EVALUATION OF HOMING AND FUNCTIONS OF UTERINE NATURAL KILLER CELLS

by

Kota Hatta

A thesis submitted to the Department of Microbiology and Immunology

In conformity with the requirements for

the degree of Master of Science

Queen’s University

Kingston, Ontario, Canada

December, 2009

Copyright © Kota Hatta, 2009
Abstract

Uterine Natural Killer cells are the major lymphocyte population in the pregnant uterus in early gestation; outnumbering both T and B cells. The numerical expansion of uterine Natural Killer cells is thought to result from the expansion of preexisting progenitor cells resident to the uterus, recruitment of Natural Killer cells from the circulation, or a combination of both pathways. Uterine Natural Killer cells are capable of cytotoxic killing and express receptors that can recognize foreign paternal antigens. Therefore, it has been argued that uterine Natural Killer cell activation can lead to killing of fetal cells and abortion. However, fetal rejection by uterine Natural Killer cells does not occur in normal pregnancies and other functions for uterine Natural Killer cells have been proposed. These include: the regulation of maternal blood supply responsible for providing oxygen to the fetus, regulation of maternal blood pressure and, in species with invasive placentation, regulation of decidualization, the process of endometrial cell expansion and transformation during the menstrual cycle and during pregnancy. These cell functions juxtapose the concept that uterine Natural Killer cell activation is harmful to the fetus and offer a new perspective that uterine Natural Killer cells regulate functions unrelated to traditional transplantation immunology. In this dissertation, work is presented showing that uterine Natural Killer cells express molecules which regulate blood pressure and decidualization. Also presented are data supporting the hypothesis that the numerical increase of uterine Natural Killer cells is due to the recruitment of Natural Killer cells from the blood. These results support roles for uterine Natural Killer cells other than cytotoxic killing and advance the understanding of uterine Natural Killer cells as dynamic players that support pregnancy-associated biological processes unrelated to traditional understandings of immune surveillance.
Co-Authorship

The data presented in this thesis were included in the publications below. The independent contributions made by KH, as indicated below, are presented in this thesis.


KH performed tissue collection; isolation of blood NK cells; adhesion assay; cell counting; histology; writing of the manuscript.

MJvdH performed duplicate adhesion assay experiments and cell counting.

BAC designed the experiments and supervised the project.


KH performed blood collection, plasma isolation and sample shipping to Queen’s University.

During his M.Sc. studies, KH performed gene annotation; Benjamini statistical analysis, interactome map construction; writing of the manuscript.

MJB established the array blotting protocol.

JKH performed False Discovery Rate statistical analysis.

J JR performed some of the blotting and all of the densitometry.

JH, MJvdH, VKH and BAC supervised the work.

BAC designed the experiments.

KH performed sample collection; peroxidase and fluorescence immunohistochemistry; immunofluorescent wet-mount microscopy; supervision of ALC; writing of the manuscript.

ZC performed polymerase chain reaction

ALC performed peroxidase immunohistochemistry of virgin mouse uteri.

ELD performed flow cytometry.

JZ performed drug treatment of mice and bone marrow transfer.

CRR, EGO, RJM and BAC supervised this work

RJM and BAC designed the experiments.


KH performed sample collection; frozen section immunohistochemistry; laser capture microdissection; RNA isolation; polymerase chain reaction; Natriuretic Peptide Precursor A targeting immunohistochemistry; Angiotensin II Receptor Types 1 and 2 targeting immunohistochemistry; cell counting; statistical analysis; writing of the manuscript.

ALC performed independent repetitions of immunohistochemistry targeting Angiotensin II Receptor Types 1 and 2 staining of uteri.

ZC performed RNA amplification and reverse transcription.
ELD performed western blotting

CRR and EGO supervised ELD

MYT and SCP developed the anti-Natriuretic Peptide Precursor A antibody.

BAC supervised this work and designed the experiments.
Acknowledgements

Dr. B. Anne Croy (Department of Anatomy and Cell Biology, Queen's University) supervised this work. Without Dr. Croy’s support, encouragement, leadership and generosity, neither my enrollment in the Master of Science program or completion would have been possible. I would like to thank her for the mentorship she provided and investment she has made in my scientific work for the past 7 years. Thank you, Dr. Croy.

Dr. Myron Szewczuk and Dr. Katrina Gee (Department of Microbiology and Immunology, Queen's University) supervised this work as Thesis Supervisory Committee Members. Head of Department Dr. R. Keith Poole, Graduate Coordinator Dr. Nancy L. Martin, Departmental Assistant Jackie Jones and Intermediate Clerk Tammy Henry (Department of Microbiology and Immunology, Queen's University) are acknowledged for their administrative support and help.

Dr. Chandrakant Tayade (Department of Anatomy and Cell Biology, Queen's University) is acknowledged for the supervision he provided during my one semester training visit at the University of Guelph’s Department of Biomedical Sciences.

Dr. Jianhong Zhang, Dr. Zhipin Chen, Dr. M. Yat Tse, Mr. Michael J. Bilinski, Ms. Suzanne D. Burke (Department of Anatomy and Cell Biology, Queen's University), Dr. R. John MacLeod, Dr. Ivan I. Pacheco (Department of Physiology, Queen’s University; Gastrointestinal Disease Research Unit, Department of Medicine, Kingston General Hospital), Dr. Marianne J. van den Heuvel, Mr. David Hilchie, Ms. Jocelyn M. Wessels, Ms. Nicola F. Linton (Department of Biomedical Sciences, University of Guelph), Dr. Patricia Mote (Westmead Millennium Institute for Medical Research, University of Sydney), Mr. Juares R. Bianco (Instituto de Biologia,
Departamento de Histología e Embriología, Universidade Estadual de Campinas) and Ms. Ester Leno-Durán (Unidad de Inmunología, IBIMER, Universidad de Granada) are acknowledged for providing consultation and technical advice.

Financial assistance was provided by a Queen's Graduate Award to Kota Hatta and NSERC and CIHR funding awarded to Dr. B. Anne Croy.
Table of Contents

Abstract .................................................................................................................................................. ii
Co-Authorship ........................................................................................................................................ iii
Acknowledgements ........................................................................................................................... vi
List of Figures ....................................................................................................................................... x
List of Tables ......................................................................................................................................... xi
List of Abbreviations .......................................................................................................................... xii
Chapter 1 Introduction ......................................................................................................................... 1
  1.1 Foreword ....................................................................................................................................... 1
  1.2 References ................................................................................................................................... 5
Chapter 2 NK cells detect changes in adaptive immunity within mouse decidua from gestation day eight ................................................................................................................................. 8
  2.1 Abstract ....................................................................................................................................... 8
  2.2 Introduction ................................................................................................................................... 9
  2.3 Materials and Methods .................................................................................................................. 11
    2.3.1 Mice ....................................................................................................................................... 11
    2.3.2 Isolation and staining of human blood NK cells ................................................................. 11
    2.3.3 Adhesion assay ................................................................................................................... 12
    2.3.4 Histology ............................................................................................................................ 13
    2.3.5 Statistics .............................................................................................................................. 13
  2.4 Results ......................................................................................................................................... 13
  2.5 Discussion .................................................................................................................................... 21
  2.6 References ................................................................................................................................... 24
Chapter 3 Cytokine array comparisons of plasma from cycling fertile women at cycle day 5 and ovulation ........................................................................................................................................... 28
  3.1 Abstract ....................................................................................................................................... 28
  3.2 Introduction ................................................................................................................................... 29
  3.3 Materials and Methods .................................................................................................................. 30
    3.3.1 Blood collection .................................................................................................................... 30
    3.3.2 Cytokine array ..................................................................................................................... 30
    3.3.3 Statistical analysis ............................................................................................................... 31
    3.3.4 Interaction map and functional annotation ........................................................................... 32
Chapter 4 Orphan receptor kinase Ror2 is expressed in the mouse uterus
4.1 Abstract
4.2 Introduction
4.3 Materials and Methods
  4.3.1 Mice and tissue collection
  4.3.2 Immunohistochemistry
  4.3.3 Wet-mount immunofluorescence
4.4 Results
  4.4.1 Ror2 is expressed in the pregnant mouse uterus
  4.4.2 Uterine NK cells express Ror2 but splenic NK cells do not
  4.4.3 Ror2 is expressed in the virgin and pregnant uterus by multiple cell types
4.5 Discussion
4.6 References

