK cells may guide branches from maternal spiral arteries to the randomly distributed sites of implantation and reinforce the neoangiogenic VEGF gradients toward trophoblast.

**UNK cell localization is a highly ordered process**

Pregnancy induces systemic changes in the mouse immune system. Numbers of lymphocytes in primary lymphoid organs (bone marrow, thymus)
decrease, while secondary sites including the uterus gain new immune cell populations (Chantakru et al. 2002). L-selectin binds addressins on activated vascular endothelial cells, tethering circulating lymphocytes and enabling closer interactions (Giblin et al. 1997). Alpha-4 integrins on lymphocytes mediate interactions with additional vascular addressins including MAdCAM-1 (α4β7 integrin) and VCAM-1 (α4β1 integrin). In vitro studies indicate L-selectin and α4 β7 integrin are involved in recruiting human and mouse pre-uNK cells from blood (Chantakru et al. 2002) although L-selectin interactions are not essential (Xie et al. 2005). UNK cells localize to DB, where endothelium expresses VCAM-1 and not MAdCAM-1 (Fernekorn et al. 2004). This vascular expression profile and uNK cell accumulation is not seen in lateral or anti-mesometrial decidua or in non-decidualized uterine segments between implantation sites (Peel 1989).

Because they are soluble factors, chemokines are more readily monitored using blood samples than vascular addressins which require tissue samples. Chemokine gradients control immune cell positioning in tissues. Human CD56Bright cells express CXCR4, which binds the stromal ligand CXCL12, previously called stromal cell-derived factor (SDF)-1α (Hanna et al. 2006). CXCL12 is strongly expressed by human intravascular trophoblast cells (Hanna et al. 2003) throughout the first trimester (Red-Horse et al. 2001) and may attract uNK cells to blood vessels. Endothelial progenitor and stem cell retention in bone marrow and thymus also utilizes CXCR4:CXCL12 (D’Apuzzo et al. 1997; Hernandez-Lopez et al. 2002). Precursor uNK and endothelial cells may have
overlapping affinities for CXCL12, promoting co-localization. CXCL14 is strongly expressed by mid and late secretory uterine glandular epithelium. \textit{In vitro}, CXCL14 promotes uNK cell migration and is likely responsible for uNK cell clustering near uterine glands (Mokhtar et al. 2010).

**Humanized mouse models**

Animal models have been essential for investigations of post-implantation stages of pregnancy. While humans and mice share many common reproductive features, important differences exist between analogous cells (differential gene expression, phenotype and function). To study human haematopoietic stem cell (HSC) differentiation and function \textit{in vivo} for other biological and medical questions, small populations of human immune cells have been established and studied in immune compromised mice. Human HSC may be isolated and purified from peripheral blood and bone marrow but higher HSC frequencies are found in human umbilical cord blood or embryonic tissues. For legal and ethical reasons, cord blood is currently the preferred source of HSC. Upon successful engraftment, the presence of such a human cell population in a genetically distinct host is referred to as chimerism. Host mice are often referred to as “humanized” or as human-to-mouse chimeras. Normal wild type (+/+) mouse strains have been examined as hosts for human cells and tissues after the recipients are immune suppressed by sub-lethal irradiation (950 cGy). This approach is inefficient and is no longer used. Rather, murine hosts with spontaneous or targeted gene mutations are preferred.
Nude mouse

Despite reports of hairless mice as early as 1856, the spontaneous recessive mutation of Foxn1\textsuperscript{nu}, a gene involved in multiple processes including hair growth, was not reported until 1966 in C57BL/6 mice (Flanagan 1966). In addition to failing to develop hair from 2 wk of age, mice of this genotype are athymic and therefore lack T cells (Pantelouris and Hair 1970). Early attempts to engraft human HSC into these mice were unsuccessful (Ganick \textit{et al.} 1980). Since only T cell-dependent processes are disrupted in these mice, their use as HSC recipients is limited, though they have been instrumental in studies of immune deficiency, tumor xenograft biology and drug responsiveness. These mice are not suitable for study of human CD56\textsuperscript{Bright} NK cell activity against spiral arteries because they retain endogenous NK cell activity (Fodstad \textit{et al.} 1984).

SCID mouse

In 1983, the Prkdc\textsuperscript{scid} spontaneous mutation was discovered in CB17 mice (Bosma \textit{et al.} 1983). Severe combined immunodeficient (scid) mice lack most mature T and B cells, though small populations of both cells develop as the animal ages, a feature known as “leakiness”. These were the first mice successfully engrafted with human HSC isolated from peripheral blood (Mosier \textit{et al.} 1988), cord blood (Yeoman H 1993), bone marrow (Lapidot \textit{et al.} 1992) and fetal tissues (McCune \textit{et al.} 1988). Engrafted cells differentiated human lymphoid cells, though in very low numbers due primarily to mouse NK cell activity (Christianson \textit{et al.} 1996). DNA repair mechanisms are disrupted in scid mice,
causing increased radiosensitivity and shortening of life (Fulop and Phillips 1990). These mice also develop spontaneous lethal thymomas at high frequency after 15-20 wk of age (Custer et al. 1985), preventing long term studies. The preserved mouse NK cells that modify spiral arteries precluded use of this genotype for my research.

Non-obese diabetic SCID (NOD-SCID) mouse

NOD is an inbred mouse strain with multiple immune aberrations, best known for spontaneous development of type 1 diabetes. Crossing NOD and CB17-scid mice generated the NOD-scid mouse (Prochazka et al. 1992). NOD-scid mice have lower innate immunity and NK cell activity and appeared to be superior recipients for human HSC engraftment. Also, since they lack adaptive immunity, they do not develop diabetes which extends lifespan (Prochazka et al. 1992). NOD mice are not suitable for study of human CD56Bright xenoengraftment because they also retain endogenous NK cells, including uNK cells and additionally have abnormal spiral artery morphology (Burke et al. 2007). Furthermore, the IL-15 gene is defective in NOD mice, resulting in disrupted NK cell function (Suwanai et al. 2010; Zekavat et al. 2010).

Recombinase activating gene (Rag) knockout mouse

In 1992, targeted genetic mutation of the Rag1 (Mombaerts et al. 1992) and Rag2 (Shinkai et al. 1992) genes was described in C57BL/6 mice. This is the same gene locus spontaneously mutated in scid mice. Rag1 and Rag2 knockout
mice both lack virtually all mature T and B cells throughout life but do not manifest the leaky phenotype seen in scid mice. *Rag1* and *Rag2* knockout mice retain high levels of innate immunity including NK cell activity (Greiner *et al.* 1998), restricting human HSC engraftment. DNA repair is unaffected in either *Rag* mutation, preserving lifespan. These mice are not suitable as they retain endogenous uNK activity and undergo spiral arterial remodelling (Burke *et al.*, submitted).

Interleukin-2 receptor gamma chain (*Il2rg*/*−−*) knockout mouse

Interleukins (IL) are cytokines involved in cellular proliferation, differentiation and survival. *Il2rg*/*−−* mice were first generated in 1995 via targeted mutation of the common cytokine receptor gamma chain (DiSanto *et al.* 1995). The gamma chain is a component of receptors for IL-2 (Noguchi *et al.* 1993a), -4 (Russell *et al.* 1993), -7 (Noguchi *et al.* 1993b), -9 (Kimura *et al.* 1995), -15 (Giri *et al.* 1994) and -21 (Asao *et al.* 2001) and is an absolute requirement for IL-15 ligand binding and signalling (Giri *et al.* 1994). Since NK cells require IL-15 signalling for differentiation and survival, *Il2rg*/*−−* mice lack NK cells (DiSanto *et al.* 1995). T and B cell development is also impaired, although progenitors are present. These mice are difficult to breed and were only sustained through further crosses. *Il2rg*/*−−* mice lack systemic and uterine NK cells and do not undergo spiral arterial remodelling (Croy *et al.* 1997). Subsequent breeding has produced multiple genotypes on various inbred genetic backgrounds. Combined
mutations of particular value include $Rag2^{+/+}/\gamma c^{+/+}$, scid-$\gamma c^{+/+}$, NOD-scid-$\gamma c^{+/+}$, NOD-\(Rag1^{+/+}/\gamma c^{+/+}\) and NOD-\(Rag2^{+/+}/\gamma c^{+/+}\) mice.

*Rag2* gamma chain double knockout ($Rag2^{+/+}/\gamma c^{+/+}$) mouse

In 1998, crossing of *Il2rg*\(^{−/−}\) and *Rag2*\(^{+/+}\) mice was reported, generating the first $Rag2^{+/+}/\gamma c^{+/+}$ double knockout in C57BL/6 mice (Goldman et al. 1998). Lacking all lymphocytes including NK cells, these mice were quickly adopted as preferred human HSC recipients. Although host immunity is practically absent, engraftment of human HSC and subsequent differentiation is highly variable and yields very few NK cells. Most human cells that differentiate in recipient mice are B cells and T cells (Goldman et al. 1998). When the combined mutation was back-crossed to BALB/c mice and human HSC were administered in the neonatal period, improved engraftment and consistent NK cell differentiation was reported (Traggiai et al. 2004). Recently, promotion of NK cell differentiation in humanized mice has been improved by administering recombinant human IL-15: IL-15RA conjugate. This mimics *trans* presentation by IL-15RA–bearing cells found *in vivo* (Huntington et al. 2009a).

