The Transcription Factor NFIL3 Is Not Required For Uterine Natural Killer Cell Differentiation But Contributes To Placental And Conceptus Development

By

Mackenzie Lynn Redhead

A thesis submitted to the Graduate Program in Anatomy and Cell Biology

In conformity with the requirements for the

Degree of Master of Science

Queen’s University

Kingston, Ontario, Canada

September, 2015

Copyright © Mackenzie Lynn Redhead, 2015
Abstract

Uterine natural killer (uNK) cells are the most abundant lymphocyte in early human and mouse decidua. UNK cell functions have been deduced by histopathologic comparisons of implantation sites between alymphoid (NK⁻T⁻B⁻), NK cell reconstituted alymphoid (NK⁻T⁺B⁻) and normal NK⁺T⁺B⁺ mice. Circulating (c)NK cells are reported to be absent from C57BL/6 mice genetically ablated for the transcription factor Nfil3 (cNK⁻T⁺B⁺) and these mice experience midgestation Th17 cell-mediated fetal loss when mated by BALB/c males. Recently, tissue resident subsets of NK cells have been described in this mouse.

A histological time-course examination of syngeneic Nfil3⁻/⁻ male x Nfil3⁻/⁻ female and allogeneic BALB/c-Tg(UBC-GFP)30Scha/J male x Nfil3⁻/⁻ female pregnancy (gestation day; GD 6.5-15.5) was performed to determine the consequences of a maternal cNK T⁺B⁺ phenotype on pregnancy with an Nfil3⁻/⁻ or Nfil3⁺/- conceptus. Whole mount immunofluorescence (WM-IF) was used as an additional technique to provide greater insights at midgestation. Nfil3 is a pleiotrophic factor; to examine Nfil3-dependent uNK cell-specific effects, pregnancies in Nfil3⁻/⁻ bone marrow engrafted alymphoid mice (BME) were studied.

UNK cells were less frequent in Nfil3⁻/⁻ pregnancies and viability of Nfil3⁻/⁻ implantation sites (IS) did not differ from wild type (WT) matings. Early gestation pathologies included impaired antimesometrial decidualization (AMD) and delays in lumen closure and embryo development (ED). WM examination revealed an enrichment of CD45⁺CD11c⁺ cells at GD 8.5 in Nfil3⁻/⁻ IS compared to WT. Mid-late gestation placental development, including labyrinth vascular space area and interhemal membrane
width, deviated significantly less from WT in allogeneic compared to syngeneic matings. BME and alymphoid placental development was similar to WT. Spiral artery (SA) remodeling did not occur in any experimental mating.

Results of the present investigation suggest Nfil3-independent uNK cells do not promote AMD or GD 8.5 ED and cannot remodel SAs. Nfil3 plays a role in placental tissue layer (specifically labyrinth) development possibly through trophoblast-dependent mechanisms. The absence of Nfil3 may promote recruitment or differentiation of CD45+CD11c+ cells in the early gestation implantation site. Although not an effective model for studying NK T+ B+ pregnancy, the Nfil3−/− mouse deepens the understanding of innate lymphoid cells and their respective subsets.
Co-Authorship

All experiments presented in this thesis are original. Mackenzie Lynn Redhead and B.A. Croy are responsible for the experimental design, interpretation of data and manuscript preparation. Mackenzie Lynn Redhead completed all experimental work and analysis of data. B.A. Croy is the primary principal investigator for these studies.
Acknowledgements

First and foremost I would like to thank Dr. Anne Croy for her mentorship throughout my Master’s Degree. I have learned so much from her expertise both in the field of reproduction and as an academic. She was instrumental in the design and execution of each of my experiments and worked tirelessly with me every step of the way to ensure my success. Her passion and incredible work ethic will continue to be an inspiration.

I would like to thank all past and present members of the Croy Lab. Specifically; I have to thank Dr. Patricia Lima for the majority of my technical skills in the lab. I also have to thank Vanessa Kay, Allison Felker, Ashley Martin, and Shuhiba Mohammad. Apart from providing me with an enormous amount of technical help in the lab, each of you made every one of my days in the lab so much fun. Thank you to Zhilin Chen, Kimberly Laverty, Yvonne Bach, Andrew Hickman and Zain Saleem for their technical assistance. Finally, I would like to thank Matthew Rätsep for his technical expertise and unwavering patience with a lab full of women.

Thank you to the members of my thesis committee Dr. Graham and Dr. Basta for their suggestions, guidance and support over the course of my degree.

Finally, I would like to extend a huge thanks to all of my friends and family who supported me throughout my degree. Adam, you shouldered my stressors right along side me and kept me smiling. I cannot thank you enough. To my mother and father, who I truly could have never have done this without. Thank you for continuing to support me in everything that I do.
Table of Contents

List of Tables ............................................................................................................................ viii
List of Figures ............................................................................................................................. ix
List of Abbreviations .................................................................................................................. xi

Chapter 1: General Introduction and Literature Review ......................................................... 1

1.1) Mammalian Placentation ................................................................................................. 1
1.2) UNK Cell Overview ........................................................................................................ 2
1.3) Mouse Pregnancy ............................................................................................................. 3
1.4) NK Cell Depletion .......................................................................................................... 9
1.5) Nfil3 Gene ....................................................................................................................... 13
1.6) Hematopoiesis ................................................................................................................ 16
1.7) NK Cell Development ..................................................................................................... 17
1.8) Innate Lymphoid Cells .................................................................................................. 20
1.9) Nfil3 expression in NK cells .......................................................................................... 21
1.10) Tissue Resident NK cells .............................................................................................. 21
1.11) UNK Cells ..................................................................................................................... 24
1.12) Tolerance ....................................................................................................................... 30

Chapter 2: Materials and Methods .......................................................................................... 34

2.1) Animals ............................................................................................................................ 34
2.2) Genotyping for Nfil3 ....................................................................................................... 35
2.3) Bone Marrow Transplantation ....................................................................................... 35
2.4) Uterine Cell Suspension ................................................................................................ 36
2.5) Mating Strategy .............................................................................................................. 37
2.6) Flow Cytometry .............................................................................................................. 38
2.7) Perfusion for collection of histological specimens ......................................................... 39
2.8) Tissue collection and preparation for histology ............................................................... 40
2.9) Cytokeratin immunohistochemistry .............................................................................. 40
2.10) Ki67 immunohistochemistry ........................................................................................ 41
2.11) Morphological and morphometrical analyses of implantation sites ......................... 42
2.12) Whole mount in-situ immunofluorescence .................................................................. 44
2.13) TUNEL Assay ................................................................................................................. 45
2.14) Statistical Analysis .......................................................... 46

Chapter 3: Results .................................................................................. 47
3.1) Litter size and viability ................................................................. 47
3.2) Nfil3\(^{-/-}\) x Nfil3\(^{-/-}\) vs. UBC-GFP x Nfil3\(^{-/-}\) implantation sites .... 52
3.3) Nfil3\(^{-/-}\) bone marrow reconstitution of alymphoid mice .......... 82
3.4) Virgin uterine cell suspension engraftment into alymphoid mice ...... 99

Chapter 4: Discussion ............................................................................ 102
4.1) Unique Approach of this Thesis .................................................. 102
4.2) Breeding and Conceptus Viability .............................................. 104
4.3) Early Gestation Developmental Delays ....................................... 106
4.4) Comparisons Between Pregnancies in Nfil3\(^{-/-}\) Females Mated by Nfil3\(^{-/-}\) or UBC-GFP Males .......................................................... 107
4.5) Nfil3\(^{-/-}\) Bone Marrow Engraftment Studies ......................... 113
4.6) Adoptive Virgin Uterine Cell Transfer Studies ......................... 118
4.7) Future Directions ..................................................................... 120
4.8) Study Limitations .................................................................... 122
4.9) Conclusion ................................................................................ 123

References ............................................................................................ 125

Appendices .......................................................................................... 139
Appendix A) Trophoblast invasion into Nfil3\(^{-/-}\) x Nfil3\(^{-/-}\) and UBC-GFP x Nfil3\(^{-/-}\) decidual tissue at GD 10.5, 12.5 and 15.5 ......................... 139
Appendix B) Rag2\(^{-/-}\)Il2rg\(^{-/-}\) dams reconstituted with C57BL/6 bone marrow have similar PAS\(^{+}\)DBA\(^{+}\) uNK cell numbers at GD 8.5, 10.5 and 12.5 .................................................. 140
Appendix C) Hematoxylin and Eosin (H&E) Staining Protocol .......... 141
Appendix D) Periodic Acid-Schiff (PAS) Staining Protocol .............. 142
Appendix E) DBA Staining Protocol .................................................... 144
Appendix F) PAS/DBA Dual Staining Protocol ................................. 146
Appendix G) Initial E4bp4\(^{-/-}\) Colony .................................................. 148
List of Tables

Chapter 1:
Table 1.1. Summary of uNK cell subset difference .......................................................... 30

Chapter 3:
Table 3.1. Tabulated implantation site data across all gestation days studied for each mating strategy ................................................................................................ 51

Chapter 4:
Table 4.1. Tabulated data of differences across all mating strategies ............................ 119
List of Figures

Chapter 1:
Figure 1.1. Comparative diagram between early and late gestation mouse implantation sites ................................................................. 6
Figure 1.2. Overview of Nfil3 functions ................................................................. 15
Figure 1.3. Development of cNK cells .................................................................. 19
Figure 1.4. NK cell subset development based on Nfil3 dependence .......................... 23

Chapter 3:
Figure 3.1. Mouse implantation site numbers and percentage of viable pups per litter . 49
Figure 3.2. PAS+DBA+ and PAS+DBA- UNK cells are present in Nfil3-/- x Nfil3-/- and UBC-GFP x Nfil3-/- implantation sites ......................................................... 54
Figure 3.3. Comparative time course study of PAS+DBA+ uNK cell size and subtype between experimental implantation sites .................................................. 56
Figure 3.4. Subtypes I, II, III and IV of PAS+DBA+ uNK cells are found in Nfil3-/- x Nfil3-/- and UBC-GFP x Nfil3-/- implantation sites ......................................................... 59
Figure 3.5. Delayed uterine lumen closure in Nfil3-/- x Nfil3-/- and UBC-GFP x Nfil3-/- implantation sites at GD 6.5 ........................................................................ 60
Figure 3.6. Antimesometrial decidualization differs in Nfil3-/- x Nfil3-/- and UBC-GFP x Nfil3-/- to C57BL/6 implantation sites at GD 8.5 .......................... 63
Figure 3.7. Differences in antimesometrial decidualization are not accounted for by frequency of cell death or proliferation in Nfil3-/- implantation sites .......... 65
Figure 3.8. Antimesometrial enrichment of unusual CD45+CD11c+ cells in the antimesometrium of Nfil3-/- x Nfil3-/- and UBC-GFP x Nfil3-/- implantation sites at GD 8.5 ............................................................................................ 67
Figure 3.9. Nfil3-/- x Nfil3-/- and UBC-GFP x Nfil3-/- embryos are developmentally delayed by 0.5-1 day at GD 8.5 .......................................................... 68
Figure 3.10. Implantation site mesometrial layers differ in surface area between Nfil3-/- x Nfil3-/- and C57BL/6 matings at GD 10.5, 12.5 and 15.5 ............ 70
Figure 3.11. Nfil3-/- x Nfil3-/- and UBC-GFP x Nfil3-/- mesometrial histology at GD 10.5, 12.5 and 15.5 ......................................................................................... 71
Chapter 4:

Figure 3.1. Summary of Nfil3-independent uNK cell-mediated pathologies.

Figure 3.2. Summary of roles of Nfil3.
List of Abbreviations

AMD; antimesometrial decidualization
ANGPT; angiopoietin
BSA; bovine serum albumin
bZIP; basic region leucine zipper
cDC; conventional dendritic cell
CLP; common lymphoid progenitor
CMP; common myeloid progenitor
cNK; circulating natural killer
DB; decidua basalis
DBA; Dolichos biflorus agglutinin
DC; dendritic cell
DLL1; delta-like ligand 1
E; embryo
E4bp4; E4-binding protein 4
ED; embryo development
EOMES; eomesoderm
EPC; ectoplacental cone
GATA3; GATA-binding protein 3
GD; gestation day
GFP; green fluorescent protein
H&E; hematoxylin and eosin
H2O2; hydrogen peroxide
HSC; hematopoietic stem cell
ICM; inner cell mass
IFNG; interferon gamma
Ig; immunoglobulin
IL; interleukin
ILC; innate lymphoid cells
iNK; immature natural killer
I.P.; intraperitoneally
I.V.; intravenously
IS; implantation site
LAB; labyrinth
MHC; major histocompatibility complex
MLAp; mesometrial lymphoid aggregate of pregnancy
mNK; mature natural killer
Nfil3; nuclear factor IL-3
NCR; natural cytotoxicity receptor
NK; natural killer
NKP; natural killer-cell precursor
PAR; proline and acidic amino acid rich
PAS; Periodic acid Schiff
PBA; phosphate buffered saline with 1% bovine serum albumin and 0.1% azide
PBS; phosphate buffered saline
PE; preeclampsia
PGF; placental growth factor
PFA; paraformaldehyde
RAG; recombination activating gene
Rag2<sup>−/−</sup>Il2rg<sup>−/−</sup> BM (C57BL/6); Rag2<sup>−/−</sup>Il2rg<sup>−/−</sup> dam reconstituted with C57BL/6 bone marrow
Rag2<sup>−/−</sup>Il2rg<sup>−/−</sup> BM (Nfil3<sup>−/−</sup>); Rag2<sup>−/−</sup>Il2rg<sup>−/−</sup> dam reconstituted with Nfil3<sup>−/−</sup> bone marrow
Rag2<sup>−/−</sup>Il2rg<sup>−/−</sup> UT (C57BL/6); Rag2<sup>−/−</sup>Il2rg<sup>−/−</sup> dam reconstituted with virgin C57BL/6 uterine cell suspension
Rag2<sup>−/−</sup>Il2rg<sup>−/−</sup> UT (Nfil3<sup>−/−</sup>); Rag2<sup>−/−</sup>Il2rg<sup>−/−</sup> dam reconstituted with virgin Nfil3<sup>−/−</sup> uterine cell suspension
RORα; retinoic acid receptor-related orphan receptor-α
SA; spiral artery
SCID; severe combined immunodeficiency
SPONG; spongiotrophoblast
TBST; tris buffered saline with 0.05% Tween 20
TCR; T cell receptor
TE; trophectoderm
TF; transcription factor
TGC; Trophoblast giant cell
Th1; T helper 1
Th2; t helper 2
TR; tissue resident
Treg; T regulatory
UBC-GFP; BALB/c-Tg(UBC-GFP)30Scha/J
UL; uterine lumen
UNK; uterine natural killer
VEGF; vascular endothelial growth factor
WM-IF; whole mount in situ immunofluorescence
WT; wild type

Mating Strategy Abbreviations

C57BL/6; C57BL/6 x C57BL/6
Nfil3+/– x Nfil3+/–; Nfil3+/– male x Nfil3+/– female
Rag2+/–Il2rg+/–; Rag2+/–Il2rg+/– male x Rag2+/–Il2rg+/– female
Rag2+/–Il2rg+/– BM (C57BL/6); Rag2+/–Il2rg+/– male x Rag2+/–Il2rg+/– BM (Nfil3+/–) female
Rag2+/–Il2rg+/– BM (Nfil3+/–); Rag2+/–Il2rg+/– male x Rag2+/–Il2rg+/– BM (Nfil3+/–) female
Rag2+/–Il2rg+/– UT (C57BL/6); Rag2+/–Il2rg+/– male x Rag2+/–Il2rg+/– UT (C57BL/6) female
Rag2+/–Il2rg+/– UT (Nfil3+/–); Rag2+/–Il2rg+/– male x Rag2+/–Il2rg+/– UT (Nfil3+/–) female
UBC-GFP x Nfil3+/–; UBC-GFP male x Nfil3+/– female
Chapter 1

General Introduction and Literature Review

1.1 Mammalian Placentation

Mammalian pregnancy is established when the hatched blastocyst comprised of outer trophectodermal cells and enclosed inner cell mass cells, interacts with the maternal uterus. These interactions vary between different species leading to the development of distinct types of placentas. The placenta is the interface between the mother and the developing embryo/fetus that performs a number of important functions throughout gestation (1). The placenta is the organ responsible for oxygen/carbon dioxide exchange, provision of nutrients to the fetus and removal of waste products during embryonic development (1). In addition to these physiological roles, the placenta also physically anchors the fetus to the uterine wall. Gross anatomical shape, and histological structure of the placenta are two methods for classifying placentas across mammalian species (1,2). There are four types of placentas based on shape. The diffuse placenta occurs over the entire surface of the uterine luminal epithelium and can be found in horses and pigs (1). The multicotyledonary placenta develops as many small oval regions that attach to caruncles within the endometrium, and is found in ruminants (1). The third type of placenta is a zonary placenta that envelops the chorionic sac like a belt and is found in carnivores (1). Finally, the discoid/bidiscoid placenta characterized by one or two roughly circular placentas is found in primates, rodents and rabbits (1).

The primary method of placental classification is based upon the invasion capabilities of the outer cells of the conceptus, the trophectodermally-derived trophoblast,
into the uterine wall. The most superficial of the three types is the epitheliocchorial placenta, which experiences little invasion into the uterine lining without any maternal tissues being destroyed or removed (3). Trophoblast loosely interacts with maternal endometrial epithelium and this placenta is found in horses, pigs and ruminants (4). The second type is the endotheliocchorial placental. Development begins with maternal uterine epithelium and connective tissue disappearing soon after implantation allowing trophoblast to come into direct contact with the maternal endometrium (4). This placenta occurs in a number of mammals including cats, dogs and bats. Most primates, rabbits and rodents have a highly invasive type of placenta, known as the hemochorial type (4). All of the maternal tissue layers disappear allowing direct communication between the trophoblast and maternal blood (4). A further distinguishing factor of hemochorial placentas is the transformation of the uterine mucosa into a highly specialized tissue known as the decidua (5).

1.2 UNK Cell Overview

In humans, uNK cells are generally considered to be confined to the decidua, as they are rarely detected in the fallopian tubes during ectopic tubal pregnancies (10). These cells are unique in that unlike their peripheral blood counterparts they secrete pro-angiogenic cytokines rather than having a primarily cytotoxic role (11). Furthermore, their recruitment to/presence in the uterus is not dependent on a conceptus as they are present 3 to 8 days after the luteinizing hormone surge of each menstrual cycle when predecidualization of stroma occurs (12). UNK cells represent 40% of the total leukocyte population during the proliferative phase, which increases to 60% by the mid-secretory
phase and up to 75% in early pregnancy (13). This is in contrast to mouse pregnancy, where uNK cells are not present until after implantation of the embryo, otherwise both cell types are similar. This thesis addresses the consequences of the absence of uNK cells during pregnancy using a murine model; therefore mouse pregnancy will be discussed in more detail.

1.3 Mouse Pregnancy

1.3.1 Implantation

Days of pregnancy are counted as gestational days (GD). The morning of the appearance of the copulation plug is GD 0.5. Fertilization of the egg leads to formation of the zygote, which undergoes a series of cleavage divisions to form an 8-cell morula (14). The 8-cell stage is when compaction is initiated, a process establishing the distinction between inner and outer cells within the embryo (14). Following this event is cavitation, where a fluid filled region called the blastocoel appears within the morula. The resulting structure is now called the blastocyst and is present at GD 3.5 (14). When the blastocoel is larger than the inner cell mass (ICM), the blastocyst is considered expanded (14). The blastocyst will then hatch from the zona pellucida (an oocyte coat that still surrounds the embryo) and implant into the endometrium on GD 4.5 (15). By this time the embryo has separated into an outer monolayer of cells termed the trophectoderm (TE), and the ICM. The TE goes on to differentiate into the placenta, while the ICM develops into the embryo proper (16,17). The position of the blastocyst during implantation is important. The area containing the ICM is termed the embryonic pole, while the opposing side is the abembryonic pole (18). The mouse endometrium also has an orientation; the mesometrial
pole (mesometrium) is associated with the highly vascular mesometrium, a ligament attaching the uterus to the abdominal cavity, while the opposite pole is the antimesometrium (18). Implantation is defined as the attachment of the abembryonic pole of the blastocyst to the antimesometrial endometrium (19). Upon implantation, the TE differentiates into trophoblast, which begins to invade the maternal tissue, creating the foundations for the placenta.

1.3.2 Decidualization

In humans, decidualization commences in the luteal phase of the menstrual cycle and continues if pregnancy is established. If pregnancy does not occur, the decidualized endometrium breaks down and is shed during menstruation (12). In mice, decidualization is not initiated until after implantation, when the uterine lumen begins to close around the embryo (Fig. 1.1A). The antimesometrial pole decidualizes first, and then the decidual reaction spreads to the mesometrial endometrium (20). The initial site of decidualization is known as the primary decidual zone and, by GD 5.5, the secondary decidual zone is formed (20). Decidualization occurs by proliferation and differentiation of new cells at the edge of the decidualized front and an increase in cell volume of decidualized cells is evident (20). The resulting uterine wall is about fivefold thicker than in a non-pregnant state (21). Decidual cells have a predetermined lifespan, and will proliferate and differentiate until GD 7.5-8.5 (22). Thereafter, decidual cells undergo apoptosis, allowing the embryo to grow. This has the consequence of regressing the antimesometrial decidua, a process called decidual involution (23). The mesometrial decidua persists and is referred to as the decidua basalis (DB) (23). Another prominent mesometrial structure
that begins to take shape at GD 8.5 is the mesometrial lymphoid aggregate of pregnancy (MLAp), a lymphocyte-enriched region that develops between the two layers of the myometrium at each implant site ([24]). Estrogen and progesterone regulate decidualization through estrogen receptors α and β and progesterone receptors A and B found on endometrial cells (25,26). A number of other changes accompany decidualization including remodeling of the extracellular matrix of the endometrial stroma, production of molecules to regulate maternal-fetal interactions, angiogenesis in order to form the vasculature necessary for nutritional supply to the conceptus, remodeling of resident endometrial blood vessels to meet the demands of the conceptus, and finally the influx of a number of CD45+ leukocytes, mainly uNK cells, which play a number of roles in the implantation site (18,27).
**Figure 1.1. Comparative diagram between early and late gestation mouse implantation sites.** Key features of post-implantation early gestation (GD 5.5-8.5) mouse implantation sites (A) is the closure of the uterine lumen, and the protrusion of the ectoplacental cone into the decidual basalis. The embryo implants in the antimesometrium, and trophoblast invasion extends mesometrially. Late gestation (GD 10.5-term) implantation sites (B) exhibiting the five layers of the placenta in the mesometrium. Labels = DB: decidua basalis; E: embryo; EPC: ectoplacental cone; LAB: labyrinth; MLAP: mesometrial lymphoid aggregate of pregnancy; SPONG: spongiotrophoblast; P-TGC: parietal trophoblast giant cell layer; UL: uterine lumen.

### 1.3.3 Trophoblast

These maternal changes are not only hormonally mediated, but are also induced by fetal trophoblast cells. The TE differentiates into a variety of trophoblast subtypes each with specific functions. Primary trophoblast stem cells arise from direct differentiation of the TE. These cells stop dividing, enlarge, undergo multiple rounds of DNA replication to become polyploid and are termed trophoblast giant cells (TGCs)(14,28,29). TGC invasion is dependent on trophoblast induced uterine epithelial cell apoptosis. TGCs phagocytose epithelial cells, permitting deeper invasion into the
endometrium (30). Proliferation of abembryonic trophoblast (termed the extraembryonic ectoderm) gives rise to the ectoplacental cone (EPC) (30). This mass of cells projecting into the mesometrial decidua is the beginning of the placenta and can be detected by GD 6.5 (30). The outer layer of the EPC is terminally differentiated secondary TGCs while the inner layer houses proliferating, undifferentiated trophoblast (14). As the proliferating trophoblast migrate towards the mesometrium they become large and irregularly shaped due to numerous cytoplasmic projections (31). These cells are called parietal TGCs (p-TGCs) and are closely associated with the decidua (31). P-TGCs remove endothelial cells from maternal vessels facilitating the maternal blood/conceptus interaction necessary for nutrient and waste exchange (31). As the placenta forms, this P-TGC rich region will develop into the labyrinth, the zone of maternal/fetal exchange analogous to placental terminal villi in humans (31).

1.3.4 Placental Development

By GD 10.5, the placenta has formed and the antimesometrial decidua has almost entirely involuted, leaving five distinct layers in the mesometrium. The layers furthest from the conceptus is the maternal MLAp and DB housing the maternal leukocytes, invading trophoblast, and maternal spiral arteries responsible for shunting blood to the developing fetus (32). Adjacent the DB is the thin layer of P-TGCs, covering the surface of the spongiotrophoblast layer (32). The final layer, closest to the developing fetus and the site of maternal/fetal exchange is the labyrinth (32). Figure 1.1B shows a schematic of the mid-late gestation implantation site. The labyrinth is made up of two highly branched vascular networks, one maternally-derived; the other conceptus-derived, that interdigitate
with one another to optimize molecular exchange (33). The maternal/conceptus capillaries are separated by the interhemal membrane, a cell barrier comprised of mononuclear TGCs adjacent to maternal blood, two layers of syncytiotrophoblast and a layer of fetal endothelial cells adjacent fetal blood (33,34). Trophoblast can also be found within maternal vessels, specifically the spiral arteries, which trophoblast invade and line. Trophoblast then direct structural remodeling of the vessels into a more venous phenotype via degradation of the smooth muscle and elastin (35). At GD 12.5 the spongiotrophoblast layer has two morphologically distinct cell types: the spongiotrophoblast, which are dense and compact and the glycogen trophoblast, which sequester glycogen in their cytoplasm giving them a large “foamy” appearance (21). Glycogen trophoblast are numerous and massively proliferate to GD 16.5 when their numbers drop by 50% (21,35-37). Spongiotrophoblast proliferate modestly, while the P-TGC layer stays stable across late gestation (36). By mid-gestation the labyrinth layer makes up the largest proportion of the placenta and is made up of the EPC, the trophoblast derived chorion and the extraembryonic allantois (36). The origin of the maternal vessels within the labyrinth begins opposite this region, in the decidua.

1.3.5 Implantation Site Vasculature

The maternal vasculature within the implantation site commences with the radial arteries that enter the uterus through the myometrium from the mesometrial pole (21). Branches of the radial arteries enter the mesometrium and then divide into several spiral arteries, which span the decidua and are the ‘supply’ arteries for the fetus (21). The diameter of these arteries increases ~90mm from GD 10.5-14.5 as a result of both
maternal uNK cell and conceptus trophoblast actions (21). Upon reaching the p-TGC layer covering the placenta, these arteries converge into one to four centrally-located arterial canals that are lined with trophoblasts (21). These relatively wide (300-600μm at GD 10.5-17.5) arteries traverse the placenta down to its base, and only then divide into the small, convoluted capillary spaces of the labyrinth (21). Maternal blood then drains from the labyrinth through peripherally located roughly “C” shaped venous sinuses in the DB (21). Conversely, the fetal circulation is a result of a single umbilical artery branching extensively within the labyrinth. The fetal capillaries initially extend outward and remain largely unbranched as they cross through the labyrinth to the spongiotrophoblast layer (21). From there, the capillaries split into the tortuous network of labyrinth vessels extending back towards the fetal side of the placenta (21). Therefore, fetal blood flow and maternal blood flow run counter to each other within the labyrinth (21).