Chapter 5 Uterine Natural Killer cells dynamically express the vasoregulatory proteins Agtr1, Agtr2 and Nppa
5.1 Abstract
5.2 Introduction
5.3 Materials and Methods
  5.3.1 Mice and tissue collection
  5.3.2 Laser capture microdissection
  5.3.3 Polymerase chain reaction
  5.3.4 Immunohistochemistry
  5.3.5 Cell counting and scoring
5.4 Results
5.5 Discussion
5.6 References

Chapter 6 Synopsis and Conclusions
6.1 Summary
6.2 References
List of Figures

Figure 1: Location of adherent human NK cells on cryosections of mouse implantation sites.... 14
Figure 2: Adhesion of human blood NK cells to decidua basalis of mouse implantation sites.... 16
Figure 3: Clustering of human CD56+CD16-CD3- NK cells in adhesion assays occurs
independently of DC cell contact in gd9 decidua but requires the presence of resident T and/or B
cells. ........................................................................................................................................ 18
Figure 4: Interactome map. ........................................................................................................ 37
Figure 5: Gestation day 10 mouse uterus was stained for Ror2 using immunohistochemistry. .... 51
Figure 6: Fluorescent immuno-detection reveals uNK cells express Ror2 while splenic NK cells
do not. ........................................................................................................................................ 53
Figure 7: Ror2 expressing uNK cells are absent in Rag2⁺/Il2rg⁻/⁻ mice, but appear after
engraftment of WT bone marrow. ............................................................................................... 55
Figure 8: Time-course fluorescent immunohistochemistry staining of the uterus................. 57
Figure 9: Uterine NK cells express angiotensin II receptors Agtr1 and Agtr2....................... 71
Figure 10: The proportion of Agtr1 and Agtr2 expressing uNK cells increases with their
numerical increase .................................................................................................................... 73
Figure 11: Uterine NK cells express Nppa transcript and protein ........................................ 75
Figure 12: Detection of Nppa expression by uNK cells is restricted to advanced stages of
pregnancy ..................................................................................................................................... 77
Figure 13: Splenic NK cells stain positively for Agtr1, Agtr2 and Nppa. ............................... 79
List of Tables

Table 1: List of proteins screened by membrane array ................................................................. 34
Table 2: Gene annotation ........................................................................................................... 36
Table 3: Summary of splenic and uterine NK cells expression of vasoregulatory proteins ........ 81
### List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>5FU</td>
<td>Fluorouracil</td>
</tr>
<tr>
<td>ACD</td>
<td>Acid citrate dextrose</td>
</tr>
<tr>
<td>ACE</td>
<td>Angiotensin I converting enzyme (peptidyl-dipeptidase A)</td>
</tr>
<tr>
<td>ADH</td>
<td>Anti-diuretic hormone</td>
</tr>
<tr>
<td>Agtr1</td>
<td>Angiotensin II receptor type 1</td>
</tr>
<tr>
<td>Agtr2</td>
<td>Angiotensin II receptor type 2</td>
</tr>
<tr>
<td>ALCAM</td>
<td>Activated leukocyte cell adhesion molecule</td>
</tr>
<tr>
<td>AngII</td>
<td>Angiotensin II</td>
</tr>
<tr>
<td>ANGPT2</td>
<td>Angiopoietin 2</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>APC</td>
<td>Adenomatous polyposis coli</td>
</tr>
<tr>
<td>B cell</td>
<td>Bursa of Fabricius-derived cell</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CaSR</td>
<td>Calcium sensing receptor</td>
</tr>
<tr>
<td>CCL19</td>
<td>Chemokine (C-C motif) ligand 19</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CMAC</td>
<td>7-amino-4-chloromethylcoumarin</td>
</tr>
<tr>
<td>CTNNB1</td>
<td>Catenin (cadherin-associated protein) beta 1</td>
</tr>
<tr>
<td>CXCL</td>
<td>Chemokine (C-X-C motif) ligand</td>
</tr>
<tr>
<td>CXCR</td>
<td>Chemokine (C-X-C motif) receptor</td>
</tr>
<tr>
<td>DAB</td>
<td>Diaminobenzadine</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DAVID</td>
<td>Database for Annotation, Visualization and Integrated Discovery</td>
</tr>
<tr>
<td>DBA</td>
<td>Dolichos biflorus agglutinin</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DC-SIGN</td>
<td>Dendritic cell-specific intercellular adhesion molecule-grabbing nonintegrin</td>
</tr>
<tr>
<td>Dkk1</td>
<td>Dickkopf-related protein 1</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>eYFP</td>
<td>Enhanced yellow fluorescent protein</td>
</tr>
<tr>
<td>ExPASy</td>
<td>Expert Protein Analysis System</td>
</tr>
<tr>
<td>FcγRIII</td>
<td>Immunoglobulin heavy chain gamma crystallizable fragment receptor 3</td>
</tr>
<tr>
<td>FDR</td>
<td>False discovery rate</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>Gapdh</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>Gd</td>
<td>Gestation day</td>
</tr>
<tr>
<td>GMG</td>
<td>Granulated metrial gland</td>
</tr>
<tr>
<td>GNDV</td>
<td>Globally normalized densitometric value</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein coupled receptor</td>
</tr>
<tr>
<td>HE</td>
<td>Hematoxylin and eosin</td>
</tr>
<tr>
<td>HPF</td>
<td>High powered field at x400 magnification</td>
</tr>
<tr>
<td>Hr</td>
<td>Hour</td>
</tr>
<tr>
<td>HUGO</td>
<td>Human Genome Organization</td>
</tr>
<tr>
<td>ICAM</td>
<td>Intercellular adhesion molecule</td>
</tr>
</tbody>
</table>
Ifng
Interferon gamma
Il
Interleukin
ITGA4
Integrin, alpha 4
KIR
Killer immunoglobulin-like receptor
Klrk1
Killer cell lectin-like receptor subfamily K, member 1
LCDV
Loading control densitometric value
LCNDV
Loading control normalized densitometric value
mDC
Myeloid dendritic cell
MAP
Mean arterial pressure
MDV
Mean densitometric value
MHC
Major histocompatibility complex
Min
Minutes
MLAp
Mesometrial lymphoid aggregate of pregnancy
MMP
Matrix metalloprotease
MW
Molecular weight
NK
Natural Killer
Nkg2d
Natural killer cell lectin-like receptor gene 2 D
NKT
Natural Killer T cell
Nppa
Natriuretic peptide precursor A
NTF
Neurotrophin
OSM
Oncoatin M
PBS
Phosphate buffer saline
PCR
Polymerase chain reaction
PE
Phycoerythrin
PGF
Placenta like growth factor
Pgr
Progesterone receptor
Rag2
Recombination activating gene 2
Ror2
Receptor tyrosine kinase-like orphan receptor 2
RPM
Rotations per minute
RPMI
Roswell Park Memorial Institute
RT
Reverse transcription
SAM
Significance analysis of microarrays
SELL
Leukocyte selectin
SINGLEC5
Sialic acid binding Ig-like lectin 5
STC1
Stanniocalcin 1
STRING
Search Tool for the Retrieval of Interacting Genes/Proteins analysis platform
TBS
Tris buffered saline
T cell
Thymus-derived cell
TNFRSF10A
Tumor necrosis factor receptor superfamily member 10a
TRAIL
Tumor necrosis factor–related apoptosis-inducing ligand
UNK
Uterine Natural Killer
Vangl2
Vang-like 2 (van gogh, Drosophila)
Vegf
Vascular endothelial growth factor
Wk
Week
WNT
Wingless
Wnt5a
Wingless-type MMTV integration site family member 5A
XCL
Chemokine (C motif) ligand