Preconditioning of mice for HSC engraftment

With any humanized mouse model, engraftment success requires ablation of residual mouse hematopoiesis in bone marrow. Conventionally, this is accomplished via sub-lethal irradiation. However, this renders the recipient reproductively sterile and precludes pregnancy studies. We previously used 5-
fluorouracil (5-FU) to effectively ablate 4 wk old host marrow and facilitate high levels of engraftment of mouse HSC engraftment including differentiation of functional mouse uNK cells in pregnancy. I hypothesized that 5-FU treated neonatal BALB/c $Rag2^{-/-}/\gamma c^{-/-}$ mice inoculated with human cord blood CD34$^+$ cells and supplemented with human IL-15:IL-15RA complex would differentiate human uNK cells that would home to spiral arteries and display their functional capability in vascular remodelling. Because BALB/c $Rag2^{-/-}/\gamma c^{-/-}$ mice have no endogenous systemic or uterine NK cells and thus do not undergo spiral arterial remodelling, analysis of spiral arteries in implantation sites would provide a read out of human uNK cell functions.
Chapter 2: Materials and Methods

Animals

C57BL/6 Rag2\(^{-/-}\)/Il2rg\(^{-/-}\) (previously termed Rag2\(^{-/-}\)/γc\(^{-/-}\)) mice deficient in NK, T and B lymphocytes and immune-competent C57BL/6J (B6) mice were bred under barrier husbandry in the Isolation Unit at the Ontario Veterinary College, University of Guelph, Guelph, Ontario. The former were derived from breeding pairs provided by Dr. J. P. Di Santo, Pasteur Institute, Paris, France. The latter were non transgenic mice culled from a breeding program employing animals provided by Dr. M. Dustin, New York University School of Medicine, New York, NY (Lindquist et al. 2004). BALB/c Rag2\(^{-/-}\)/Il2rg\(^{-/-}\) mice were generated from original breeding pairs provided by Dr. Mamuro Ito at the Central Institute for Experimental Animals, Kawasaki, Japan. BALB/c Rag2\(^{-/-}\)/Il2rg\(^{-/-}\) mice were bred under barrier husbandry in a dedicated facility at Queen’s University (BioBubble\(^{TM}\)). At both sites, autoclaved water was acidified (pH 2.8) or supplemented with apo-sulfonamide on alternate wks. The barrier facilities were maintained at 20\(^\circ\)C, air was exchanged 15x per hr and a 14:10 hr dark:light cycle was employed. Additionally, sentinel mice were serologically-negative for common viral and mouse pathogens (2-4x/year; Charles River Laboratories Comprehensive Serology Panel, Pointe-Claire, QC). Wild type BALB/c mice were purchased from Charles River and were bred in conventional (non-sterile) animal care facility at Queen’s University. All mice were housed in autoclaved microisolator cages containing nesting materials (Nestlets\(^{TM}\), Ancare, Bellmore, NY), and had access to sterile mouse chow (Charles River, Pointe-Claire, QC))
and water *ad libitum*. All procedures were conducted in full compliance with local Animal Care Committee guidelines (University of Guelph, Queen’s University).

**Animal Procedures**

For breeding, adult (8+ wk) female and male mice were housed together and then the females were examined daily for the presence of a copulatory plug. At detection of the copulatory plug, a gestational age of gd0.5 was assigned and males were removed. Some females were euthanized on timed gestational days; others were used to deliver and rear litters. During the period of lactation, pups were not handled except to record weights (weekly) or to administer treatments. Handled mice were promptly returned to the nursing dam. Pups were weaned at 3 wk of age and sexed by urogenital distance inspection. Females and males were transferred to separate cages with a maximum of 4 total occupants. At the University of Guelph, euthanasia was carried out via CO₂ asphyxiation followed by cervical dislocation. At Queen’s University, euthanasia (BALB/c study) was carried out via cervical dislocation. Animal carcasses were removed from the barrier unit for dissection in the research laboratory and disposal.

**Tissue fixation and processing**

Uterus, spleen, liver and kidneys were dissected and fixed in fresh 4% paraformaldehyde (Fisher Scientific, Whitby, ON, Canada) in 0.1M phosphate buffer. Uteri from animals killed at gd8 or earlier were fixed for 6 hr and uteri from animals killed at later gestational times and all other tissues were fixed for 8 hr.
Following fixation, tissues were moved to 70% ethanol and processed in an automated ATP1 tissue processor (Triangle Biomedical Sciences, Inc., Durham, NC, USA) then embedded in paraffin using standard methodology.

**Preconditioning of graft recipients**

Previous studies established that the optimal 5-FU preconditioning dose for 4 wk old mice is 150 mg/kg bodyweight (Guimond et al. 1998b). To determine the appropriate dose for neonates, mated C57BL/6 Rag2\(^{-/-}\) Il2rg\(^{-/-}\) females were allowed to deliver. On the morning of delivery, cages were placed in a Class II laminar flow hood under a heat lamp and the dam was temporarily removed to a clean cage away from sight of the pups. The pups were enumerated and weighed, then received an intra-peritoneal injection of 5-FU (Mayne Pharma, Montreal, QC, Canada) and were returned to the nest. The various doses of the drug were administered in a 20 μL volume of sterile saline using a 30 gauge needle on a Hamilton syringe. The dam was returned to her pups immediately upon completion of injection of the litter. The pups were monitored closely over the next 48 hr by animal care staff to monitor survival. One litter was selected for titration of 5-FU, using two pups per group and dosages of 125 mg/kg, 75 or 25 mg/kg. From these studies, a dose of 25mg/kg was selected and used for the BALB/c series of neonatal studies.
Preparation of bone marrow suspensions

On the days of reconstitution, C57BL/6 mice (n=32 used in total) were euthanized using CO₂ followed by cervical dislocation. Femurs and humeri were immediately dissected and placed in sterile saline in a dish resting on ice. The condyles of the long bones were removed with scissors; then the marrow cavities were flushed into a clean receptacle using a tuberculin syringe filled with 0.6 ml sterile saline. The flushed marrows were centrifuged gently (300× g) for 2 min 4°C to pellet cells. The supernatant was removed, cells were re-suspended in 1 ml sterile phosphate buffered saline pH 7.4 and an aliquot was stained for viability using 0.4% trypan blue dye exclusion and cell concentration was calculated using a hemacytometer. Cells were then re-centrifuged, the supernatant was removed and cells were resuspended in a volume of sterile PBS to provide the final concentration (usually 5 x10⁸ cells/ml). The prepared cell suspension was drawn into a Hamilton syringe fitted with a 26 gauge needle. Decreased needle gauge (increased bore diameter) was selected to prevent cell shearing.

Preparation of enriched human CD34⁺ cell suspensions

Human umbilical cord blood was obtained from 10 placentae from singleton elective caesarean sections at Kingston General Hospital. Blood was collected in acid citrate dextrose (ACD) blood tubes and immediately placed on ice for transport to Queen’s University. All subsequent work was carried out in a laminar flow hood. CD34⁺ cells were enriched using RosetteSep™ (Stem Cell
Technologies Inc, Vancouver, BC) according to manufacturer’s protocol to remove lineage-committed cells (negative selection). Flow cytometric analysis of 30,000 cells for CD34 (CD34 antibody [B-C34] (FITC); Abcam Inc, Cambridge, MA) revealed ~60% CD34+ cells in the enriched suspension. Viable cell number was determined using 0.4% trypan blue dye exclusion and a hemacytometer. If cells were being inoculated on the day of acquisition and enrichment, they were centrifuged and resuspended to a concentration of 2x10^7 cells per ml in 37°C PBS. Otherwise, enriched fractions were resuspended to a concentration of 5x10^7 cells/ml in 10% DMSO/10% FBS in PBS and placed in a container of pre-chilled (-80°C) 2-bromomethanol (isopentane) and stored at -80°C. On the day of inoculation, frozen CD34+ cells were thawed in a 37°C water bath, diluted drop-wise with 15 ml warm (37°C) PBS and immediately centrifuged at 500 g for 10 minutes followed by 2 washes with 15 ml of 37°C PBS. A 20 µl aliquot of the thawed cells was stained with 0.4% trypan blue and viable cell number was determined. Thawed cells were then resuspended to a final concentration of 2x10^7 cells/ml.

**Inoculation Procedures**

Syngeneic mouse bone marrow inoculation of *Rag2^−/−/Il2rg^−/−* mice

Bone marrow inoculations were performed under laminar flow on 48-72 hr old *Rag2^−/−/Il2rg^−/−* mice that survived 5-FU treatment (some C57BL/6; all BALB/c). In all inoculations, a dose of 5 x 10^6 bone marrow cells (selected from our previous studies of 4 wk old transplants) was given in a 20 µL volume to each
litter member. To address the optimal site for neonatal inoculation, some C57BL/6 \textit{Rag2}\(^{-/-}\)/\textit{Il2rg}\(^{-/-}\) neonates were inoculated intrasplenically; all other mice were inoculated intrahepatically (including all BALB/c \textit{Rag2}\(^{-/-}\)/\textit{Il2rg}\(^{-/-}\)). Dams were returned to the litters immediately. At 3 wk of age, surviving pups were sexed and weaned. Usually only females were raised to adulthood.

From inoculations of C57BL/6 \textit{Rag2}\(^{-/-}\)/\textit{Il2rg}\(^{-/-}\) mice, 19 inoculated adult females were available for breeding to assess engraftment of uNK progenitor cells. 12 inoculated females became pregnant and were euthanized at gd6, 8, 10, 12 and 13. From inoculations of BALB/c \textit{Rag2}\(^{-/-}\)/\textit{Il2rg}\(^{-/-}\) mice, 5 inoculated adult females were available for breeding and became pregnant. All inoculated BALB/c \textit{Rag2}\(^{-/-}\)/\textit{Il2rg}\(^{-/-}\) mice were euthanized on gd12. Uterus, spleen, liver and kidneys were collected from all inoculated mice.

Human CD34\(^+\) cell inoculation

Neonatal BALB/c \textit{Rag2}\(^{-/-}\)/\textit{Il2rg}\(^{-/-}\) mice were weighed individually and preconditioned at 24 hr of age with 25 mg/kg 5-FU. They were inoculated with human CD34\(^+\) cell suspension on the following day. Each pup received \(10^6\) cells intrahepatically in a 50 \(\mu\)l volume. CD34\(^+\) cell-inoculated mice were weighed weekly until 8 wk. At adulthood (8+ wk), inoculated female mice were paired with unmanipulated BALB/c \textit{Rag2}\(^{-/-}\)/\textit{Il2rg}\(^{-/-}\) males for mating. Inoculated mated females were then either allowed to deliver or euthanized at timed gestational points.
From 6 wk of age, 5 female BALB/c $\textit{Rag2}^{-/-} \textit{Il2rg}^{-/-}$ mice that received human CD34$^+$ cells as neonates were given weekly 100 µl injections (IP) of freshly conjugated recombinant human IL-15/RA complex (2.5 µg rhIL-15 + 7.5 µg rhIL-15RA-Fc) in PBS. At 7 wk, they were paired with unmanipulated BALB/c $\textit{Rag2}^{-/-} \textit{Il2rg}^{-/-}$ males for mating. Of these mice, 4 became pregnant. 2 additional unmanipulated BALB/c $\textit{Rag2}^{-/-} \textit{Il2rg}^{-/-}$ females were given only the human IL-15/RA complex for control purposes. Both of these mice mated and were pregnant when sacrificed at gd12.