### 1.4 NK Cell Depletion

In order to more deeply understand processes disturbed in human pregnancy complications, normal and pathological mouse pregnancies are frequently studied. To assess the functions of different cell types in pregnancy, the consequences of depletion experiments are commonly used. Conclusions are typically validated by subsequent reconstitution experiments. Techniques to achieve cell lineage specific depletion include antibody-mediated depletion and gene ablation in mice. A number of investigators have applied these approaches to define function of the most abundant leukocyte in the decidua during pregnancy, the uNK cell. Initial studies treated CD1 mice intraperitoneally (i.p.) with Ly-49G2 antibody in an attempt to deplete uNK cells, as Ly-
49G2 is a receptor on peripheral NK cells (38). Although NK cells were depleted in peripheral blood and spleen, pregnancies in the treated females appeared normal and there was no reduction in uNK cell numbers (38). Thereafter, a number of gene knockout strains were tested for a reduction or depletion of uNK cells including $I_{L2}^{-/-}$ and $I_{L2}^{-/-} \beta_2m^{-/-}$ without success (38). Finally, a transgenic mouse strain that carries the human CD3e gene called $T_{ge}26$ was revealed to be T cell deficient and have only 3% of normal uNK cell levels (NK$^{-}$T$^{-}$B$^{-}$) (38). There were a number of pathologies associated with the pregnancy including a 67% fetal resorption rate, a 45% decrease in placental area and a complete absence of the MLAp (38). Spiral artery wall thickness persisted, never achieving a venous phenotype. When $T_{ge}26$ mice were mated with immune competent (NK$^{-}$T$^{+}$B$^{+}$) CD1 mice, the implantation sites mirrored those from $T_{ge}26$ homozygous matings, indicating the conceptus could not correct a maternal phenotype (38). This also held true for CD1 embryo transfers into $T_{ge}26$ dams. This study determined that uNK cells are not essential for implantation, or differentiation of trophoblast however they do play an important role in normal maternal decidual vascular development during pregnancy (38).

1.4.1 Bone Marrow Transplants

A follow-up study to the $T_{ge}26$ work included bone marrow grafts to effectively reconstitute NK cells into the dams. Severe combined immunodeficient (SCID; NK$^{-}$T$^{-}$B$^{-}$) bone marrow was injected intravenously (i.v.) into $T_{ge}26$ females to determine if the presumed uNK dependent pregnancy pathologies could be corrected. These included a loss of uNK cells to $<1\%$ of normal, absence of MLAp development, acellularity and
edema in the decidua, increased wall:lumen ratios in the spiral arteries, small placenta size, and mid-gestational fetal death (39). UNK cells were 50% reconstituted in the pregnant bone marrow recipients and the MLAp was well developed. No major decidual or placental anomalies were found and spiral artery wall:lumen ratios became similar to controls (39). The placentas were larger than the homozygous Tge26 implantation sites but still smaller than controls and fetal viability was equivalent to immune-competent controls (39). Results from these experiments supported the idea that uNK cells somehow deplete vascular smooth muscle cells during pregnancy to promote spiral artery remodeling, are instrumental to the development of the MLAp, and participate in decidual cell maturation (39).

Historically, there have been three strains of mice that have a marked deficiency in NK cells. In each, the NK cell deficiency was accompanied by a co-deficiency in T cells: Tge26, discussed above, interleukin (IL)-2Rβ+xp56c+/- and IL-2Rγ−/− (40). In all three strains, uNK cells are at less than 1% of normal levels, the MLAp fails to develop, the decidua and its vasculature are compromised and the placenta is very small (40). These strains are difficult to maintain due to death during infancy, or poor reproductive performance. The development of the alymphoid (NK− T− B−) Rag2−/−Il2rg−/− genotype was an important advance in the study of uNK cells because these mice breed well when reared using barrier husbandry (40). There are some similarities between the Tge26 and Rag2−/−Il2rg−/− strains, however there are also a number of striking differences. Both strains experience decidual cell and vessel pathology beginning at GD 7.5 (40). However the placental sizes of the Rag2−/−Il2rg−/− implantation sites are the same as controls and mid-gestational fetal loss is not observed (40). UNK cell functions were taken to play a role
in the overlapping pathologies between the two strains of mice. Therefore primary uNK cell function is likely initiated at GD 6.5 as no implantation site pathology was evident before this time point in either strain and uNK cells seem to sustain but not initiate development of normal mesometrial decidua (40). Furthermore uNK cells are not essential for fetal survival but may enhance fetal growth (40). Finally \( \text{Rag}^{2/-}\text{Il2rg}^{-/-} \) mice were considered to be superior to \( Tge26 \) for continued analysis of uNK cell function due not only to their absolute deficiency in uNK cells but also to the stability of their genetic modification (40).

1.4.2 \( \text{Rag}^{2/-}\text{Il2rg}^{-/-} \) mice

The absence of lymphocytes in the \( \text{Rag}^{2/-}\text{Il2rg}^{-/-} \) strain (NK–T–B–) creates an ideal environment for assessing the lymphoid lineage differentiation potential of grafts (41). Exploiting this phenomenon allows the use of syngeneic or congenic grafts to explore the impact of reconstitution on the well-characterized phenotype of \( \text{Rag}^{2/-}\text{Il2rg}^{-/-} \) pregnancy. Ashkar et al. reported outcomes from a number of pre-conception bone marrow transplants in \( \text{Rag}^{2/-}\text{Il2rg}^{-/-} \) pregnancy (42). All of the graft recipients were syngeneically mated to \( \text{Rag}^{2/-}\text{Il2rg}^{-/-} \) males regardless of the bone marrow donor. C57BL/6 mice were used as control mice (N+T+B+). GD 11.5 (midgestation) was chosen for study as this is when uNK cell numbers are at their peak. From GD12.5, uNK cell numbers decline until term. Reconstitution of \( \text{Rag}^{2/-}\text{Il2rg}^{-/-} \) dams with C57BL/6 bone marrow induced high number of uNK cells, decidual vessel modification and normal decidual cellularity during pregnancy (42). The SCID mouse mutant is NK cell competent without having T or B lymphocytes (NK–T–B–) (42). Reconstitution using SCID bone marrow generated
pregnancies similar to C57BL/6 reconstituted and control pregnancies (42). Using this same adoptive transfer approach, others showed progenitors of uNK cells were also present in thymus, spleen, fetal liver and lymph nodes (43).

Adoptive bone marrow transplant studies were also carried out to identify the early pregnancy actions of uNK cells in alymphoid dams on the BALB/c background (44). The novel technique of decidual whole mount in situ immunofluorescence (WM-IF) was used in parallel with paraffin-embedded tissue sections to assess implantation sites. WM-IF allows examination of live tissue after incubation with fluorescently-conjugated antibodies. At GD 6.5, the DB of Rag2⁻/⁻Il2rg⁻/⁻ implantation sites displayed active angiogenesis, but failed to achieve the mature vessel pruning seen in controls (44). The vascular insufficiencies persisted to GD 8.5 where mesometrial vessels were thin and resembled GD 6.5 control vessels. Outside of vascular effects alymphoid implantation sites experienced a delay in uterine lumen closure, delayed trophoblast migration and a 24-hour delay in embryo development compared to controls (44). Reconstitution of NK cells in Rag2⁻/⁻Il2rg⁻/⁻ dams using SCID (NK⁺T⁺B⁻) bone marrow provided uNK cells and normalized the vascular modifications and rate of implantation site development (44). In all reported cases, the reconstituted Rag2⁻/⁻Il2rg⁻/⁻ dams were either NK⁺T⁺B⁻ or NK⁺T⁻B⁻. Thus, the gestational roles of NK cells have not been assessed under the conditions of a pure NK cell deficit in the presence of normal T and B cells.

1.5 Nfil3 Gene

A strain of mouse that is NK cell deficient, T and B cell competent (NK⁺T⁺B⁻) was recently developed independently in two laboratories by knocking out the gene for a
transcription factor (TF). The TF was described in 1992 by Cowell et al. by its ability to recognize the proximal activating transcription factor binding site of the adenovirus E4 promoter and was called E4-binding protein 4 (E4BP4)(45). Subsequently, Zhang et al. identified the same protein as nuclear factor IL-3 (NFIL3), which binds the 5’ flanking region of the human IL-3 promoter and is expressed in T cells (46). NFIL3 is a TF that is part of the proline and acidic amino acid rich (PAR) basic region leucine zipper (bZIP) subfamily. This subfamily also includes hepatic leukemia factor, a TF involved in B cell lineage commitment (47), implying NFIL3 may have similar actions. It is an IL-3 responsive gene whose expression is dependent on de novo protein synthesis. *Nfil3* is almost ubiquitously expressed and present at relatively high levels in the lungs, liver and bone marrow, and at low levels in the spleen (48). *Nfil3* has been implicated in a wide range of processes. It plays a role in circadian rhythm working antagonistically to other PAR bZIP proteins (49,50). *Nfil3* is a regulator of neuron growth and survival (51,52), osteoblast function (53) and ovulation (54). Its expression has also been detected in endothelial cells (55). Most importantly *Nfil3* is expressed in specific immune cell lineages revealing its important role in the immune system. *Nfil3*−/− mice have been widely used as a model to deepen this understanding (56). The roles of *Nfil3* are summarized in Figure 1.2.
Figure 1.2. Overview of Nfil3 functions. Nfil3 plays a role in neuron growth and survival, circadian rhythm, ovulation, osteoblast function, immune cell differentiation and function, and is expressed in the endothelium.

1.5.1 Nfil3−/− mice

Nfil3−/− mice are also deficient in CD8α+ conventional dendritic cells (cDCs) while numbers of CD8α−, circulating plasmacytoid dendritic cells (DCs), and DC progenitors are normal (57). Conventional DCs are important in the presentation of peptides from endocytosed particles on major histocompatibility complex (MHC) class I molecules (a cell surface molecule responsible for displaying peptide fragments of pathogens). This action is called cross-presentation (58). NFIL3 mediates the IL-4 driven process of immunoglobulin (Ig) heavy chain class switching necessary for IgE production and this is impaired in B cells of Nfil3−/− mice (59,60).

Nfil3 expression increases in response to macrophage activation in vitro and Nfil3−/−
mice over-produce IL-12 (61,62). The gene may play a role in polarizing T helper cell responses via direction of macrophage cytokine production (56). *Nfil3* also has a direct effect on cytokine production in CD4+ T cells. *Nfil3−/−* expression increases over the course of in vitro T helper 2 (Th2) differentiation and *Nfil3−/−* cells produce less IL-10 than wild type (WT) cells (57,63). NFIL3 is also upregulated in chronically stimulated T helper 1 cells (Th1) that secrete the Th2 cytokines IL-10 and IL-13. NFIL3 binds the *Il13* promoter directly but modulates IL-10 transcription indirectly by affecting chromatin structure at the *Il10* locus (63).

The most striking finding in *Nfil3−/−* mice is their severe deficiency in circulating NK (cNK) cells; they retain normal numbers of T, B and NKT cells in blood, spleen, liver, lung and bone marrow (48,64). This NK cell deficiency is the result of a defect in NK cell progenitors as in vitro cultured lineage-negative (Lin−) bone marrow cells from *Nfil3−/−* mice could not give rise to NK cells (48). Irradiated *Nfil3−/−* mice were successfully reconstituted with NK cells using wild type bone marrow, indicating the bone marrow microenvironment in *Nfil3−/−* is able to support NK cell development (48). Additionally, *Nfil3−/−* bone marrow is able to reconstitute T, B and NKT cells proving the NK cell deficiency is specifically due to alterations in NK cell progenitors and does not result from an *Nfil3*-deficient tissue microenvironment (48). To determine the precise point at which *Nfil3* is necessary in lymphocyte and NK cell development requires an understanding of hematopoiesis, or the formation of blood cells.

1.6 Hematopoiesis

Hematopoietic stem cells (HSCs) are pluripotent stem cells that give rise to and
replenish mature blood cells (65). In adults, HSCs reside in the bone marrow and are capable of both self-renewal and differentiation into the progressively more restricted precursors of red blood cells, myeloid cells and lymphocytes (66). There is a small population of self-renewing HSCs that divide relatively infrequently, therefore the lineage-restricted progenitors are responsible for the extensive proliferation required to maintain blood cell homeostasis. HSCs commit to differentiation into either common lymphoid progenitors (CLPs) or common myeloid progenitors (CMPs) (67).

1.7 NK Cell Development

The NK-cell precursor (NKP) is generated from CLPs and these, by definition, have the potential to develop into mature NK cells but not any other hematopoietic lineage. NKPs give rise to immature NK (iNK) cells, which express some, but not all, NK-cell specific markers (68). NKPs are found in the bone marrow, which is the main site for NK-cell development from NKP to mature, fully differentiated and functional NK-cells (69-71). The only NK cell restricted murine bone marrow subset (therefore a true NKP) that was described historically was the Lin^CD49b^CD161^-CD122^+ population that can differentiate into mature NK cells in vitro or in fetal thymic organ cultures (72). However many lymphoid-restricted bone marrow subsets that have NK-cell potential have been characterized (68,72,73). Furthermore the Lin^CD49b^-CD161^-CD122^+ population is heterogeneous, as less than 30% of cells can differentiation into NK cells in vitro (72). NKP cells are characterized by expression of both CD122 and NKG2D (68). A pure NKP population was successfully isolated in 2011 by selecting Lin^-CD27^CD244^+CD122^-IL7Rα^+Flt3^- cells from mouse bone marrow (74).
INK cells express CD161, a family of C-type lectin receptors that includes CD161c, which is recognized by the antibody NK1.1. All immature and mature NK cells express CD161. CD161 however, is not an NK cell specific marker as it is also expressed on NKT and subsets of T cells (75,76). NKp46, a member of the family of natural cytotoxicity receptors (NCRs), is highly expressed on NK cells beginning at the iNK-cell stage (77). INK cells do not express CD49b (which is recognized by the antibody DX5) or CD11c and express very low levels of CD11b and CD43 (70,78). INKs begin to express CD94 and some express Ly49 receptors, a family of NK cell lectin receptors that binds host MHC class I molecules (72,78). The functionality of iNKs is fairly low; they are not cytotoxic and they do not secrete interferon gamma (IFNG) (72). Mature NK (mNK) cells express the markers DX5, CD122, CD161, NK 1.1, in addition to high levels of CD11b and CD43 (70,79,80). Additionally they express activating receptors including the NCRs (NKp30, NKp44 and NKp46), NKG2D and the Fc receptor CD16, as well the family of Ly49 receptors with both activating and inhibitory members (70,79,80). The activating receptors recognize self-encoded ligands expressed poorly on normal cells but strongly by unhealthy cells such as tumor cells or virally-infected cells. The inhibitory receptors bind to classical and non-classical MHC class I antigens (81,82). The development of NK cells is outlined in Figure 1.3.
Figure 1.3. Development of cNK cells. CNK cell development is initiated in the bone marrow. Maturation is dependent on cytokine stimulation, transcription factor expression and surface cell receptor acquisition. Labels = HSC: hematopoietic stem cell; CLP: common lymphoid progenitor; NKP: NK-cell precursor; iNK: immature NK cell; mNK: mature NK cell; IFNG: interferon gamma.

CNK cells express high levels of the integrin DX5, and secrete high levels of IFNG and IL-13 (83). Their cytolytic effects are achieved via use of perforin and granzymes, and can be triggered within minutes without requiring transcription, translation or cell proliferation (83). Activation of cNK cells is directed by the “missing-self hypothesis”. NK cells provide immune surveillance for cells that have down regulated MHC class I, an event often associated with cellular transformation (as in tumor cells) or infection with viruses (83). If an NK cell interacts with a cell possessing MHC class I, the receptor engages an inhibitory receptor on the surface of the NK cell.
Alternatively NK cells may be activated if an engaging somatic cell overexpresses ligands for NK cell activating receptors (81).

### 1.8 Innate Lymphoid Cells

CNK cells are a subset within a large family of lymphocytes termed innate lymphoid cells (ILCs) that are expected to share a common progenitor that has yet to be fully elucidated. ILCs represent a relatively newly defined family of hematopoietic cells that serve protective roles in innate immune responses to infectious microorganisms, in lymphoid tissue formation, in tissue remodeling after injury or infection induced damage and in the homeostasis of tissue stromal cells (84). Three main features define ILCs: the absence of recombination activating gene (RAG)-dependent rearranged antigen receptors; a lack of myeloid and DC markers, and lymphoid morphology (85). There are three distinct groups of ILCs, with cNK cells being the prototypical population (85). Group 1 (ILC1) includes ILCs that produce interferon gamma (IFNG; which includes NK cells) (85). Group 2 (ILC2) is made up of ILCs that produce a humoral response inducing type 2 cytokines (including IL-5 and IL-13) and are dependent on the transcription factor (TF) GATA-binding protein 3 (GATA3) and the retinoic acid receptor-related orphan receptor-α (RORα) (85). Group 3 (ILC3) is comprised of all ILCs that produce IL-17 and/or IL-22 and depend on the TF RORγt for their development and function (85). The role of Nfil3-dependent development extends beyond ILC1 NK cells. It is also expressed in ILC2 and ILC3 subsets and Nfil3−/− mice have compromised development of these subsets in the lung and gut (80).
1.9 Nfil3 expression in NK cells

Experiments using Nfil3<sup>−/−</sup> mice have determined that this strain has dramatically reduced numbers of NKPs and unchanged numbers of CLPs. Experiments using other strains of mice with severe NK cell deficiencies (i.e. Il15ra<sup>−/−</sup>) did not have reduced numbers of NKPs (86), identifying NFIL3 as the only TF identified as essential for NKP production. Furthermore Nfil3 is required for expression of the IL-15 receptor, and so acts upstream of IL-15 signaling which previously had been considered the definitive factor required for the production of NK and uNK cells (86,87). CLPs sorted from Nfil3<sup>−/−</sup> bone marrow transduced with an Nfil3 vector were able to differentiate into NK cells; therefore NFIL3 is required specifically at the lineage commitment point (48,87). NFIL3 is not, however, required for maintenance of cells that are already committed to the NK lineage (88).

1.10 Tissue Resident NK Cells

A number of different subsets exist within the NK lineage. Although bone marrow is the principal site of NK cell production, fetal liver and thymus are also sites of NK cell development (76). Thymic NK cells are derived from early thymic precursors and differ from cNK cells in their retention of high levels of IL-7Rα, their dependence on GATA3, and their poor cytotoxicity (89). The liver houses cNK cells, however there is also a CD49b<sup>+</sup>TRAIL<sup>+</sup> NK cell population that secretes limited IFNG and IL-13 (90). TRAIL is a death ligand and also a marker of NK cell immaturity (90). TRAIL<sup>+</sup> NK cells are the main NK cell population in fetal and neonatal mice then TRAIL<sup>+</sup> NK cells decline with age (90). However, a small TRAIL<sup>+</sup> population resides in the liver of adult mice(90).
It is thought that differential expression of TFs drives the formation of NK cell subsets. The TFs TBET and Eomesoderm (EOMES) direct fate and function of bone marrow-derived cNK cells (91). Without TBET and EOMES, hematopoietic progenitors do not support NK development and mNKs revert to a more immature state and lose classical NK cell antigens (91). However EOMES is not necessary for TRAIL⁺ NK cell development (91). Both Id2 and Nfil3 are important regulators of cNK cell development as deletion of either gene results in a severe reduction of the cNK population (80). Nfil3 has been shown to be critical for the development of thymic NK cells however Nfil3 is dispensable for TRAIL⁺ liver NK cells (80). Furthermore experiments restoring EOMES expression in Nfil3⁻/⁻ progenitors rescued cNK cell development (80). Therefore the mechanism of action of Nfil3 in cNK development is through induction of EOMES expression (80). Other subsets within the NK cell lineage that are not reliant on Nfil3 are the TRAIL⁺ tissue resident (tr) salivary gland NK cell (92) and a CD49a⁺DX5⁻ subset found in virgin mouse uterus (93). The impact of Nfil3 on NK cell development is summarized in Figure 1.4.
**Figure 1.4. NK cell subset development based on Nfil3 dependence.** Nfil3 is necessary for the development of peripheral thymic and circulating NK (cNK) cells through expression of EOMES. This gene also acts upstream of IL-15, a cytokine necessary for peripheral NK cell development. TRAIL⁺ liver and salivary gland (SG) NK cells along with CD49a⁺DX5⁻ uNK cells are Nfil3 independent and defined as tissue resident NK cells.

The question of how uNK cells are related to other ILCs remains outstanding as does the issue of the actions of T cells and B cells in normal pregnancies when NK cells are absent. It has been postulated that interactions between T and NK cells are critical to T cell conceptus tolerance (94). Development of Nfil3⁻/⁻ mice appeared to offer the appropriate tool to answer these questions. In an initial report by Fu et al., expansion of proinflammatory decidual Th17 cells and fetal losses (~50% of each litter) later in gestation (GD 14.5) were found in Nfil3⁻/⁻ females mated allogeneically by BALB/c males (94). These authors postulated that in normal pregnancies, uNK cell regulatory
functions promoted T cell tolerance of conceptuses and they extended their findings and conclusions to deciduas collected from women who experienced spontaneous pregnancy failures (94). The data collected over the course of this thesis studies, and simultaneously developed in other laboratories (93) challenge the conclusions of Fu et al.

1.11 UNK Cells

1.11.1 UNK Cell Subsets

UNK cells were first discovered by Mathias Duval in 1891 in rabbit decidua, and later described as granulated cells found in the human endometrium, eventually termed granulated metrial gland cells (95). Most historical studies of uNK cells have been histological and the cells have been regarded as a single population with subsets discriminated on the basis of maturity (24,96). Studies of the receptor repertoire on uNK cells began ~25 years ago. Currently available information in humans and mice shows that uNK cells have a receptor repertoire distinct from cNK cells. In humans, uNK cells predominantly exhibit a CD56\textsuperscript{bright}CD16\textsuperscript{+}CD57\textsuperscript{−} phenotype while cNK cells are typically CD56\textsuperscript{dim}CD16\textsuperscript{+}CD57\textsuperscript{+} although small percentages (<10%) of the inverse phenotypes exist in the decidua and periphery (97). In mice, two distinct subsets of uNK cells are defined by antibodies to surface markers. At GD 9.5, a smaller population of NK1.1\textsuperscript{+}DX5\textsuperscript{+} cells is present that mirrors the phenotype of cNK cells. The larger population is defined as NK1.1\textsuperscript{−}DX5\textsuperscript{−} (79). Mature cNK cells are functionally distinct based on their expression of CD11b and CD27, however all NK1.1\textsuperscript{−}DX5\textsuperscript{−} uNK cells are CD27\textsuperscript{−} and positive for varying levels of CD11b (79). CNK-like NK1.1\textsuperscript{−}DX5\textsuperscript{−} uNK cells are CD11b\textsuperscript{low} while the more granular cells are CD11b\textsuperscript{high} (79). uNK cells are CD69\textsuperscript{+}.
which is a marker of activation in cNK cells however uNK cells lack the common mature NK cell marker CD43 (98). UNK cells share expression of CD117 (a marker of hematopoietic precursors, upregulated in IL2 activated cNK cells) and CD9 with cNK cells (79). The acquisition of Ly49 receptors before CD94, NK1.1 and DX5 is unique to mouse uNK cells and the resulting Ly49 repertoire on NK1.1\textsuperscript{+}DX5\textsuperscript{−} uNK cells is qualitatively and quantitatively different from cNK cells. Ly49C/I and CD49D are selected against, while Ly49G2 is expanded (79). Ly49C/I binds to self-MHC therefore the down regulation of this receptor may play a role in developing tolerance at the maternal/fetal interface (79).

1.11.2 UNK Cell Maturation

Terminally differentiating uNK cells are first recognized at GD 5.5 in the mesometrial decidua of mouse implantation sites. Initially there are very few uNK cells and they are small in size and agranular ([24]). Peel has estimated that only 5 cells would be sufficient to achieve peak populations at approximately GD 12.5 due to the rapid proliferation of uNK cells that initially occurs in the DB and from GD 8.5 continues in the newly developed MLAp, a transient lymphoid structure that develops between the uterine smooth muscle layers immediate apposed to each placenta (24). The uNK cell population then undergoes apoptosis and cell numbers decline gradually to term. As uNK cells mature and differentiate, they enlarge from ~10mm to approximately 50mm in diameter. The mature cells have many cytoplasmic granules and accumulate in the DB while less mature cells are found in the MLAp (99). The development of double compartment cytoplasmic granules is the hallmark of NK cell and uNK cell maturation,
and they contain cytolytic and secretory proteins such as perforin and granzymes (96). There are four morphological subtypes of uNK cells. Subtype I cells are round, small (9µm) agranular lymphocyte-like cells (96). Subtype II is slightly larger (13µm) with some granules, while subtype III cells are round, much larger (26µm), heavily granulated and have round euchromatic nuclei (96). Finally subtype IV cells are the largest cells (30µm) with abnormally condensed chromatin in the nucleus (signaling senescence or apoptosis) and granules scattered amongst vacuoles in the cytoplasm (96). The mature subtype III and IV accumulate closer to the placenta during mid-pregnancy while the immature, proliferative subtypes I and II are nearer the myometrium in the MLAp (96). This difference in distribution suggests a gradient dependent on uNK cell maturation moving from the myometrium towards the placenta (96). It is of note in human studies that senescent NK cells have been regarded as highly active secretory cells that promote vascular remodeling (100,101).

1.11.3 UNK Cell Markers In Mice

The lectin *Dolichos biflorus agglutinin* (DBA) marks both the plasma membrane and granule enclosing membranes of NK1.1⁺DX5⁻ uNK cells (96). Periodic acid Schiff (PAS) staining is a universal uNK cell marker, staining both NK1.1⁺DX5⁻ and NK1.1⁺DX5⁺ subsets. The glycol-conjugate contents in uNK cytoplasmic cell granules react with the Schiff reagent after treatment with periodic acid. Initially, at GD 6.5 PAS⁺DBA⁻ uNK cells and PAS⁺DBA⁺ uNK cells are present in the implantation site in equal proportions. However by mid-gestation PAS⁺DBA⁻ uNK cells are outnumbered 1:9 due not to loss in abundance of PAS⁺DBA⁻ cells but due to the large expansion in
PAS⁺DBA⁺ uNK cells (102). When BALB/c mouse bone marrow was transplanted into alymphoid BALB/c-<sup>Rag2<sup>−/−</sup></sub><sup>Il2rg<sup>−/−</sup></sub> females who were subsequently mated with BALB/c-<sup>Rag2<sup>−/−</sup></sub><sup>Il2rg<sup>−/−</sup></sub> males and sacrificed at GD 12.5, the reconstituted population was overwhelmingly PAS⁺DBA⁺ uNK cells, characterizing PAS⁺DBA⁺ uNK cells as the only transplantable subset (103). These two subsets differ not only in their expression of DBA throughout pregnancy, but also in their functional potential based on differing cytokine profiles (108).