Chapter 1

Introduction

1.1 Foreword

In 1953, Sir Peter Medawar postulated that the fetus is a semi-allogeneic graft [1]. The fetus expresses foreign, paternal major histocompatibility complex (MHC) antigens and is therefore theoretically at risk of immune rejection. In 1889, the presence of granulated uterine immune cells was first described [2]. Later re-named uterine Natural Killer (uNK) cells [3], these lymphocytes express killer immunoglobulin-like receptors (KIR) that interact with MHC [4]. In humans, both peripheral and uterine Natural Killer (NK) cells are classified by their expression of CD56 [4]. In mice, uNK cells are identified by reactivity with Dolichos biflorus agglutinin (DBA) lectin [5]. In transplantation immunology, blood NK cell KIR recognize graft MHC and, if an alloreactivity threshold is reached, kill the foreign cells [6]. This concept led to research asking whether uNK cell cytotoxicity contributed to pregnancy complications such as recurrent spontaneous abortion [7]. From their first description, a large body of the primary literature has asked questions about the potential harmful effects of uNK cells for the conceptus. However in 1996, Croy et al. used knock-out and transgenic mouse models to show that although uNK cells are equipped with cytolytic killing machinery, they have limited killing activity [8]. Based on the observation that uNK cells secrete a plethora of cytokines and matrix metalloproteases (MMP), the report concluded that uNK cells may instead have functions related to the development of the placenta. Since then, a number other cytokines and growth factors have been found to be produced by uNK cells, providing evidence for ideas that argue uNK cells contribute to biological processes other than classical immune surveillance and cytotoxic killing.
The major biological function postulated today for human and mouse uNK cells is their contribution to implantation site angiogenesis. Gene microarray [9;10] and explant studies [9] characterize uNK cells as highly pro-angiogenic cells that produce angiogenic factors such as members of the vascular endothelial growth factor (VEGF) family, including placenta growth factor (PGF) [9-11]. Mouse knock-out models devoid of uNK cells have shown that uNK cells not only participate in angiogenesis, but in regulating the structure of blood vessels in the uterus [12]. During pregnancy, spiral arteries arising from branches of the maternal uterine artery and feeding the placenta, dilate and their vascular smooth muscle is lost [13]. The result of this change is increased blood volume flowing to the placenta, the organ where gases and other molecules exchange between the maternal and fetal blood [13]. This change called spiral artery modification does not occur in mice in the absence of uNK cells [12]. Lack of spiral artery modification and poor angiogenesis are often features of gestational diseases such as preeclampsia, fetal growth retardation and in some cases of recurrent spontaneous abortion [13;14]. Previously, the poor placentation observed in these diseases led investigators to address the hypothesis that uNK cells obstructed the proper development and support of the placenta because of their cytotoxic killing [15]. However, more consideration now is given to the growth factors and cytokines that are produced by uNK cells based on results that narrate uNK cells as key players that regulate placentation [7].

A new functional property of uNK cells hypothesized and addressed in this dissertation is their ability to contribute to blood pressure regulation. Blood pressure is controlled by the kidney via the Renin Angiotensin System (RAS). However, in 2007, it was shown that human peripheral blood T and NK cells also express all components of RAS. Specifically, T and NK cells express angiotensin II receptor types 1 (AGTR1) and 2 (AGTR2) [16]. Both AGTR1 and AGTR2 are G-protein coupled receptors (GPCR) but they have opposite effects; AGTR1 receptor activation leads to vasoconstriction while AGTR2 activation leads to vasodilation [17]. In this dissertation,
it was hypothesized that murine uNK cells also express these receptors. Data are presented on the expression of these molecules in uNK cells, as well as the expression of natriuretic peptide precursor A (Nppa), an antagonist of Agtr1 signalling.

Uterine NK cell numbers increase with the proliferation of endometrial stromal cells in a process called decidualization, an expansion and transient differentiation of endometrial tissue. Decidualization in humans occurs during each menstrual cycle, and if implantation occurs, continues until late first trimester of pregnancy. If implantation does not occur, the decidual tissue is shed during menses. In mice, decidualization occurs in response to implantation, but can be induced artificially by physical stimuli of the appropriately hormone-stimulated uterus. Artificially induced decidual tissue is referred to as deciduomata to distinguish it from decidua of pregnancy. Because uNK cells numerically increase during decidualization regardless of the presence or absence of the conceptus [4;18], their enrichment in the uterus is not dependant on fetal antigen. Two opinions exist on the origin of uNK cells: i) that they proliferate from a resident precursor cell in the uterus, or ii) they are actively recruited from the peripheral blood. Indeed, CD34+ hematopoietic cells exist in human uterus [19], although their differentiation into uNK cells has not been observed. Female knock-out mice devoid of uNK cells can be grafted with syngeneic bone marrow to restore uNK cells [20], suggesting, at least in mice, cell trafficking as a plausible source. Uterine NK cells are heterogeneous [20], and therefore a combination of the two possibilities is also attractive. The trafficking potential of blood NK cells to migrate to the uterus can be measured using in vitro assays of lymphocyte adhesion to frozen tissue sections under shear forces [21]. To address whether NK cell trafficking was determinant on fetal antigen recognition by uNK cells or the adaptive immune system, trafficking potential of human blood NK cells using implantation sites from normal, lymphocyte-deficient and dendritic cell tagged mice was measured.
The hypothesis that uNK cells differentiate from NK cell progenitors that egress from blood to the uterus is of clinical relevance. A series of experiments have demonstrated that the \textit{in vitro} trafficking potential of human blood NK cells for migration to the uterine tissue fluctuates over the course of the menstrual cycle and pregnancy, with trafficking potential peaking at ovulation [21] and week (wk) 2 of pregnancy [22]. These time points match periods when uNK cells are numerically increasing in the uterus [4]. Furthermore, this fluctuating pattern of peak NK cell trafficking potential at ovulation does not occur in infertile women with recurrent implantation failure [21]. It is postulated that blood NK cells egress to the uterus at ovulation and by their production of pro-angiogenic factors create a lush, vascularized endometrium receptive to the embryo in anticipation of implantation [21]. In patients with low NK cell potential for uterine trafficking, decreased amounts of uNK cell-derived angiogenic factors acting on the endometrium are postulated to result in a poorly vascularized surface that does not support implantation [21]. In this dissertation, plasma samples from fertile women at time points in the menstrual cycle corresponding to periods of low and high NK cell egress to the uterus were screened for the expression of 174 cytokines and chemokines. Differential changes detected in the expression of selected immunoregulatory molecules were expected to offer clues to the immune regulated changes that occur in the blood related to female fertility.

Regardless of whether uNK cells are derived from a precursor cell resident in the uterus or derived from blood uNK progenitor cells, uNK cells are expected to be mobile within the uterus. In pregnant mice, small, young uNK cells are anatomically separated from older, large, granulated uNK cells by the thin circular smooth muscle layer of the uterus. The lymphoid space containing the less developed uNK cells is called the mesometrial lymphoid aggregate of pregnancy (MLAp) and encircles branches of the uterine artery entering each implantation site. During murine pregnancy, uNK progenitor cells are thought to migrate from the blood initially to the central decidua basalis and then to the MLAp for maturation before migration to the decidua
basalis [23]. The mouse decidua expresses Wingless (Wnt)5a, a Wnt signalling protein. In bone development, Wnt5a gradients direct chondrocyte migration via its receptor, receptor tyrosine kinase-like orphan receptor 2 (Ror2) [24]. To address the hypothesis that uNK cells in the MLAp migrate towards the decidua by Wnt5a mediated chemoattraction, experiments were performed to ask if uNK cells expressed Ror2.

In summary, this thesis presents four investigations as briefly introduced above: the investigation of the contribution of fetal antigen recognition by uterine lymphocytes to the trafficking potential of blood NK cells; the investigation of differential plasma protein expression during the menstrual cycle by screening 174 cykines and chemokines; the investigation of vasoregulatory protein expression by uNK cells; and the investigation of Wnt signalling-related molecule expression by uNK cells during decidualization. New data that gives further insights into the functions of both human and mouse uNK cells are reported.