**Histological evaluation of syngeneic bone marrow-inoculated C57BL/6 $\textit{Rag2}^{-/-} \textit{Il2rg}^{-/-}$ and BALB/c $\textit{Rag2}^{-/-} \textit{Il2rg}^{-/-}$ mice**

Twelve C57BL/6 mice mated and were euthanized at gd 6, 8, 10, 12, or 13. Seven of the adult treated females did not conceive over a mating period of 35 days. Five BALB/c mice mated and were euthanized at gd12. Pregnant uterus, spleen, liver and kidney were dissected from each mouse and processed as outlined above. Sections of spleen, liver and kidney were stained with hematoxylin and eosin for routine histopathology. Seven micron serial sections were cut from all uteri. When available, serial sections were studied from each of a minimum of three implantation sites. Values for uNK cells in gd-matched normal C57BL/6 mice were derived from new analyses of archival samples we previously described (Ashkar et al. 2000a). These tissues were used to estimate the extent to which graft-derived cells had achieved normal repopulation of recipients’ implantation sites.
To identify mouse uNK cells, slides were stained with periodic acid Schiff’s (PAS) reagent. This reacts with glycoproteins found in cytoplasmic granules of mouse uNK cells. Eleven serial sections spaced at least 7 sections (49µm) apart were stained. 49 µm exceeds the diameter of most uNK cells and thus prevents duplicate counting. Using a Zeiss AxioImager M1 motorized microscope (Carl Zeiss Canada Ltd), uNK cells were enumerated in the MLAp (when present) and in the decidua basalis. A 400x magnification field of view (37,500 µm²) was enumerated and uNK cells/mm² was calculated for each site. Field area was adjusted to omit areas of vascular wall and lumen. Only cells in which a nucleus was clearly visible in the plane of section were enumerated.

To evaluate the extent of spiral arterial remodelling, spiral arteries were identified in mid-sagittal sections of implantation sites stained with hematoxylin and eosin. These sections alternated with those used for uNK cell enumeration. Spiral arteries were located in the decidua basalis and maximum outside and luminal diameters (D) of at least 3 round spiral arteries were measured in each of 11 sections that were again separated by 49 µm. The ratio of $D_{\text{Wall}}:D_{\text{Lumen}}$ was calculated to index the extent of spiral arterial remodelling. The means of 33 measurements per implantation site were calculated and pooled with means of other gestation-matched implantation sites.
**DNA isolation and polymerase chain reaction (PCR)**

Genomic DNA was extracted from liver of gd10 human CD34$^+$ cell-inoculated *Rag2$^{-/-}$Il2rg$^{-/-}$* mice and from human placenta using Qiagen QIAamp DNA Blood Mini Kit. The resulting genomic DNA (500ng) was used as a template for PCR. Sequences of primers were as follows: human chromosome 17-specific α-satellite sequences, 5’- ACACTCTTTTTGCAGGATCT-3’ (forward) and 5’- AGCAATGTGAAACTCTGGGA-3’ (reverse); PCR was performed under the following conditions: 94°C for 3 minutes (1 cycle); 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 1 minute (33cycles); and 72°C for 10 minutes (1 cycle). PCR products were separated on 1.0% agarose gel and visualized by ethidium bromide staining.

**Immunohistochemistry**

Immunohistochemistry was used in an attempt to identify human cells in implantation sites of human CD34$^+$ cell-inoculated female mice (Table 1). Three antibodies were used: mouse anti-human mitochondria [MTC02] (Abcam, ab80649), monoclonal mouse anti-human CD45 common leukocyte antigen (Dako, M0701), and mouse anti-human nuclei (Millipore, MAB1281). Titrations and positive reactivity were first established by staining 5 µm sections of frozen or paraffin-embedded human placenta and human tonsil. Additionally, implantation sites from human CD34$^+$ cell-inoculated mice were stained with DBA lectin according to published protocols (Croy *et al.* 2010), which reacts with *N*-acetyl-galactosamine on mouse uNK cell membrane and cytoplasmic granules.
Table 1: Immunohistochemistry protocol

1. Slides deparaffinized in 2 changes of xylene for 10 minutes each
2. Slides hydrated in graded ethanol series; 2 minutes each:
   - 100%, 95%, 70%, 0.1M PBS pH 7.4
3. Incubation for 30 minutes at RT in 3% peroxidase quench prepared in 0.1M PBS pH 7.4
4. Sections washed 2-3 times with 0.1M PBS pH 7.4
5. Antigen retrieval
6. Sections washed 2-3 times with 0.1M PBS pH 7.4
7. Sections circled using waterproof pen
8. Sections incubated for 30 minutes at RT with ~100 µl commercial serum-free protein block (Dako North America Inc., Carpinteria, CA, USA)
9. Sections covered with ~100 µl blocking solution (negative control) or primary antibody working solution and placed in a humidified chamber at 4°C overnight
10. Sections washed 2-3 times with 0.1M PBS pH 7.4
11. Sections incubated in ~100 µl secondary antibody working solution (when applicable) for 30 minutes at RT
12. Sections washed 2-3 times with 0.1M PBS pH 7.4
13. Sections incubated in Extravidin-peroxidase™ (Dako North America Inc., Carpinteria, CA, USA) for 30 minutes at RT
   - 1:80 dilution in commercial Antibody Diluent (Dako North America Inc., Carpinteria, CA, USA)
14. Detection using commercial DAB kit (Dako North America Inc., Carpinteria, CA, USA)
15. Sections washed 2-3 times with 0.1M PBS pH 7.4
16. Slides dehydrated in graded ethanol series; 2 minutes each
   - 70%, 95%, 100%
17. Slides moved to fresh 100% ethanol for 10 minutes
18. Slides cleared in 2 changes of xylene for 10 minutes each
19. Slides mounted with coverslips using Permount™ (Fisher Scientific Company, Ottawa, ON)

<table>
<thead>
<tr>
<th>Primary antibody + titration</th>
<th>Antigen retrieval</th>
<th>Secondary antibody + incubation time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse anti-human mitochondrion Neat: 1:1000</td>
<td>EDTA-Tris 20 minutes @ 90-100°C</td>
<td>1:250 Biotinylated goat anti-mouse IgG1 for 30 minutes @ RT</td>
</tr>
<tr>
<td>Mouse anti-human CD45 Neat: 1:1000</td>
<td>Citrate buffer 30 minutes @ 90-100°C</td>
<td>1:250 Biotinylated goat anti-mouse IgG1 for 30 minutes @ RT</td>
</tr>
<tr>
<td>Mouse anti-human nucleus Neat: 1:1000</td>
<td>EDTA-Tris 20 minutes @ 90-100°C</td>
<td>1:250 Biotinylated goat anti-mouse IgG1 for 30 minutes @ RT</td>
</tr>
</tbody>
</table>

*Summary of antibody-specific conditions*
Human Ig ELISA

Plates were coated (100 µl per well) with 0.4 µg/ml rabbit anti-human IgG (Accurate Chemical & Scientific; cat# JZH005003), and incubated overnight at 4ºC. Coating antibody was removed and wells were washed 3 times with wash solution (0.1% tween-20 in PBS), once with quench solution (0.1% BSA, 0.2% gelatin, 0.5% Tween-20 in PBS). 100 µl of quench solution was added to each well and the plate was incubated for 1 hr at room temperature. Quench solution was removed and 100 µl of diluted (1:10) mouse serum samples was added to each well and incubated for 1 hr at 37 ºC. Serum was removed and the plate was washed 3 times with wash solution then once with the quench solution. 100 µl of rabbit anti-human IgG-HRP (Accurate Chemical & Scientific; cat# JOH000003, 1:75000) was added to each well and incubated for 1 hr at room temperature. The plate was then washed 4 times and 150 µl of OPD substrate was added to each well. The plate was then incubated for 6 minutes at room temperature in the dark. 30 µl of 6N HCl was then added to each well to stop the reaction. Absorbance was read at an optical density of 490nm.

Statistics.

Data were analyzed using GraphPad Prism 5 software package (GraphPad, San Diego, CA, USA). One way ANOVA with Bonferroni post-testing was used to analyze differences in means of uNK cells/mm² in deciduas basalis and for comparison of spiral artery modification. P<0.05 was considered statistically significant.
Chapter 3: Results

Implantation sites of untreated C57BL/6 mice

Implantation sites of unmanipulated C57BL/6 pregnancies were examined at gd6 (not shown), 8, 10 and 12 (Figure 1). These sites are normal and had well-developed decidual, trophoblast and fetal tissues. UNK cells were present and localized to both the MLAp and decidua basalis. Spiral arteries were identified that changed morphologically after gd8.

At gd6, ectoplacental cone trophoblast cells were apparent at the mesometrial end of the embryonic cavity. In the decidua basalis, mean uNK cell number/mm$^2$ was 32.3±8.2. At gestation day 8, MLAp were identified between smooth muscle layers of the uterine wall, at the mesometrial pole of the implantation site. Mean uNK cell number/mm$^2$ was 36.4±8.7 in the MLAp and 55.1±12.2 in the decidua basalis. At gd10, mean uNK cell number/mm$^2$ was 98.9±14.2 cells/mm$^2$ in the MLAp and 65.6±13.6 uNK cells/mm$^2$ in decidua basalis. At gd12, decidua had compact and loose zones and large bilateral venous sinuses. Mean uNK cell number/mm$^2$ was 150.0±30.7 in the MLAp and 51.8±6.3 in decidua basalis.

Perivascular smooth muscle surrounding spiral arteries in decidua basalis was reduced after gd8 and spiral artery modification was complete by gd12 ($D_{Wall}:D_{Lumen} = 1.42±0.19$). Average litter size in this strain is 6.2 (http://www.informatics.jax.org/external/festing/mouse/docs/C57BL.shtml).
Figure 1: Implantation sites of C57BL/6 mice at gestation day 8(A), 10(B) and 12(C). Hematoxylin and eosin stain.