1.11.4 UNK Cell Function

PAS⁺DBA⁺ uNK cells play a primarily angiogenic role via secretion of pro-angiogenic cytokines including placental growth factor (PGF), vascular endothelial growth factor (VEGF)-A, VEGFC, delta-like ligand 1 (DLL1; a NOTCH signaling molecule that induces endothelial tip cell differentiation), angiopoietin 1 (ANGPT1), ANGPT2 and inducible nitric oxide synthase (104-110). VEGFA is among the most important of these cytokines as it is highly expressed in the implantation site during early pregnancy and is essential for decidual angiogenesis (111). PAS⁺DBA⁺ uNK cells are also strong IL22 producers, a cytokine that may support trophoblast survival and invasion into the decidua (108). Conversely, PAS⁺DBA⁻ uNK cells show dominant expression of Ifng, despite being the underrepresented population in mid-pregnancy when uNK cell derived IFNG levels rapidly increase (108). Using eYFP-tagged IFNG, the exclusive production of IFNG by PAS⁺DBA⁻ uNK cells was confirmed on GD 9 by flow cytometry. UNK cells also secrete transforming growth factor-beta 1, a cytokine involved in cell proliferation and differentiation, and matrix metalloproteinases, enzymes capable
of degrading extracellular matrix and play a role in a number of cell behaviours including angiogenesis (112,113).

UNK cells are particularly important during early pregnancy as demonstrated by the normalization of alymphoid $Rag^{2-/-}Il2rg^{-/-}$ mouse implantation sites after reconstitution with uNK cells via transplantation of SCID or $Rag^{2-/-}$ (NK$^+$T$^-$B$^-$) bone marrow (44). The onset of decidual angiogenesis was restored, the impaired angiogenesis in the lateral vascular sinuses was normalized and the 24-hour delay in embryo development was corrected (44). In addition to the presence of uNK cells, uNK cell activation status is functionally important. Mice disrupted in the gene for NCR1 ($Ncr1^Gfp/Gfp$), which codes for activating receptors on NK cells experience deficient decidual angiogenesis similar to $Rag^{2-/-}Il2rg^{-/-}$ mouse implantation sites at GD 6.5 (114). Treatment of mice with antibodies to NKG2D, a different NK cell activation receptor, on GD 6.5 and 7.5 of pregnancy depleted PAS$^+$DBA$^+$ angiogenic uNK cells, decreased blood vessel density and reduced lateral vascular sinus folding in the mesometrial decidua (111). NKG2D is an activation receptor on PAS$^+$DBA$^+$ uNK cells, which seemingly plays a role in early pregnancy angiogenesis (111). Studies involving pan knockdown of the mouse activation and inhibitory Ly49 receptor gene family that ligates class I MHC (B6.Ly49$^{KD}$) also support angiogenic roles for uNK cells in early pregnancy. UNK cell numbers were not reduced from those in pregnant GD 9.5 congenic controls, however Ly49 receptor expression was reduced by 50% in PAS$^+$DBA$^-$ uNK cells and 90% in DP uNK cells at GD 9.5 (115). Most B6.Ly49$^{KD}$ were infertile and this was subsequently established to have an association with poor development of the implantation crypt and lack of uterine lumen closure around the hatched blastocyst (Leon,
Kay, Croy, *MS in preparation*). If pregnancy was established the implantation site experienced lagging decidual angiogenesis at GD 6.5 and insufficient spiral artery remodeling and VEGFA production at GD 10.5 (115). Interestingly IFNG and perforin production were at normal levels in B6.Ly49KD, therefore MHC recognition appears to be important for VEGF regulation while other receptor pathways control induction of the former proteins (115).

While PAS+DBA+ uNK cells direct early decidual angiogenesis, it appears that PAS+DBA− uNK cells direct remodeling of maternal spiral arteries via the secretion of IFNG. This was elucidated in an elegant series of experiments. IFNG, a cytokine that is secreted by activated cNK cells, has regulatory effects on a variety of different cell types (116). UNK cells express IFNG mRNA and are positive for IFNG protein (117). IFNG levels are significantly lower in early Rag2−/Il2rg− mice suggesting uNK cells are a major source in the implantation site and responsible for the GD 9.5 increase of IFNG in the mesometrium (118). It is important to note that non-lymphoid cells are also capable of producing this protein (118). Ifng−/− mice have increased numbers of immature uNK cells, impaired spiral artery remodeling and altered decidual differentiation leading to necrosis later in gestation (118). Ifng−/− bone marrow grafted into Rag2−/Il2rg− mice generates normal numbers of uNK cells and decidua resembling Ifng−/− implantation sites. This indicates that the low levels of IFNG expressed in the decidua of Rag2−/Il2rg− are sufficient to regulate population size of uNK cells, but not adequate to influence the vascular actions of uNK cells (118). Implantation sites from IfngRa−/− grafted Rag2−/Il2rg− dams and Rag2−/Il2rg− dams treated with mouse recombinant IFNG experience normal spiral artery remodeling confirming that PAS+DBA− uNK cell dependent vascular
modifications are IFNG driven (42,119). IFNG levels in the DB are initially detectable at GD 6.5, increase four- to six-fold and peak at GD 10.5 (just before spiral artery remodeling), then drop from GD 12.5-14.5, (the period after SA remodeling) (42,120,121). The differences between PAS⁺DBA⁻ and PAS⁺DBA⁺ uNK cell subset are outlined in Table 1.1.

<table>
<thead>
<tr>
<th>Antibody Surface Receptors</th>
<th>PAS⁺DBA⁻ Subset</th>
<th>PAS⁺DBA⁺ Subset</th>
</tr>
</thead>
<tbody>
<tr>
<td>NK1.1⁺DX5⁺</td>
<td>NK1.1⁺DX5⁻</td>
<td></td>
</tr>
<tr>
<td>GD 6.5 frequency</td>
<td>50%</td>
<td>50%</td>
</tr>
<tr>
<td>Mid gestation frequency</td>
<td>10%</td>
<td>90%</td>
</tr>
<tr>
<td>Transplant Success</td>
<td>Rare (1:300)</td>
<td>Strong (&gt;99.5% of graft)</td>
</tr>
<tr>
<td>Cytokine Secretion</td>
<td>IFNG</td>
<td>PGF/VEGFA/VEGFC DLL1/ANGPT1 ANGPT2/iNOS/IL-22</td>
</tr>
<tr>
<td>Function</td>
<td>Spiral artery remodeling</td>
<td>Decidual angiogenesis</td>
</tr>
</tbody>
</table>

Table 1.1. Summary of uNK cell subset difference. PAS⁺DBA⁻ uNK cells are the minor subset and promote IFNG-mediated spiral artery remodeling. The dominant PAS⁺DBA⁺ uNK cell subset promotes decidual angiogenesis through the secretion of a number of angiogenic cytokines.

1.12 Tolerance

Interactions between uNK cells, other maternal leukocytes (particularly T cells) and fetal trophoblast exist in a balance to maintain growth of the semi-allogeneic fetus in the mother. This balance involves immunological tolerance mediated via a number of different mechanisms that are not entirely understood. T cell tolerance is more fully understood and begins early in life through a process called ‘education’. T cells are educated in the thymus using the cell’s unique surface antigen-specific T cell receptors (TCRs) (122). Most T cells that recognize self MHC molecules presented by thymic
stromal cells undergo apoptosis, but some (~1%) self-recognizing T cells survive. This is called MHC-restriction (122). TCR and MHC molecules developed to interact with each other in the body through education of T cells in the thymus. Once T cells have left the thymus and their TCRs interact with non-self MHCs, a vigorous T cell response is provoked against the cells expressing the mismatched MHC (122). Such a response would be fatal to the developing allogeneic fetus. The uterine environment somehow promotes tolerance as decidual T cells are present throughout gestation (123). T cells can be classified into cytotoxic T cells, T helper cells and T regulatory (Treg) cells. Treg cells are CD4\(^+\)CD25\(^+\) and are key players in the induction of tolerance to paternal antigens during pregnancy (123). BALB/c nude mice (CByJ.Cg-Foxn\(^{nu}\)/J) are normally fertile, T cell deficient, NK\(^-\)B\(^+\) mice. Adoptive cell transfer of BALB/c CD25\(^-\) lymphocytes into BALB/c nude females results in mice with conventional T cells, however no Treg cells (124). These mice show spontaneous abortion when mated with allogeneic C57BL/6 males, however have normal pregnancies when mated with syngeneic BALB/c mice. This suggests allogeneic fetuses are rejected when CD4\(^-\)CD25\(^+\) Treg cells are absent (124).

UNK cells work closely with trophoblast and placental cells throughout gestation to maintain an ideal environment for the growing fetus (97). There is limited evidence to support the notion that uNK cells directly associate with trophoblast in early pregnancy prior to completion of placental development. However, uNK cells are thought to regulate trophoblast invasion indirectly through secretion of cytokines, matrix proteins and chemokines (97). In this way, uNK cells regulate access to the maternal blood resources that the fetoplacental unit taps into while also promoting growth and structural
changes to the maternal vessels themselves through production of a multitude of angiogenic factors discussed previously (97,122). Interactions between conceptus MHC molecules and maternal Ly49 receptors likely also play roles in regulation of uNK cell function although this relationship is not yet elucidated and is quite complicated due to unusually limited expression of MHC molecules by trophoblast, and shedding of trophoblast fragments into maternal circulation (125,126). In mice, the presence of an MHC class 1 allele that binds to more inhibitory than activating NK cell receptors can result in suppressed uNK cell function, resulting in reduced fetal growth and decidual vascular insufficiencies (125). In humans, down-regulation of MHC class I proteins on trophoblast cells is thought to help evade cytotoxic T cell killing. However, down-regulated MHC does not appear to render these cells susceptible to NK cell attack (126). HLA-G is a non-classical MHC class 1 protein with immunosuppressive qualities that is expressed at high levels by extravillous human trophoblast (126). This MHC inhibits NK cell effector functions as well as cytotoxic T cell responses \textit{in vitro} (126,127). In contrast, genetic studies suggest activation of NK cells by HLA-C is critical for normal human pregnancy (128). Clearly integration of a complex mixture of signals, transmitted through both activating and inhibitory receptors, regulates uNK function during pregnancy.

To clearly understand the independent roles of NK cells and T cells in pregnancy, \textit{in vivo} studies in a mouse model lacking NK cells but retaining full capacity of the T and B cell adaptive immune system would be ideal. The \textit{Nfil3} \textsuperscript{−/−} mice appeared at the outset of my thesis studies to provide this model. It was my hypothesis that the adaptive immune system plays no role in early decidual angiogenesis or in mid gestational spiral arterial remodeling. This hypothesis predicts that early implantation sites from matings of \textit{Nfil3} \textsuperscript{−/−}
males x Nfil3−/− females would phenocopy early implantation sites from matings of Rag2−/− Il2rg−/− males x Rag2−/− Il2rg−/− females (NK-T-B-) mice regarding angiogenesis and conceptus growth, due to the absence of uNK cells despite the presence of T and B cells.

My second hypothesis was that later in pregnancy, conceptus losses would occur in allogeneically mated Nfil3−/− females due to disturbed regulation of T and B cells resulting from the absence of NK cells. Neither hypothesis proved to be correct because a unique lineage of NFIL3-independent uNK cells was identified. Over the course of the study, this lineage was reported in virgin C57BL/6 mice by Sojka et al. (93). For our studies and those of the other labs to sustain Nfil3 breeding colonies, heterozygote females had to be used for breeding. Nfil3−/− females developed dystocia at term implicating a role of the Nfil3 gene in pregnancy that is not seen in alymphoid mice. Because Nfil3 has other roles beyond a lymphocyte-based role, adoptive cell transplants into alymphoid mice were also undertaken.
Chapter 2

Materials and Methods

2.1 Animals

All animal usage was covered by animal utilization protocols approved by Queen’s University and compliant with guidelines of the Canadian Council on Animal Care for Care and Use of Laboratory Animals. Immune competent (WT or +/+ ) C57BL/6 mice were purchased from Charles River Canada, St. Constant, QU, Canada. BALB/c-Tg(UBC-GFP)30Scha/J (UBC-GFP), +/+ mice with ubiquitous expression of green fluorescent protein (GFP) were purchased from The Jackson Laboratory, Bar Harbor, ME, USA. Both strains were bred under conventional animal husbandry at Queen’s University. C57BL/6-Rag2^{−/−}Il2rg^{−/−} mice (NK\( ^{−}\)B\(^{−}\)) were purchased from Taconic Biosciences (Hudson, New York, USA). Breeding pairs of C57BL/6 mice deleted for expression of NFIL3 (Nfil3^{−/−}; also called E4bp4^{−/−}) (NK\( ^{+}\)B\(^{+}\)) were generously provided by Dr. Tak Mak, University of Toronto, Toronto, ON, Canada. Both immune-deficient strains were bred under barrier husbandry (bioBUBBLE\textsuperscript{TM}, Fort Collins CO; Positive Ventilation Caging with HEPA filtering, Allentown Inc., Allentown NY) at Queen’s University. Rag2^{−/−}Il2rg^{−/−} mice received autoclaved reverse osmosis water that was acidified (pH 2.9) or supplemented with sulfamethoxazole and trimethoprim on alternating weeks. Nfil3^{−/−} mice received autoclaved reverse osmosis water. Mice were housed in autoclaved microisolator cages containing nesting materials and received sterile chow. The barrier facilities were maintained at 23°C, air was exchanged 8x per hour and a 12:12 hour dark:light cycle was employed.
2.2 Genotyping for *Nfil3*

*Nfil3<sup>-/-</sup>* male x *Nfil3<sup>-/-</sup>* female breeding pairs frequently experience peripartum reproductive failure. Therefore, the breeding colony was maintained using matings of *Nfil3<sup>-/-</sup>* males x *Nfil3<sup>+/+</sup>* females. The resulting litters were genotyped using the Accustart<sup>TM</sup> II Mouse Genotyping Kit (Quanta Biosciences, Gaithersburg, MD, USA). 2mm ear snips were collected from each mouse and the tissue was submerged in 50µL of Extraction Reagent. The samples were heated to 95°C for 30 minutes, cooled to room temperature and 50µL of Stabilization Buffer were added. 2µL of the resulting DNA extract were used to run a 13µL PCR reaction. The PCR products were resolved using a 1% agarose gel made with 1X Tris-acetate-EDTA buffer and ethidium bromide, run for 20 minutes and visualized under UV light. DNA Primers; Seg05: 5’- GCC TTA CCG CAC AAG CTT CG -3’; Neo 469: 5’- TAG CCG GAT CAA GCG TAT GC -3’; Ex 2, 1659L: 5’- ACA CCC AGA CAG ACG CCG TT -3’ (Integrated DNA Technologies, Coralville, IA, USA).

2.3 Bone Marrow Transplantation

Six to eight week old *Rag2<sup>-/-</sup> Il2rg<sup>-/-</sup>* females were used as recipients of bone marrow. Six to eight week old *Nfil3<sup>-/-</sup>* or C57BL/6 mice were used as bone marrow donors. Bone marrow cells were collected by flushing both femurs, using a 25-gauge needle, of each donor mouse with sterile saline. The suspended marrow was centrifuged at 400g for five minutes and the pellet was resuspended in sterile saline. Bone marrow from one donor was given i.p. to one recipient in a 0.4mL inoculum (a minimum of 1.4x10<sup>7</sup> cells). I.V. administration of marrow was not routinely successful due to the
small diameter of \( \text{Rag}^{2/\text{Il2rg}} \) tail veins. Reconstitution of T and B cells, but not NK cells was confirmed by flow cytometry. Subsequent confirmation of engraftment was the presence of uNK cells within a minimum of three implantation sites. All i.p. recipients were successfully engrafted. Three weeks were allotted for graft expansion, and then bone marrow recipient females were mated to \( \text{Rag}^{2/\text{Il2rg}} \) males.

### 2.4 Uterine Cell Suspensions

Virgin \( Nfil3^{+/} \) or C57BL/6 females were anesthetized to a deep surgical plane using Pentobarbital\textsuperscript{®} Sodique (6742145, Ceva Santé Animale, Libourne, France) administered i.p. Then, a 5-6cm lateral incision was made through the abdominal wall just caudal to the rib cage. The rib cage was removed using scissors exposing the heart. A 23 gauge blood collection needle (368656, BD, Franklin Lanes, NJ, USA) attached to a 60mL syringe filled with 20 mL of sterile saline containing 1mg/mL heparin connected to a volume-regulated perfusion pump was inserted into the left ventricle and an incision was made in the right atrium to create an outlet for drainage. The perfusion rate was fixed at 1mL/minute. Following perfusion, the uterus was isolated and washed in 4°C saline. The mesometrium was removed and the uterus was cut longitudinally and then minced into small pieces using razor blades. The tissue was transferred into a 50mL conical tube containing 4°C saline and washed twice for five minutes to remove blood contamination. The tissue was then digested in 10mL of saline containing 2mg/mL of Collagenase from \textit{Clostridium histolyticum} Type IA (C9891, Sigma-Aldrich, St. Louis, MO, USA) for 60 minutes at 37°C. The cell suspension was centrifuged at 180g for 5 minutes at 4°C. The supernatant was discarded, the pellet was resuspended in 5mL of
4°C saline, and the resulting suspension was centrifuged at 180g for 5 minutes at 4°C. This was repeated two more times to ensure no collagenase remained. The pellet was then passed through a 70mm Falcon™ Cell Strainer (08-771-2; Fisher Scientific, Hampton, NH, USA) the resulting suspension was resuspended in 0.4mL of sterile saline and inoculated i.p. to \( \text{Rag}^{2^{-/-}}\text{Il2rg}^{r/-} \) females. Uterine cell recipient dams were allotted three weeks for graft expansion, and then dams were mated to \( \text{Rag}^{2^{-/-}}\text{Il2rg}^{r/-} \) males.

### 2.5 Mating Strategy

The morning of the appearance of the copulation plug was taken as GD 0.5 of pregnancy. A number of \( \text{Nfil3}^{/-/-} \) females were mated to \( \text{Nfil3}^{/-/-} \) males and euthanized at GD 6.5, 8.5, 10.5, 12.5 and 15.5. Other \( \text{Nfil3}^{/-/-} \) females were mated to GFP males and euthanized at GD 6.5, 8.5, 10.5, 12.5 and 15.5. C57BL/6 females were mated to C57BL/6 males and euthanized at GD 6.5, 8.5, 10.5, 12.5 and 15.5. \( \text{Rag}^{2^{-/-}}\text{Il2rg}^{r/-} \) females engrafted with \( \text{Nfil3}^{/-/-} \) or C57BL/6 bone marrow were mated to \( \text{Rag}^{2^{-/-}}\text{Il2rg}^{r/-} \) males, perfused (described below) and euthanized at GD 8.5, 10.5 and 12.5. A new set of perfused C57BL/6 females mated by C57BL/6 males was used as the WT comparison to the \( \text{Nfil3}^{/-/-} \) bone marrow recipients. Each mating combination had 3 pregnancies per time point except C57BL/6 bone marrow recipients that had 1 pregnancy per time point (See Appendix B). The full set of C57BL/6 bone marrow recipients is in the process of being completed. Of note, in published work (42) and for the bone marrow recipients studied here, no differences appear to be present between uNK cell numbers in engrafted \( \text{Rag}^{2^{-/-}}\text{Il2rg}^{r/-} \) females and C57BL/6 females when enumerated on matched days of gestation. Mice that were not euthanized by perfusion were euthanized by CO\(_2\) inhalation, followed...
by cervical dislocation.

2.6 Flow Cytometry

After euthanasia, spleens of three *Nfil3*−/− bone marrow recipients were dissected and homogenized using scissors over ice in a petri dish containing cold sterile phosphate buffered saline (PBS). The homogenate was then passed through a fine screen mesh and the resulting suspension was collected in a petri dish. 5mL of the cell suspension were layered over 5mL of Lympholyte® Cell Separation Media (CL5030, Cedarlane Burlington, ON, Canada) and centrifuged at 2400 rpm for 20 minutes at 20°C. The cells were aspirated from the interface, and washed (10mL of PBS; 2300 rpm; 10 minutes; 20°C). Pellet was vortexed to resuspend, and 1mL of Red Blood Cell Lysing Buffer Hybri-Max™(R7757, Sigma-Aldrich, St. Louis, MO, USA) was added to the cell pellet, centrifuged for 3 minutes at 37°C, 10mL of PBS were added and the tube was centrifuged again for 10 minutes at 2400 rpm at 20°C. The supernatant was discarded and the resultant pellet was resuspended in 1% bovine serum albumin in PBS (BSA-PBS) for 30 minutes at room temperature. 100µL of the suspension were then incubated with NK1.1-PE (NK cells; PK136, eBioscience, San Diego, CA, USA), CD3e-PEcy5 (T cells; 145-2c11, eBioscience, San Diego, CA, USA), CD19-FITC (B cells; eBio1D3; eBioscience, San Diego, CA, USA) or all three antibodies for 15 minutes. The samples were then washed with 300µL of 1%BSA-PBS and centrifuged for 1200 rpm for 5 minutes. The supernatant was discarded and the wash step was repeated. Finally, the cells were resuspended in 500µL 1%BSA-PBS for analysis.

The Beckman Coulter EPICS Altra Flow Cytometer (Mississauga, ON, Canada)
and EXPO32 software (Beckman Coulter, Mississauga, ON, Canada) were used for analysis. Gating was set using a blank negative control and isotype controls Mouse IgG2a κ-PE (eBM2a; eBioscience, San Diego, CA, USA), Armenian Hamster IgG-PEcy5 (eBio299Arm; eBioscience, San Diego, CA, USA), and Rat IgG2a κ-FITC (R35-95, BD Pharmingen, Mississauga, ON, Canada). 10 000 cells were counted per sample. Single stained cell suspensions were used to attain fluorescence compensation to avoid spectral overlap. All fluorochromes were excited with a 488nm laser and the detection ranges varied (FITC –525(±20)nm; PE – 575(±20)nm, PEcy5 – 675(±20)nm).

2.7 Perfusion for collection of histological specimens

Bone marrow recipients and uterine cell suspension recipients were anesthetized to a deep surgical plane using Pentobarbital® Sodique (6742145, Ceva Santé Animale, Libourne, France) administered i.p. Then, a 5-6cm lateral incision was made through the abdominal wall just caudal to the rib cage. The thoracic cavity was entered to expose the heart. A 23 gauge blood collection needle (368656, BD, Franklin Lanes, NJ, USA) attached to a 60mL syringe filled with 4% paraformaldehyde (PFA) connected to a volume-regulated perfusion pump was inserted into the left ventricle and an incision was made in the right atrium to create an outlet for drainage. One mL of fixative was perfused at a rate of 1mL/minute for the first minute and the flow rate was then decreased to 0.5mL/minute until the full 20mL of PFA had been administered. The implantation sites were then harvested, immersed in PFA for 1 hour, then place in 70% ethanol until processed into paraffin using standard methodology.
2.8 Tissue collection and preparation for histology

At specific days of gestation, pregnant mice were euthanized by CO\textsubscript{2} inhalation, followed by cervical dislocation. Uterine horns were dissected, grossly examined for fetal viability (assessed by implant size and colour). Resorbing implant sites (smaller and a deeper red or paler than healthy sites) were collected and recorded but were not further studied. Implantation sites were fixed in 4\% PFA for six hours (gd6.5 and 8.5) or overnight (16h; GD 10.5+), then placed in 70\% ethanol until processed into paraffin using standard methodology. Three paraffin-embedded implantation sites from each pregnant mouse (3 pregnant females per study group; total of 9 implantation sites/data point presented) were serially sectioned (6\,\mu m). Some slides were stained with hematoxylin and eosin (H&E; see Appendix C); alternate sections were stained with periodic acid Schiff’s (PAS; see Appendix D) reagent for glycoproteins and with the lectin *Dolichos biflorus agglutinin* (DBA; Appendix E; L6533, Sigma-Aldrich, St. Louis, MO, USA) that detects terminal N-acetyl galactosamine. In some experiments, dual staining using PAS and DBA lectin was undertaken (see Appendix F).

2.9 Cytokeratin immunohistochemistry

Serially sectioned slides (from the same series as above) were immersed in three changes of xylene and rehydrated in 100\%, 95\% and 70\% ethanol for 5 minutes each. Slides were then rinsed in distilled water for 2 minutes and washed with tris buffered saline with 0.05\% Tween 20 (TBST). Sections were digested with Proteinase K at room temperature for 5 minutes and incubated with 3\% hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) at room temperature for 30 minutes. Sections were washed with TBST and incubated with CAS-
block™ (00-8120, Thermo Fisher Scientific, Burlington, ON, Canada) for 10 minutes at room temperature. Sections were incubated with polyclonal rabbit anti-cytokeratin, Wide Spectrum Screening (Z0622, Dako, Burlington, ON, Canada) at a 1:500 dilution in CAS-block™ or purified rabbit polyclonal isotype control (910801, Biolegend, San Diego, CA, USA) at a 1:500 dilution in CAS-block™ overnight at 4°C. Sections were washed with TBST and incubated with polyclonal goat anti-rabbit immunoglobulins/biotinylated (E0432, Dako, Burlington, ON, Canada) at a 1:200 dilution in TBST at room temperature for 1 hour. Sections were washed with TBST and incubated with ExtrAvidin Peroxidase (E2886, Sigma-Aldrich, St. Louis, MO, USA) at a dilution of 1:100 in TBST at room temperature for 30 minutes. Sections were washed with TBST and incubated with DAB (ab64238, Abcam, Toronto, ON, Canada) for 15 seconds. Slides were rinsed with distilled water and immersed in hematoxylin for 90 seconds. Slides were rinsed under running tap water for 5 minutes, rehydrated in 70%, 95% and 100% ethanol for 5 minutes, and cleared in 2 changes of xylene for 3 minutes each. Slides were mounted using glass coverslips and Entellan™ Mounting Medium (14800, Electron Microscopy Sciences, Hatfield, PA, USA). N = 1 implantation site per time point, per mating combination. Data shown in Appendix B.