1.2 References


Chapter 2

NK cells detect changes in adaptive immunity within mouse decidua from gestation day eight

2.1 Abstract

Viable human CD56+CD16- peripheral blood NK cells show specific in vitro binding under shear forces to ligands expressed by endothelial cells in cryostat sections of gestation day (gd)7 mouse decidua basalis. In serial assays, numbers of cells adhering to gd7 tissue are constant for men but have cyclical variation for fertile women, suggesting a brief gain in functional decidual homing potential of this NK cell subset during the menstrual cycle. Regardless of gender, numbers of adhering cells from an individual donor increase dramatically when the substrate is decidua basalis from a later gestational timepoint. Results show that human blood CD56+CD16- NK cells which adhere as single cells over gd7 decidua basalis, adhere as large clusters over gd8 and gd9 tissues, suggestive of antigen recognition and lymphocyte activation. Next, experiments were performed to ask which cells within mouse decidua basalis trigger this response in CD56+CD16- cells. Using decidua from mice transgenic for myeloid dendritic cell (mDC) expression of enhanced yellow fluorescent protein (eYFP), it was found that cluster formation was independent of mDC contact. Use of decidua from alymphoid mice showed clustering behavior required substrate lymphocytes. By use of decidua containing NK cells but lacking T and B cells, decidual T and/or B lymphocytes were identified as the cells altered after gd7 in a manner that activates CD56+CD16- cell clustering. This time point is just prior to mouse spiral arterial modification and its detection by these indicator cells implicates adaptive, decidual immune responses in the regulation of NK cell function.
2.2 Introduction

NK cells, identified by expression of CD56 (neural cell adhesion molecule, NCAM), are ~7-12% of human blood lymphocytes. CD56+ NK cells can be phenotyped into two subsets based on the expression on CD16 (FcγRIII). The first subset, CD56+CD16+ NK cells, comprise ~90% of blood NK cells. The second low abundance subset, CD56+CD16- NK cells, express much more CD56. Because of this difference in the level of CD56 expression, these two subsets are termed CD56\textsuperscript{Dim} and CD56\textsuperscript{Bright} cells, respectively. Another lymphocyte, the NKT cell, co-expresses CD56, CD3 and a T cell receptor and is considered a T cell. While most mammalian NK cells are found in the circulation or spleen, human CD56+CD16- NK cells are transiently the dominant lymphocytes in post-ovulatory uterus and persist in decidua during the first half of pregnancy [1, 2]. These cells, called uNK cells, appear with endometrial decidualization in mice, persist to mid gestation and decline numerically once they have established the essential environment for physiological modification of spiral arteries [3]. While the origins of uNK cells are not firmly established, it is hypothesized that at least some traffic from blood to the uterus [4]. They may additionally develop from hematopoietic stem cells that reside in the uterus [5, 6].

The trafficking potential of lymphocytes from circulation into different tissues can be compared using an assay of leukocyte adhesion to cytostat sections of the test tissues under shear forces (Stamper-Woodruff adhesion assay) [7]. This \textit{in vitro} assay takes advantage of the fact that indicator cells demonstrate specific binding to ligands expressed by endothelial cells of frozen sections of murine substrate tissue. Although this assay does not measure mechanisms of tethering and diapedesis, enumeration of adherent cells has been shown to be indicative of their potential to extravasate. For example, numbers of cells adhering to mouse lymph node high endothelial venules \textit{in vitro} increase with fever range hyperthermia and have been directly correlated with fever-induced changes in \textit{in vivo} homing [8-10]. Similarly, T and NK cells from autoimmune diabetic patients show preferential functional adhesion to pancreatic islets in mouse
tissue sections, consistent with the pathologic lymphoid cell infiltrates that lead to type 1 diabetes [11]. Previous work, using gd6-7 mouse implantation sites as substrates, reported that the adhesion patterns of human blood NK cells at ovulation is predictive of successful embryo implantation [12]. Serial studies of women successfully undergoing fertility treatment showed a rapid loss of CD56+CD16- blood NK cell adhesion to mouse decidua after ovulation that was followed by a second period of adhesive gain up to wk 6 of pregnancy, then declined over the next 26 wks [13]. This prediction of high recruitment of uNK cells between 2-6 wks of gestation followed by less recruitment potential is consistent with the known time course for CD56+CD16- (u)NK cell appearance in human decidua. In all the above reports, gains in adhesion were linked to gains in functional SELL (L-selectin, CD62L) and ITGA4 (α4-integrin, CD49D) by lymphocytes and gains in their counter-receptors in the substrate tissue [12-15].

In previous studies employing human lymphocyte adhesion to mouse decidua substrate, it was noted that use of sections from more advanced pregnancies (gd 8-12) led to huge gains in numbers of adherent NK cells from a common blood sample, even when analysed on a single slide [16]. NK cells from the same blood adhered to gd6-7 tissue as single cells but adhered in progressively larger clusters to sections from tissues of more advanced gestational ages [16]. The clusters did not adhere over the fetus or placenta but were found broadly across the decidua basalis, suggesting a major functional change within the decidua basalis was being detected by the blood indicator lymphocytes. Lymphocyte clustering has been described in intact, antigen activated lymph nodes in organ bath cultures using two-photon confocal imaging or intravital microscopy [17-19]. Clustering of the viable indicator blood lymphocytes on sections of gd8-9 decidua could represent immune cell recognition of DC activated by conceptus antigens since co-localization of these cell types has been reported in early human decidua [20]. Alternately, it could represent onset of a specific change in murine uNK cells as they initiate the process of spiral arterial modification, in endothelia within the decidua, in invasive trophoblast or in other
cell types found within decidua. Using genetically altered mice, this experiment was undertaken to identify the cell type within gd9 mouse decidua basalis responsible for transforming decidua into a substrate competent for induction of clustering behavior in human blood NK cells and to define the subclasses of human NK cells participating in the clustering phenomenon.

2.3 Materials and Methods

2.3.1 Mice

Four strains of mice were homozygously mated to provide timed implantation sites; C57BL6, the wild type control; $Rag2^{-/-}$ on the BALB/c background as mice lacking T and B cells; alymphoid $Rag2^{-/-}/Il2rg^{-/-}$ on the BALB/c background as mice lacking NK, T and B cells and a transgenic mouse strain whose mDC express eYFP under control of $CD11c$ [21] on a C57BL6 background. The morning of copulation plug detection was designated gd0. Uteri were collected the mornings of gd6 to 9. All use of mice was approved by Queen’s University Animal Care Committee.

2.3.2 Isolation and staining of human blood NK cells

Fifteen male and female (not selected for menstrual cycle day) healthy adult subjects were recruited to participate in the study and provided informed consents approved by the Queen’s University Health Sciences Human Subjects Research Ethics Board. Participants donated 20mL of blood that were collected into evacuated vials containing the anti-coagulant acid citrate dextrose (ACD). Mononuclear cells were obtained after density centrifugation over Histopaque 1077 (Sigma, Oakville, ON, Canada). Cells were then washed and labeled with anti-CD56 antibodies conjugated with magnetic beads (CD56 Multisort kit, Miltenyi Biotec, Auburn, CA, USA). Labeled cells were isolated using AutoMACS (Miltenyi Biotec, Auburn, CA, USA) using the “Posselds” program setting. To identify the three CD56+ subsets, a tri-colour stain was
performed. The NK cells collected from magnetic isolation were labelled with CellTracker Blue CMAC (7-amino-4-chloromethylcoumarin) (Invitrogen, Burlington, ON, Canada), anti-CD16-PE (Beckman Coulter, Mississauga, ON, Canada) and anti-CD3-FITC (Miltenyi Biotec, Auburn, CA, USA) and incubated at 37°C for 30min. Blocking experiments were also performed by treating cells with function blocking antibodies to SELL (10µg/mL anti-CD62L; BD Pharmingen, Mississauga, ON, Canada) and ITGA4 (10µg/mL anti-CD49D; BD Pharmingen, Mississauga, ON, Canada). Cells were then washed and resuspended at 5x10^5 cells per 100µL of unsupplemented RPMI media (Sigma, Oakville, ON, Canada) and used for the adhesion assay. For experiments using wild type control tissue, tissue from n=3 mice for each gd (gd6, 7, 8, 9) were used with each gd paired with blood from n=3 individuals (a total of 12 individual blood donors for pairing with wild type mouse tissue). For experiments using tissue from Rag2−/− mice, Rag2−/−Il2rg−/− mice and eYFP mice, gd9 tissue from n=3 mice for each type of mouse were used. The same blood from additional blood donor individuals (n=3) were used for adhesion assays using tissue from Rag2−/− mice, Rag2−/−Il2rg−/− mice and eYFP mice.