DB: decidua basalis; E: embryo; F: fetus; MLAp: mesometrial lymphoid aggregate of pregnancy; Myo: myometrium; P: Placenta; SA: spiral artery; Sp: spongiotrophoblast; Lab: labyrinthine placenta YS: yolk sac
Implantation sites of unmanipulated C57BL/6 \( Rag2^{-/-}/Il2rg^{-/-} \) mice

Implantation sites from C57BL/6 \( Rag2^{-/-}/Il2rg^{-/-} \) mice were examined at gd7, 9 and 12 (Figure 2). These sites differed from normal C57BL/6 mice and were consistent with previous descriptions (Greenwood et al. 2000). The implantation sites lacked uNK cells. At gd12, no MLAp had developed and spiral arteries remained unmodified; \( D_{\text{Wall}}:D_{\text{Lumen}} = 2.12 \pm 0.35 \) versus \( 1.42\pm 0.19 \) in syngeneic C57BL/6 mice (\( P>0.05 \)).

Selection of 5-FU dosage for treatment of neonatal C57BL/6 \( Rag2^{-/-}/Il2rg^{-/-} \) mice

The 2 neonatal C57BL/6 \( Rag2^{-/-}/Il2rg^{-/-} \) mice that received 125 mg/kg 5-FU plus C57BL/6 bone marrow at 48 hr via injection in either liver or spleen survived to 8 wk (1 male; 1 female). However, this female did not become pregnant. The 2 neonatal C57BL/6 \( Rag2^{-/-}/Il2rg^{-/-} \) mice that received 75 mg/kg 5-FU with 48 hr intrahepatic injection of C57BL/6 bone marrow died unexpectedly at 4 wk of age. The 4 neonates that received 25 mg/kg 5-FU followed by C57BL/6 bone marrow at 48 or at 72 hr survived. Pregnancies were achieved in these females upon reaching adulthood. Thus, the dosage of 25 mg/kg was used for all subsequent preconditioning of neonatal mice. No histological difference was found between 48 and 72 hr graft recipients and 48 hr injections were used in all subsequent studies.
Figure 2: Implantation sites of C57BL/6 Rag2"/"Il2rg"/ mice at (A) gd8, (B) gd10 and (C) gd12 (fetus not shown). Hematoxylin and eosin stain.

DB: decidua basalis; E: embryo; EPC: ectoplacental cone; F: fetus; Lab: labyrinthine placenta; M: mesometrium; Myo: myometrium; P: placenta; SA: spiral artery; Sp: spongiotrophoblast; VS: venous sinus; YS: yolk sac
Differentiation of uNK cells from C57BL/6 syngeneic bone marrow transplantation

Twelve pregnant uteri were available from the series of C57BL/6 Rag2\(-/-\)/Il2rg\(-/-\) females preconditioned with 25 mg/kg 5-FU and receiving C57BL/6 bone marrow at 24 or 48 hr (Table 1). Only one of the two mice studied at gd6 had uNK cell engraftment (not shown). The number of uNK cells/mm\(^2\) in this mouse (8.7±1.9 versus 32.3±8.2 in C57BL/6 mice) was significantly lower than in normal mice at gd6 (P<0.05). All 10 recipients studied later in pregnancy had numerous PAS\(^+\) uNK cells. Since all normal mice have detectable uNK cells by gd6, this result suggests that the gd6 graft recipients have delayed uNK cell differentiation, perhaps due to reduced uNK progenitor cell numbers, rather than engraftment failure.

Quantitatively, decidual uNK cell numbers were lower at gd8 (Figure 3A) in both graft recipients than in C57BL/6 mice. MLAp had not developed in gd8 graft recipients. In the decidua basalis (Figure 3B), mean uNK cells/mm\(^2\) was 17.2±9.3 (versus 55.1±12.2 in C57BL/6). In normal mice at midgestation (gd10), uNK cells are found in two distinct microdomains, the MLAp and the decidua basalis. The MLAp requires uNK cell differentiation to induce its development in the central region of each implantation site between the two layers of myometrium. The presence of a MLAp signifies a normal mid-gestation implantation site (Guimond et al. 1998b). Gd10 bone marrow recipients (Figure 4A) had uNK cells in both the MLAp and in the decidua basalis (Figure 4B).
Table 2: Distribution of 5-FU-preconditioned (25 mg/kg) female neonatal bone marrow recipients studied.

<table>
<thead>
<tr>
<th>Bone marrow injection sites and interval after 5-FU</th>
<th>Did not become pregnant</th>
<th>Gd6</th>
<th>Gd8</th>
<th>Gd10</th>
<th>Gd12</th>
<th>Gd13</th>
</tr>
</thead>
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<tr>
<td>Liver @ 24 hr</td>
<td>3</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Liver @ 48 hr</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Spleen @ 24 hr</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>0</td>
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<td>Spleen @ 48 hr</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
Figure 3: (A) Implantation site of gestation day 8 C57BL/6 Rag2^{-/-}/Il2rg^{-/-} mouse treated with 25 mg/kg 5-FU at 24 hr of age followed by 5 x 10^7 C57BL/6 bone marrow cells at 48 hr. Periodic acid Schiff’s stain; (B) 400x image of decidua basalis stained with Periodic acid Schiff’s. Arrows indicate small, granulated uNK cells. (C) 200x image of unmodified spiral arteries stained with hematoxylin and eosin. Perivascular smooth muscle is apparent.

DB: decidua basalis; EPC: ectoplacental cone; F: fetus; SA: spiral artery; VS: venous sinus
Figure 4: (A) Implantation site of gestation day 10 C57BL/6 Rag2^{−/−}/Il2rg^{−/−} mouse treated with 25 mg/kg 5-FU at 24 hr of age followed by $5 \times 10^5$ C57BL/6 bone marrow cells at 48 hr. Hematoxylin and eosin stain. (B) 400x image of MLAp stained with periodic acid Schiff’s to identify uNK cells. (C) 200x image of spiral arteries stained with hematoxylin and eosin. Perivascular smooth muscle is reduced, producing large, flaccid vessels.

DB: decidua basalis; F: fetus; MLAp: mesometrial lymphoid aggregate of pregnancy; P: placenta;
At gd10, the mean uNK cell number/mm² in the recipients (n=3) was comparable to that in syngeneic C57BL/6 mice in both microdomains. In the MLAp, mean uNK cell number/mm² was 90.4±12.7, versus 98.9±14.2 in C57BL/6 mice (P<0.05). In the decidua basalis, mean uNK cell number/mm² was 59.9±10.6, versus 65.6±13.6 in C57BL/6 (P<0.05). At gd12 (Figure 5A), mean uNK cell number/mm² in the MLAp (Figure 5B) was 148.0±15.2, versus 150.0±30.1 in C57BL/6 mice (P<0.05). In the decidua basalis, mean uNK cell number/mm² was 51.1±6.3, versus 51.8±6.3 in C57BL/6 mice (P<0.05). Numbers of uNK cells appeared to decline in implantation sites of the single gd13 female studied (118.3±12.5 uNK cells/mm² in the MLAp; 42.9±7.5 uNK cells/mm² in the decidua basalis) as is reported for uNK cell numbers in normal mice (Delgado et al. 1996b). The data from this series of animals is summarized in Figure 6.

Injection of the neonatal spleen is technically difficult due its small width and thinness at 48 hours of age. Thus, intra-peritoneal injection and/or leakage to the peritoneum after intrasplenic inoculation introduced a degree of variability in this protocol and potentially in the number of hematopoietic stem cells (HSC) reaching recipient’s bloodstream. Most (9/12) C57BL/6 Rag2−/− Il2rg−/− mice inoculated with C57BL/6 bone marrow that survived to adulthood and became pregnant were injected via the liver. Intrahepatic injection of HSC was therefore selected for all subsequent studies based on the proven efficacy and reliability of
Figure 5(A) Implantation site of gestation day 12 C57BL/6 Rag2−/−/Il2rg−/− mouse treated with 25 mg/kg 5-FU at 24 hr of age followed by $5 \times 10^5$ C57BL/6 bone marrow cells at 48 hr. Hematoxylin and eosin stain (B) 400x image of MLAp stained with periodic acid Schiff’s to identify uNK cells. (C) 400x image of large, modified spiral artery. Hematoxylin and eosin stain.

DB: decidua basalis; F: fetus; MLAp: mesometrial lymphoid aggregate of pregnancy; P: placenta; SA: spiral artery
Figure 6: Comparison of mean uNK cells/mm² in MLAp and decidua basalis (DB) of C57BL/6 Rag2⁻/⁻/Il2rg⁻/⁻ mice inoculated with syngeneic bone marrow and wild type C57BL/6 mice. No gd13 C57BL/6 implantation was available for control data. No statistically significant difference was found between gd12 and gd13 values.
injection. No significant gross histological pathologies were apparent in the liver or kidneys as a result of treatment (not shown).

**Assessment of spiral arterial modification in C57BL/6 Rag2\(^{-/-}\)/Il2rg\(^{-/-}\) inoculated with C57BL/6 bone marrow**

Spiral artery modification occurred in pregnant C57BL/6 Rag2\(^{-/-}\)/Il2rg\(^{-/-}\) mice inoculated with syngeneic bone marrow as neonates. At gd8, before spiral arterial modification is normally detected histologically in C57BL/6 mice (Figure 3C), the spiral arteries of the treated mice had a \(D_{wall}/D_{lumen}\) ratio of 1.78 ±0.10. This is similar to unmanipulated C57BL/6 mice. Spiral artery modification had occurred in gd10 (Figure 4C) and in gd12 (Figure 5C) transplant recipients and was comparable to unmanipulated C57BL/6 mice. At gd10, the mean diameter wall:lumen ratio was 1.34 ±0.09 (versus 1.32±0.11 in gd10 C57BL/6 mice) and at gd12 was 1.40 ±0.18 (versus 1.42 ±0.19 in gd12 C57BL/6 mice; \(P<0.05\) at both time points).