2.10 Ki67 immunohistochemistry

Serially sectioned slides were immersed in three changes of xylene and rehydrated in 100%, 95%, 70% ethanol and distilled water for 5 minutes each. Slides were incubated in sodium citrate (0.01M, pH 6.0) in a humidity chamber at 37°C for 30 minutes. Slides were washed in PBS and incubated with 1% H₂O₂ in PBS for 30 minutes.
Slides were washed with PBS and incubated with 10% goat serum in distilled water for 1 hour. Slides were washed with PBS and incubated with rabbit polyclonal anti-Ki67 (ab15580, Abcam, Toronto, ON, Canada) at a dilution of 1:200 in PBS or purified rabbit polyclonal isotype control (910801, Biolegend, San Diego, CA, USA) at a 1:500 dilution in PBS for 1 hour at room temperature. Sections were washed with PBS and incubated with polyclonal goat anti-rabbit immunoglobulins/biotinylated (E0432, Dako, Burlington, ON, Canada) at a 1:500 dilution in PBS at room temperature for 1 hour. Sections were washed with PBS and incubated with ExtrAvidin Peroxidase (E2886, Sigma-Aldrich, St. Louis, MO, USA) at a dilution of 1:500 in PBS at room temperature for 30 minutes. Sections were washed with PBS and incubated with DAB (ab64238, Abcam, Toronto, ON, Canada) for 15 seconds. Slides were rinsed with distilled water and immersed in hematoxylin for 90 seconds. Slides were rinsed under running tap water for 5 minutes, rehydrated in 70%, 95% and 100% ethanol for 5 minutes, and cleared in 2 changes of xylene for 3 minutes each. Slides were mounted using glass coverslips and Entellan™ Mounting Medium (14800, Electron Microscopy Sciences, Hatfield, PA, USA). N = 1 implantation site per pregnancy, 3 pregnancies per time point, per mating strategy.

2.11 Morphological and morphometrical analyses of implantation sites

All stained slides were examined by light microscopy. The center section of each serially sectioned implantation site was identified and 6 median tissue sections on each side of the central section were also analyzed. Sections were at least 42μm apart to avoid duplicate counting of individual uNK cells. The number of uNK cells in the field of view at 200X in the middle of the implantation site section was counted. Three implantation
sites per pregnancy and 3 pregnancies per time point per mating strategy were counted. PAS$^+$DBA$^-$ uNK cells were scored at GD 6.5 and GD 8.5, PAS$^+$DBA$^+$ uNK cells were scored at GD 8.5, 10.5, 12.5 and 15.5. Field area was adjusted to omit areas of vascular space and lumen. Only cells in which a nucleus was clearly visible in the plane of section were enumerated. Note: uNK cell counts for pregnancies of Rag2$^{-/-}$Il2rg$^{-/-}$ dams that received C57BL/6 bone marrow were conducted in 3 implantation sites per pregnancy and 1 pregnancy per time point (Appendix B).

UNK cell diameter measurements were conducted at 400X on 10 random cells per implantation site, 3 implantation sites per pregnancy, and 3 pregnancies per time point per mating strategy were. The same cells were used to determine the subtype classification by examination of size, shape, and granularity.

GD 10.5, 12.5 and 15.5 total vascular area measurements of labyrinths were conducted on mid-sagittal sections of implantation sites stained with H&E. Three images at 400X per labyrinth, 3 non overlapping labyrinthine regions per implantation site, 3 implantation sites per pregnancy and 3 pregnancies per mating combination were analyzed. GIMP Software Version 2.6.11 pixel analysis was used to determine vascular area. Erythrocytes were included in the vascular space pixel selection in non-perfused animals. Maternal and fetal labyrinth vascular area and interhemal membrane width measurements were conducted on 3 images at 400X per implantation site, 3 implantation sites per pregnancy and 3 pregnancies per mating combination. The same images were used for this analysis as were used for total vascular area measurements. Maternal and fetal labyrinth areas were distinguished by the presence of enucleated and nucleated erythrocytes respectively.
To evaluate the extent of spiral arterial remodeling, spiral arteries were identified in mid-sagittal sections of implantation sites stained with H&E. These sections alternated with those used for uNK cell scoring. For each implantation site, all visible spiral arteries were measured at 100X in three sections separated by at least 42µm. The ratio of wall:lumen diameters was calculated to index the extent of spiral artery remodeling. Cross-sectional area measurements for DB, spongiotrophoblast, and placental labyrinth were taken at 25X for each of three sections separated by at least 42µm for each implantation site studied (n=9 or more per data point). Measurements of the interhemal membrane of the placental labyrinths were taken at 400X in 3 areas of the labyrinth in three sections separated by at least 42µm of each implantation site. All measurements were on the same slides that were used for spiral artery analysis and analysed using ImageJ software (Bethesda, MD, USA).

2.12 Whole mount in-situ immunofluorescence

At GD 8.5, pregnant Nfil3−/− mice mated by UBC-GFP or Nfil3−/− males were euthanized by CO₂ inhalation, followed by cervical dislocation. The uterus was transected between implantation sites, and washed in PBS. Then, the myometrium of each implantation site was gently peeled back using watchmaker’s forceps from the antimesometrial side over the mesometrial side and removed using a scalpel blade. A small tag of myometrium was left on the mesometrial side to serve as an orientation landmark. The implantation site was then cut midsagittally using a scalpel while being viewed under a stereoscope. Sample tissues were placed into a 1.5mL tube containing 200µL of chilled PBS with 1% bovine serum albumin and 0.1% azide (PBA), 3µL of Fc
blocking antibody (anti-CD16/CD32; supernatant clone 2.4G2 ATCC, Manassas, Virginia, USA), and 5-10µg/mL of fluorescently-conjugated primary antibodies; PE tagged CD31 (MEC13.3, BD Pharmingen, Mississauga, ON, Canada), APC tagged CD45 (30-F11, BD Pharmingen, Mississauga, ON, Canada), PE tagged CD11c (N418, eBioscience, San Diego, CA, USA) and fluorescein tagged DBA (FL-1031, Vector Laboratories, Burlington, ON, Canada). Incubating samples were placed on an orbital shaker in a 4°C fridge, and mixed at medium intensity for 1 hour. Samples were then mounted onto glass slides using PBA, coverslipped and examined and photographed as live tissue under epifluorescence microscopy using AxioVision SE64 Rel. 4.8 (Zeiss, North York, ON, Canada).

2.13 TUNEL Assay

Tissue sections were stained using the TACS® 2 TdT-DAB In Situ Apoptosis Detection kit (4810-30-K, Trevigen, Gaithersburg, MD, USA). Slides were hydrated according to H&E protocols (Appendix C) and washed with 1X PBS. Samples were covered with 50µL of Proteinase K Solution for 23 minutes, washed with deionized water and immersed in the Quenching Solution for 5 minutes. The samples were washed in PBS, and then immersed in 1X TdT Labeling Buffer for 5 minutes. Next the samples were covered with 50µL of Labeling Reaction Mix and incubated for 60 minutes at 37°C in a humidity chamber. Then the samples were immersed in 1X TdT Stop Buffer for 5 minutes, washed twice with deionized H2O and covered with 50µL of Strep-HRP Solution and incubated at 37°C in a humidity chamber. Subsequently samples were washed twice in PBS, immersed in DAB, washed in deionized H2O, immersed in 1%
Methyl Green for 1 minute and dipped ten times in 2 changes of deionized H₂O, 95% ethanol, 100% ethanol and xylene. Slides were then mounted using glass coverslips and Entellan™ Mounting Medium (14800, Electron Microscopy Sciences, Hatfield, PA, USA). N = 1 implantation site per pregnancy, 3 pregnancies per time point, per mating strategy.

2.14 Statistical Analysis

One-way ANOVA and Tukey’s multiple comparisons test was used to compare mating strategies at different GDs in each data set with the following exceptions: Student’s unpaired T test was used to compare Rag2⁻/⁻ Il2rg⁻/⁻ BM(Nfil3⁻/⁻) and C57BL/6 NK cell frequency (Fig. 17) and to compare Rag2⁻/⁻ Il2rg⁻/⁻ BM(C57BL/6) and C57BL/6 NK cell frequency (Supplemental Fig. 2). Statistical significance was indicated as follows; *: p<0.05, **: p<0.01, ***:p<0.005, ****:p<0.001
Chapter 3

Results

3.1 Litter size and viability

Syngeneically and allogeneically mated \( Nfil3^{-/-} \) females produce litters of normal numbers and pup viability.

At least three syngeneic \( Nfil3^{+/} \) male \( \times \) \( Nfil3^{+/} \) female \( (Nfil3^{-/-} \times Nfil3^{-/-}) \) and allogeneic UBC-GFP males (on a BALB/c background) \( \times \) \( Nfil3^{-/-} \) female \( (UBC-GFP \times Nfil3^{-/-}) \) matings were studied and compared to \( C57BL/6 \times C57BL/6 \) (C57BL/6) WT matings at GD 6.5, 8.5, 10.5, 12.5 and 15.5. No significant differences between implantation site numbers per uterus (Fig. 3.1A) or implantation site viability (Fig. 3.1B) were found between \( Nfil3^{+/} \times Nfil3^{+/} \), UBC-GFP \( \times Nfil3^{+/} \) and \( C57BL/6 \) pregnancies. The average implantation site number for \( Nfil3^{-/-} \times Nfil3^{-/-} \) pregnancies \( (n= 26 \text{ litters}) \) was 6.73 ± 2.05 with a range of 2 to 11 implantation sites and 90.83% ± 14.98 viability. The average implantation site number for UBC-GFP \( \times Nfil3^{-/-} \) pregnancies \( (n= 20 \text{ litters}) \) was 7.65 ± 1.23 with a range of 5 to 10 fetuses and 94.22% ± 11.10 viability. The average implantation site number for \( C57BL/6 \) females \( (n = 24 \text{ litters}) \) was 8.04 ± 1.97 with a range of 1 to 10 fetuses and 94.18% ± 9.92 viability. 87% of \( Nfil3^{-/-} \) \( \times Nfil3^{-/-} \) breeders went into dystocia at ~GD 18.5 \( (n = 13) \). Therefore, \( Nfil3^{+/} \) male \( \times Nfil3^{+/} \) female breeding pairs were used to generate experimental \( Nfil3^{-/-} \) mice. These breeding pairs produced an average of \( n = 4.7 \pm 2.1 \) live born pups/litter.
Figure 3.1. Mouse implantation site numbers and percentage of viable pups per litter. (A) There were no significant differences between numbers of implantation sites in any of the matings. N = a minimum of 3 to a maximum of 7 mice per mating per time point. (B) The number of viable implantation sites assessed by gross examination of the uterine horns at euthanasia did not differ between matings with the exception of the Rag2−/−Il2rg−/− BM (Nfil3−/−) mice which differed significantly from C57BL/6 viable implantation sites (p<0.05). Previous studies reported no significant differences in litter sizes between Rag2−/−Il2rg−/− implantation sites and in congenic normal (+/+) mice.
Figure 3.1.

A

Implantation Site Numbers

B

Percent Conceptus Viability by GD
Matings of $Rag2^{+/}I12rg^{-/-}$ male x $Rag2^{+/}I12rg^{-/-}$ females inoculated with $Nfil3^{-/-}$ bone marrow ($Rag2^{+/}I12rg^{-/-}$ BM ($Nfil3^{-/-}$)) were also studied at GD 8.5, 10.5 and 12.5. The number of implantation sites in $Rag2^{+/}I12rg^{-/-}$ BM ($Nfil3^{-/-}$) pregnancies ($n = 11; 5.64 \pm 3.14$; viability = 88.94% ± 5.19; range = 1-10) did not differ from reported litter sizes in $Rag2^{+/}I12rg^{-/-}$ male x $Rag2^{+/}I12rg^{-/-}$ females ($Rag2^{+/}I12rg^{-/-}$) (129) or from those observed for $Nfil3^{-/-}$ x $Nfil3^{-/-}$, UBC-GFP x $Nfil3^{-/-}$, or C57BL/6 pregnancies (Fig. 3.1A). Implantation site viability in $Rag2^{+/}I12rg^{-/-}$ BM ($Nfil3^{-/-}$) pregnancies did not differ from $Nfil3^{-/-}$ x $Nfil3^{-/-}$, UBC-GFP x $Nfil3^{-/-}$ and C57BL/6 pregnancies at GD 8.5 and GD 10.5. At GD 12.5 $Rag2^{+/}I12rg^{-/-}$ BM ($Nfil3^{-/-}$) implantation sites had significantly more fetal death than C57BL/6 pregnancies (p<0.05; Fig 3.1.B). Table 1.1 summarizes the above mating strategies.
Table 1. Tabulated implementation data across all estimation days studied for each mating strategy.

<table>
<thead>
<tr>
<th>Mating Strategy</th>
<th>GD 15.5</th>
<th>GD 12.5</th>
<th>GD 10.5</th>
<th>GD 8.5</th>
<th>GD 6.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>GD 15.5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>GD 12.5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>GD 10.5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>GD 8.5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>GD 6.5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

---

Legend:
- R: Response
- V: Variability

Mating Day:
- GD: Gestation Day
- GD 15.5
- GD 12.5
- GD 10.5
- GD 8.5
- GD 6.5

Note: The table should be read from left to right, following the rows and columns as specified.
3.2 Nfil3\(^{-/-}\) x Nfil3\(^{-/-}\) vs. UBC-GFP x Nfil3\(^{-/-}\) implantation sites

**PAS\(^{+}\)DBA\(^{-}\) and PAS\(^{+}\)DBA\(^{+}\) uNK cells are present in Nfil3\(^{-/-}\) x Nfil3\(^{-/-}\) and UBC-GFP x Nfil3\(^{-/-}\) matings.**

Both PAS\(^{+}\)DBA\(^{-}\) and PAS\(^{+}\)DBA\(^{+}\) uNK cells were found in Nfil3\(^{-/-}\) x Nfil3\(^{-/-}\) and UBC-GFP x Nfil3\(^{-/-}\) implantation sites at GD 8.5 (Fig. 3.2A). Although present, fewer PAS\(^{+}\)DBA\(^{-}\) and PAS\(^{+}\)DBA\(^{+}\) uNK cell numbers were present in syngeneic Nfil3\(^{-/-}\) x Nfil3\(^{-/-}\) or allogeneic UBC-GFP x Nfil3\(^{-/-}\) implantation sites compared to C57BL/6 implantation sites across all GDs (Fig. 3.2B and F). At GD 10.5, 12.5 and 15.5 the MLAp (a lymphocyte rich region between the two layers of myometrium) was distinguishable from the DB (Fig. 3.2C, D, E). PAS\(^{+}\)DBA\(^{+}\) uNK cells were present in both the DB and MLAp in Nfil3\(^{-/-}\) x Nfil3\(^{-/-}\) and UBC-GFP x Nfil3\(^{-/-}\) implantation sites. PAS\(^{+}\)DBA\(^{+}\) uNK cells were significantly decreased in both the MLAp (Fig. 3.2G) and the DB (Fig. 3.2H) across all GD. Because uNK cells were detected in Nfil3\(^{-/-}\) pregnancies, Nfil3 expression does identify or discriminate between circulating and tissue resident uNK cells. The Nfil3\(^{-/-}\) uNK cell populations appeared to expand more slowly than C57BL/6 uNK cell populations and never achieved normal numbers (Fig. 3.2B).

At GD 6.5 uNK cells in UBC-GFP x Nfil3\(^{-/-}\) implantation sites were significantly smaller than GD-matched C57BL/6 uNK cells. Average uNK cell diameter was significantly smaller during midgestation (GD 10.5 and 12.5) in Nfil3\(^{-/-}\) x Nfil3\(^{-/-}\) (p<0.001 and p<0.01) and UBC-GFP x Nfil3\(^{-/-}\) (GD 10.5: p<0.001) implantation sites compared to C57BL/6 implantation sites. By GD 15.5 uNK cell diameters did not differ between Nfil3\(^{-/-}\) x Nfil3\(^{-/-}\), UBC-GFP x Nfil3\(^{-/-}\) and C57BL/6 matings (Fig. 3.3A). These data suggest
Figure 3.2. PAS⁺DBA⁻ and PAS⁺DBA⁺ uNK cells are present in $Nfil3^{−/−} \times Nfil3^{−/−}$ and UBC-GFP $\times Nfil3^{−/−}$ implantation sites. (A) PAS/DBA dual staining of the mesometrium in a paraffin embedded GD 8.5 $Nfil3^{−/−} \times Nfil3^{−/−}$ implantation site. PAS⁺DBA⁻ uNK cells are stained magenta (yellow arrows); PAS⁺DBA⁺ uNK cells are stained brown (black arrows). (B) Sections reacted with DBA lectin were used for total uNK cell enumeration of $Nfil3^{−/−} \times Nfil3^{−/−}$ (red circles), UBC-GFP $\times Nfil3^{−/−}$ (blue squares) and C57BL/6 (black triangles) matings. PAS⁺DBA⁺ $Nfil3^{−/−}$ uNK cell populations expand and decline in frequency in a pattern identical to uNK cells in C57BL/6. The frequency of PAS⁺DBA⁻ uNK cells (200X field of view) in $Nfil3^{−/−} \times Nfil3^{−/−}$ and UBC-GFP $\times Nfil3^{−/−}$ implantation sites is lower than in C57BL/6 at GD 6.5 (p<0.005). The frequency of PAS⁺DBA⁺ uNK cells in $Nfil3^{−/−} \times Nfil3^{−/−}$ and UBC-GFP $\times Nfil3^{−/−}$ implantation sites is lower than in C57BL/6 at GD 8.5, 10.5, 12.5 and 15.5 (p<0.001). Photomicrographs are shown of position-matched, DBA lectin-reacted GD 12.5 sections from implantation sites in $Nfil3^{−/−} \times Nfil3^{−/−}$ (C), UBC-GFP $\times Nfil3^{−/−}$ (D), and C57BL/6 (E) matings. (F) PAS⁺DBA⁻ uNK cell numbers were significantly lower in $Nfil3^{−/−}$ matings compared to C57BL/6 at GD 6.5 (p<0.005) and at GD 8.5 ($Nfil3^{−/−} \times Nfil3^{−/−}$; p<0.01; UBC-GFP $\times Nfil3^{−/−}$; p<0.005). PAS⁺DBA⁺ uNK cell numbers were significantly lower than C57BL/6 numbers (p<0.01 or less) in both the MLAp (G) and DB (H) in $Nfil3^{−/−}$ matings across all GDs. PAS⁺DBA⁺ uNK cell frequency in $Nfil3^{−/−} \times Nfil3^{−/−}$ was significantly higher in in the MLAp and lower in the DB compared to UBC-GFP $\times Nfil3^{−/−}$ matings (p<0.001) at GD 12.5. N = all uNK cells in field of view at 200X in one section, 3 sections per implantation site, 3 implantation sites per pregnancy, 3 pregnancies per mating. Labels = DB: decidua basalis; MLAp: mesometrial lymphoid aggregate of pregnancy.
Figure 3.2.
Figure 3.3. Comparative time course study of PAS+DBA+ uNK cell size and subtype between experimental implantation sites. (A) PAS+DBA+ uNK cell diameter at GD 6.5, 8.5, 10.5, 12.5 and 15.5 in the three experimental matings determined by measuring 10 random cells at 400X per implantation site, 3 implantation sites per pregnancy and three pregnancies per mating. Diameter of UBC-GFP x Nfil3−/− uNK cells but of not Nfil3−/− x Nfil3−/− is significantly smaller than C57BL/6 uNK cell diameter at GD 6.5 (p<0.05). There were no significant differences in uNK cell diameters at GD 8.5 and GD 15.5 of the two experimental matings. However, uNK cells in Nfil3−/− x Nfil3−/− were significantly smaller at GD 10.5 and GD 12.5 (p<0.001) than uNK cells in C57BL/6. Nfil3−/− x Nfil3−/− uNK cells are significantly smaller than UBC-GFP x Nfil3−/− at GD 12.5 (p<0.01). UBC-GFP x Nfil3−/− uNK cells were significantly smaller than C57BL/6 uNK cells at GD 10.5 (p<0.001). Measurements were an average of cells in both the DB and MLAp.

Proportions of the four subtypes of PAS+DBA+ uNK cells were calculated using the same cells as those used for the diameter measurements at GD 6.5 (B), GD 8.5 (C), GD 10.5 (D), GD 12.5 (E) and GD 15.5 (F). Nfil3−/− x Nfil3−/− and UBC-GFP x Nfil3−/− PAS+DBA+ uNK cells tend to have proportions similar to PAS+DBA+ uNK cells in C57BL/6 at GD 6.5 and 8.5. However, more immature subtypes are found at GD 10.5, 12.5 and 15.5 in Nfil3−/− implantation sites than in C57BL/6 (p<0.01).
Figure 3.3.
that a delay occurs in maturation of Nfil3\(^{-/-}\) uNK cells but show that full maturity is indeed achieved.

\(Nfil3^{+/+}\) x \(Nfil3^{+/+}\) and UBC-GFP x \(Nfil3^{+/+}\) uNK cells differentiated into all four classically-defined subtypes of DBA lectin-reactive uNK cells. The ratio of the subtypes did not differ between \(Nfil3^{+/+}\) x \(Nfil3^{+/+}\), UBC-GFP x \(Nfil3^{+/+}\) and C57BL/6 implantation sites at GD 6.5 and 8.5 (Fig. 3B and C). \(Nfil3^{+/+}\) x \(Nfil3^{+/+}\) and UBC-GFP x \(Nfil3^{+/+}\) implantation sites had a significantly larger proportion (p<0.01) of more immature subtypes at GD 10.5 (Fig. 3.3D), 12.5 (Fig. 3.3E) and 15.5 (Fig 3.3F) compared to C57BL/6 implantation sites. Figure 3.4 shows subtype I (yellow circle), subtype II (black circle), subtype III (red circle) and subtype IV (blue circle) in each mating combination at GD 8.5 and 10.5.

\(Nfil3^{+/+}\) x \(Nfil3^{+/+}\) and UBC-GFP x \(Nfil3^{+/+}\) implantation sites have abnormal development and delayed early embryonic growth

Analysis of paraffin embedded, H&E-stained, sections (Fig. 3.5A) revealed that in \(Nfil3^{+/+}\) x \(Nfil3^{+/+}\) (Fig. 3.5B) and UBC-GFP x \(Nfil3^{+/+}\) (Fig. 3.5C) implantation sites the uterine lumen persisted at GD 6.5. The lumen should be residual and/or entirely closed at this time point as illustrated by the gestation length-matched C57BL/6 implantation site (Fig. 3.5D). The delay in closure was less severe in the UBC-GFP x \(Nfil3^{+/+}\) implantation sites than in \(Nfil3^{+/+}\) x \(Nfil3^{+/+}\) implantation sites.
Figure 3.4. Subtypes I, II, III and IV of PAS⁺DBA⁺ uNK cells are found in Nfil3−/− x Nfil3−/− and UBC-GFP x Nfil3−/− implantation sites. Photomicrographs of PAS/DBA dual stained paraffin embedded implantation sites of Nfil3−/− x Nfil3−/− (Ai), UBC-GFP x Nfil3−/− (Aii) and C57BL/6 (Aiii) matings at GD 8.5. UNK cells were subtyped by maturity according to Paffaro et al. (96) Subtype I uNK cells are small round cells (black circles) and subtype II uNK cells are slightly larger cells with a small number of DBA⁺ cytoplasmic granules (yellow circles). Photomicrographs of DBA lectin reacted sections of Nfil3−/− x Nfil3−/− (Bi), UBC-GFP x Nfil3−/− (Bii) and C57BL/6 (Biii) matings at GD 10.5. Subtype III uNK cells are large round heavily granulated cells (red circles) and subtype IV uNK cells are the largest cells with DBA⁺ granules in the vacuolated-like cytoplasm (blue circles).
Figure 3.4.
Figure 3.5. Delayed uterine lumen closure in \textit{Nfil3}^{+/+} \times \textit{Nfil3}^{+/+} and UBC-GFP x \textit{Nfil3}^{+/+} implantation sites at GD 6.5. Paraffin sections of GD 6.5 implantation sites from (A) \textit{Nfil3}^{−/−} \times \textit{Nfil3}^{−/−} mating. An open uterine lumen (UL) and viable conceptus (yellow oval) are both visible at low power. The black box in (A) denotes the region from which image B, C and D are taken for each mating. The uterine lumen persisted in \textit{Nfil3}^{−/−} \times \textit{Nfil3}^{−/−} implantation sites (B) and was 80% larger than in C57BL/6 (D). In UBC-GFP x \textit{Nfil3}^{−/−} implantation sites (C), the uterine lumen was of intermediate size and 10% larger than in C57BL/6 implantation sites. The lumen has almost apposed in C57/BL6 mice (D). Black arrows = uterine lumen. All high power images were stained with H&E and taken in a section with a visible conceptus not visible in image.
At GD 8.5, differences were present in the antimesometrial pole, the side of embryo implantation that is opposite to the mesometrial pole, where leukocytes (including the uNK cells) infiltrate (Fig 3.6A). Antimesometial cells in Nfil3\textsuperscript{-/-} x Nfil3\textsuperscript{-/-} (Fig. 3.6B) and UBC-GFP x Nfil3\textsuperscript{-/-} (Fig. 3.6C) implantation sites are more compact and less swollen than C57BL/6 cells (Fig. 3.6D). The area of swollen cells was the area of decidualization. The decidualized area relative to the total area of the antimesometrium was 74.91±10.49% in C57BL/6 implantation sites. This area was significantly smaller in Nfil3\textsuperscript{-/-} x Nfil3\textsuperscript{-/-} and UBC-GFP x Nfil3\textsuperscript{-/-} implantation sites by 18.65% and 30.73% respectively (p<0.005; Fig. 3.6E).

TUNEL staining was undertaken to determine whether increased cell death in Nfil3\textsuperscript{-/-} x Nfil3\textsuperscript{-/-} and UBC-GFP x Nfil3\textsuperscript{-/-} antimesometrial decidua accounted for the absence of decidualization. Figure 7A and B is an enzyme treated C57BL/7 implantation site at GD 8.5 and represents a positive signal. No cell death was evident in the deciduas of Nfil3\textsuperscript{-/-} x Nfil3\textsuperscript{-/-} (Fig. 3.7Di), UBC-GFP x Nfil3\textsuperscript{-/-} (Fig. 3.7Dii), or C57BL/6 matings (Fig. 3.7Diii), although the positive staining controls reacted in each of two replicate experiments.