2.3.3 Adhesion assay

A modified Stamper-Woodruff assay [7] was performed using gd6 to 9 mouse uterine tissue [11-15]. Tissue was embedded and frozen in Shandon Cryomatrix (Thermo Scientific, Ottawa, ON, Canada) then cut at 12µm using a cryostat and melted onto charged glass slides (Fisher, Ottawa, ON, Canada). Tissue sections were encircled using a hydrophobic Dako Pen (DAKO, Mississauga, ON, Canada) and 5x10^5 lymphocytes in a 100µL suspension of unsupplemented RPMI medium were applied onto the tissue while rotating on an orbital shaker (60rpm at 4°C). Once the lymphocytes had been applied, rotation was increased to 112rpm for 30min at 4°C. The slides were then rinsed in PBS to remove non-adherent cells, fixed for 30min in 4% paraformaldehyde, washed, mounted, and coverslipped before being visualized under
ultraviolet illumination. Numbers of adherent cells were independently counted in 25 high power fields (HPF; x400) by two people and the counts were averaged.

2.3.4 Histology

Standard hematoxylin and eosin (HE) staining was done on a cryosection from each of the implantation site tissue blocks used for the adhesion assays. These sections were photographed for reference alignment with the fluorescent images using Zeiss Axiomat and Axiovision image analysis software (Zeiss; Toronto, ON, Canada).

2.3.5 Statistics

One way ANOVA on ranks (Microsoft Excel; Toronto, ON, Canada) was used to analyze data from adhesion experiments. Tukey’s test (non-parametric) was used to further identify which groups differed from others. A value of p<0.05 was considered significant.

2.4 Results

Experiments first sought to determine which subsets of NK cells were adherent to mouse implantation sites on gd6, 7, 8 and 9. Figure 1 shows the number of adherent cells as subdivided by cell subset to each area of the implantation site. Black bars represent adherent CD56+CD16-CD3- cells, hatched bars show CD56+CD16+CD3- cells and gray bars depict NKT cells. Due to the large clusters at gd9, enumeration became inaccurate (not shown). Thus, focus was put on differences within and between gd7 (Figure 1A) and gd8 (Figure 1B). At gd7, the number of CD56+CD16-CD3- cells adhering to decidua basalis exceeded those cells adhering to other regions of the implantation site (p=0.003). There were no differences in the numbers of CD56+CD16+CD3- cells or NKT cells adhering to any area of the implantation site. Similarly, no differences were found amongst subsets in the anti-mesometrial or lateral areas, but there was a significant increase in the number of adherent CD56+CD16-CD3- cells in the decidua basalis.
Figure 1: Location of adherent human NK cells on cryosections of mouse implantation sites.

Panel A shows number of adherent cells per high power field (HPF) on gd7 implantation sites, panel B shows number of adherent cells HPF on gd8 implantation sites. Black bars represent CD56+CD16-CD3- cells, hatched bars represent CD56+CD16+CD3- cells and gray bars represent CD56+CD16-CD3+ cells. A p value <0.05 was considered significant. Numbers of adherent cells were independently counted in 25 HPF (x400) by two people and the counts were averaged.
Figure 2: Adhesion of human blood NK cells to decidua basalis of mouse implantation sites.

Panels A to H show CD56+CD16-CD3- cells stained blue, CD56+CD16+CD3- cells stained red and CD56+CD16-CD3+ cells stained green. Variable tissue autofluorescence is present. Panels A to H illustrate the decidua basalis, the preferred site of human CD56+CD16-CD3- cell adherence. Panels E-H; pre-treatment of cells with anti-ITGA4 and anti-SELL prevent cluster formation. Panels I-L; decidua basalis is boxed in hematoxylin and eosin stained sections.
Figure 3: Clustering of human CD56+CD16-CD3- NK cells in adhesion assays occurs independently of DC cell contact in gd9 decidua but requires the presence of resident T and/or B cells.

Panel A; formation of clusters of CD56+CD16-CD3- NK cells (stained blue) over decidua of mice whose DC express eYFP. Panel B; CD56+CD16-CD3- NK clusters were not associated with DC. Panel C; human NK cells do not display clustering function over gd9 implantation sites from \textit{Rag2}^{−/−}/\textit{Il2rg}^{−/−} mice (NK-, T-, B-). Panel D; human NK cells do not display clustering function over gd9 implantation sites from \textit{Rag2}^{−/−} mice (NK+, T-, B-). Bar =100µm (A,C,D), bar =50µm (B)
(p=0.017) relative to the other two subsets. At gd8 (Figure 1B), there were no differences in the numbers of adherent cells in any of the three subsets in anti-mesometrial or lateral areas, but there was a significant increase in the number of CD56+CD16-CD3- cells adhering to decidua basalis as compared to the other two subsets (p=0.006). The number of adherent cells increased significantly between gd7 and gd8 (p<0.001). Specifically, there was increased adhesion of the CD56+CD16-CD3- subset to decidua basalis at gd8 as compared to the same cells at gd7 or of CD56+CD16+CD3- and NKT cells to decidua basalis, anti-mesometrial and lateral areas of the implant site at gd7 (p<0.05).

Next, whether clumping adhesion of human blood NK cell to mouse decidua was dependant upon the gestational age of the substrate tissue was addressed. When gd6 and gd7 tissues were used, NK cells were seen as individual adherent cells across maternal tissue, but with preferential attachment to the decidua basalis. Preferential attachment to decidua basalis was also seen for gd8 and gd9 substrates. However, when gd8-9 tissue was used there were many more adherent CD56+CD16-CD3- NK cells. These cells occasionally formed large clusters when gd8 tissue was used, and when gd9 tissue was used clusters appeared more often and were larger and individual cells within them became impossible to enumerate. Figure 2 (A-D) shows representative images of cells adhering to decidua basalis for each gd. The clusters of cells consisted of groups of closely associated cells that densely packed together in an aggregate fashion. Figure 2D shows such groups of densely aggregated cells. Figure 2 (E-H) demonstrates that the formation of clusters is prevented when the applied cells are pretreated with function blocking antibodies to SELL and ITGA4. Figure 2 (I-L) shows HE stained matched tissue sections. The boxed areas represent the regions of preferential human blood NK cell adhesion shown in Figure 2 (A-D).
To determine whether human blood NK cells were forming clusters over uterine mDC, substrate decidua from CD11c-eYFP transgenic mice were used in assays. As shown in Figure 3A, CD56+CD16-CD3- cells form clusters over eYFP gd9 decidua. However, no clusters were associated with eYFP expressing mDC. On rare occasions, an association between individual CD56+CD16-CD3- NK cells and gd9 uterine mDC could be observed as shown in Figure 3B.

Whether clustering adhesion depended upon changes to decidual lymphocytes was addressed. Assays were conducted using substrate from the alymphoid Rag2−/−Il2rg−/− mouse to address this question. As shown in Figure 3C, human blood NK cells did not form clusters over uterine sections from gd9 alymphoid mice, suggesting that activation of lymphocytes found in mouse decidua was essential for promotion of clustering in the indicator cells. Whether changes in mouse uNK cells in the substrate were necessary to trigger indicator NK cell clustering by using implantation sites from Rag2−/− mice (NK+, T-, B-) was also asked. Normal activation of uNK cells and spiral arterial modification occur in mice spontaneously mutant at this locus [22, 23, 24]. When gd9 Rag2−/− tissue was used as substrate, no indicator cell clustering was seen (Figure 3D). This suggests that gestationally-induced changes in T and/or B cells underlie indicator cell clustering and may have importance in activation of uNK cells in vivo.