Preconditioning C57BL/6 Rag2\(^{-/-}\)/Il2rg\(^{-/-}\) mice with 25 mg/kg followed by inoculation with C57BL/6 bone marrow is compatible with female growth to adulthood with retention of fertility and the ability to differentiate uNK cells with normal function.
Implantation sites of unmanipulated BALB/c mice

To determine if the BALB/c $\text{Rag}^{2-/-}\text{Il2rg}^{-/-}$ mice that would be used for human CD34$^+$ cell inoculation were fertile after 25 mg/kg 5-FU and HSC engraftment and could develop functional uNK cells, it was necessary to establish measurements of uNK cell numbers and extent of spiral artery remodelling in implantation sites of unmanipulated BALB/c mice. In general, implantation site histology of BALB/c (Figure 7) and C57BL/6 (Figure 1) mice was similar.

In the MLA$p$, uNK cell number/mm$^2$ was highest at gd12 (159.8± 21.4 cells/mm$^2$) and decidual uNK cells/mm$^2$ peaked at gd10 (41.9± 9.0 cells/mm$^2$). At gd12, spiral artery modification in BALB/c mice was comparable to that seen in C57BL/6 mice, with a $D_{\text{Wall}}:D_{\text{Lumen}}$ ratio of 1.43± 0.9 versus 1.42± 0.19 in C57BL/6 mice (P<0.05). No significant strain differences were found between implantation sites of normal BALB/c and C57BL/6 mice. The average litter size observed in BALB/c mice born at Queen’s University was 6.4± 2.4.

Implantation sites of unmanipulated BALB/c $\text{Rag}^{2-/-}\text{Il2rg}^{-/-}$ mice

Implantation sites in unmanipulated female BALB/c $\text{Rag}^{2-/-}\text{Il2rg}^{-/-}$ mice had no uNK cells and gd12 spiral arteries retained significant perivascular smooth muscle (Figure 8A). The $D_{\text{Wall}}:D_{\text{Lumen}}$ ratio was 1.78± 0.37 versus 2.12± 0.35 in C57BL/6 $\text{Rag}^{2-/-}\text{Il2rg}^{-/-}$ mice (P<0.05) (Figure 8A inset). The average litter size of BALB/c $\text{Rag}^{2-/-}\text{Il2rg}^{-/-}$ mice bred at Queen’s University was 7.4± 2.1
Figure 7: Implantation sites of unmanipulated BALB/c mice at gestation day 7 (A), 9 (B) and 12 (C). No fetus is shown in (C). All sections were stained with hematoxylin and eosin.

DB: decidua basalis; E: embryo; EPC: ectoplacental cone; F: fetus; MLAp: mesometrial lymphoid aggregate of pregnancy; P: placenta; SA: spiral artery;
Figure 8: Implantation sites of (A) unmanipulated gestation day 12 BALB/c and (B) gestation day 12 BALB/c $\text{Rag2}^{-/-}/\text{Il2rg}^{-/-}$ treated with 25 mg/kg 5-FU at 24 hr of age followed by inoculation with $5 \times 10^6$ BALB/c bone marrow cells at 48 hr. Hematoxylin and eosin stain.

DB: decidua basalis; F: fetus; MLAp: mesometrial lymphoid aggregate of pregnancy; P: placenta;
Differentiation of uNK cells in BALB/c \( \text{Rag2}^{-/-}/\text{Il2rg}^{-/-} \) mice inoculated with syngeneic BALB/c bone marrow

BALB/c \( \text{Rag2}^{-/-}/\text{Il2rg}^{-/-} \) mice treated with 25 mg/kg 5-FU and inoculated with BALB/c bone marrow survived to adulthood. If graft did not follow 5-FU treatment, BALB/c \( \text{Rag2}^{-/-}/\text{Il2rg}^{-/-} \) mice lived to weaning age but did not reach adulthood.

From grafted animals, 5 pregnant uteri became available at gd12. Histological assessment revealed normal implantation sites resembling those in unmanipulated BALB/c mice (Figure 8B). In the MLAp, uNK cells were present at 173.3 ± 19.5 cells/mm\(^2\) versus 159.8± 21.4 in unmanipulated BALB/c mice (P<0.05). In the decidua basalis, uNK cells were present at 48.9± 2.7 cells/mm\(^2\) versus 53.7± 6.2 in unmanipulated BALB/c mice (P<0.05). No significant strain differences in uNK cells/mm\(^2\) were found between implantation sites of BALB/c \( \text{Rag2}^{-/-}/\text{Il2rg}^{-/-} \) and C57BL/6 \( \text{Rag2}^{-/-}/\text{Il2rg}^{-/-} \) mice inoculated with syngeneic bone marrow. The uNK cells in BALB/c \( \text{Rag2}^{-/-}/\text{Il2rg}^{-/-} \) mice inoculated with BALB/c bone marrow showed all stages of uNK cell morphological differentiation (Figure 9).

Assessment of spiral arterial modification in BALB/c \( \text{Rag2}^{-/-}/\text{Il2rg}^{-/-} \) mice inoculated with syngeneic BALB/c bone marrow

Spiral artery modification occurred in pregnant BALB/c \( \text{Rag2}^{-/-}/\text{Il2rg}^{-/-} \) mice syngeneically engrafted as neonates (Figure 8B inset). At gd12, spiral arteries of
Figure 9: Stages of uNK cell maturation in unmanipulated BALB/c (Ai-iii) and BALB/c mice treated with 25 mg/kg 5-FU at 24 hr of age followed by inoculation with $5 \times 10^5$ BALB/c bone marrow cells at 48 hr. UNK cells matured from (i) small to (ii) large, heavily granulated cells to (iii) large, granulated and vacuolated cells. Implantation sites were stained with periodic acid Schiff’s to identify uNK cells.
the treated mice had a $D_{\text{Wall}}:D_{\text{Lumen}}$ ratio of 1.31± 0.26, statistically equivalent to that seen in spiral arteries of unmanipulated pregnant BALB/c mice (1.42± 0.17; P<0.05). No significant strain differences in spiral artery modification were apparent between BALB/c $\text{Rag2}^{-/-}/\text{Il2rg}^{-/-}$ mice ($D_{\text{Wall}}:D_{\text{Lumen}} = 1.31± 0.26$) and C57BL/6 $\text{Rag2}^{-/-}/\text{Il2rg}^{-/-}$ mice ($D_{\text{Wall}}:D_{\text{Lumen}} = 1.40± 0.18$; P<0.05) that received 25 mg/kg 5-FU followed by $10^6$ syngeneic bone marrow cells injected intrahepatically. Having established a protocol for preconditioning neonatal BALB/c $\text{Rag2}^{-/-}/\text{Il2rg}^{-/-}$ mice with 5-FU that preserves fertility and enables differentiation of functional uNK cells, experiments with human to mouse xenografting were undertaken.

**Growth trajectory of mice inoculated as neonates with enriched human cord blood CD34$^+$ cells**

Eight litters of BALB/c $\text{Rag2}^{-/-}/\text{Il2rg}^{-/-}$ mice (18 female, 19 male) received 25 mg/kg 5-FU at 24 hr of age and were inoculated intrahepatically with $10^6$ enriched human cord blood cells at 48 hr. To evaluate potential developmental consequences of this protocol, treated animals were weighed weekly and compared to unmanipulated BALB/c $\text{Rag2}^{-/-}/\text{Il2rg}^{-/-}$ mice. Mean litter sizes between the inoculated and control groups remained statistically equivalent during the interval of lactation (4.6±1.8 inoculated versus 7.4±2.1 untreated). Inoculated mice showed a transient pattern of growth restriction at one wk of age (P= 0.046). This was anticipated due to the anti-proliferative action of 5-FU. At 2 and 3 wk post-partum, differences in weights of inoculated versus unmanipulated
BALB/c *Rag2<sup>−/−</sup>/Il2rg<sup>−/−</sup> mice were not statistically significant (P>0.05). From weaning (3 wk) to adulthood (8 wk), weights for females and males were collected independently. In both inoculated and unmanipulated control mice, weights of females and males were statistically different after weaning, as is typical in mice.

**Gestation day 7–12 implantation sites of BALB/c *Rag2<sup>−/−</sup>/Il2rg<sup>−/−</sup> mice inoculated as neonates with enriched human cord blood CD34<sup>+</sup> cells**

To study human uNK cell differentiation, nine pregnancies were established in the above 18 females. The number of implantation sites was highly variable and no statistically significant difference was detected in comparison to syngeneic control mice. The number of implants at gd7 was 16, at gd9 the mean was 12 (n=3 pregnancies) and at gd12 the mean was 5.5 (n=5 pregnancies). Despite this decline over gestation, the frequency of advanced resorption was not notably elevated over syngeneic controls (8.13% in BALB/c *Rag2<sup>−/−</sup>/Il2rg<sup>−/−</sup> mice bred at Queen's University).

Ten of the gd7 implants were examined histologically. In these, decidua appeared normal in comparison to syngeneic controls. No lymphoid or other inflammatory cells were apparent. Unexpectedly, implantation sites appeared heterogeneous. Only one implantation site appeared to contain a mature, appropriate for gestation age embryo (Figure 10A). All other sites analyzed
Figure 10: (A) Gestation day 7 implantation site of Rag2−/−Il2rg−/− mouse inoculated with 10⁶ human CD34⁺ cells. (B) 50x image of mature for gestation age embryo. (C-D) Heterogeneity of embryonic development. In (C) there is some evidence of trophoblast invasion despite no apparent embryo. In (D) only minor decidual disruption is apparent (arrow). All sections stained with hematoxylin and eosin.

DB: decidua basalis; E: embryo; EPC: ectoplacental cone.
lacked identifiable embryos despite decidualized uterus and histological evidence of implantation (Figure 10B).

At gd9 (Figure 11), decidua in implantation sites from all 3 dams had developed large vascular spaces and an aggregate of cells (MAp) was present between the smooth muscle layers at the mesometrial pole. While some cellularity in the mesometrial region is normal in gd9 BALB/c $\textit{Rag2}^{-/-} \textit{Il2rg}^{-/-}$ implantation sites, it was exaggerated in implantation sites of inoculated mice, with increased cellularity in comparison to unmanipulated BALB/c $\textit{Rag2}^{-/-} \textit{Il2rg}^{-/-}$ mice. Cells in the MAp did not have a lymphoid appearance. Rather, they were strongly eosinophilic cells.