To determine whether there was increased cell proliferation in the C57BL/6 antimesometrial decidualia, sections were stained with the proliferation marker Ki67. A positive Ki67 signal reacts as a dark brown nuclear stain seen in the mesometrial decidua of a C57BL/6 implantation site (Fig. 3.7C). No differences were apparent between Nfil3\textsuperscript{-/-} x Nfil3\textsuperscript{-/-} (Fig. 3.7Ei), UBC-GFP x Nfil3\textsuperscript{-/-} (Fig. 3.7Eii), and C57BL/6 antimesometrium (Fig. 3.7Eiii). The dark brown haze in the antimesometrial pole of each mating
Figure 3.6. Antimesometrial decidualization differs in Nfil3−/− x Nfil3−/− and UBC-GFP x Nfil3−/− to C57BL/6 implantation sites at GD 8.5. (A) Illustrates a typical C57BL/6 mouse implantation site at GD 8.5. The black box denotes the orientation of the subsequent images. The area from the decidualized border (yellow line) to the antimesometrial trophoblast (*) is the decidualized area (double headed yellow arrow). The total area (double headed black arrow) was measured from the edge of the myometrium (black line) to the antimesometrial trophoblast. Nfil3−/− x Nfil3−/− (B; 56.35% ± 10.49) and UBC-GFP x Nfil3−/− (C; 44.18% ± 8.127) implantation sites have significantly less antimesometrial decidual tissue (double headed yellow arrow) compared to C57BL/6 (D; 74.91% ± 10.29; p<0.005). (E) Nfil3−/− x Nfil3−/− and UBC-GFP x Nfil3−/− antimesometrial decidual areas are decreased by 18.65% and 30.73% respectively (p<0.0005). Photomicrographs are stained with H&E. Labels = Mes: mesometrial; Anti: antimesometrial.
Figure 3.6.
Figure 3.7. Differences in antimesometrial decidualization are not accounted for by frequency of cell death or proliferation in Nfil3−/− implantation sites. (A) Representative TUNEL stained C57BL/6 implantation site treated with TACS-Nuclease™ was the positive control. (B) Brown nuclei represent nicked DNA in the positive control. (C) Dark brown staining represents a positive reactivity for Ki67 in the mesometrium of GD 8.5 C57BL/6 implantation site (yellow arrows). See panel A for localization of image C. TUNEL staining was negative in the antimesometrium of Nfil3−/− x Nfil3−/− (D1), UBC-GFP x Nfil3−/− (Dii), and C57BL/6 (Diii) implantation sites. Ki67 staining was also interpreted as negative with high background due to lack of specific cell staining in the antimesometrium of Nfil3−/− x Nfil3−/− (Ei), UBC-GFP x Nfil3−/− (Eii), and C57BL/6 (Eiii) implantation sites. For both stains n = 3; 1 implantation site per pregnancy, 3 pregnancies per mating.
Figure 3.7.
combination represents background staining; the nuclei were blue from the hematoxylin counterstain.

Prominent, unique looking cells were identified in the antimesometrium of Nfil3−/− x Nfil3−/− (Fig. 3.8A) and UBC-GFP x Nfil3−/− (Fig. 3.8B) pregnancies using WM-IF. These cells are present in C57BL/6 implantation sites (Fig. 3.8C) however the population looks to be larger in Nfil3−/− implantation sites. These cells have numerous cytoplasmic projections (Fig. 3.8D) and are both CD45+ (a pan-leukocyte marker) and CD11c+ (a dendritic cell marker).

Finally, embryos of Nfil3−/− x Nfil3−/− and UBC-GFP x Nfil3−/− matings were 0.5-1 day developmentally delayed at GD 8.5. The Nfil3−/− x Nfil3−/− embryo illustrated is at Theiler stage 12, equivalent to GD 8 (Fig. 3.9A), the UBC-GFP x Nfil3−/− embryo is at Theiler stage 11, equivalent to GD 7.5 (Fig. 3.9B) and the C57BL/6 embryo is at Theiler stage 13, equivalent to GD 8.5 and typical for normal development (Fig. 3.9C). Figure 3.9D represents tabulated data of the Theiler stage of each embryo per mating combination.
Figure 3.8. Antimesometrial enrichment of unusual CD45<sup>+</sup>CD11c<sup>+</sup> cells in the antimesometrium of Nfil3<sup>−/−</sup> x Nfil3<sup>−/−</sup> and UBC-GFP x Nfil3<sup>−/−</sup> implantation sites at GD 8.5. Whole mount in situ immunofluorescence of the antimesometrial pole in Nfil3<sup>−/−</sup> x Nfil3<sup>−/−</sup> (A), UBC-GFP x Nfil3<sup>−/−</sup> (B), and C57BL/6 (C) implantation sites at GD 8.5 stained with fluorescently conjugated CD11c-PE (dendritic cell marker; white). (D) High power image of CD11c<sup>+</sup> cells in C57BL/6 antimesometrium.
Figure 3.9. *Nfil3<sup>+/−</sup> x Nfil3<sup>+/−</sup>* and UBC-GFP x *Nfil3<sup>+/−</sup>* embryos are developmentally delayed by 0.5-1 day at GD 8.5. Implantation sites from *Nfil3<sup>+/−</sup> x Nfil3<sup>+/−</sup>* (A), UBC-GFP x *Nfil3<sup>+/−</sup>* (B), and C57BL/6 (C) matings. (A) 67% of *Nfil3<sup>+/−</sup> x Nfil3<sup>+/−</sup>* embryos were at Theiler stage 12 (equivalent to GD 8.0). (B) 56% UBC-GFP x *Nfil3<sup>+/−</sup>* embryos were at Theiler stage 11 (equivalent to GD 7.5). (C) 78% of C57BL/6 embryos were at Theiler stage 13 (typical for normal development). (D) Table depicting number of embryos at each Theiler stage per mating (n = 9 embryos/mating). N = 3 implantation sites per pregnancy, 3 pregnancies per mating. Photomicrographs of paraffin embedded sections stained with H&E at GD 8.5. Labels = 1: notocord, 2: pharyngeal region of foregut diverticulum, 3: allantois, 4: myocardial tissue forming outer wall of primitive heart tube, 5: condensation of tissue in caudal region of notocord, 6: neural groove, 7: neuroepithelium, 8: optic eminence, 9: trigeminal neural crest.
Late gestation implantation site layers are aberrant in $Nfil3^{-/-} \times Nfil3^{-/-}$ and UBC-GFP $\times Nfil3^{-/-}$ matings

From GD 10.5 onward the typical mesometrium of a normal implantation site has 4 distinct layers: the maternal derived decidua basalis (inclusive of the MLAp), fetal derived trophoblast giant cell layer, fetal derived spongiotrophoblast and fetal derived labyrinth. The trophoblast giant cell layer and spongiotrophoblast layer were combined for cross sectional surface area measurements. Organization of these layers was disturbed in both $Nfil3^{-/-} \times Nfil3^{-/-}$ and UBC-GFP $\times Nfil3^{-/-}$ implantation sites. The more severe $Nfil3^{-/-} \times Nfil3^{-/-}$ phenotype was compared to C57BL/6 implantation sites at GD 10.5 (Fig. 3.10A), GD 12.5 (Fig. 3.10B), and GD 15.5 (Fig. 3.10C). $Nfil3^{-/-} \times Nfil3^{-/-}$ implantation site layers merged into one another, while C57BL/6 implantation site layers were distinctively defined. The disparity was greatest at GD 10.5 and GD 12.5 and appeared to be normalized by GD 15.5. The total implantation site areas did not differ significantly between $Nfil3^{-/-} \times Nfil3^{-/-}$, UBC-GFP $\times Nfil3^{-/-}$ and C57BL/6 pregnancies (Fig. 3.10D) except for UBC-GFP $\times Nfil3^{-/-}$ implantation sites that were significantly smaller compared to C57BL/6 implantation sites but only at GD 12.5 (p<0.001).

Figure 11 shows a placental histology time course grid of $Nfil3^{-/-} \times Nfil3^{-/-}$, UBC-GFP $\times Nfil3^{-/-}$ and C57BL/6 implantation sites at GD 10.5 (Fig. 3.11Ai, Aii, Aiii), GD 12.5 (Fig. 3.11Bi, Bii, Biii) and GD 15.5 (Fig. 3.11Ci, Cii, Ciii). Figure 3.12A shows GD 10.5 mesometrial tissue surface area analysis. $Nfil3^{-/-} \times Nfil3^{-/-}$ and UBC-GFP $\times Nfil3^{-/-}$ had significantly larger decidua basalis (DB) surface areas compared to C57BL/6 areas (p<0.001). The spongiotrophoblast occupied area did not differ between $Nfil3^{-/-} \times Nfil3^{-/-}$ and C57BL/6 matings. UBC-GFP $\times Nfil3^{-/-}$ spongiotrophoblast area was significantly
Figure 3.10. Implantation site mesometrial layers differ in surface area between \textit{Nfil3}\textsuperscript{-/-} x \textit{Nfil3}\textsuperscript{-/-} and C57BL/6 matings at GD 10.5, 12.5 and 15.5. Halved \textit{Nfil3}\textsuperscript{-/-} x \textit{Nfil3}\textsuperscript{-/-} implantation sites compared to C57BL/6 implantation sites at GD 10.5 (A), GD 12.5 (B) and GD 15.5 (C). Yellow lines demarcate the transition between the classic mesometrial tissue layers. The \textit{Nfil3}\textsuperscript{-/-} x \textit{Nfil3}\textsuperscript{-/-} implantation sites became more distinct and approach normal by GD 15.5. (D) Total mesometrial areas did not differ between matings at GD 10.5 or 15.5. UBC-GFP x \textit{Nfil3}\textsuperscript{-/-} but not \textit{Nfil3}\textsuperscript{-/-} x \textit{Nfil3}\textsuperscript{-/-} implantation sites were smaller than C57BL/6 implantation sites at GD 12.5 (p<0.01). N = 3 placentas per implantation site, 3 implantation sites per pregnancy and 3 pregnancies per matings. Photomicrographs of paraffin embedded sections stained with H&E. Labels = DB: decidua basalis, SP: spongiotrophoblast, LB: labyrinth.
Figure 3.11. Nfil3<sup>−/−</sup> x Nfil3<sup>−/−</sup> and UBC-GFP x Nfil3<sup>−/−</sup> mesometrial histology at GD 10.5, 12.5 and 15.5. Time course images from Nfil3<sup>−/−</sup> x Nfil3<sup>−/−</sup> implantation sites are in the left column (Ai, Bi, Ci). UBC-GFP x Nfil3<sup>−/−</sup> implantation sites are in the center column (Aii, Bii, Cii). C57BL/6 implantation sites are in the right column (Aiii, Biii, Ciii). Images of Nfil3<sup>−/−</sup> x Nfil3<sup>−/−</sup> and C57BL/6 implantation sites are the full images of Figure 10 halves. The most atypical mesometrial tissues were present in Nfil3<sup>−/−</sup> x Nfil3<sup>−/−</sup> implantation sites with indistinct tissue boundaries at GD 10.5. UBC-GFP x Nfil3<sup>−/−</sup> had an intermediate phenotype but tissue boundaries were still more indistinct and irregular than in C57BL/6 implantation sites. Photomicrographs of paraffin embedded sections stained with H&E.
larger than either Nfil3<sup>−/−</sup> x Nfil3<sup>−/−</sup> (p<0.001) or C57BL/6 areas (p<0.01). Nfil3<sup>−/−</sup> x Nfil3<sup>−/−</sup> and UBC-GFP x Nfil3<sup>−/−</sup> labyrinthine areas were significantly larger (p<0.001) than C57BL/6 labyrinth areas.

Figure 3.12B shows GD 12.5 mesometrial tissue surface area analyses. Nfil3<sup>−/−</sup> x Nfil3<sup>−/−</sup> DB area was significantly larger than UBC-GFP x Nfil3<sup>−/−</sup> or C57BL/6 decidua (p<0.005). The UBC-GFP x Nfil3<sup>−/−</sup> DB area was significantly smaller than the C57BL/6 DB area (p<0.005). Nfil3<sup>−/−</sup> x Nfil3<sup>−/−</sup> and UBC-GFP x Nfil3<sup>−/−</sup> spongiotrophoblast areas were significantly smaller than C57BL/6 spongiotrophoblast area (p<0.005) but did not differ significantly from each other. Nfil3<sup>−/−</sup> x Nfil3<sup>−/−</sup> labyrinthine tissue area was significantly smaller than UBC-GFP x Nfil3<sup>−/−</sup> and C57BL/6 labyrinth areas (p<0.005). UBC-GFP x Nfil3<sup>−/−</sup> and C57BL/6 labyrinthine areas did not differ from each other.

Figure 3.12C shows GD 15.5 mesometrial tissue surface area analysis. DB tissue area did not differ between Nfil3<sup>−/−</sup> x Nfil3<sup>−/−</sup>, UBC-GFP x Nfil3<sup>−/−</sup> and C57BL/6. Nfil3<sup>−/−</sup> x Nfil3<sup>−/−</sup> spongiotrophoblast was significantly larger than UBC-GFP x Nfil3<sup>−/−</sup> and C57BL/6 spongiotrophoblast (p<0.005). UBC-GFP x Nfil3<sup>−/−</sup> spongiotrophoblast area was significantly smaller than C57BL/6 spongiotrophoblast (p<0.005). Nfil3<sup>−/−</sup> x Nfil3<sup>−/−</sup> labyrinths did not differ from UBC-GFP x Nfil3<sup>−/−</sup> and C57BL/6 labyrinths. The surface area of UBC-GFP x Nfil3<sup>−/−</sup> labyrinth was significantly larger than for C57BL/6.
Figure 3.12. Surface area analysis of Nfil3+/ x Nfil3−/ and UBC-GFP x Nfil3−/ mesometrial implantation site regions at GD 10.5, 12.5 and 15.5. (A) Mesometrial measurements of tissue layer surface area at GD 10.5. Nfil3+/ x Nfil3−/ and UBC-GFP x Nfil3−/ decidua basalis (DB) layers are significantly larger than the DB in C57BL/6 matings (p<0.01) but do not differ significantly from each other. Nfil3+/ x Nfil3−/ spongiotrophoblast (spongio) is significantly smaller than the spongiotrophoblast in UBC-GFP x Nfil3−/ matings (p<0.001) but not significantly different from C57BL/6 spongiotrophoblast. UBC-GFP x Nfil3−/ spongiotrophoblast is significantly larger than C57BL/6 spongiotrophoblast layers (p<0.01). Nfil3+/ x Nfil3−/ and UBC-GFP x Nfil3−/ labyrinthine layers are significantly larger than C57BL/6 labyrinthine layers (p<0.001) but do not differ significantly from each other. (B) Mesometrial measurements of tissue surface area at GD 12.5. Nfil3+/ x Nfil3−/ DB layers are significantly larger than both UBC-GFP x Nfil3−/ and C57BL/6 DB layers (p<0.005). UBC-GFP x Nfil3−/ DB layers are significantly smaller than C57BL/6 DB layers (p<0.005). Nfil3+/ x Nfil3−/ and UBC-GFP x Nfil3−/ spongiotrophoblast layers are significantly smaller than C57BL/6 spongiotrophoblast layers (p<0.005) and do not differ significantly from each other. Nfil3+/ x Nfil3−/ labyrinth layers are significantly smaller than UBC-GFP x Nfil3−/ and C57BL/6 layers (p<0.005). UBC-GFP x Nfil3−/ and C57BL/6 labyrinth layers do not differ significantly from each other. (C) Mesometrial measurements of tissue surface area at GD 15.5. Nfil3+/ x Nfil3−/, UBC-GFP x Nfil3−/ and C57BL/6 DB layers do not significantly differ from each other. Nfil3+/ x Nfil3−/ spongiotrophoblast layers are significantly larger than UBC-GFP x Nfil3−/ and C57BL/6 spongiotrophoblast layers (p<0.005). UBC-GFP x Nfil3−/ spongiotrophoblast layers are significantly smaller than C57BL/6 spongiotrophoblast layers (p<0.005). Nfil3+/ x Nfil3−/ labyrinthine layers do not differ significantly from UBC-GFP x Nfil3−/ and C57BL/6 labyrinthine layers. UBC-GFP x Nfil3−/ labyrinthine layers are significantly larger than C57BL/6 labyrinthine layers (p=0.005).
Figure 3.12.

A

GD 10.5 Tissue Surface Area

B

GD 12.5 Tissue Surface Area

C

GD 15.5 Tissue Surface Area
To determine whether there was a difference in the amount of trophoblast relative to the decidua, cytokeratin immunohistochemistry was undertaken. Only one paraffin-embedded section was used per time point per mating strategy (See Appendix A). Trophoblast appeared to occupy a smaller proportion of mesometrial tissue in *Nfil3<sup>−/−</sup> x Nfil3<sup>−/−</sup>* implantation sites than in UBC-GFP x *Nfil3<sup>−/−</sup>* and C57BL/6 implantation sites at GD 10.5 and GD 12.5. The proportion of trophoblast to decidua was less in *Nfil3<sup>−/−</sup> x Nfil3<sup>−/−</sup>* implantation sites compared to UBC-GFP x *Nfil3<sup>−/−</sup>* and C57BL/6 implantation site at GD 15.5 (Appendix C-D) implicating impaired trophoblast invasion. Because each mating strategy had only one measurement per time point, statistical analyses were not performed.

The placental labyrinth of *Nfil3<sup>−/−</sup> x Nfil3<sup>−/−</sup>* and UBC-GFP x *Nfil3<sup>−/−</sup>* shows vascular abnormalities

The placental labyrinth is the site of maternal/fetal nutrient exchange (Fig. 3.13A). High power analysis of the placental labyrinth revealed no differences between *Nfil3<sup>−/−</sup> x Nfil3<sup>−/−</sup>*, UBC-GFP x *Nfil3<sup>−/−</sup>* and C57BL/6 labyrinthine vascular spaces at GD 10.5 (Fig. 3.13B). Significantly larger vascular spaces were found in the placentae from *Nfil3<sup>−/−</sup> x Nfil3<sup>−/−</sup>* (Fig. 3.13Ci, Di) and UBC-GFP x *Nfil3<sup>−/−</sup>* (Fig. 3.13Cii, Dii) matings at GD 12.5 (p<0.0001) and GD 15.5 (p<0.0001) compared to C57BL/6 matings (Fig. 3.13Ciii, Diii).

Maternal and fetal vascular areas were then analyzed separately to determine whether the fetal genotype had an impact on the vascular abnormalities. Nucleation of
Figure 3.13. The labyrinthine regions in *Nfil3*<sup>−/−</sup> x *Nfil3*<sup>−/−</sup> and UBC-GFP x *Nfil3*<sup>−/−</sup> placentas at GD 10.5, 12.5 and 15.5. (A) Low power photomicrograph of *Nfil3*<sup>−/−</sup> x *Nfil3*<sup>−/−</sup> implantation site at GD 12.5. Black box denotes the region from which image Ci, Cii, Ciii, Di, Dii and Diii are taken for each mating. No distinction was made between maternal and conceptus vessels; both were randomly included in each labyrinthine section studied using erythrocyte morphology for identification. (B) *Nfil3*<sup>−/−</sup> x *Nfil3*<sup>−/−</sup> and UBC-GFP x *Nfil3*<sup>−/−</sup> labyrinthine vascular spaces did not differ significantly from C57BL/6 spaces at GD 10.5. However, both matings with *Nfil3*<sup>−/−</sup> mothers had significantly larger spaces than C57BL/6 at GD 12.5 and 15.5 (p<0.001). Photomicrographs of GD 12.5 labyrinthine vascular spaces of *Nfil3*<sup>−/−</sup> x *Nfil3*<sup>−/−</sup> (Ci), UBC-GFP x *Nfil3*<sup>−/−</sup> (Cii) and C57BL/6 (Ciii) implantation sites. Photomicrographs of GD 15.5 labyrinthine vascular spaces of *Nfil3*<sup>−/−</sup> x *Nfil3*<sup>−/−</sup> (Di), UBC-GFP x *Nfil3*<sup>−/−</sup> (Dii) and C57BL/6 (Diii) implantation sites. Vascular spaces outlined in black. This analysis used n = 3 random areas in one labyrinth at 400X, 3 labyrinth sections per pregnancy, 3 pregnancies per implantation site. Paraffin embedded sections stained with H&E representative of images used for measurements.
Figure 3.13.
erythrocytes was used to assess vessel origin. Only vessels containing either maternal or fetal red blood cells were analyzed, which introduced variability to the data.

Maternal labyrinthine vascular areas in Nfil3+/− x Nfil3+/− (Fig. 3.14Ai) were significantly larger than in UBC-GFP x Nfil3+/− (Fig. 3.14Aii) and C57BL/6 (Fig. 3.14Aiii; p<0.01) at GD 10.5, 12.5 and 15.5. UBC-GFP x Nfil3+/− maternal vascular areas were larger than C57BL/6 spaces at GD 10.5 only (Fig. 3.14B).

Fetal labyrinthine vascular areas were significantly larger in Nfil3+/− x Nfil3+/− than C57BL/6 spaces at GD 10.5 (p<0.05), 12.5 (p<0.001) and 15.5 (p<0.001). UBC-GFP x Nfil3+/− fetal labyrinthine vascular areas did not differ from C57BL/6 areas at GD 10.5 but were significantly larger than Nfil3+/− x Nfil3+/− (p<0.05) and C57BL/6 (p<0.001) areas at GD 12.5 and 15.5 (Fig. 3.14C). The interhemal membrane width (separating the maternal and fetal vessels) was significantly thicker in Nfil3+/− x Nfil3+/− (p<0.001) and UBC-GFP x Nfil3+/− (p<0.01) compared to C57BL/6 membranes at GD 10.5 and 12.5. The interhemal membrane width was also thicker in Nfil3+/− x Nfil3+/− compared to UBC-GFP x Nfil3+/− at GD 10.5 (p<0.001) and 12.5 (p<0.05). These differences did not persist to GD 15.5 when interhemal membrane widths in Nfil3+/− x Nfil3+/− and UBC-GFP x Nfil3+/− matched those in C57BL/6 (Fig. 3.14D).

**Nfil3+/− x Nfil3+/− and UBC-GFP x Nfil3+/− spiral arteries fail to remodel**

Spiral arteries of both Nfil3+/− x Nfil3+/− (Fig. 3.15A) and UBC-GFP x Nfil3+/− (Fig. 3.15B) implantation sites failed to remodel by GD 12.5 (p<0.001) compared to C57BL/6 spiral arteries (Fig. 3.15C, D). The wall:lumen ratio is significantly greater in Nfil3+/− x Nfil3+/− implantation sites compared to UBC-GFP x Nfil3+/− (p<0.001).
Figure 3.14. Maternal and fetal labyrinthine vascular area and interhemal membrane measurements. Photomicrographs of Nfil3<sup>−/−</sup> x Nfil3<sup>−/−</sup> (Ai), UBC-GFP x Nfil3<sup>−/−</sup> (Aii), and C57BL/6 (Aiii) labyrinths at GD 12.5 representative of images used for measurements. (B) Maternal labyrinthine vascular area is significantly larger in Nfil3<sup>−/−</sup> x Nfil3<sup>−/−</sup> and UBC-GFP x Nfil3<sup>−/−</sup> compared to C57BL/6 matings at GD 10.5 and 12.5. Nfil3<sup>−/−</sup> x Nfil3<sup>−/−</sup> areas are larger compared to UBC-GFP x Nfil3<sup>−/−</sup> and C57BL/6 matings at GD 15.5. (C) Fetal labyrinthine vascular area is significantly larger in Nfil3<sup>−/−</sup> x Nfil3<sup>−/−</sup> compared to UBC-GFP x Nfil3<sup>−/−</sup> and C57BL/6 matings at GD 10.5. Nfil3<sup>−/−</sup> x Nfil3<sup>−/−</sup> and UBC-GFP x Nfil3<sup>−/−</sup> areas are significantly larger compared to C57BL/6 matings at GD 12.5 and 15.5. (D) The interhemal membrane is significantly thicker in Nfil3<sup>−/−</sup> x Nfil3<sup>−/−</sup> and UBC-GFP x Nfil3<sup>−/−</sup> compared to C57BL/6 matings at GD 10.5 and 12.5. At GD 15.5 there is no significant difference in width of the interhemal membrane between matings. Maternal vascular space is outlined in black and fetal vascular space is outlined in yellow. Interhemal membranes are denoted by *. N = 3 random areas in one labyrinth at 400X, 3 labyrinths per pregnancy, 3 pregnancies per matings. Paraffin embedded sections stained with H&E.
Figure 3.14.
Figure 3.15. *Nfil3*<sup>-/-</sup> x *Nfil3*<sup>-/-</sup> and UBC-GFP x *Nfil3*<sup>-/-</sup> spiral arteries fail to remodel. Photomicrographs show typical spiral arteries of *Nfil3*<sup>-/-</sup> x *Nfil3*<sup>-/-</sup> (A), UBC-GFP x *Nfil3*<sup>-/-</sup> (B) and C57BL/6 (C) GD 12.5 implantation sites. (D) Morphometry of spiral arteries in *Nfil3*<sup>-/-</sup> x *Nfil3*<sup>-/-</sup>, UBC-GFP x *Nfil3*<sup>-/-</sup>, and C57BL/6 implantation sites. Spiral arteries in *Nfil3*<sup>-/-</sup> x *Nfil3*<sup>-/-</sup> (0.5165 pixels ± 0.2359), and UBC-GFP x *Nfil3*<sup>-/-</sup> (0.4440 pixels ± 0.2109) implantation sites have a greater wall:lumen ratio compared to C57BL/6 arteries (0.2001 pixels ± 0.1035 p<0.001). Spiral arteries in *Nfil3*<sup>-/-</sup> x *Nfil3*<sup>-/-</sup> have a greater wall:lumen ratio compared to UBC-GFP x *Nfil3*<sup>-/-</sup> arteries (p<0.001). N = every spiral artery in one section, 3 sections per implantation site, 3 implantation sites per pregnancy, 3 pregnancies per mating. Paraffin embedded sections stained with H&E.
3.3 *Nfil3*⁻/⁻ bone marrow reconstitution of alymphoid mice

*Nfil3*⁻/⁻ bone marrow engraftment into alymphoid mice reconstitutes PAS⁺DBA⁻ and PAS⁺DBA⁺ uNK cells

To determine whether the irregularities seen in the *Nfil3*⁻/⁻ pregnancies were attributable to modifications of uNK cell function and number or resulted from confounding effects induced by the absence of NFIL3 on non-lymphoid cells, alymphoid *Rag2*⁻/⁻*Il2rg*⁻/⁻ dams were reconstituted with *Nfil3*⁻/⁻ bone marrow (*Rag2*⁻/⁻*Il2rg*⁻/⁻ BM (*Nfil3*⁻/⁻)) and mated with *Rag2*⁻/⁻*Il2rg*⁻/⁻ males. Reconstitution of T (CD3) and B (CD19) cells, but not NK (NK1.1) cells was confirmed by flow cytometry (Fig. 3.16). Subsequent confirmation of engraftment was the presence of uNK cells within implantation sites. PAS⁺DBA⁻ and PAS⁺DBA⁺ uNK cells were found at GD 8.5 in *Rag2*⁻/⁻*Il2rg*⁻/⁻ BM (*Nfil3*⁻/⁻) (Fig. 17 Ai, Bi). PAS⁺DBA⁻ uNK cells were noted at 1/15 cells (Fig. 3.17C). This is considerably higher than the bone marrow engraftment studies using C57BL/6 marrow that had previously reported 1/230 SP cells at GD 6. PAS⁺DBA⁺ uNK cell numbers were significantly lower in *Rag2*⁻/⁻*Il2rg*⁻/⁻ BM (*Nfil3*⁻/⁻) implantation sites compared to C57BL/6 implantation sites at GD 8.5, 10.5 and 12.5 (*p*<0.005; Fig. 3.17D).
Figure 3.16. *Rag2*<sup>−/−</sup> *Il2rg*<sup>−/−</sup> mice reconstituted with *Nfil3*<sup>−/−</sup> bone marrow have T and B cells, but lack peripheral NK cells. Leukocytes isolated from the spleen of a *Rag2*<sup>−/−</sup> *Il2rg*<sup>−/−</sup> mouse reconstituted with *Nfil3*<sup>−/−</sup> bone marrow. Flow cytometric contour plots show successful T cell (CD3) and B cell (CD19) reconstitution, but limited NK cell (NK1.1) reconstitution.
Figure 3.17. Progenitors for PAS$^+$DBA$^-$ and PAS$^+$DBA$^+$ uNK cells can be adoptively transferred from Nfil3$^{-/-}$ bone marrow. Photomicrographs of PAS/DBA dual stained sections from perfused Rag2$^{-/-}$Il2rg$^{-/-}$ BM (Nfil3$^{-/-}$) (Ai) and C57BL/6 (Aii) implantation sites at GD 8.5. Black boxes denote region of high power inset. Photomicrographs of DBA lectin reacted sections from perfused Rag2$^{-/-}$Il2rg$^{-/-}$ BM (Nfil3$^{-/-}$) (Bi) and C57BL/6 (Bii) implantation sites at GD 12.5. (C) PAS$^+$DBA$^-$ uNK cells are present but significantly fewer in Rag2$^{-/-}$Il2rg$^{-/-}$ BM (Nfil3$^{-/-}$) compared to C57BL/6 (p<0.001). (D) PAS$^+$DBA$^+$ uNK cells are present but significantly fewer in Rag2$^{-/-}$Il2rg$^{-/-}$ BM (Nfil3$^{-/-}$) implantation sites compared to C57BL/6 at GD 8.5 (p<0.005), GD 10.5 (p<0.001) and GD 12.5 (p<0.001). N = all uNK cells in field of view at 200X in one section, 3 sections per implantation site, 3 implantation sites per pregnancy, 3 pregnancies per mating. Implantation sites from Rag2$^{-/-}$Il2rg$^{-/-}$ BM (C57BL/6) at GD 8.5, 10.5 and 12.5 (n = all uNK cells in field of view at 200X in one section, 3 sections per implantation site, 3 implantation sites per pregnancy, 1 pregnancy per GD) stained with PAS/DBA are reported in Appendix B. Values were similar to those in nonmanipulated C57BL/6 pregnancies as previously reported.
Figure 3.17.