2.5 Discussion

In this investigation it was observed that human CD56+CD16-CD3- blood NK indicator cells form clusters over mature mouse decidua basalis that contains T and B cells, and that this functional behavior is independent of mDC and uNK cells. Clustering behavior depends on upon both the day of gestation of the substrate decidual tissue and either SELL and/or ITGA4 expression by the CD56+CD16-CD3- blood NK cells. The CD56+CD16+CD3- subset gained in cell adhesion but did not respond to the changes in the substrate by cluster formation. The changed behaviour was most notable in cells overlying decidua basalis. These findings reveal that
a significant immunological change occurs in mouse decidua basalis after gd7 that is detected functionally by human NK cells.

Murine uNK cells promote angiogenesis via secretion of the Vegf family of cytokines, they relax the decidual stroma by secretion of matrix metalloproteases and synthesis of inducible nitric oxide synthase [24] which allows deeper invasion by trophoblast and vascular relaxation, and they secrete interferon gamma (Ifng) which triggers modification of spiral arteries [22]. Because uNK cells dynamically change the architectural structure and milieu of the uterus during pregnancy [25], it was to our surprise that clustering was not observed over Rag2−/− implantation site sections in which uNK cell function remains (Figure 3D).

One possible explanation of the clustering observed is that it detects an antigen presentation phase. Studies by Chtanova et al. have shown that neutrophils form clusters in the subcapsular sinus of the mouse draining lymph node after infection with Toxoplasma gondii [26]. There have been numerous reports that T cells also form clusters, stable for up to 24 hr, with DC in the lymph node during initiation of immune responses leading to activation or to tolerance [21, 27-29]. Whether the clusters of NK cells that were observed formed over DC in a similar fashion to the manner in which T cells form clusters in vivo around DC was also asked. Uterine DC have the potential to present fetal antigen to local leukocytes, and in early human decidua, some DC-SIGN (dendritic cell-specific ICAM-grabbing nonintegrin, CD209) expressing antigen-presenting cells have intimate contact with uNK cells [30]. Locksley et al. have shown in mice that DC activation by Listeria seeded rapid clustering of Ifng producing NK cells in the spleen [31]. Clustering was necessary for Ifng production in these NK cells, which were responsive to pertussis toxin. However, changes in fluorescence emission indicating overlay of blood NK cell clusters on mDC was not seen. This suggests direct mDC-NK cell contact was not responsible for the shift in function of the viable NK cells.
In previous studies this adhesion assay was used with gd6-7 substrate to study trafficking potential of human blood NK cells and mouse splenocytes to the uterus [12-14, 32, 33]. Specific attachment of cells was achieved via elevated SELL and ITGA4 expression [12-14, 33]. In the current investigation it was demonstrated that both adhesion and clustering of CD56+CD16-CD3- NK cells depended on either SELL and/or ITGA4, suggesting the alternative or supplementary explanation that clustered attachment of CD56+CD16-CD3- NK cells is a response to elevated expression of trafficking ligands in the substrate. This explanation is consistent with reports that peak uNK cell numbers are achieved in mouse uterus between gd8-14 depending on the strain [34-36] with gd10-12 usually reported as their numerically most abundant days in C56BL6 [34].

A third explanation is that there are other factors that contribute to this phenomenon. Interleukin 15 (Il15), a progesterone-regulated, essential growth factor for NK cells, might play a role in CD56+CD16-CD3- NK cell clustering. Since Il15 is presented via cell to cell contact in trans, this mechanism may have the potential to restrict clustering to specific Il15-producing locations in the decidua basalis as it is involved in regulating the differentiation of uNK cells [37]. While only a minority of mouse uNK cells associate in vivo, with progesterone receptor positive (Pgr+) stromal cells [38], Pgr+ mouse decidual cells still might be capable of triggering clustering through synthesis of molecules other than Il15. How NK cell clusters were sustained was not investigated. However, in the case of T cell clustering, it has been reported that microtubule organizing centers become polarized and IL2 is secreted to adjacent cells in a cell-synapse delivery mechanism [39]. CD56+CD16-CD3- NK cells may be participating in similar paracrine communication, or in homophilic interactions [40-42] such as CD56 dimerization to form the large aggregates observed. It should also be noted that adhesion and clustering occurred at 4°C, therefore a mechanism that allows for immediate hyperactivation of SELL or ITGA4 is more likely to be responsible for this response than transcriptional or translational mechanisms.
It was demonstrated that specific CD56+CD16-CD3- human NK cell clustering over mouse decidua basalis is dependent on day of gestation and occurs only in the presence of endometrial T and B lymphocytes. This novel observation further highlights the immunological gestational changes that occur to the mouse uterus. This work provides evidence of an early function of adoptive immune responses across the mouse decidua basalis. It is unknown if during human pregnancy endometrial T and B lymphocytes also induce similar functional changes in NK cells. The timing of this increased adhesion and NK cell clumping provides further support for the hypothesis that blood NK cells have the capacity to home to the uterus during pregnancy and that they are regulated to undergo functional changes by the decidual environment.

2.6 References


Chapter 3

Cytokine array comparisons of plasma from cycling fertile women at cycle day 5 and ovulation

3.1 Abstract

To identify plasma immuno-regulatory molecules up or down regulated between the follicular phase and ovulation of the human menstrual cycle, RayBio® cytokine arrays were used to screen 174 immuno-regulatory molecules in plasma collected during the follicular phase at menstrual cycle day 5 and at ovulation from 5 healthy, non-smoking, fertile women of reproductive age not using hormonal contraception. A total of 23 differentially expressed molecules were found: 10 molecules were differentially up-regulated and 13 down-regulated at ovulation compared to the follicular phase (α=0.05, false discovery rate of 0.45). Circulating immuno-regulatory molecules fluctuate over the menstrual cycle in healthy women. The combination of differentially expressed molecules suggests roles in cyclical regulation of angiogenesis of and immune cell trafficking.
3.2 Introduction

The immune system responds to pregnancy in dynamic ways. Although much attention has been given to the immunology of the fetal-maternal interface, systemic maternal immune responses also occur during pregnancy [1]. For example, men and non-pregnant women have a systemic cytokine profile that is type 1 biased (pro-inflammatory). However during late second trimester of a normal pregnancy, women switch to an overall type 2 biased (anti-inflammatory) cytokine profile [2].

The immune system is also regulated hormonally by the menstrual cycle [3]. Many investigators have considered whether potential dysregulation of blood cytokines might participate in female infertility. It has been previously reported that, in fertile women, the trafficking potential of the minor subset of blood Natural Killer (NK) cells, CD56\textsuperscript{Bright} cells, for egress to the uterus had menstrual cyclical variation. Trafficking potential, as detected in the in vitro cell adhesion assay to frozen decidual tissue sections under shear force, peaked at ovulation. Adhesion detected changes in endothelial cells within the section tissue and depended upon L-selectin (SELL) and \textalpha4-integrin (ITGA4)-based changes in the lymphocytes [4-7]. In women who successfully conceived from controlled hormonal ovulation and transfer of fresh embryos or transfer of frozen embryos in a monitored natural cycle, in vitro NK cell trafficking potential was dynamic and peaked on the day of ovulation [7]. This cyclical pattern of blood NK cell behavior was not found in menstrual cycles of women receiving frozen embryos that did not implant [7].

Lymphocytes from male blood incubated in plasma collected from fertile women at ovulation also showed gains in adhesive function of CD56\textsuperscript{Bright} NK cells in comparison to cells from the same blood sample incubated in plasma collected at cycle day 5 [8]. From this observation, it was hypothesized that, at ovulation, circulating immune cells respond to menstrual cycle-regulated immuno-regulatory signals in plasma to egress from vessels and into
endometrium. To advance this hypothesis, a screening study of 174 soluble cytokines, chemokines, growth factors and angiogenic molecules was undertaken. Test plasma was donated twice, during the follicular phase and at ovulation by each of five healthy, fertile women of reproductive age. Statistical analyses were performed to look for differentially expressed molecules during the menstrual cycle.