At gestation day 12 (n=5), implantation sites remained heterogeneous within and between litters (Figure 12). Some implantation sites in inoculated mice had excessively vascularized decidua basalis. Venous blood vessels were enlarged, often occupying large sections of the central decidua basalis. While venous sinuses are commonly found in lateral decidua basalis of unmanipulated BALB/c $\textit{Rag2}^{-/-} \textit{Il2rg}^{-/-}$ mice, extension to the central zone of the decidua is abnormal. In contrast to the inflated, sinusoidal veins, spiral arteries were unmodified and were often surrounded in an eosinophilic material, possibly fibrinoid in nature. Other implantation sites in the same dam had relatively normal vasculature and did not possess the perivascular deposition. MAp were apparent between the smooth muscle layers of the uterine wall in some implantation sites
Figure 11: Gestation day 9 implantation site of Rag2\(^{-/-}\)/Ii2rg\(^{-/-}\) mouse inoculated with \(10^6\) human CD34\(^+\) cells. Large, bilateral venous sinuses are apparent. Fetus not shown. Hematoxylin and eosin stain.

DB: decidua basalis; EPC: ectoplacental cone; FC: fetal cavity; MAp: mesometrial aggregate of pregnancy; U: uterine lumen; VS: venous sinus
Figure 12 Gestation day 12 heterogeneous implantation sites of Rag2⁻/⁻/Il2rg⁻/⁻ mice inoculated with 10⁶ human CD34⁺ cells. In (A) no abnormal phenotype is apparent. In (B), MAp and extracellular vascular deposition seen around spiral arteries. Extreme phenotype is seen in (C), with heavy arterial extracellular deposition (box) and large venous sinuses. All sections stained with hematoxylin and eosin.
but did not contain readily identified lymphoid cells. Despite the abnormal vascular phenotype seen at midgestation, 2 litters of inoculated mice that were allowed to proceed to parturition gave birth to large litters (average litter size 12.5) of healthy pups.

These studies suggested that my protocol permitted survival of human cells but that they were unable to differentiate into lymphocytes in the uterus.

**Implantation sites of mice inoculated as neonates with enriched human cord blood CD34+ cells and supplemented with IL-15/RA complex**

Since no lymphocytes were readily identified in the implantation sites of pregnant, human CD34+ cell-inoculated BALB/c Rag2⁻/⁻/Il2rg⁻/⁻ mice, weekly injections (IP) with recombinant human IL-15/RA complex were administered to inoculated female BALB/c Rag2⁻/⁻/Il2rg⁻/⁻ mice using a newly reported protocol (Huntington et al. 2009b). IL-15 supports differentiation, survival and proliferation of NK cells. Of 5 treated animals, 4 became pregnant. These animals were sacrificed at gd 10 (Figure 13A), gd11 (not shown), gd12 (Figure 14A) and gd13 (not shown) for histological analysis. Implantation sites in all 4 animals possessed pregnancy-induced MAp where the MLAp normally develops in immune-competent mice (Figures 13B and 14B). However, cells in the aggregate were non-lymphoid in appearance. Additionally, prominent laminae of smooth muscle (Figure 13C) were found between the Map and decidua basalis. Decidua was heterogeneous between implantation sites, often with excessively large
Figure 13: (A) Gestation day 10 implantation site of human CD34+ cell-engrafted BALB/c $Rag2^{-/-} Il2rg^{-/-}$ mouse treated with IL-15/RA complex. (B) 100x MAp featuring multiple layers of smooth muscle and unidentified cells. (C) 200x lateral uterine wall with multiple layers of smooth muscle. (D) 400x uterine lumen and late ectoplacental cone stage placentation. All sections stained with hematoxylin and eosin.

DB: decidua basalis; EPC: ectoplacental cone; F: fetus; MAp: mesometrial aggregate of pregnancy
Figure 14 (A) Gestation day 12 implantation site of BALB/c Rag2−/−Ii2rg−/− mouse inoculated with human CD34+ cells and treated with IL-15/RA complex. Arrow indicates caudal neuropore. (B) 100x image of mesometrial aggregate showing multiple cells and layers of smooth muscle. (C+D) 400x view of unidentified perivascular cells (arrows). All sections stained with hematoxylin and eosin.

DB: decidua basalis; F: fetus; MAp: mesometrial aggregate of pregnancy; P: placenta; U: uterine lumen;
venous sinuses extending to the central decidua basalis. Spiral arteries were constricted (Figure 14C) but numerous throughout the decidua and had distinct perivascular depositions that stained strongly with eosin.

At gs10 and gd11, the implantation sites were developmentally retarded. A uterine lumen was present (Figure 13D). Ectoplacental cone trophoblast cells were apparent and only a rudimentary placenta had developed. In unmanipulated BALB/c *Rag2<sup>-/-</sup>/Il2rg<sup>-/-</sup> mice, the ectoplacental cone is normally found at gestation day 6-8.

At gd12, placental development was more advanced but still appeared immature for gestational age. Uterine lumen was still present in many implantation sites (Figure 14A). The fetuses in viable implantation sites were also notably restricted in development, with neural tube closure at variable stages of advancement/completion between implantation sites.

At gd13 (not shown), implantations sites lacked the abnormal vascular phenotype seen in inoculated animals at earlier timepoints. Both veins and spiral arteries in the decidua basalis were comparable to unmanipulated BALB/c *Rag2<sup>-/-</sup>/Il2rg<sup>-/-</sup> mice. The placentae and fetuses appeared to have undergone normal development. This suggested an absence of human cell engraftment.
Effects of IL-15/RA complex alone

To determine if IL-15/RA complex contributes independently to implantation site phenotype, otherwise untreated female BALB/c $\text{Rag}2^{-/-}/\text{Il}2\text{rg}^{-/-}$ mice ($n=2$) received weekly injections (IP) of IL-15/RA complex beginning at 6 wk and continued through gestation. Cost of human IL-15 was a limiting factor in this study (~$800/mouse). Both mice became pregnant. At gd12, implantation sites in these females differed from all others in the study and were similar to each other (Figure 15). The decidua basalis appeared to be more vascularized and excessive amounts of smooth muscle were present in the uterine wall, particularly at the mesometrial pole, though in cases extending to the lateral uterine wall. Distinctly eosinophilic deposition was concentrated around spiral arteries and was more extensive than seen in the human cell-inoculated BALB/c $\text{Rag}2^{-/-}/\text{Il}2\text{rg}^{-/-}$ mice treated with IL-15/RA complex.

Based on these data, some but not all of the changes seen in inoculated BALB/c $\text{Rag}2^{-/-}/\text{Il}2\text{rg}^{-/-}$ mice treated with IL-15/RA complex should be attributed to a mouse response to the human cytokine complex.

Growth trajectory of mice born to dams inoculated as neonates with human CD34\(^+\) cells

To assess gestational consequences of 5-FU treatment, neonatal inoculation with human CD34\(^+\) cells and the induced uterine phenotype, two pregnancies were allowed to deliver and pups were weighed weekly to
Figure 15: (A) Gestation day 12 implantation site of BALB/c Rag2⁻/⁻/Il2rg⁻/⁻ mouse treated with IL-15/RA complex without preconditioning or hematopoietic stem cell inoculation. (B) 400x view of eosinophilic deposit surrounding spiral arteries. Hematoxylin and eosin stain.

DB: decidua basalis; F: fetus; MAp: mesometrial aggregate of pregnancy; P: placenta;
adulthood. Average litter size was 4.5± 1.5. After weaning at 4 wk, males were significantly larger than females. In contrast to the transient restriction pattern seen in the first generation human CD34⁺ cell recipients, pups born to inoculated mice were significantly larger throughout postnatal development than pups born to unmanipulated female BALB/c Rag2⁻/⁻/Il2rg⁻/⁻ mice (Figure 16). Thus, despite the massive disruption of typical implantation site organization, some female mice that received human CD34⁺ cells could carry healthy litters to term. All mice in these litters were successfully nursed and survived to adulthood.

Detection of Chimerism

PCR analysis detected the presence of human cells in the liver of three of the 15 (20%) BALB/c Rag2⁻/⁻/Il2rg⁻/⁻ mice inoculated with human CD34⁺ cells (Figure 17). The PCR amplicon was 1.171 kb. ELISA for human Ig revealed a higher rate of chimerism. In nine of the 22 (40.9%) human CD34⁺ cell-inoculated mice, human Ig was detected in the sera of inoculated animals, two of which were females treated with IL-15/RA complex. These data suggested that human cells were present in at least 40% of the human CD34⁺ cell-inoculated mice, although lower numbers were indicated by the liver PCR study. Because histological and molecular evidence indicated the presence of human cells in human CD34⁺ cell-inoculated mice, immunohistochemical staining was performed to identify human cells in the implantation sites of human CD34⁺ cell-engrafted females. Immunohistochemistry did not detect human mitochondria or human CD45 in the MAp or within the deciduas of human CD34⁺ cell-inoculated
Figure 16: Growth trajectories of mice born to female BALB/c Rag2/Il2rg mice inoculated with human CD34+ cells. After 3 wk, both female and male mice born to human CD34+ cell-inoculated BALB/c Rag2−/−Il2rg−/− mice were statistically larger than unmanipulated BALB/c Rag2−/−Il2rg−/− mice.
Figure 17: Gel electrophoresis of PCR product following amplification of DNA using a primer specific to the alpha satellite region of human chromosome 17. Tissues used were from (A) Human placenta (positive control), (B) Liver from BALB/c Rag2⁻/⁻/Il2rg⁻ mouse inoculated with human CD34⁺ cells and treated with IL-15/RA complex and (C) negative control (liver from unmanipulated BALB/c Rag2⁻/⁻/Il2rg⁻ mouse). Lane (L) is the DNA ladder.
mice (including those that tested positive in molecular assays for chimerism), although positive controls (human placenta, human tonsil) stained readily. Staining for human nuclei revealed antibody cross-reactivity with mouse cells. Representative images of stained tissues from inoculated mice and positive controls are presented in Figure 18.
Figure 18: Immunohistochemistry to detect human cells.
(A+B) Staining of human mitochondria in (A) gd12 decidua basalis in human CD34+ cell-inoculated Rag2−/Il2rg− mouse decidua basalis and (B) human tonsil. Reactive cells are stained brown. Counterstained with hematoxylin.
(C+D) Staining of human CD45 in gd12 decidua basalis of human CD34+ cells-inoculated Rag2−/Il2rg− mouse and (D) human endometrium. Reactive cells are stained brown. Counterstained with hematoxylin.
(E) Staining of human nucleus in liver of unmanipulated Rag2−/Il2rg− mice showing cross-reactivity of anti-human nuclei antibody.
(F) Overlay of staining for human nuclei and DAPI (ubiquitous nuclear stain) showing all mouse hepatocytes cross-reactive for human nucleus staining.
Chapter 4: Discussion