Ai

Rag2⁻/⁻Il2rg⁻/⁻
BM(Nfe13⁻/⁻)

Aii

C57BL/6

Bi

GD 8.5

Bii

GD 10.5

C

D

**Unk Cell Numbers**

- **Gestation Day**

---

85
Implantation sites from \textit{Rag2}^/-\textit{Il2rg}^/- BM (\textit{Nfil3}^/-) and \textit{Rag2}^/-\textit{Il2rg}^/- matings have similarities to implantation sites from \textit{Nfil3}^/- x \textit{Nfil3}^/- and UBC-GFP x \textit{Nfil3}^/- at GD 8.5.

\textit{NOTE}: In the remaining studies implantation sites from \textit{Rag2}^/-\textit{Il2rg}^/- BM (\textit{Nfil3}^/-) and \textit{Rag2}^/-\textit{Il2rg}^/- are compared to C57BL/6 implantation sites as controls. A superior study would have been to use \textit{Rag2}^/-\textit{Il2rg}^/- x \textit{Rag2}^/-\textit{Il2rg}^/- BM (C57BL/6) for these comparisons. Others have reported no differences in uNK cell numbers between these two control groups (42). I prepared a small series of \textit{Rag2}^/-\textit{Il2rg}^/- x \textit{Rag2}^/-\textit{Il2rg}^/- BM (C57BL/6) pregnancies (n=1 pregnancy for each of GD 8.5, 10.5 and 12.5 and 3 implantation sites per pregnancy). These showed no differences to the C57BL/6 controls used for the rest of this study (See Appendix B). Expansion of C57BL/6 graft recipient numbers is in the process to obtain 3 pregnancies per time point for comparisons and submission of a manuscript for peer review.

\textit{Rag2}^/-\textit{Il2rg}^/- BM (\textit{Nfil3}^/-) (Fig. 3.18A) and \textit{Rag2}^/-\textit{Il2rg}^/- x \textit{Rag2}^/-\textit{Il2rg}^/- (\textit{Rag2}^/-\textit{Il2rg}^-; Fig. 18B) implantation sites have smaller areas of antimesometrial decidualization compared to C57BL/6 (Fig. 18C) at GD 8.5 (-29.39\% and -33.7\% respectively, p<0.001) (Fig. 3.18D). TUNEL staining for cell death was negative in the antimesometrium of \textit{Rag2}^/-\textit{Il2rg}^/- BM (\textit{Nfil3}^/-) (Fig. 3.19Ai), \textit{Rag2}^/-\textit{Il2rg}^- (Fig. 3.19Aii) and C57BL/7 (Fig. 3.19Aiii) implantation sites at GD 8.5. Staining for the cell proliferation marker Ki67 was negative in the antimesometrium of \textit{Rag2}^/-\textit{Il2rg}^- BM (\textit{Nfil3}^/-) (Fig. 3.19Bi), \textit{Rag2}^/-\textit{Il2rg}^- (Fig. 3.19Bii) and C57BL/7 (Fig. 3.19Biii) implantation sites at GD 8.5 although the positive control slide was heavily stained (Fig. 3.7C).
Figure 3.18. Antimesometrial decidualization differs between \( \text{Rag}^{2-/-}\text{Il}2\text{rg}^{-/-} \) BM (Nfil3^{-/-}) and \( \text{Rag}^{2-/-}\text{Il}2\text{rg}^{-/-} \) implantation sites at GD 8.5. Photomicrographs of antimesometrial decidua from \( \text{Rag}^{2-/-}\text{Il}2\text{rg}^{-/-} \) BM (Nfil3^{-/-}) (A) \( \text{Rag}^{2-/-}\text{Il}2\text{rg}^{-/-} \) (B) and C57BL/6 (C) perfused implantation sites at GD 8.5. Areas from the decidualized border (yellow line) to the antimesometrial trophoblast are defined as the decidualized area (marked by a double headed yellow arrow). The total area (marked by a double headed black arrow) was measured from the edge of the myometrium (black line) to the antimesometrial trophoblast. (D) Antimesometrial decidua is significantly underdeveloped (-29.4%; \( p<0.001 \)) in \( \text{Rag}^{2-/-}\text{Il}2\text{rg}^{-/-} \) BM (Nfil3^{-/-}) and \( \text{Rag}^{2-/-}\text{Il}2\text{rg}^{-/-} \) (-33.7%; \( p<0.001 \)) compared to C57BL/6 antimesometrial decidua. Paraffin embedded section stained with H&E. N = 3 sections per implantation site, 3 implantation sites per pregnancy, 3 pregnancies per mating.
Figure 3.19. Differences in antimesometrial decidualization are not accounted for by frequency of cell death or proliferation in \textit{Rag2}^{−/−}\textit{Il2rg}^{−/−} BM (\textit{Nfil3}^{−/−}) and \textit{Rag2}^{−/−}\textit{Il2rg}^{−/−} implantation sites. TUNEL staining for cell death is negative in the antimesometrium of \textit{Rag2}^{−/−}\textit{Il2rg}^{−/−} BM (\textit{Nfil3}^{−/−}) (Ai) \textit{Rag2}^{−/−}\textit{Il2rg}^{−/−} (Aii) and C57BL/6 (Aiii) GD 8.5 implantation sites. The DNAse digested positive control for TUNEL staining is shown in Figure 7A and B. The proliferation marker Ki67 is negative in the antimesometrium of \textit{Rag2}^{−/−}\textit{Il2rg}^{−/−} BM (\textit{Nfil3}^{−/−}) (Bi) \textit{Rag2}^{−/−}\textit{Il2rg}^{−/−} (Bii) and C57BL/6 (Biii) GD 8.5 antimesometrium. A positive signal is shown in the mesometrium of a C57BL/6 pregnancy in Figure 7C.
Embryos of \( \text{Rag}^{2-/-}\text{Il2rg}^{+/-} \) BM (\( \text{Nfil}^{3/-} \)) and \( \text{Rag}^{2-/-}\text{Il2rg}^{+/-} \) matings were 0.5-1 day developmentally delayed at GD 8.5. The \( \text{Rag}^{2-/-}\text{Il2rg}^{+/-} \) BM (\( \text{Nfil}^{3/-} \)) embryo is at Theiler stage 12 equivalent to GD 8 (Fig. 3.20A), mirroring the \( \text{Nfil}^{3/-} \times \text{Nfil}^{3/-} \) GD 8.5 embryo. The \( \text{Rag}^{2-/-}\text{Il2rg}^{+/-} \) embryo is at Theiler stage 11, equivalent to GD 7.5 (Fig. 3.20B), mirroring the UBC-GFP \( \times \text{Nfil}^{3/-} \) GD 8.5 embryo. The C57BL/6 embryo is at Theiler stage 13, equivalent to GD 8.5 and typical for normal development (Fig. 3.20C). Staging of all conceptuses scored for developmental stage are given in Fig. 3.20D.

\( \text{Rag}^{2-/-}\text{Il2rg}^{+/-} \) BM (\( \text{Nfil}^{3/-} \)) and \( \text{Rag}^{2-/-}\text{Il2rg}^{+/-} \) implantation sites normalize at midgestation

\( \text{Rag}^{2-/-}\text{Il2rg}^{+/-} \) BM (\( \text{Nfil}^{3/-} \)) implantation sites were compared to C57BL/6 implantation sites at GD 10.5 (Fig. 3.21A) and GD 12.5 (Fig. 3.21B). Unlike the \( \text{Nfil}^{3/-} \times \text{Nfil}^{3/-} \) implantation site layers the \( \text{Rag}^{2-/-}\text{Il2rg}^{+/-} \) BM (\( \text{Nfil}^{3/-} \)) implantation site layers are well defined and mirror those of the C57BL/6. This indicates that conceptus deletion of the \( \text{Nfil}^{3} \) gene, rather than the maternal environment, accounts for the disturbed placental histopathology identified in the \( \text{Nfil}^{3/-} \times \text{Nfil}^{3/-} \) mating. These data also appear to exclude a role for uNK cells in normalizing the structural layers of the placenta as \( \text{Rag}^{2-/-}\text{Il2rg}^{+/-} \) mesometrial tissue layers are similar to C57BL/6. Total implantation site areas did not differ significantly between \( \text{Rag}^{2-/-}\text{Il2rg}^{+/-} \) BM (\( \text{Nfil}^{3/-} \)), \( \text{Rag}^{2-/-}\text{Il2rg}^{+/-} \) and C57BL/6 matings at GD 10.5. At GD 12.5, \( \text{Rag}^{2-/-}\text{Il2rg}^{+/-} \) BM (\( \text{Nfil}^{3/-} \)) implantation sites were significantly smaller than C57BL/6 implantation sites (\( p<0.05 \)). \( \text{Rag}^{2-/-}\text{Il2rg}^{+/-} \) GD 12.5 implantation site size did not differ from \( \text{Rag}^{2-/-}\text{Il2rg}^{+/-} \) BM (\( \text{Nfil}^{3/-} \)) or C57BL/6 (Fig. 3.21C).
Figure 3.20. \textit{Rag2}^{−/−}\textit{Il2rg}^{−/−} BM (\textit{Nfil3}^{−/−}) and \textit{Rag2}^{−/−}\textit{Il2rg}^{−/−} embryos are developmentally delayed by 0.5-1 day at GD 8.5. GD 8.5 implantation sites from \textit{Rag2}^{−/−}\textit{Il2rg}^{−/−} BM (\textit{Nfil3}^{−/−}) (A), \textit{Rag2}^{−/−}\textit{Il2rg}^{−/−} (B), and C57BL/6 (C) matings. (A) 56\% of \textit{Rag2}^{−/−}\textit{Il2rg}^{−/−} BM (\textit{Nfil3}^{−/−}) embryos were at Theiler stage 12 (equivalent to GD 8.0). (B) 67\% of \textit{Rag2}^{−/−}\textit{Il2rg}^{−/−} embryos were at Theiler stage 11 (equivalent to GD 7.5). (C) 78\% of C57BL/6 embryos were at Theiler stage 13 (typical for normal development). (D) Table depicting number of embryos at each Theiler stage per mating. N = 3 implantation sites per pregnancy, 3 pregnancies per mating. Photomicrographs of paraffin embedded sections stained with H&E. Labels = 1: condensation of tissue in caudal region of notocord, 2: lateral extent of body wall, in continuity with the amnion, 3: notocord, 4: pharyngeal region of foregut diverticulum, 5: primitive streak region, 6: amnion, 7: mesoderm, 8: rostral extremity of neural tube closure, 9: entrance to foregut diverticulum.
Figure 3.21. \( \text{Rag}^{2-} \text{Il2rg}^{-/-} \text{BM} \ (\text{Nfil3}^{-/-}) \) mesometrial tissue layers are similar to controls at GD 10.5 and 12.5. Halved \( \text{Rag}^{2-} \text{Il2rg}^{-/-} \text{BM} \ (\text{Nfil3}^{-/-}) \) implantation sites are compared to C57BL/6 implantation sites at GD 10.5 (A) and GD 12.5 (B). Yellow lines demarcate the transition between three layers. The layers between the two matings are similar, both having distinct regular tissue. (C) Total mesometrial tissue area does not differ between matings at GD 10.5. \( \text{Rag}^{2-} \text{Il2rg}^{-/-} \text{BM} \ (\text{Nfil3}^{-/-}) \) mesometrial tissue area is significantly smaller than C57BL/6 mesometrial tissue at GD 12.5 (p<0.05). Photomicrographs of paraffin embedded sections stained with H&E. N = 3 sections per implantation site, 3 implantation sites per pregnancy, 3 pregnancies per mating. Labels = DB: decidua basalis, SP: spongiotrophoblast, LB: labyrinth.
Figure 3.21.
Figure 3.22A and B represent histological photomicrographs of GD 10.5 and 12.5 implantation sites of \textit{Rag2}\textsuperscript{−/−}\textit{Il2rg}\textsuperscript{−/−} BM (\textit{Nfil3}\textsuperscript{−/−}), \textit{Rag2}\textsuperscript{−/−}\textit{Il2rg}\textsuperscript{−/−} and C57BL/6 mating strategies. Figure 3.22C shows GD 10.5 mesometrial tissue surface area analysis. \textit{Rag2}\textsuperscript{−/−}\textit{Il2rg}\textsuperscript{−/−} BM (\textit{Nfil3}\textsuperscript{−/−}) and \textit{Rag2}\textsuperscript{−/−}\textit{Il2rg}\textsuperscript{−/−} DB were significantly smaller (p<0.001 and p<0.005) than C57BL/6 DB in surface area but did not differ from each other. This result implicates contributions from functionally normal uNK cells to the appropriate normal expansion of early decidua. Since T and B cells are normal in \textit{Nfil3}\textsuperscript{−/−} mice, this outcome supports early decidual expansion as a function of normal uNK cells. At GD 10.5, \textit{Rag2}\textsuperscript{−/−}\textit{Il2rg}\textsuperscript{−/−} BM (\textit{Nfil3}\textsuperscript{−/−}), \textit{Rag2}\textsuperscript{−/−}\textit{Il2rg}\textsuperscript{−/−} and C57BL/6 spongiotrophoblast and labyrinthine tissue areas do not differ from each other. Thus, the variances in placental layer structure reported above in \textit{Nfil3}\textsuperscript{−/−} x \textit{Nfil3}\textsuperscript{−/−} and UBC-GFP x \textit{Nfil3}\textsuperscript{−/−} (Figures 3.10-12) do not result from the actions of maternal hematopoietic cells but must result from disruption of NFIL3 signaling in the concepts, perhaps within trophoblast cells.

Figure 3.22D shows GD 12.5 mesometrial tissue area analysis. \textit{Rag2}\textsuperscript{−/−}\textit{Il2rg}\textsuperscript{−/−} BM (\textit{Nfil3}\textsuperscript{−/−}) DB surface area is significantly smaller than C57BL/6 DB surface area (p<0.001). \textit{Rag2}\textsuperscript{−/−}\textit{Il2rg}\textsuperscript{−/−} DB area does not differ from \textit{Rag2}\textsuperscript{−/−}\textit{Il2rg}\textsuperscript{−/−} BM (\textit{Nfil3}\textsuperscript{−/−}) or C57BL/6 DB areas. \textit{Rag2}\textsuperscript{−/−}\textit{Il2rg}\textsuperscript{−/−} BM (\textit{Nfil3}\textsuperscript{−/−}) spongiotrophoblast surface area is significantly larger than C57BL/6 spongiotrophoblast area (p<0.001). \textit{Rag2}\textsuperscript{−/−}\textit{Il2rg}\textsuperscript{−/−} spongiotrophoblast tissue area does not differ from \textit{Rag2}\textsuperscript{−/−}\textit{Il2rg}\textsuperscript{−/−} BM (\textit{Nfil3}\textsuperscript{−/−}) or C57BL/6 spongiotrophoblast area. \textit{Rag2}\textsuperscript{−/−}\textit{Il2rg}\textsuperscript{−/−} BM (\textit{Nfil3}\textsuperscript{−/−}) and \textit{Rag2}\textsuperscript{−/−}\textit{Il2rg}\textsuperscript{−/−} labyrinthine tissue areas are significantly smaller (p<0.001 and p<0.05) than C57BL/6 labyrinthine tissue area however they do not differ from each other.
Figure 3.22. Rag2<sup>−/−</sup>Il2rg<sup>−/−</sup> BM (Nfil3<sup>−/−</sup>) and Rag2<sup>−/−</sup>Il2rg<sup>−/−</sup> mesometrial tissues are similar to C57BL/6 at GD 10.5, and 12.5. Photomicrographs of Rag2<sup>−/−</sup>Il2rg<sup>−/−</sup> BM (Nfil3<sup>−/−</sup>) (Ai), Rag2<sup>−/−</sup>Il2rg<sup>−/−</sup> (Aii), and C57BL/6 (Aiii) implantation sites at GD 10.5. Photomicrographs of Rag2<sup>−/−</sup>Il2rg<sup>−/−</sup> BM (Nfil3<sup>−/−</sup>) (Bi), Rag2<sup>−/−</sup>Il2rg<sup>−/−</sup> (Bii), and C57BL/6 (Bii) implantation sites at GD 12.5. (C) At GD 10.5, decidua basalis (DB) tissue areas of Rag2<sup>−/−</sup>Il2rg<sup>−/−</sup> BM (Nfil3<sup>−/−</sup>) and Rag2<sup>−/−</sup>Il2rg<sup>−/−</sup> are significantly smaller than C57BL/6 tissue area (p<0.001 and p<0.005 respectively). Rag2<sup>−/−</sup>Il2rg<sup>−/−</sup> BM (Nfil3<sup>−/−</sup>) and Rag2<sup>−/−</sup>Il2rg<sup>−/−</sup> DB tissue areas do not significantly differ from each other. Spongiotrophoblast (spongio) and labyrinth tissue areas do not significantly differ between the three matings. (D) At GD 12.5 Rag2<sup>−/−</sup>Il2rg<sup>−/−</sup> BM (Nfil3<sup>−/−</sup>) DB area is significantly smaller than C57BL/6 DB (p<0.001). Rag2<sup>−/−</sup>Il2rg<sup>−/−</sup> BM (Nfil3<sup>−/−</sup>) and Rag2<sup>−/−</sup>Il2rg<sup>−/−</sup>, and Rag2<sup>−/−</sup>Il2rg<sup>−/−</sup> and C57BL/6 DB areas do not significantly differ from each other. Rag2<sup>−/−</sup>Il2rg<sup>−/−</sup> BM (Nfil3<sup>−/−</sup>) spongiotrophoblast tissue area is significantly larger than C57BL/6 spongiotrophoblast area (p<0.005). Rag2<sup>−/−</sup>Il2rg<sup>−/−</sup> BM (Nfil3<sup>−/−</sup>), Rag2<sup>−/−</sup>Il2rg<sup>−/−</sup> and C57BL/6 spongiotrophoblast areas do not significantly differ from each other. Rag2<sup>−/−</sup>Il2rg<sup>−/−</sup> BM (Nfil3<sup>−/−</sup>) and Rag2<sup>−/−</sup>Il2rg<sup>−/−</sup> labyrinthine areas are significantly smaller than C57BL/6 tissue area (p<0.001 and p<0.05 respectively). Rag2<sup>−/−</sup>Il2rg<sup>−/−</sup> BM (Nfil3<sup>−/−</sup>) and Rag2<sup>−/−</sup>Il2rg<sup>−/−</sup> labyrinth tissue areas do not significantly differ from each other. Paraffin embedded sections stained with H&E. N = 3 sections per implantation site, 3 implantation sites per pregnancy, 3 pregnancies per mating. Labels: spongio = spongiotrophoblast.
The placental labyrinth of \textit{Rag2}\textsuperscript{-/-}II2rg\textsuperscript{-/-} BM (\textit{Nfil3}\textsuperscript{-/-}) and \textit{Rag2}\textsuperscript{-/-}II2rg\textsuperscript{-/-} shows less vascular aberration compared to \textit{Nfil3}\textsuperscript{-/-} x \textit{Nfil3}\textsuperscript{-/-} and UBC-GFP x \textit{Nfil3}\textsuperscript{-/-} labyrinths.

Representative high power images of \textit{Rag2}\textsuperscript{-/-}II2rg\textsuperscript{-/-} BM (\textit{Nfil3}\textsuperscript{-/-}), \textit{Rag2}\textsuperscript{-/-}II2rg\textsuperscript{-/-} and C57BL/6 GD 12.5 labyrinths are shown in Figures 3.23A, B and C respectively. At GD 10.5, high power analysis of the placental labyrinth revealed significantly larger labyrinthine vascular spaces in \textit{Rag2}\textsuperscript{-/-}II2rg\textsuperscript{-/-} BM (\textit{Nfil3}\textsuperscript{-/-}) matings compared to C57BL/6 labyrinthine vascular spaces (p<0.01) however labyrinth vascular area did not differ between \textit{Rag2}\textsuperscript{-/-}II2rg\textsuperscript{-/-} BM (\textit{Nfil3}\textsuperscript{-/-}) and \textit{Rag2}\textsuperscript{-/-}II2rg\textsuperscript{-/-} or \textit{Rag2}\textsuperscript{-/-}II2rg\textsuperscript{-/-} and C57BL/6 (Fig. 3.23D). At GD 12.5, significantly larger vascular spaces were found in the placentae from \textit{Rag2}\textsuperscript{-/-}II2rg\textsuperscript{-/-} BM (\textit{Nfil3}\textsuperscript{-/-}) compared to \textit{Rag2}\textsuperscript{-/-}II2rg\textsuperscript{-/-} and C57BL/6 matings at GD 12.5 (p<0.05). Although the differences in the labyrinth vascular spaces reach significance, they do so to a lesser degree than seen in \textit{Nfil3}\textsuperscript{-/-} x \textit{Nfil3}\textsuperscript{-/-} and UBC-GFP x \textit{Nfil3}\textsuperscript{-/-} labyrinths. GD 12.5 \textit{Rag2}\textsuperscript{-/-}II2rg\textsuperscript{-/-} and C57BL/6 labyrinth spaces did not differ. \textit{Rag2}\textsuperscript{-/-}II2rg\textsuperscript{-/-} BM (\textit{Nfil3}\textsuperscript{-/-}) and \textit{Rag2}\textsuperscript{-/-}II2rg\textsuperscript{-/-} dams were perfused, clearing the placenta of maternal red blood cells, making the distinction between maternal and fetal vessels impossible. Therefore these measurements were not conducted.

\textit{Rag2}\textsuperscript{-/-}II2rg\textsuperscript{-/-} BM (\textit{Nfil3}\textsuperscript{-/-}) and \textit{Rag2}\textsuperscript{-/-}II2rg\textsuperscript{-/-} spiral arteries fail to remodel

\textit{Rag2}\textsuperscript{-/-}II2rg\textsuperscript{-/-} BM (\textit{Nfil3}\textsuperscript{-/-}) (Fig. 3.23A) and \textit{Rag2}\textsuperscript{-/-}II2rg\textsuperscript{-/-} (Fig. 3.24B) implantation sites do not show evidence for spiral arterial remodeling by GD 12.5 (p<0.001) compared to modified C57BL/6 spiral arteries (Fig. 3.24C). The wall:lumen ratios for \textit{Rag2}\textsuperscript{-/-}II2rg\textsuperscript{-/-} BM (\textit{Nfil3}\textsuperscript{-/-}) and \textit{Rag2}\textsuperscript{-/-}II2rg\textsuperscript{-/-} spiral arteries were 2.6 and 2.4 fold greater than for
Figure 3.23. The labyrinthine regions in $\text{Rag2}^{+/\text{Il2rg}^{-/-}}\text{BM (Nfil3^{-/-})}$ and $\text{Rag2}^{+/\text{Il2rg}^{-/-}}$ placentas at GD 10.5 and 12.5. High power photomicrographs of labyrinthine vascular spaces of $\text{Rag2}^{+/\text{Il2rg}^{-/-}}\text{BM (Nfil3^{-/-})}$ (A), $\text{Rag2}^{+/\text{Il2rg}^{-/-}}$ (B) and C57BL/6 (C) implantation sites at GD 12.5. (D) Total labyrinth vascular area in $\text{Rag2}^{+/\text{Il2rg}^{-/-}}\text{BM (Nfil3^{-/-})}$ matings were significantly larger than C57BL/6 areas at GD 10.5 ($p<0.01$). Labyrinth vascular area did not differ between $\text{Rag2}^{+/\text{Il2rg}^{-/-}}\text{BM (Nfil3^{-/-})}$ and $\text{Rag2}^{+/\text{Il2rg}^{-/-}}$ or $\text{Rag2}^{+/\text{Il2rg}^{-/-}}$ and C57BL/6 at GD 10.5. Total labyrinth vascular areas in $\text{Rag2}^{+/\text{Il2rg}^{-/-}}\text{BM (Nfil3^{-/-})}$ matings were significantly larger than $\text{Rag2}^{+/\text{Il2rg}^{-/-}}$ and C57BL/6 areas at GD 12.5 ($p<0.05$). Labyrinth vascular area did not differ between $\text{Rag2}^{+/\text{Il2rg}^{-/-}}$ and C57BL/6 at GD 12.5.
Figure 3.24. *Rag2<sup>−/−</sup>Il2rg<sup>−/−</sup> BM (Nfil3<sup>−/−</sup>) and *Rag2<sup>−/−</sup>Il2rg<sup>−/−</sup> spiral arteries fail to remodel.* Photomicrographs show typical spiral arteries of *Rag2<sup>−/−</sup>Il2rg<sup>−/−</sup> BM (Nfil3<sup>−/−</sup>) (A), *Rag2<sup>−/−</sup>Il2rg<sup>−/−</sup> (B), and C57BL/6 (C) implantation sites at GD 12.5. (D) Morphometry of spiral arteries in *Rag2<sup>−/−</sup>Il2rg<sup>−/−</sup> BM (Nfil3<sup>−/−</sup>), *Rag2<sup>−/−</sup>Il2rg<sup>−/−</sup>, and C57BL/6 implantation sites. Spiral arteries in *Rag2<sup>−/−</sup>Il2rg<sup>−/−</sup> BM (Nfil3<sup>−/−</sup>) (0.5139 pixels ± 0.2827) and *Rag2<sup>−/−</sup>Il2rg<sup>−/−</sup> (0.4680 pixels ± 0.2735) implantation sites have a greater wall:lumen ratio than C57BL/6 arteries (0.1971 pixels ± 0.1388; p<0.001). N = every spiral artery in one section, 3 sections per implantation site, 3 implantation sites per pregnancy, 3 pregnancies per mating.
C57BL/6 (p<0.001)(Fig. 3.24D). Thus, expression of *Nfil3* in uNK cells is essential for uNK cell-induced spiral arterial remodeling.