3.3 Materials and Methods

3.3.1 Blood collection

Five healthy, non-smoking, adult women with proven fertility (conceived at least one healthy child within the past two years without medical intervention), and normal menstrual cycles, who were seronegative for Hepatitis B and C and HIV and had not used any form of hormonal contraception within the past year were recruited. Ten mL of blood were collected by venipuncture into vacuum tubes on menstrual cycle day 5 and on the day of ovulation. The menstrual cycle day 5 blood sample will hereafter be referred to as the follicular phase sample. The first day of menses was designated cycle day 1. The day of ovulation was determined using an ovulation detection kit (Ovulation Indicator, Life Brand Shopper’s Drug Mart; Toronto, Ontario, Canada). Participants were instructed to urinate on the ovulation detection strips in the morning and in the evening beginning on cycle day 12. Blood was collected on the morning of a positive detection, or the morning following a positive evening detection. All participants provided informed written consent approved by the Human Research Ethic Board, University of Western Ontario.

3.3.2 Cytokine array

Blood was layered over Histopaque 1077 (Sigma; Oakville, Ontario, Canada) and centrifuged (400xg 4°C, 30 min). The resulting plasma supernatant was aspirated and stored at -
80°C. Using the RayBio® Human Cytokine Antibody Array 6, 7 and 8 (catalogue numbers AAH-CYT-6, AAH-CYT-7 and AAH-CYT-8, respectively, RayBiotech Inc.; Norcross, Georgia, U.S.A.), a total of 174 immuno-regulatory molecules were simultaneously screened for each plasma sample. These molecules are listed in Table 1. The membranes were pre-coated with antibodies against target peptides anchored onto the membrane for sandwich-ELISA detection. Each unique target molecule was assessed in duplicate. Manufacturer’s directions were followed. Briefly, membranes were individually placed in chambers of 8-well tissue culture plates and blocked with the kit’s blocking buffer. Following blocking, plasma diluted 1:10 in blocking buffer was added. After 2 hr of incubation, membranes were repeatedly washed and then incubated with biotin-conjugated antibodies for 2 hr. Following a further set of washes, horse radish peroxidase-conjugated streptavidin was added and incubated for 1 hr followed by a final set of washes. The membranes were then placed in the manufacturer’s chemiluminescence detection buffer and incubated for 2 min. Membranes were sandwiched between clean plastic sheets and excess detection buffer was carefully squeezed out before being exposed to Kodak x-omat AR film (MarketLINK Scientific; Burlington, Ontario, Canada). The film was then developed and scanned using a high resolution scanner (Hewlett Packard HP PSC1315; Mississauga, Ontario, Canada) and saved digitally. The densitometric value of each locus on the array was measured using ImageJ software (National Institutes of Health; Bethesda, Maryland, U.S.A.).

3.3.3 Statistical analysis

Statistical analyses were performed to interpret 174 different cytokine readings at two time points. Normalization was performed to account for any variation between one membrane and another to permit valid cross membrane comparisons. Briefly, the mean value of the manufacturer’s positive control replicates on each membrane, called the loading control
densitometric value (LCDV), was determined. The LCDV value for each membrane was subtracted from all experimental cytokine densitometric values on that membrane. The resulting value is referred to as the loading control normalized densitometric value (LCNDV) of the cytokine. To reduce variation between membranes within a plasma sample, all three membranes (comprising all 174 cytokines) were processed simultaneously. Hence, a second normalization was done for each plasma sample by taking the mean densitometric value of all 174 experimental cytokines for that sample and subtracting it from LCNDV. The resulting value is the globally normalized densitometric value (GNDV) of the loci. Because each membrane detected unique cytokines in duplicate, the GNDV values of the duplicates (i.e. two loci detecting the same molecule) were averaged and used for all of the analyses. This value will be referred to hereafter as the mean densitometric value (MDV) of each cytokine, and was the value used for interpretation of data.

To look for differential expression between menstrual cycle days, significance analysis of microarrays (SAM, Microsoft Excel; Mississauga, Ontario, Canada) analysis was performed using one class response format for comparison between menstrual cycle days. For SAM analysis, cutoff values for significance were chosen based on the false discovery rate (FDR). The application, advantages and prevalence of the use of FDR to analyze array data, such as microarray, have been described by others [9;10]. Use of FDR is also applicable for analyzing membrane based protein arrays [11].

3.3.4 Interaction map and functional annotation

An interaction map was created to show the relationship between the differentially expressed molecules. Gene symbols of the up- and down-regulated proteins were imported into the Search Tool for the Retrieval of Interacting Genes/Proteins analysis platform (STRING; http://string.embl.de/) [12] and an interaction map was created by using STRING’s high
confidence setting (score=0.7) and by setting the maximum number of predicted interactors to 20. Gene ontology functional annotation was also performed using the differentially expressed molecules to characterize the biological processes regulated. Annotation was performed using The Database for Annotation, Visualization and Integrated Discovery (DAVID; http://david.abcc.ncifcrf.gov/) [13].

3.4 Results

Using FDR-based SAM analysis, we discovered 23 significant results at a FDR cut-off of 45%. The appropriate FDR cut-off value was chosen using SAM analysis and was based on how distributed the molecules were in terms of their individual FDR values (not shown). There were 10 differentially up-regulated and 13 down-regulated molecules that were identified on the day of ovulation compared to the follicular phase (cycle day 5). Figure 4 shows the STRING generated predicted interaction map of these 23 molecules with predicted interacting partners. Table 2 summarizes the gene ontology functional annotation results using DAVID. Functional annotation revealed that the differentially expressed molecules participated in biological processes listed in DAVID as having relationship with “immune system processes”, “cell communication”, “growth factor activity”, “locomotory behavior” and “positive regulation of cell proliferation”.
Table 1: List of proteins screened by membrane array.

**RayBio® Human Cytokine Antibody Array 6**

<table>
<thead>
<tr>
<th>Protein</th>
<th>ADAM11</th>
<th>CCL23</th>
<th>GDNF</th>
<th>IL10</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ANG</td>
<td>CCL24</td>
<td>IFNG</td>
<td>IL13</td>
</tr>
<tr>
<td></td>
<td>BDNF</td>
<td>CCL26</td>
<td>IGF1</td>
<td>IL15</td>
</tr>
<tr>
<td></td>
<td>BMP4</td>
<td>CNTF</td>
<td>IGFBP1</td>
<td>IL16</td>
</tr>
<tr>
<td></td>
<td>BMP6</td>
<td>CSF1</td>
<td>IGFBP2</td>
<td>KITLG</td>
</tr>
<tr>
<td></td>
<td>CCL1</td>
<td>CSF2</td>
<td>IGFBP4</td>
<td>LEP</td>
</tr>
<tr>
<td></td>
<td>CCL2</td>
<td>CX3CL1</td>
<td>IL1A</td>
<td>LTA</td>
</tr>
<tr>
<td></td>
<td>CCL5</td>
<td>CXCL6</td>
<td>IL1B</td>
<td>NAP1L4</td>
</tr>
<tr>
<td></td>
<td>CCL7</td>
<td>CXCL9</td>
<td>IL1R1</td>
<td>NTF3</td>
</tr>
<tr>
<td></td>
<td>CCL8</td>
<td>CXCL12</td>
<td>IL2</td>
<td>PDGFB</td>
</tr>
<tr>
<td></td>
<td>CCL11</td>
<td>CXCL13</td>
<td>IL3</td>
<td>PPBP</td>
</tr>
<tr>
<td></td>
<td>CCL13</td>
<td>EGF</td>
<td>IL4</td>
<td>TGFβ1</td>
</tr>
<tr>
<td></td>
<td>CCL15</td>
<td>FGF6</td>
<td>IL5</td>
<td>TGFβ3</td>
</tr>
<tr>
<td></td>
<td>CCL17</td>
<td>FGF7</td>
<td>IL6</td>
<td>TNF</td>
</tr>
<tr>
<td></td>
<td>CCL18</td>
<td>FLT3LG</td>
<td>IL7</td>
<td>TNFSF14</td>
</tr>
</tbody>
</table>