UNK cells are the dominant leukocytes in the gestational uteri of women and mice. Although it has been shown that in mice, uNK cells participate in endometrial remodelling that promotes successful pregnancy, studies of uNK cells in women are limited by restriction in available sampling times and for ethical reasons. Human to mouse xenograft studies in alymphoid mice have provided insight into the functions and regulatory mechanisms of immune cells outside of the context of pregnancy. Sublethal irradiation of recipient immune-compromised mice has been used to precondition graft recipients, killing endogenous mouse HSC residing in the bone marrow to create a niche for human HSC engraftment. However, irradiating mice renders them sterile and as such precludes studies of engrafted uterine lymphocyte effector and regulatory functions in pregnancy. The goal of my study was to develop a protocol that permitted engraftment of human HSC in alymphoid mice while preserving the recipient’s fertility and to use this model to evaluate the functions of human uNK cells. A neonatal recipient model was successfully developed using syngeneic mouse-to-mouse bone marrow engrafting. However, when human cord blood CD34+ cells were used as the graft rather than mouse bone marrow, non-lymphoid cells homed to sites where, classically, uNK cells are found.

C57BL/6 $\text{Rag2}^{-/-}/\text{Il2rg}^{-/-}$ mice were initially chosen as hosts for HSC engraftment. Lacking all lymphocytes including NK cells, adults of this genotype have high engraftment of HSC as irradiated recipients. In lieu of radiation, 5-FU
was chosen to ablate endogenous HSC in mouse bone marrow. 150 mg/kg 5-FU successfully ablates 4-6 wk old C57BL/6 Rag2\textsuperscript{−/−}\textsuperscript{Il2rg\textsuperscript{−/−}} mouse bone marrow and enables syngeneic bone marrow engraftment. However, this dose was not compatible with survival to reproductive age when administered to neonatal C57BL/6 Rag2\textsuperscript{−/−}\textsuperscript{Il2rg\textsuperscript{−/−}} mice that were subsequently inoculated with 5x10\textsuperscript{5} syngeneic bone marrow cells. To determine a dose of 5-FU that would enable HSC engraftment in neonatal C57BL/6 Rag2\textsuperscript{−/−}\textsuperscript{Il2rg\textsuperscript{−/−}} mice and survival to adulthood, a dose response study was undertaken. C57BL/6 Rag2\textsuperscript{−/−}\textsuperscript{Il2rg\textsuperscript{−/−}} mice were treated at 24 hr of age with 125, 75 or 25 mg/kg 5-FU and inoculated with 5x10\textsuperscript{5} syngeneic bone marrow cells at 24 or 48 hours post-5-FU via either intrahepatic or intrasplenic injection. 100% survival was only achieved in the 25 mg/kg dosage group. From these animals, 12 pregnancies were established and mice were sacrificed at gd6, gd8, gd10, gd12 and gd13 for histological analysis of implantation sites.

PAS staining of uNK cell cytoplasmic granules was used to detect mouse uNK cells. This method provides a more comprehensive assessment of uNK cells than DBA lectin since a PAS\textsuperscript{+}DBA\textsuperscript{−} subset of mouse uNK cells has been identified (Zhang et al. 2009). C57BL/6 Rag2\textsuperscript{−/−}\textsuperscript{Il2rg\textsuperscript{−/−}} mice that received 25 mg/kg 5-FU followed by 5x10\textsuperscript{5} bone marrow cells that as adults mated and became pregnant underwent uNK cell differentiation. At gd6-8, statistically fewer mouse uNK cells were present in the decidua of treated mice versus syngeneic C57BL/6 controls (P>0.05). However, in the animals killed at gd10 and gd12,
statistically equivalent numbers of uNK cells were detected in treated mice versus syngeneic controls. This may reflect a transient delay in uNK cell differentiation or homing potential or may be attributable to lower numbers of uNK progenitor cells in engrafted mice. The former explanation would imply a functional deficit in uNK cells that differentiated in treated mice. The progenitor of uNK cells is not specifically defined and was therefore not assessed in the whole bone marrow leukocyte inoculations. Morphologically, there was no evidence that the uNK cells themselves were different if they arose from a graft (Figure 9).

It was previously established that mouse uNK cells are the predominant source of uterine IFNG and that uNK cell-derived IFNG is both necessary and sufficient for the induction of spiral arterial remodelling (Ashkar et al. 2000a). Therefore, uNK cell function was assessed indirectly by indexing the degree of spiral artery modification. Hematoxylin and eosin staining was used to stain smooth muscle surrounding spiral arteries and a ratio of the wall:lumen arterial diameters was used to index the extent of spiral arterial remodelling. At gd8, prior to the initiation of vascular smooth muscle reduction in unmanipulated C57BL/6 mice, treated mice had statistically equivalent amounts of perivascular smooth muscle in comparison to syngeneic C57BL/6 controls. Similarly, at gd10 and 12, spiral artery remodelling was statistically equivalent to that seen in unmanipulated syngeneic C57BL/6 controls. Since spiral arterial remodelling is normally complete by gd12 in unmanipulated syngeneic controls, these results indicate that differentiated uNK cells in treated mice were functionally equivalent.
to endogenous uNK cells in C57BL/6 mice. This functional equivalence suggests that the numerical deficit of uNK cells seen at gd6 and gd8 was not due to reduced functionality of graft-derived uNK cells.

This study began prior to the report that BALB/c Rag2$^{-/}$/Il2rg$^{-/}$ mice support differentiation of human NK cells from cord blood HSC (Traggiai et al. 2004). C57BL/6 Rag2$^{-/}$/Il2rg$^{-/}$ mice primarily support human T cell and B cell lineages, with almost no NK cell differentiation. Since the ultimate goal of my study was to develop a human to mouse xenograft model in which to study human uNK cell functions, the BALB/c Rag2$^{-/}$/Il2rg$^{-/}$ mouse was adopted. Experiments were undertaken to determine whether refinement of the neonatal 5-FU treatment protocol was required to permit HSC engraftment in BALB/c Rag2$^{-/}$/Il2rg$^{-/}$ mice.

BALB/c Rag2$^{-/}$/Il2rg$^{-/}$ mice were preconditioned with 25 mg/kg 5-FU at 24 hr of age and subsequently inoculated with 5x10$^5$ syngeneic BALB/c bone marrow cells at 48 hr. Five pregnancies became available and all treated mice were sacrificed at gd12 for histological assessment of all five recipients’ uterine implantation sites. At gd12, uNK cells of normal morphology were present in both the MLAp and decidua basalis in numbers that were statistically equivalent to the numbers in unmanipulated BALB/c controls. Spiral artery remodelling in the engrafted BALB/c Rag2$^{-/}$/Il2rg$^{-/}$ mice was calculated from spiral artery wall:lumen diameters and compared to gestation-matched syngeneic BALB/c controls. The
extent of spiral artery remodelling was statistically equivalent between the groups. This study established the protocol of 25 mg/kg 5-FU at 24 hr, HSC at 48 hr and expectation of normalized spiral artery remodelling at gd12 as the protocol I would use for human cord blood CD34⁺ cell inoculations.

Human CD34⁺ cells were isolated from cord blood obtained from placentae of elective caesarian sections at Kingston General Hospital. Initial preliminary studies used magnetic bead and flow cytometric positive selection of human CD34⁺ cells but consultation suggested that negative selection yielded superior engraftment. Therefore, in this thesis, CD34⁺ cells were prepared using the commercial kit, RosetteSep™, in which cells bearing lineage-specific surface markers were removed via linkage to red blood cells and density gradient centrifugation. Supernatants contained enriched (~60%) human CD34⁺ cells. Following preconditioning with 25 mg/kg 5-FU at 24 hr, BALB/c Rag2⁻/⁻/Il2rg⁻/⁻ mice were inoculated with 10⁶ CD34⁺ cells and returned to the nursing dam for normal rearing.

To evaluate the developmental consequences of preconditioning with 5-FU followed by inoculation with enriched human cord blood CD34⁺ cells, treated animals were weighed weekly from the date of inoculation through to adulthood (8wk). Treated animals showed a transient pattern of growth restriction at the first wk when compared to unmanipulated BALB/c Rag2⁻/⁻/Il2rg⁻/⁻ mice. This was likely due to the anti-proliferative effects of 5-FU. From 2 wk through to adulthood, no
statistically significant difference was detected between the groups, suggesting that the human CD34+ cell-inoculated mice, like those receiving syngeneic bone marrow, were healthy and that they would mate and conceive.

Upon reaching adulthood, human CD34+ cell-inoculated females were mated with unmanipulated BALB/c \( \text{Rag2}^{+/-}\text{Il2rg}^{-/-} \) mice. Nine pregnancies became available and treated females were sacrificed at gd7 (n=1), gd9 (n=3) and gd12 (n=5). From gd7 to gd12, inconsistent implantation site morphology was seen within and between litters. Heterogeneous vascular phenotypes predominated. In many implantation sites large venous sinuses were apparent and spiral arteries had an unusual perivascular eosinophilic extracellular matrix. At the mesometrial pole of many midgestation gd9-12 implantation sites where the MLAp develops in immune-competent mice, the intermuscular space between the layers of smooth muscle contained variable numbers of large, unidentified cells. While some non-lymphoid cellularity at this site is common in BALB/c \( \text{Rag2}^{+/-}\text{Il2rg}^{-/-} \) mice, several implantation sites had dramatically increased numbers of cells. The heterogeneity between littermates (Figure 12) was seen in all pregnancies, with the excessively vascular phenotype and non-lymphoid MAp apparent in some implantation sites but not others.