3.4 Virgin uterine cell suspension engraftment into alymphoid mice

**Assessment of C57BL/6 and *Nfil3*−/− uterine cell suspensions for progenitors of PAS⁺DBA⁺ uNK cells**

A pilot study was undertaken to assess the capacity of cells resident in virgin uterus for differentiation into uNK cells. Well-perfused uteri were employed to remove contamination of any circulating progenitor cells. *Rag2+/−Il2rg−/−* dams inoculated with a virgin uterine cell suspension from either an *Nfil3*−/− (Fig. 3.25A) or C57BL/6 (Fig. 3.25B) female have PAS⁺DBA⁺ but not PAS⁺DBA− uNK cells. Two out of three C57BL/6 uterine cell suspensions generated uNK cells at GD 8.5. Four *Nfil3*−/− cell suspension pregnancies were studied; two at GD 8.5 and two at GD 10.5. One pregnancy per time point generated uNK cells. Additional experiments to enlarge animal numbers are ongoing. These data not only show that transplantable uNK progenitor cells reside within the uterine tissue of adult mice but also that these apparently tissue resident uNK progenitor cells have no absolute requirement for *Nfil3* gene usage during uterine homing or during maturation to fully mature uNK cells.
Figure 3.25. Virgin Nfil3− and C57BL/6 uterine cell suspensions contain transplantable PAS−DBA+, but not PAS+DBA− uNK cell progenitors. Photomicrographs of a Rag2−/Il2rg− UT (Nfil3−) GD 8.5 implantation site at increasing magnifications (Ai, Aii, Aiii). PAS−DBA+ uNK cells are subtype I (immature DBA+ cells with a DBA+ plasma membrane and no cytoplasmic granules; black circle). Photomicrographs of a Rag2−/Il2rg− UT (C57BL/6) GD 8.5 implantation site at increasing magnification (Bi, Bii, Biii). PAS−DBA+ uNK cells are subtype I (black circle) and II (DBA+ cells with some DBA+ cytoplasmic granules; yellow circle). (D) Table depicting uterine transplant experiment data. Each implantation site of a pregnancy was sectioned and stained for uNK cells. Paraffin embedded section stained with PAS/DBA.
Figure 3.25.

<table>
<thead>
<tr>
<th>C</th>
<th>No. of inoculated dams</th>
<th>No. of pregnancies</th>
<th>No. of pregnancies uNK cells were present</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rag2^{-/-}I2rg^{-/-} UT(Nfil3^{-/-})</td>
<td>8</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Rag2^{-/-}I2rg^{-/-} UT(C57BL/6)</td>
<td>5</td>
<td>3</td>
<td>2</td>
</tr>
</tbody>
</table>
Chapter 4

Discussion

4.1 Unique Approach of this Thesis

Investigations into a mouse pregnancy model lacking NK cells but retaining the capacities of T and B lymphocytes have been missing from efforts to fully define, in vivo, the actions of decidual lymphocytes. This model has been sought to complement understandings of the functions of uNK cells from studies of pregnant mice that lacked NK cells and also lacked T cells and/or B cells. By use of uNK cell reconstitution in alymphoid mice, understanding of the functions of uNK cells arising from transplantable progenitor cell populations that differentiate all lymphocytes or only NK cells has also been advanced. The latter studies are limited by not including the study of uNK cells that may differentiate from tissue resident progenitors but also because interpretation of uNK cell functions is made in the absence of T and B cells. The first two papers reporting the Nfil3\textsuperscript{−/−} (also known as E4bp4\textsuperscript{−/−}), NK\textsuperscript{T+}B\textsuperscript{+} mouse appeared to provide the desired model (48,64).

At the outset of this thesis project, there was no report on the virgin or pregnant uterus of the Nfil3\textsuperscript{−/−} mouse. At the time of thesis submission, there are 2 published reports and one manuscript in review that the authors have shared with me. One of the published reports describes the presence of CD49a\textsuperscript{+}DX5\textsuperscript{−} uNK cells in virgin Nfil3\textsuperscript{−/−} uteri by flow cytometry (93). The other describes elevation of Th17 cells in the GD 14.5 decidua of Nfil3\textsuperscript{−/−} females crossed allogeneically to normal BALB/c males and reports elevated fetal deaths in this cross (i.e. Nfil3\textsuperscript{+/−} conceptuses) (94). In a personal communication from Dr.
F. Colucci, University of Cambridge, May 2015, and work presented by his laboratory in abstracts and posters, heterozygote conceptuses were again used with Nfil3\(^{-/-}\) females mated to syngeneic normal C57BL/6 males. This study focused upon GD 9.5-10 for flow cytometric analyses but did include histologic studies quantified using stereology at GD 14.5, 16.5 and 18.5. This work by S. Boulenouar et al., describes the presence of uNK cells that differentiate with no dependence on the Nfil3\(^{-/-}\) transcription factor. Comparisons in this study were to implantation sites of Rag2\(^{-/-}\)/Il2rg\(^{-/-}\) and the conclusion was reached that similar pathology occurs in the implantation sites of Nfil3\(^{-/-}\) and Rag2\(^{-/-}\)/Il2rg\(^{-/-}\). The results of my thesis concur with those from Sojka et al. and the Colucci laboratory that Nfil3\(^{-/-}\) mice differentiate uNK cells. The work from these 3 laboratories challenge the conclusions made by Fu et al., that absence of uNK cells drives the elevation of decidual Th17 cells in mouse decidua and elevated fetal death rates (94). These findings expand on those of the Colucci laboratory because of the novel investigation into pregnancies in Nfil3\(^{-/-}\) x Nfil3\(^{-/-}\) and in alymphoid (Rag2\(^{-/-}\)/Il2rg\(^{-/-}\)) recipients of Nfil3\(^{-/-}\) bone marrow. It was determined that Nfil3\(^{-/-}\) implantation site histopathology does not match that of Rag2\(^{-/-}\)/Il2rg\(^{-/-}\) pregnancies at midgestation.

In this thesis, syngeneic implantation sites in Nfil3\(^{-/-}\) x Nfil3\(^{-/-}\) mice are described for the first time. Experiments were conducted from GD 6.5 to 15.5 and thus report the first information concerning the early post implantation, pre-placental phase in Nfil3\(^{-/-}\) females. Nfil3\(^{-/-}\) females mated syngeneically (C57BL/6) as well as allogeneically (BALB/c) were studied, and adoptive cell transfer studies were conducted. In the first instance, this was to remove outcomes from Nfil3\(^{-/-}\) deletion in all tissues except transplantable hematopoietic cells. In the second place, this was to address whether the
progenitors of Nfil3-independent uNK cells were transplantable. The uNK cells were transplantable when either marrow or virgin, well perfused uterus was the progenitor cell source. In conclusion, the Nfil3"− strain is not an appropriate model to address decidual lymphocyte functions that are independent of uNK cells. The Nfil3"− strain does provide the opportunity to investigate a new subset of innate lymphoid cells that seems to be an NK cell subset.

4.2 Breeding and Conceptus Viability

4.2.1 Nfil3"− x Nfil3"−

Initially, the Queen’s University Nfil3"− breeding colony was maintained using Nfil3"− x Nfil3"− breeding pairs to generate experimental Nfil3"− mice. It was difficult to obtain births and to wean those pups born so the breeding colony was moved to the use of Nfil3"− males to breed Nfil3"+/− females. I now know that both of the original laboratories developing these mice and the 3 other laboratories who are studying pregnancies in this strain use the current Queen’s University breeding strategy. In our hands, most Nfil3"− x Nfil3"− pregnancies ended in apparent dystocia at ~GD 18.5-19.5 and the request from the University Veterinarian for euthanasia of the pregnant Nfil3"− female. In contrast, Nfil3"+/− dams mated by Nfil3"− males deliver successfully and dystocia has not been seen over the 2 year interval of my studies.

This suggests the dystocia did not result from the absence of Nfil3"− uNK cells or from unusual activities in the Nfil3-independent uNK cells found in these mice. Rather, the dystocia is due to a maternal deficit. In mice, declining ovarian progesterone is the key signal for parturition, followed by increased prostaglandin synthesis in maternal
decidua (130-132). Since parturition was initiated, compromised decidual function would be a more likely explanation for this phenotype. In rats, NFIL3 binding is reported to regulate the promoters of PTGS2, PGR, AREG, EREG, and HPGD, implicating both ovarian and decidual aberrations in the signaling of parturition in \( Nfil3^{-/-} \) dams (133). No blood samples were collected from the \( Nfil3^{-/-} \) mice with dystocia for hormone assays.

Dystocia is also seen in transgenic mice developed to overexpress the estrogen receptor (134). \( Nfil3 \) is a known circadian clock gene and increased expression of the gene has been linked to a better prognosis in estrogen receptor (ER) positive breast cancers (135). Hormonal treatment of ER+ breast cancer can include lowering estrogen or blocking estrogen receptors within the body (136). A direct interaction between \( Nfil3 \) and the ER has not been described however it is possible \( Nfil3 \) may directly or indirectly inhibit ER expression. Therefore the absence of \( Nfil3 \) in the mother during pregnancy may allow overexpression of the ER leading to dystocia.

4.2.2 Allogeneic UBC-GFP x \( Nfil3^{-/-} \) pregnancies

Allogeneic, normal male BALB/c x female \( Nfil3^{-/-} \) pregnancies were reported to have significantly fewer numbers of live allogeneic fetuses per pregnancy at GD 14.5 (137). The investigators attributed this to the absence of NK cells in \( Nfil3^{-/-} \) mice. I have shown that the number of viable fetuses in pregnancies from male UBC-GFP mice on a BALB/c background mated to \( Nfil3^{-/-} \) females does not significantly differ from wild type C57BL/6 mice, or from \( Nfil3^{-/-} \) x \( Nfil3^{-/-} \) pregnancies up to GD 15.5. I have also shown that uNK cells develop in this allogeneic mating, therefore the reported fetal loss cannot be due to the absence of uNK cells during gestation. It is possible animal husbandry differed between our colonies in endogenous room and animal microbiological flora, diet
and/or the temperature leading to the high GD 14.5 losses reported by Fu et al. Fu et al. measured the percentage of T\textsubscript{H}17 but not NK cells in the decidua of BALB/c x \textit{Nfil3}\textsuperscript{-/-} mice. T\textsubscript{H}17 cells were significantly more abundant than in BALB/c x \textit{Nfil3}\textsuperscript{+/+} allogeneic matings (94). \textit{Nfil3}-independent uNK cells may be unable to moderate T\textsubscript{H}17 cell expansion; however, T\textsubscript{H}17 cell percentage was not quantified in my study. My finding of late fetal viability in \textit{Nfil3}\textsuperscript{-/-} is further supported by the study of GD18.5 implantation sites by the Colucci group in \textit{Nfil3}\textsuperscript{-/-} females mated syngeneically by normal males (138).

4.3 Early Gestation Developmental Delays

Conceptuses of an \textit{Nfil3}\textsuperscript{-/-} mother experience developmental delay regardless of the paternal genotype. Implantation sites of \textit{Nfil3}\textsuperscript{-/-} females mated by \textit{Nfil3}\textsuperscript{-/-} or UBC-GFP males showed signs of up to a one-day delay in post-implantation development up to GD 8.5. Delayed closure of the uterine lumen may have contributed to the observed conceptus delay in both \textit{Nfil3}\textsuperscript{-/-} x \textit{Nfil3}\textsuperscript{-/-} and UBC-GFP x \textit{Nfil3}\textsuperscript{-/-}. \textit{Nfil3} is detected weakly in the mouse embryo at GD 9.5 (139). No earlier gestation days have been reported. The data suggests deficient NFIL3 in the early maternal decidual environment underlies the conceptus growth retardation observed. If \textit{Nfil3} expression does not begin in conceptuses until GD 9.5, this conclusion would be strengthened. Assessment of early conceptus expression of \textit{Nfil3} is therefore identified as a priority area for future research.

The finding of pre-placental stage conceptus growth retardation in \textit{Nfil3}\textsuperscript{-/-} implantation sites is similar to observations made in my laboratory by others studying implantation sites from \textit{Rag2}\textsuperscript{-/-}\textit{Il2rg}\textsuperscript{-/-} females mated syngeneically by UBC-GFP males. These implantation sites had persisting uterine lumens and conceptuses developmentally
delayed by one day at GD 8.5, despite a heterozygous fetal genotype (140). The heterozygous conceptuses in UBC-GFP x Nfil3+/− matings were more developmentally delayed (-1 day) than those in Nfil3+/− x Nfil3+/− (-0.5 days). However, the Nfil3+/- conceptuses studied in this thesis were semi-allogeneic (BALB/c x C57BL/6 mating) rather than syngeneic. The mismatching MHC in concert with a small population of Nfil3-independent uNK cells may contribute to the more severe developmental delay due to an imbalance in the cells (i.e. uNK and Treg) that promote fetal tolerance. Other investigators reported antibody-mediated depletion of CD25+ Treg cells at GD 4.5 and 7.5 significantly increased resorption rates in allogeneic pregnant mice but not in syngeneic pregnant mice (141). Fu et al. reported increased numbers of T_H17 cells in BALB/c x Nfil3+/− implantation sites at GD 14.5 (94). It is well documented that Treg cells are important in the generation of tolerance to paternal antigens during pregnancy (123). A uNK^low Treg^low T_H17^high allogeneic environment during gestation may be as detrimental to the growing conceptus as an alymphoid decidual environment.

4.4 Comparisons Between Pregnancies in Nfil3+/− Females Mated by Nfil3+/− or UBC-GFP Males

4.4.1 UNK Cells

NK cells can be broken into a number of different subsets. Most cNK cells arise from the bone marrow however there are also a number of tissue resident subsets (76). TrNK cells are found in the thymus (89), liver (90), salivary glands (92) and most recently in the uterus (93,138). The surface marker CD49a was designated as a specific marker of liver-resident DX5− NK cells (142) and has been used to define the tissue resident NK
population in the uterus (138). Nfil3 has been shown to be critical for the development of cNK and thymic NK cells (80). However Nfil3 is not required by tissue resident liver, salivary or uterine NK cells, all of which are present in Nfil3−/− mice (80). The phenotype of Nfil3−/− uNK cells was explored in this thesis.

The uNK cell population that was present in Nfil3−/− females mated by Nfil3−/− or normal males had a higher proportion of immature uNK cells from mid to late pregnancy. Ifng−/− mouse implantation sites are also reported to have increased numbers of immature uNK cells and to have implantation site abnormalities including impaired spiral artery remodeling and abnormal decidual differentiation (42). Increased numbers of immature uNK cells and altered placental architecture are also reported in homozygous Hectd1 (a HECT domain E3 ubiquitin ligase) mutant implantation sites. Hectd1 is highly expressed in the chorion, which differentiates into labyrinth trophoblast, but is not expressed in uNK cells. Hectd1 is required for normal development of the labyrinth, and this aberrant phenotype appears to impact uNK cell maturity. Therefore the abnormal placental development may be suppressing Nfil3-independent uNK cell maturation because a higher proportion of immature uNK cells in Nfil3−/− implantation sites is not realized until GD 10.5 when the mature placenta begins to function. (143).

In the present study, morphometric assessments of greater uNK cell immaturity in Nfil3−/− implantation sites is in line with the stereological findings of S. Boulenouar et al. (144). The authors assessed both WT and Nfil3−/− tr-uNK cells at midgestation by flow cytometry and found the cells to be less mature as defined by CD11b and CD27 staining. Nfil3−/− dams had a much larger population of these immature trNK cells than the normal controls. UNK cell immaturity may affect uNK cell functional capability in terms of their
cytokine and angiokine secretion profiles. Degaki et al found DLL1 expression was dependent on uNK cell maturation (109). UNK cells are known for their role in spiral artery remodeling (42) and although present, Nfil3-independent uNK cells are unable to fulfill this role.

IFNG levels have been compared between CD49a+ tr-uNK cell and CD49a- circulating uNK cells from pregnant C57BL/6 mice. CD49a+ tr-uNK cells produced significantly less IFNG than the cNK cells (138). IFNG production has not been measured in Nfil3−/− uNK cells but is expected to be very low since Nfil3−/− mice have significantly fewer PAS+DBA− uNK cells that produce IFNG and a larger proportion of their uNK cells are immature. Gestational time course studies that quantified IFNG in decidua basalis found highest levels when mature uNK cells were present (118). This theory is supported by the lack of IFNG mediated spiral artery remodeling (42) seen in Nfil3−/− implantation sites at GD 12.5.

4.4.2 Antimesometrial decidua

UNK cells are found in the mesometrial pole of the implantation site, and therefore uNK cell-related findings are generally focused to this region. A novel finding in Nfil3−/− pregnancies was incomplete antimesometrial decidualization at GD 8.5. Disparity between Nfil3−/− pregnancies and C57BL/6 antimesometrial decidualization could not be explained by increased apoptosis in Nfil3−/− decidua or increased proliferation of C57BL/6 decidual cells. Bany and Cross used gene expression arrays of GD 7.5 decidua and decidualoma (artificially induced decidua) to determine which decidualization-associated genes were conceptus-dependent. Of the conceptus-dependent genes they identified, interferon alpha-inducible (G1p2), decidual/trophoblast prolactin-related
protein (Dtprp), and angiopoietin 2 (Angpt2) transcripts were concentrated in antimesometrial decidua (145). Investigation into the expression of these genes would be valuable in Nfil3\(^{-/-}\) implantation sites to determine if these transcripts are decreased in Nfil3\(^{-/-}\) decidua. Optimally such studies would be complimented by secretion assays to see whether Nfil3\(^{-/-}\) decidua is functionally impaired. It would also be of considerable interest to know if genes whose expression in decidua is not dependent on a conceptus are dysregulated in the antimesometrial decidua of Nfil3\(^{-/-}\) as both Nfil3\(^{-/-}\) mating combinations are affected.

Another important finding concerning the antimesometrial decidua was the identification of CD45\(^+\)CD11c\(^+\). These cells had previously been identified in our laboratory in C57BL/6 implantation sites however they are much more prominent in Nfil3\(^{-/-}\) pregnancies. CD11c is a DC marker and NFIL3 is a key transcription factor for CD8\(^+\) cDC development. Nfil3\(^{-/-}\) mice still possess CD11c\(^+\) CD8\(^-\) cDCs and plasmacytoid DCs in lymphoid tissue however populations in the uterus have not been studied (57). CD8\(^-\) cDCs are considered lymphoid (146) and are thought to prime naïve CD4\(^+\) T cells to synthesize Th1 cytokines (i.e. IL-12) while CD8\(^+\) cDCs are considered myeloid (146) and are thought to prime naïve CD4\(^+\) T cells to synthesize Th2 cytokines (i.e. IL-10) (147,148). Blois et al. showed the majority of uterine DCs are the NFIL3-independent CD8\(^-\) and a transient peak of systemic CD11c\(^+\) cells was found at GD 8.5 (149). The enrichment of these CD11c\(^+\) cells may be an increased population of CD8\(^-\) cDCs. This subset may be filling the empty niche due to the absence of the CD8\(^+\) cDCs, or they may be a protective response to a potentially inflammatory or weakened Nfil3\(^{-/-}\) decidual environment (94).
4.4.3 Trophoblast

Many of the differences in the phenotypic anomalies in *Nfil3*\(^{-/-}\) females mated by *Nfil3*\(^{-/-}\) versus UBC-GFP males strongly suggest abnormal trophoblast behavior in conceptuses that are *Nfil3*\(^{-/-}\). It is unknown whether *Nfil3* is expressed by mouse trophoblast, however *Nfil3*\(^{-/-}\) is not expressed in human trophoblast or decidua (150). Trophoblast cells are key players in ensuring a healthy gestational environment.

Placental pathologies were found in implantation sites of *Nfil3*\(^{-/-}\) females mated syngeneically or allogeneically. Many of these pathologies were less severe by late gestation. From mid to late gestation UBC-GFP x *Nfil3*\(^{-/-}\) implantation sites presented a phenotype that often had less severe pathology than *Nfil3*\(^{-/-}\) x *Nfil3*\(^{-/-}\) or had become similar to C57BL/6 wild type implantation sites. Therefore, the influences of *Nfil3*\(^{+/+}\) conceptuses may not become important until later in gestation once placental circulation is established.

Total implantation site area largely did not differ between *Nfil3*\(^{-/-}\) x *Nfil3*\(^{-/-}\), UBC-GFP x *Nfil3*\(^{-/-}\) and C57BL/6 crosses, however the proportions of the 3 mesometrial implantation site tissue layers did (trophoblast giant cell and spongiotrophoblast layers were amalgamated). At GD 10.5 implantation sites from syngeneically and allogeneically-mated *Nfil3*\(^{-/-}\) females had more decidua basalis than C57BL/6, implicating impaired trophoblast invasion. Although there is no evidence from WM-IF studies of uNK cells interacting directly with trophoblasts, trophoblast invasion was also impaired in UBC-GFP x *Rag2*\(^{-/-}\)*Il2rg*\(^{-/-}\) (NK\(^{+}\)T\(^{-}\)B\(^{-}\)) implantation sites at GD 6.5 and corrected upon pre-conception restoration of uNK cells during pregnancy through transplantation of SCID (NK\(^{-}\)T\(^{+}\)B\(^{-}\)) bone marrow into the dams (140). However by GD 12.5 trophoblast
invasion was normal in Rag2<sup>−/−</sup>Il2rg<sup>−/−</sup> and the SCID bone marrow transplant mice (42). Early gestation trophoblast invasion has been proposed to be uNK cell mediated (11). GD 6.5 trophoblast invasion was not investigated in this thesis. The study of GD 6.5 UBC-GFP (for a fluorescent conceptus) x Nfil3<sup>+/−</sup> implantation sites would determine whether early (GD 6.5-10.5) trophoblast invasion is Nfil3-dependent uNK cell-regulated. The larger decidua basalis persisted to GD 12.5 in the Nfil3<sup>+/−</sup> x Nfil3<sup>+/−</sup> matings. The depth of Nfil3<sup>−/−</sup> trophoblast invasion does not normalize until GD 15.5, implicating Nfil3 in mid gestation trophoblast invasion.

Trophoblast-derived placental tissue layer areas varied between Nfil3<sup>−/−</sup> x Nfil3<sup>+/−</sup>, UBC-GFP x Nfil3<sup>+/−</sup> and C57BL/6 implantation sites at GD 10.5, 12.5 and 15.5. This highlights the complex influences conceptuses have on placental development. There was no distinct pattern between the placenta layer, area and mating combination at each gestation day. Although the cross sectional area measurements are variable, the organization of the placental layers was improved in UBC-GFP x Nfil3<sup>+/−</sup> implantation sites. Hough et al. reported Nfil3 transcript expression in endothelial cells, although there was variation across different cell types. Their focus was the interaction of NFIL3 with the von Willebrand factor (VWF) promoter and, depending on the cell type, NFIL3 could be either phosphorylated or unphosphorylated, which dictated repression or promotion of VWF translation (55). Nfil3 has not been investigated in the context of placental endothelium, however the findings of Hough et al, may implicate this gene in normal vascular development of the placenta.

Nfil3<sup>−/−</sup> placentas have larger labyrinthine vascular spaces with Nfil3<sup>−/−</sup> x Nfil3<sup>−/−</sup> placentas again having a more severe phenotype than UBC-GFP x Nfil3<sup>−/−</sup> implantation
sites. These spaces resemble labyrinths found in \( Pgf^{-/} \) pregnancies where deficient labyrinthine vascular branching is present at GD 15.5-18.5 (151). Although uNK cells produce PGF, this phenotype may indicate a trophoblast deficiency, since trophoblasts are the predominant source of PGF in the placenta (152).

Fetal genotype influenced labyrinthine vascular branching. \( Nfil3^{-/-} \times Nfil3^{-/-} \) labyrinths had larger maternal vascular spaces than UBC-GFP x \( Nfil3^{-/-} \) labyrinths between GD 10.5-15.5. Fetal vascular spaces of the labyrinth however, were larger in UBC-GFP x \( Nfil3^{-/-} \) labyrinths at GD 12.5 and 15.5 than in \( Nfil3^{-/-} \times Nfil3^{-/-} \). This suggests than an \( Nfil3^{+/} \) conceptus promotes maternal vascular branching but impairs fetal vascular branching within the placental labyrinth. The latter would be expected to compromise maternal-fetal exchange. In addition, the interhemal membrane separating the maternal and fetal labyrinthine vessels was significantly thicker in \( Nfil3^{-/-} \), independent of fetal genotype, before GD 15.5, potentially adding compromise to nutrient and waste exchanges between mother and fetus. Micro computed tomography (CT) of the maternal and fetal placental vessels as well as placental perfusion analyses, could add further understanding to these observations (153,154).

4.5 \( Nfil3^{-/-} \) Bone Marrow Engraftment Studies

\( Nfil3 \) plays roles in the circadian rhythm (49,50), neuron growth and survival (51,52), osteoblast function (53), ovulation (54) and is expressed in endothelium (55). To eliminate extraneous effects of \( Nfil3 \) deletion in cell lineages other than hematopoietic cells during pregnancy, preconception engraftment studies were conducted using \( Nfil3^{-/-} \) marrow or uterine cell suspensions inoculated into \( Rag2^{-/-} Il2rg^{-/-} \), a strain with a well-
characterized implantation site phenotype. Alterations to the Rag2<sup>−/−</sup>Il2rg<sup>−/−</sup> phenotype should reflect the activities of Nfil3-independent uNK cells. Persisting phenotypic characteristics should reflect the effects of Nfil3-dependent uNK cells. In addition, comparisons of these phenotypes with Nfil3<sup>−/−</sup> x Nfil3<sup>−/−</sup> pregnancies were expected to define Nfil3-dependent/uNK cell independent features of implantation sites.