Poor fixation was suspected given the perivascular staining pattern seen in implantation sites of human CD34+ cell-inoculated mice. However, fixation times were consistent with those used to fix tissues obtained from unmanipulated
BALB/c $Rag2^{-/-}l2rg^{-/-}$ pregnancies and cellular detail was preserved throughout implantation sites. Furthermore, other experiments in which 4 wk old BALB/c $Rag2^{-/-}l2rg^{-/-}$ mice were inoculated with human CD34+ cells also manifested the same perivascular staining pattern.

These results suggest differential homing of putative human cells to individual implants within a given pregnancy. Numerous attempts to identify these cells via immunohistochemistry against human mitochondria, human CD45 and human nuclei failed despite validation of the staining protocols in positive control tissues (Figure 18). Because immunohistochemical detection of human cells in the implantation sites of treated animals failed, other experiments were undertaken in an attempt to assay for human chimerism.

PCR amplification of liver and spleen revealed the presence of human DNA in three of the fifteen human CD34+ cell-inoculated mice examined (20%). However, human immunoglobulin (Ig) ELISAs detected human Ig in the sera of 9 of the 22 animals assayed (40.9%). These data, alongside the histological evidence of chimerism, suggest that human cells were present in human CD34+ cell-inoculated mice at frequencies below the sensitivity of PCR detection.

Histological assessment of midgestation implantation sites from human CD34+ cell-inoculated mice indicated fewer viable implantation sites than scored by gross inspection. At gd7, 16 implants were identified; 12 at gd9 and 6 at gd12.
Histological assessment of the gd7 implantation sites revealed only one viable, mature for gestational age embryo (Figure 12). At gd9 and gd12, no significant increase in resorption rate was apparent. It is possible that the vascular phenotype induced in many of the implantation sites increased uterine receptivity to implantation. Such an increase would potentially enable implantation of genetically inferior embryos. Very early death and subsequent resorption would account for the decidualization of the endometrium and the increased number of implants seen at earlier time points. An early selection process could also explain why only 1 viable embryo was detected at gd7 despite normal decidualization and the absence of recognizable resorption sites. This does not account for the apparently high number of implants and lack of higher than normal resorption sites at gd9. Since no statistically significant difference in implantation rate emerged between inoculated mice and syngeneic control mice, the apparent pattern of embryonic reduction through gestation may be artifactual.

To evaluate the developmental consequences of the vascular phenotype seen in the uteri of inoculated female mice, 2 pregnancies were taken to term and growth trajectories of the offspring of these inoculated females were calculated. Despite the dramatic disruption to normal uterine vasculature and cellular organization, large litters of healthy mice were delivered. The weights of the offspring of inoculated female BALB/c $Rag2^{-/-}/Il2r{gamma}^{-/-}$ mice were statistically larger than syngeneic BALB/c $Rag2^{-/-}/Il2r{gamma}^{-/-}$ controls. It is possible that the
increased decidual vascularity presented a highly nourishing environment for embryonic and/or fetal development and accelerated growth in utero.

Given my inability to identify human lymphocytes in implantation sites of BALB/c \(Rag2^{-/-}/Il2rg^{-/-}\) mice preconditioned with 5-FU and subsequently inoculated with enriched human cord blood CD34\(^+\) cells, I refined the protocol by incorporation of an IL-15 cytokine complex to support the differentiation and survival of human NK cells. IL-15 is necessary for NK cell differentiation, proliferation and survival. In fact, deletion of \(Il2rg^{-/-}\) gene, and consequent loss of an essential component of the IL-15R endogenously expressed by NK cells, is responsible for the lack of NK cells in \(Il2rg^{-/-}\) mice. The common cytokine receptor \(\alpha\) chain shared between receptors for IL-2, -4, -7, -9 and -21 is an absolute requirement for IL-15 signaling. Huntington reported improved NK cell engraftment in irradiated humanized mice upon weekly supplementation with human IL-15 bound to its receptor \(\alpha\) chain (Huntington et al. 2009b). This mimics trans presentation of IL-15, the process by which human NK cells are stimulated by IL-15-bearing cells in vivo (Huntington et al. 2009b; Mortier et al. 2008). Treatment with human IL-15 has also been shown to promote both antigen presentation (Oh et al. 2008) and the development of GvHD (Miyagawa et al. 2008).

Graft-versus-host disease (GvHD) does not develop in irradiated BALB/c \(Rag2^{-/-}/Il2rg^{-/-}\) mice that receive neonatal inoculation with human CD34\(^+\) cells. Mixed lymphocyte reactions of donor-derived human T cells, isolated from mouse...
lymph nodes, and host BALB/c *Rag2*<sup>-/-</sup>*Il2rg*<sup>-/-</sup> or C57BL/6 dendritic cells suggest that graft recipients undergo tolerogenic selection on host mouse MHC (Traggiai *et al.* 2004). It was unknown whether treatment of inoculated BALB/c *Rag2*<sup>-/-</sup>*Il2rg*<sup>-/-</sup> mice with IL-15/RA would induce GvHD.

Five female BALB/c *Rag2*<sup>-/-</sup>*Il2rg*<sup>-/-</sup> mice were preconditioned with 25 mg/kg 5-FU at 24 hr of age, followed by inoculation with 10<sup>6</sup> enriched human cord blood CD34<sup>+</sup> cells at 48 hr. All 5 mice survived to adulthood. From six wk, IL-15/RA complex was administered (IP) weekly. No skin rashes or lesions (potential indicators of GvHD) developed in treated mice. At eight wk, these mice were mated with syngeneic BALB/c *Rag2*<sup>-/-</sup>*Il2rg*<sup>-/-</sup> males. Four pregnancies became available. These four females were sacrificed at midgestation (gd10-13) and tissues were collected for analysis. On histological evaluation of implantation sites, liver and kidneys from these four mice, no evidence of GvHD was found. Lymphocyte infiltration appeared to be completely absent and neutrophils were not found in abnormally high numbers. Treatment with IL-15/RA complex did not appear to promote GvHD in BALB/c *Rag2*<sup>-/-</sup>*Il2rg*<sup>-/-</sup> mice neonatally preconditioned with 5-FU and inoculated with human CD34<sup>+</sup> cells.

All four treated females that became pregnant developed the excessive vascular uterine phenotype seen in the mice that were inoculated but not supplemented with IL-15/RA complex. In addition to the venous dilation and peri-arterial deposition of matrix, non-lymphoid MAp of increased cellularity were
found. In these mice, extensive smooth muscle was present between the MAP and the decidua basalis. Several additional layers of smooth muscle were often apparent between the MAP and decidua basalis. The lateral uterine walls also appeared to have excessive layers of smooth muscle in comparison to unmanipulated syngeneic BALB/c Rag2−/−Il2rg−/− controls. Serum IL-15 level has been associated with increased systemic blood pressure (Kaibe et al. 2005) and may underlie the vascular changes induced in mice treated with IL-15/RA. In contrast to the normal inhibitory effect that IL-15 has on smooth muscle cell proliferation (Baker and Abel 1995), BALB/c Rag2−/−Il2rg−/− mice inoculated with human CD34+ cells and treated with the IL-15/RA complex showed increased smooth muscle in the uterus. It is possible that treatment with IL-15 affected smooth muscle cell proliferation indirectly via induction of a response in other types of stromal cells.

To determine whether treatment with IL-15 complex induced effects independent of human HSC inoculation, two female BALB/c Rag2−/−Il2rg−/− mice were given weekly injections (IP) of IL-15/RA complex without 5-FU preconditioning or HSC inoculation. Treated animals were mated with syngeneic BALB/c Rag2−/−Il2rg−/− males and both became pregnant. They were sacrificed on gd12 and tissues were collected for histological analysis. The decidua basalis of both treated animals developed excessive vascular sinuses and numerous spiral arteries were found in the decidua. Perivascular depositions were present around the spiral arteries. A distinctly large MAP was present. Several layers of smooth
muscle lined the entirety of the uterine wall, and between the MAp and the decidua basalis. Thus, major changes in implantation sites are induced by IL-15/RA complex, which confound the outcome interpretation in CD34⁺ cell-inoculated and IL-15/RA-treated mice. Since some of the uterine features induced by IL-15/RA treatment alone were seen in CD34⁺ cell-inoculated mice, it is possible that the negative selection enrichment of CD34⁺ cells included progenitors of cells that produce IL-15.

The goal of this study was to refine the neonatal human-to-mouse xenogeneic engraftment protocol to develop fertile chimeric mice to study graft-derived uNK cell functions. My work established that 5-FU is an effective replacement for irradiation in preconditioning neonatal recipient for syngeneic bone marrow-derived HSC engraftment. The protocol was robust across two inbred mouse strains. Upon transition to human HSC grafts, engraftment was seen that supported viability of the preconditioned mice but did not establish recognizable lymphocytes within implantation sites. This differs from treatment of non-obese diabetic-γc⁻/⁻ (NOG) mice with human bone marrow, liver and thymus (BLT), in which human lymphocytes differentiate and populate the reproductive tract (cervix, vagina, and endometrium) of non-pregnant recipient mice (Denton et al. 2008). Despite human lymphocyte differentiation and homing to the reproductive tract in NOG-BLT mice, this model was not suitable for my research since it has recently been shown that NOD mice have deficient IL-15 signaling that would impair uNK cell differentiation (Suwanai et al. 2010).
In contrast to NOG-BLT mice, in my study, non-lymphoid cells appeared to have engrafted. The CD34+ enrichment protocol leaves non-lymphoid human cells in the inoculum, including endothelial progenitor cells and mesenchymal stem cells. It is most probable that this non-lymphoid cell is being detected and that it is rare, since not all implantation sites within a litter were identical. Others in our laboratory have extended these studies to inoculate 4-6 wk 5-FU-preconditioned BALB/c $Rag2^{-/-}/Il2rg^{-/-}$ mice with human CD34+ cells. In these mice, identical phenotypes developed (heterogeneity, extracellular matrix deposition, MAp) and PCR revealed the presence of human cells in both the MLAp and decidua basalis.

The successful development of a neonatal preconditioning protocol that enables long term engraftment and differentiation of functional uNK cells from HSC represents an important landmark in the study of uNK cell functions. However, selection of appropriate human NK progenitor cells and a treatment protocol that supports differentiation of uNK cells in graft recipient mice requires further refinement. To date, a xenograft model of human uNK cell functions remains elusive.