4.5.1 Nfil3-independent uNK cell regulated pathology

Nfil3<sup>−/−</sup> uNK cells were transplantable but fewer in number than in C57BL/6 pregnancies. Antimesometrial decidualization was impaired in the bone marrow recipients however this phenotype was also seen in alymphoid Rag2<sup>−/−</sup>Il2rg<sup>−/−</sup> mice. Therefore this feature cannot be attributed to absence of the Nfil3 gene but could be due to a functional or absolute uNK cell deficit. Mesometrial decidualization elevates a number of cytokines including IL-15 and IL-18 (155). IL-15 is an essential cytokine to uNK cell survival in the implantation site (86). IL-18 (in concert with additional pro-inflammatory signals) induces uNK cell activation and production/release of IFNG. IFNG alters gene transcription in uterine stromal/decidual cells (155).

Rag2<sup>−/−</sup>Il2rg<sup>−/−</sup> and UBC-GFP x Nfil3<sup>−/−</sup> GD 8.5 embryos were developmentally delayed by 1 GD. Rag2<sup>−/−</sup>Il2rg<sup>−/−</sup> BM (Nfil3<sup>−/−</sup>) and Nfil3<sup>−/−</sup> x Nfil3<sup>−/−</sup> GD 8.5 embryos were delayed by 0.5 GD. Therefore Nfil3-independent uNK cells are able to enhance alymphoid embryo development by 0.5 days, but only with syngeneic, not allogeneic conceptuses. This may be due to a possible increased inflammatory environment previously discussed that impairs Nfil3-independent uNK cell function.

Rag2<sup>−/−</sup>Il2rg<sup>−/−</sup> BM (Nfil3<sup>−/−</sup>) and Rag2<sup>−/−</sup>Il2rg<sup>−/−</sup> uterine spiral arteries were not remodeled at GD 12.5. This is unsurprising as PAS<sup>−</sup>DBA<sup>−</sup> uNK cells are thought to be
responsible for spiral artery remodeling as described above. \( \text{Rag}2^{+/-} \text{Il2rg}^{-/-} \) BM (\( \text{Nfil3}^{-/-} \)) have very low numbers of \( \text{PAS}^{+} \text{DBA}^{-} \) uNK cells while \( \text{Rag}2^{+/-} \text{Il2rg}^{-/-} \) are alymphoid. \( \text{Nfil3} \)-independent uNK cell mediated pathology is summarized in Figure 4.1.

4.5.2 \( \text{Nfil3} \)-gene regulated pathology

\( \text{Rag}2^{+/-} \text{Il2rg}^{-/-} \) BM (\( \text{Nfil3}^{-/-} \)) and \( \text{Rag}2^{+/-} \text{Il2rg}^{-/-} \) mesometrial implantation site layers are similar to C57BL/6 implantation site layers. The placental layer anomalies seen in \( \text{Nfil3}^{-/-} \times \text{Nfil3}^{-/-} \) and UBC-GFP \( \times \text{Nfil3}^{-/-} \) are likely a product of the \( \text{Nfil3} \) gene and not likely attributable to \( \text{Nfil3} \)-independent uNK cells. The placental layer areas at GD 10.5 and 12.5 significantly differ mostly between \( \text{Rag}2^{+/-} \text{Il2rg}^{-/-} \) BM (\( \text{Nfil3}^{-/-} \)) and C57BL/6. Therefore \( \text{Nfil3} \)-independent uNK cells may play a small role in placental layer organization. \( \text{Nfil3} \) independent-uNK cells also may play a small role in subdivision of the labyrinthine vascular space. \( \text{Rag}2^{+/-} \text{Il2rg}^{-/-} \) BM (\( \text{Nfil3}^{-/-} \)) labyrinthine vascular space is significantly larger than C57BL/6 however the disparity between these spaces and \( \text{Nfil3}^{-/-} \) \( \times \text{Nfil3}^{-/-} \) and UBC-GFP \( \times \text{Nfil3}^{-/-} \) compared to C57BL/6 is less. The published and my newly discovered roles of \( \text{Nfil3} \) are summarized in Figure 4.2.

I hypothesize the \( \text{Nfil3}^{-/-} \) related pathologies are driven by irregular trophoblast cells for the reasons cited above. However, it is worth noting that mid gestation placentas fare better without any lymphocytes. \( \text{Nfil3}^{-/-} \) placental layer organization and labyrinth vascular space may also be a product of \( \text{Nfil3}^{-/-} \) T and B lymphocytes. Apart from \( \text{T}_{11}17 \) cells (94), the decidual lymphocyte profile of \( \text{Nfil3}^{-/-} \) mice during pregnancy has not been studied. It would be of interest to determine if T and B cell subset populations are present in different proportions in these mice, and whether that may be influencing the
Figure 4.1. Summary of Nfil3-independent uNK cell-mediated pathologies. Nfil3-independent uNK cells inhibit or are unable to promote antimesometrial decidualization. They are unable to initiate spiral artery remodeling or promote a normal rate of embryo development. Labels = DB: decidua basalis; E: Embryo.
Figure 4.2. Summary of roles of *Nfil3*. *Nfil3* plays a number of roles throughout the body. This thesis has demonstrated its role in labyrinthine branching and placental tissue layer development. In addition, its absence may allow a larger population of *Nfil3*-independent CD45<sup>+</sup>CD11c<sup>+</sup> dendritic cells to appear in the decidua. Labels = MLAP: mesometrial lymphoid aggregate of pregnancy; DB: decidua basalis; SPONG: spongio-trophoblast; LAB: labyrinth; E: embryo. Red circles: fetal red blood cells; purple circles: stromal nuclei; salmon circles: maternal red blood cells.
4.6 Adoptive Virgin Uterine Cell Transfer Studies

*Rag2*⁻/⁻*Il2rg*⁻/⁻ mice inoculated with C57BL/6 and *Nfil3*⁻/⁻ virgin uterine cell suspensions were euthanized at GD 8.5 or 10.5. PAS⁺DBA⁺ but not PAS⁺DBA⁻ uNK cells were present in the decidua basalis. These cells likely represent the tissue resident uNK cells described by Sojka et al. Tissue resident uNK cells were described to be CD49a⁺DX5⁻. This is in line with my findings as DBA marks DX5⁻ uNK cells (115). These investigators also found both circulating (CD49a⁺DX5⁻) and tissue resident (CD49a⁺DX5⁺) NK cells in the uterus, though they report the NFIL3-independent tissue resident NK cells were the dominant population (93). It should be noted that Boulenouar found a CD49a⁺DX5⁺ tissue resident uNK cell population in virgin *Nfil3*⁻/⁻ uterus. DX5⁺ cells are typically PAS⁺DBA⁻ and this subset was not found in my dually stained tissue sections from the implantation sites of either *Nfil3*⁻/⁻ or C57BL/6 uterine cell transplanted mice. Therefore the PAS⁺DBA- subset may not be transplantable from a uterine cell suspension. If CD49a⁺DX5⁻ *Nfil3*-independent uNK cells are the dominant population during pregnancy, data from my thesis could support two different arguments. First, CD49a⁺DX5⁺ circulating NK cells need to be present to facilitate expansion and function of the tissue resident population. The second argument would be that the additional effects of *Nfil3* inhibit normal expansion and function of *Nfil3*-independent tissue resident NK cells.
<table>
<thead>
<tr>
<th>Conceptus Viability</th>
<th>No Difference</th>
<th>No Difference</th>
<th>GD 12.5: &lt;C57BL/6</th>
<th>No Difference</th>
<th>No Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>UNK Cell Frequency</td>
<td>&lt; C57BL/6</td>
<td>&lt; C57BL/6</td>
<td>&lt; C57BL/6</td>
<td>None</td>
<td>Normal</td>
</tr>
<tr>
<td>GD 6.5 Uterine Lumen Closure</td>
<td>Lumen persisted</td>
<td>Lumen persisted, less open than (Nfil3^{--}) x (Nfil3^{--})</td>
<td>N/A</td>
<td>N/A</td>
<td>Lumen apposing</td>
</tr>
<tr>
<td>GD 8.5 Antimesometrial Decidualization</td>
<td>&lt; C57BL/6</td>
<td>&lt; C57BL/6</td>
<td>&lt; C57BL/6</td>
<td>&lt; C57BL/6</td>
<td>Normal</td>
</tr>
<tr>
<td>GD 8.5 Embryo Development</td>
<td>~0.5 day delay</td>
<td>~1 day delay</td>
<td>~0.5 day delay</td>
<td>~1 day delay</td>
<td>Normal</td>
</tr>
<tr>
<td>Mesometrial Tissue Layer Development</td>
<td>Highly aberrant</td>
<td>Aberrant</td>
<td>Aberrant</td>
<td>Similar to C57BL/6</td>
<td>Normal</td>
</tr>
<tr>
<td>Labyrinth Area</td>
<td>&gt; C57BL/6</td>
<td>&gt; C57BL/6</td>
<td>&gt; C57BL/6</td>
<td>No difference to C57BL/6</td>
<td>Normal</td>
</tr>
<tr>
<td>Interhemal Membrane Width</td>
<td>&gt; C57BL/6</td>
<td>&gt; C57BL/6</td>
<td>N/A</td>
<td>N/A</td>
<td>Normal</td>
</tr>
<tr>
<td>Spiral Artery Wall:Lumen Ratio</td>
<td>&gt; C57BL/6</td>
<td>&gt; C57BL/6</td>
<td>&gt; C57BL/6</td>
<td>&gt; C57BL/6</td>
<td>Normal</td>
</tr>
</tbody>
</table>

**Table 4.1. Tabulated data of differences across all mating strategies.** \(Nfil3^{--}\) x \(Nfil3^{--}\) and UBC-GFP x \(Nfil3^{--}\) early gestation pathologies are similar. \(Nfil3^{--}\) x \(Nfil3^{--}\) mid gestation pathologies are more severe than UBC-GFP x \(Nfil3^{--}\) compared to C57BL/6 implantation sites. N/A = Not applicable.
4.7 Future Directions

As discussed above, many points need to be established to support further interpretations of my findings. Two lines of investigation should be pursued. The first line would define the role of Nfil3 in pregnancy. To determine the etiology of dystocia estrogen and progesterone assays should be conducted on term plasma from Nfil3−/− females mated by Nfil3+/− males. Quantification of the CD45+CD11c+ cells as well as determining other surface markers for this lineage has merit. Knowledge of whether these cells are inflammatory or anti-inflammatory could implicate them in the early implantation site pathologies observed, especially the process of antimesometrial decidualization. In order to verify whether Nfil3−/− is expressed in the trophoblast of early conceptuses and the impact of its deficit, RNA analysis of GD 7.5 ectoplacental cones dissected from normal and Nfil3−/− conceptuses should be conducted.

The second line of investigation would more directly address uNK cells and the role of Nfil3 in separation of lineages found in the peripheral versus mucosal tissues. The functional capacity of Nfil3-independent uNK cells needs to be assessed. The dominant population in both wild type and Nfil3−/− mice is the PAS+DBA+ subset. This population typically secretes pro-angiogenic factors including VEGF, PGF and DLL1 (108). Assays to determine the secretory profile of this subset would help determine whether these cells are functionally similar to wild type PAS+DBA+ uNK cells and represent a small fraction of the total population, or are entirely independent with different secretory capacity.

PAS+DBA− uNK cells are the primary producers (90%) of IFNG in the decidua (42). This population was present but severely reduced in Nfil3−/− implantation sites. IFNG production during pregnancy has not been measured in Nfil3−/− implant sites or
uNK cells. Thus, there is no measurement of IFNG in a NK T+ strain and it is well known that activated CD4+ Th1 cells produce IFNG (156). This cytokine is important in maintaining healthy murine pregnancy; Ifng−/− mouse implantation sites have increased numbers of immature uNK cell, necrotic decidua at GD 8.5, maternal spiral arteries did not remodel and primiparous mothers experienced significant fetal loss (118). IFNG has been shown to potentially regulate genes important for normal trophoblast invasion (mouse α2-M; MAM)(157). IFNG regulates expression of more than 0.5% of the mouse genome. Nfil3-independent uNK cells not secreting, or secreting low levels of IFNG would likely have widespread effects. Subsequent experiments would include the addition of Nfil3-dependent uNK cells to an Nfil3-independent population and determining if concordant expression of both cells impacts their functions.
4.8 Study Limitations

The research presented in this thesis is a comprehensive histological time course study that provides a foundation for further study of Nfil3−/− pregnancies. However, there are limitations to what has been achieved in this dissertation. All experiments were carried out in the morning however during early time points a difference in the time of copulation could vary developmental stages. The morphometry studies were not conducted blinded or repeated by different members of the laboratory. However due to the number of mating strategies and the subjectivity of measurements (especially when using pixel quantification), this would have been difficult. Many of the measurements were subjective; therefore in the future specific markers for decidualization, and placental layers should be used.

Bone marrow engraftment studies were carried out before the Nfil3−/− time course was undertaken. Initially all animals were perfused to ensure accurate spiral artery quantification. When Nfil3−/− experiments commenced, some of the implantation sites were also used for whole mount immunofluorescence. Because whole mount immunofluorescence is conducted using live tissue, perfusion of these animals with PFA was not possible. Therefore Nfil3−/− x Nfil3−/− and UBC-GFP x Nfil3−/− implantation sites are not perfused, while Rag2−/−Il2rg−/− BM(Nfil3), Rag2−/−Il2rg−/− BM(C57BL/6) and Rag2−/−Il2rg−/− implantation sites were perfused. Separate, non-perfused and perfused C57BL/6 implantation sites were used as the wild type comparison.

The wild type C57BL/6 control animal for the Rag2−/−Il2rg−/− BM(Nfil3) data is inadequate. C57BL/6 implantation sites were used because previous work in our laboratory had shown uNK cell numbers to be similar in both Rag2−/−Il2rg−/−
BM(C57BL/6) and C57BL/6 implantation sites. These studies gave 2 x 10^7 bone marrow cells intravenously to mice preconditioned with 5-fluorouracil. I used the equivalent of 2 femurs of bone marrow administered i.p. in non-conditioned mice. Three Rag2<sup>−/−</sup>Il2rg<sup>−/−</sup> BM (C57BL/6) pregnancies (one at each time point) were investigated in this thesis, and uNK cell numbers were found to be similar to C57BL/6. A full complement of pregnancies (3 pregnancies at each time point) of i.p. C57BL/6 bone marrow administered non-preconditioned Rag2<sup>−/−</sup>Il2rg<sup>−/−</sup> mice is currently being developed to provide implantation sites for use in submission of a peer-reviewed manuscript.

4.9 Conclusion

Initially, the Nfil3<sup>−/−</sup> mouse was thought to be an ideal model to describe NK<sup>T</sup>B<sup>+</sup> pregnancies. Instead, Nfil3<sup>−/−</sup> mice established the differentiation of Nfil3-independent uNK cells. These may be trNK cells similar to those in liver, and salivary glands. Using multiple mating combinations, I was able to differentiate between Nfil3-independent uNK cell effects, Nfil3<sup>+/−</sup> conceptus effects and global Nfil3 effects on pregnancy. Although Nfil3-independent uNK cells are present, the total uNK cell population is significantly lower than in normal mice and functionally deficient. Implantation sites with an Nfil3<sup>+/−</sup> conceptus experience less severe placental tissue layer aberrations that are still variant from wild type implantation sites. Nfil3 has a number of effects on pregnancy that are not uNK cell related. These effects include placental layer organization, normal labyrinthine vascular branching, and possibly altering the leukocyte population in the maternal decidua (CD11c<sup>+</sup> cells).
Elucidating the development of NK cells is important to the field of immunology and this mouse strain provides a foundation for new insights into the emerging classification of innate lymphoid cells (ILCs). NK cells belong to IFNG producing group 1 ILCs (85). Additionally, through further investigation into the pregnancy complications of *Nfil3*−/− mice, understanding of human pregnancy complications may emerge. Although human and mice pregnancies differ, many fundamental cell types, cytokines and tissue medication processes are similar. Specifically, human decidual NK cell roles were largely discovered initially in the mouse. Therefore a more in depth understand of murine reproduction has conceptual value in human reproduction especially due to the ease and freedom of manipulation in murine models.
References


35. Simmons DG. Postimplantation development of the chorioallantoic placenta. 2014.


102. Felker A, Croy BA. Uterine natural killer cell partnerships in early mouse decidua basalis.


114. Felker AM, Chen Z, Foster WG, Croy BA. Receptors for non-mhc ligands contribute to uterine natural killer cell activation during pregnancy in mice. Placenta 2013, Sep;34(9):757-64.


119. Functions of uterine natural killer cells are mediated by interferon gamma production during murine pregnancy; Seminars in immunology. 2001.


144. Leon L, Kay VR, Felker AM, Tu MM, Makrigiannis AP, Croy BA. LY49 knockdown in mice leads to aberrant uterine crypt formation and impaired blastocyst.


Appendices

Appendix A

Appendix A. Trophoblast invasion into \textit{Nfil3}^{-/-} \times \textit{Nfil3}^{-/-} and UBC-GFP \times \textit{Nfil3}^{-/-} maternal decidual tissue at GD 10.5, 12.5 and 15.5. Time course images from \textit{Nfil3}^{-/-} \times \textit{Nfil3}^{-/-} implantation sites are in the left column (Ai, Bi, Ci). UBC-GFP \times \textit{Nfil3}^{-/-} implantation sites are in the center column (Aii, Bii, Cii). C57BL/6 implantation sites are in the right column (Aiii, Biii, Ciii). (D) Percentage of the area (in pixels) of mesometrial implantation site invaded by trophoblast. There is less trophoblast invasion in \textit{Nfil3}^{-/-} \times \textit{Nfil3}^{-/-} implantation sites relative to UBC-GFP \times \textit{Nfil3}^{-/-} and C57BL/6 implantation sites at GD 10.5 and 12.5. There is more trophoblast invasion in \textit{Nfil3}^{-/-} \times \textit{Nfil3}^{-/-} and UBC-GFP \times \textit{Nfil3}^{-/-} implantation sites relative to C57BL/6 implantation sites at GD 15.5. N = 1 implantation site per time point. Photomicrographs of paraffin embedded sections stained with cytokeratin.
Appendix B

*Rag2*/*Il2rg* /- dams reconstituted with C57BL/6 bone marrow have similar PAS"DBA" uNK cell numbers at GD 8.5, 10.5 and 12.5. Photomicrographs of *Rag2*/*Il2rg* /- BM (C57BL/6) (Supplemental Fig. 2A) and C57BL/6 (Supplemental Fig 2B) implantation sites at GD 12.5. PAS"DBA" uNK cell frequency is significantly lower (*p*<0.001) in *Rag2*/*Il2rg* /- BM (C57BL/6) compared to C57BL/6 implantation sites at GD 8.5. PAS"DBA" uNK cell frequency does not significantly differ between *Rag2*/*Il2rg* /- BM (C57BL/6) and C57BL/6 implantation sites at GD 8.5, 10.5 and 12.5. N for *Rag2*/*Il2rg* /- BM (C57BL/6) = 3 implantation sites per pregnancy, 1 pregnancy per time point. N for C57BL/6 = 3 implantation sites per pregnancy, 3 pregnancies per time point. Statistical values (mean ± standard deviation) for each cross are noted below expressed as *Rag2*/*Il2rg* /- BM (C57BL/6); C57BL/6. Total PAS"DBA" cells at GD 8.5: 3.111 ± 2.261; 34.96 ± 17.71. Total PAS"DBA" cells at GD 8.5: 150.3 ± 43.74; 180.4 ± 66.15, GD 10.5: 221.4 ± 87.69; 253.7 ± 71.13, and GD 12.5: 120.1 ± 24.24; 137.4 ± 47.00.
Appendix C

Hematoxylin and Eosin (H&E) Staining Protocol

1- Deparaffinization/Hydration:
   - Xylene 1 – 5 minutes
   - Xylene 2 – 5 minutes
   - Xylene 3 – 5 minutes
   - 100% Ethanol – 5 minutes
   - 95% Ethanol – 5 minutes
   - 80% Ethanol – 5 minutes
   - 70% Ethanol – 5 minutes
   - Distilled H₂O (dH₂O) – 1 minute

2- Stain with Harris Hematoxylin (26108-01, Electron Microscopy Sciences, Hatfield, PA, USA) – 10 seconds

3- Wash with tap water – 10 minutes

4- Stain with Eosin Y (26051-10, Electron Microscopy Sciences, Hatfield, PA, USA) – 3 minutes

5- Dehydration and Clearing:
   - 70% Ethanol – 5 minutes
   - 80% Ethanol – 5 minutes
   - 95% Ethanol – 5 minutes
   - 100% Ethanol I – 5 minutes
   - 100% Ethanol II – 5 minutes
   - Xylene I – 3 minutes
   - Xylene II – 3 minutes
Appendix D

Periodic Acid-Schiff (PAS) Staining Protocol

1- Deparaffinization/Hydration:

   Xylene 1 – 5 minutes
   Xylene 2 – 5 minutes
   Xylene 3 – 5 minutes
   100% Ethanol – 5 minutes
   95% Ethanol – 5 minutes
   80% Ethanol – 5 minutes
   70% Ethanol – 5 minutes
   Distilled H₂O (dH₂O) – 1 minute

2- Wash with PBS – 1 minute

3- Incubate with 1% amylase (0.1g amylase in 10mL PBS) – 30 minutes at 37°C

4- Wash with PBS – 1 minute

5- Incubate in 1% periodic acid (0.5g periodic acid; BCBB7710V, Sigma-Aldrich, St. Louis, MO, USA; in 50mL dH₂O) – 5 minutes

6- Wash with dH₂O – 1 minute

7- Cover with Schiff’s reagent (3952016, Sigma-Aldrich, St. Louis, MO, USA) – 3 minutes

8- Wash with dH₂O – 1 minute

9- Wash with sulphurous acid solution – 5 minutes

10- Wash with dH₂O – 1 minute

11- Stain with Harris’s hematoxylin – 10 seconds

12- Wash with tap water – 10 minutes
13- Dehydration and Clearing:

70% Ethanol – 5 minutes
80% Ethanol – 5 minutes
95% Ethanol – 5 minutes
100% Ethanol I – 5 minutes
100% Ethanol II – 5 minutes
Xylene I – 3 minutes
Xylene II – 3 minutes
Appendix E

DBA Staining Protocol

1- Deparaffinization/Hydration:

- Xylene 1 – 5 minutes
- Xylene 2 – 5 minutes
- Xylene 3 – 5 minutes
- 100% Ethanol – 5 minutes
- 95% Ethanol – 5 minutes
- 80% Ethanol – 5 minutes
- 70% Ethanol – 5 minutes

Distilled H₂O (dH₂O) – 1 minute

2- Wash with PBS – 1 minute

3- Incubate with 1% H₂O₂ (33µL of 30% H₂O₂ in 967µL PBS) – 30 minutes

4- Wash with PBS – 1 minute

5- Incubate with 1%BSA/PBS (0.5g Bovine Fraction Albumin, BioShop, Burlington, ON, Canada in 50mL PBS) – 30 minutes

6- Incubate with biotinylated-DBA (L6533, Sigma-Aldrich, St. Louis, MO, USA) in 1%BSA/PBS (1:200) – 1 hour

7- Wash with PBS three times for 1 minute each

8- Incubate with ExtrAvidin Peroxidase (2.5µL in 1mL PBS; E2886, Sigma-Aldrich, St. Louis, MO, USA2) – 1 hour

9- Wash with PBS three times for 1 minute each

10- Incubate with DAB (ab64238, Abcam, Toronto, ON, Canada) – 1 minute
11- Stop reaction by immersing in dH₂O – 1 minute

12- Stain with Harris’s hematoxylin – 10 seconds

13- Wash with tap water – 10 minutes

14- Dehydration and Clearing:

    70% Ethanol – 5 minutes
    80% Ethanol – 5 minutes
    95% Ethanol – 5 minutes
    100% Ethanol I – 5 minutes
    100% Ethanol II – 5 minutes
    Xylene I – 3 minutes
    Xylene II – 3 minutes
Appendix F
PAS/DBA Dual Staining Protocol

1- Deparaffinization/Hydration:
   Xylene 1 – 5 minutes
   Xylene 2 – 5 minutes
   Xylene 3 – 5 minutes
   100% Ethanol – 5 minutes
   95% Ethanol – 5 minutes
   80% Ethanol – 5 minutes
   70% Ethanol – 5 minutes
   Distilled H\textsubscript{2}O (dH\textsubscript{2}O) – 1 minute

2- Wash with PBS – 1 minute

3- Incubate with 1% amylase (0.1g amylase; BCBG9314V, Sigma-Aldrich, St. Louis, MO, USA; in 10mL PBS) – 30 minutes at 37°C

4- Wash with PBS – 1 minute

5- Incubate with 1% H\textsubscript{2}O\textsubscript{2} (33µL of 30% H\textsubscript{2}O\textsubscript{2} in 967µL PBS) – 30 minutes

6- Wash with PBS – 1 minute

7- Incubate with 1%BSA/PBS (0.5g Bovine Fraction Albumin, BioShop, Burlington, ON, Canada in 50mL PBS) – 30 minutes

8- Incubate with biotinylated-DBA (L6533, Sigma-Aldrich, St. Louis, MO, USA) in 1%BSA/PBS (1:200) – 1 hour

9- Wash with PBS three times for 1 minute each

10- Incubate with ExtrAvidin Peroxidase (2.5µL in 1mL PBS; E2886, Sigma-Aldrich, St. Louis, MO, USA2) – 1 hour
11- Wash with PBS three times for 1 minute each

12- Cover with DAB (ab64238, Abcam, Toronto, ON, Canada) – 1 minute

13- Stop reaction by immersing in dH$_2$O – 1 minute

14- Incubate in 1% periodic acid (0.5g periodic acid; BCBB7710V, Sigma-Aldrich, St. Louis, MO, USA; in 50mL dH$_2$O) – 5 minutes

15- Wash with dH$_2$O – 1 minute

16- Cover with Schiff’s reagent (3952016, Sigma-Aldrich, St. Louis, MO, USA) – 3 minutes

17- Wash with dH$_2$O – 1 minute

18- Wash with sulphurous acid solution – 5 minutes

19- Wash with dH$_2$O – 1 minute

20- Stain with Harris Hematoxylin (26108-01, Electron Microscopy Sciences, Hatfield, PA, USA) – 10 seconds

21- Wash with tap water – 10 minutes

22- Dehydration and Clearing:

- 70% Ethanol – 5 minutes
- 80% Ethanol – 5 minutes
- 95% Ethanol – 5 minutes
- 100% Ethanol I – 5 minutes
- 100% Ethanol II – 5 minutes
- Xylene I – 3 minutes
- Xylene II – 3 minutes
Appendix G

Initial $E4bp4^{-/-}$ colony

The original experimental colony was the $E4bp4^{-/-}$ colony donated by Dr. Hugh JM Brady from the University College London Institute of Child Health, London, UK. This was replaced with the $Nfib3^{-/-}$ colony from Dr. Tak Mak at the University of Toronto due to infertility in the strain.