**RayBio® Human Cytokine Antibody Array 7**

<table>
<thead>
<tr>
<th>Protein</th>
<th>ADIPOQ</th>
<th>CXCL1,2,3</th>
<th>IL2RA</th>
<th>THPO</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AGRP</td>
<td>CXCL5</td>
<td>IL6R</td>
<td>TIMP1</td>
</tr>
<tr>
<td></td>
<td>ANGPT2</td>
<td>CXCL11</td>
<td>IL6ST</td>
<td>TIMP2</td>
</tr>
<tr>
<td></td>
<td>AREG</td>
<td>EGFR</td>
<td>IL8</td>
<td>TMPRSS13</td>
</tr>
<tr>
<td></td>
<td>AXL</td>
<td>FGF4</td>
<td>IL11</td>
<td>TNFRSF1A</td>
</tr>
<tr>
<td></td>
<td>BTC</td>
<td>FGF9</td>
<td>IL12A</td>
<td>TNFRSF1B</td>
</tr>
<tr>
<td></td>
<td>CCL3</td>
<td>FIGF</td>
<td>IL12B</td>
<td>TNFRSF6</td>
</tr>
<tr>
<td></td>
<td>CCL4</td>
<td>HGF</td>
<td>IL17</td>
<td>TNFRSF10C</td>
</tr>
<tr>
<td></td>
<td>CCL16</td>
<td>ICAM1</td>
<td>MIF</td>
<td>TNFRSF10D</td>
</tr>
<tr>
<td></td>
<td>CCL19</td>
<td>ICAM3</td>
<td>NGF</td>
<td>TNFRSF11B</td>
</tr>
<tr>
<td></td>
<td>CCL25</td>
<td>IGFR1</td>
<td>NTF4</td>
<td>TNFRSF18</td>
</tr>
<tr>
<td></td>
<td>CCL27</td>
<td>IGFBP3</td>
<td>NUDT6</td>
<td>TNFSR18</td>
</tr>
<tr>
<td></td>
<td>CCL28</td>
<td>IGFBP6</td>
<td>OSM</td>
<td>TYRO3</td>
</tr>
<tr>
<td></td>
<td>CSF3</td>
<td>IL1R1</td>
<td>PGF</td>
<td>VEGFA</td>
</tr>
<tr>
<td></td>
<td>CXCL1</td>
<td>IL1RL1</td>
<td>PLAUR</td>
<td>XCL1</td>
</tr>
</tbody>
</table>

34
**RayBio® Human Cytokine Antibody Array 8**

<p>| | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>ALCAM</td>
<td>FASLG</td>
<td>INHBA</td>
<td>PDGFRB</td>
</tr>
<tr>
<td>BMP5</td>
<td>FLT4</td>
<td>KDR</td>
<td>PECAM1</td>
</tr>
<tr>
<td>BMP7</td>
<td>ICAM2</td>
<td>KIT</td>
<td>PRL</td>
</tr>
<tr>
<td>CCL23</td>
<td>IGF2</td>
<td>LAP3</td>
<td>SELE</td>
</tr>
<tr>
<td>CD14</td>
<td>IL1R2</td>
<td>LEPR</td>
<td>SELL</td>
</tr>
<tr>
<td>CD80</td>
<td>IL2RA</td>
<td>LIF</td>
<td>SIGLEC5</td>
</tr>
<tr>
<td>CDH5</td>
<td>IL2RB</td>
<td>MMP1</td>
<td>TEK</td>
</tr>
<tr>
<td>CSF1R</td>
<td>IL5RA</td>
<td>MMP3</td>
<td>TGFA</td>
</tr>
<tr>
<td>CTF1</td>
<td>IL9</td>
<td>MMP9</td>
<td>TGFB2</td>
</tr>
<tr>
<td>CXCL10</td>
<td>IL10RB</td>
<td>MMP13</td>
<td>TIE1</td>
</tr>
<tr>
<td>CXCL12</td>
<td>IL13RA2</td>
<td>NGFR</td>
<td>TIMP4</td>
</tr>
<tr>
<td>CXCL16</td>
<td>IL18BP</td>
<td>PDGFA</td>
<td>TNFRSF21</td>
</tr>
<tr>
<td>ENG</td>
<td>IL18RAP</td>
<td>PDGFA/B</td>
<td></td>
</tr>
<tr>
<td>ERBB3</td>
<td>IL21R</td>
<td>PDGFRA</td>
<td></td>
</tr>
</tbody>
</table>
Table 2: Gene annotation.

<table>
<thead>
<tr>
<th>Biological process</th>
<th>Count</th>
<th>Percent</th>
<th>Molecules</th>
<th>Significance (Benjamini)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immune system process</td>
<td>12</td>
<td>52%</td>
<td>CCL19, CD14, CSF3, CXCL10, CXCL11, CXCL16, FASLG, IHNBA, KIT, LIF, OSM, XCL1</td>
<td>3.1x10^-6</td>
</tr>
<tr>
<td>Cell communication</td>
<td>19</td>
<td>83%</td>
<td>ALCAM, ANGPT2, CCL19, CD14, CSF1R, CSF3, CXCL10, CXCL11, LIF, KIT, FASLG, FIGF, IHNBA, NTF4, OSM, PDGFA, TNFRSF10D, VEGFA, XCL1</td>
<td>2.5x10^-5</td>
</tr>
<tr>
<td>Growth factor activity</td>
<td>7</td>
<td>30%</td>
<td>CSF3, FIGF, IHNBA, LIF, NTF4, PDGFA, VEGFA, CCL19, CXCL10, CXCL11, CXCL16, NTF4, VEGFA, XCL1</td>
<td>6.1x10^-5</td>
</tr>
<tr>
<td>Locomotory behavior</td>
<td>7</td>
<td>30%</td>
<td>CSF3, CXCL10, FIGF, KIT, LIF, PDGFA, VEGFA</td>
<td>7.4x10^-5</td>
</tr>
<tr>
<td>Positive regulation of cell proliferation</td>
<td>7</td>
<td>30%</td>
<td>CSF3, CXCL10, FIGF, KIT, LIF, PDGFA, VEGFA</td>
<td>6.2x10^-4</td>
</tr>
</tbody>
</table>

Functional annotation using DAVID revealed that the differentially expressed molecules participated in a number of biological processes. “Count” denotes the number of molecules participating in each biological process. “Percent” refers to the proportion of significantly expressed molecules belonging to each biological process.
Figure 4: Interactome map.

First level interaction map of differentially expressed molecules, as well as predicted interacting partners. The map was created using seven lines of evidence selected provided by STRING: “neighborhood”, “gene fusion”, “cooccurrence”, “coexpression”, “experiments”, “databases” and “textmining”.

3.5 Discussion

The molecules potentially up-regulated in fertile women at ovulation can be grouped functionally. Three molecules, Angiopoietin 2 (ANGPT2), VEGFA and c-fos induced growth factor (FIGF) strongly promote angiogenesis through their actions on endothelium. Three molecules, chemokines ligands 1, 11 and 19 (XCL1, CXCL11 and CCL19, respectively) would be expected to increase cell homing under pro-inflammatory conditions. The latter two are specifically active on CD56\textsuperscript{Bright} blood NK cells [14;15]. The structurally-related molecules oncostatin M (OSM) and granulocyte colony stimulating factor 3 (CSF3) are cytokines of the leukemia inhibitory factor/interleukin 6 (LIF/IL6) family that support the viability of primitive cell types [16]. The former is a cytokine-induced early response gene [17]. Neurotrophin 4 (NTF4; formally called NTF5) is a product of pre-implantation trophoblast and is detected in mouse embryos from the 2 cell stage. In mice, both the oviduct and uterus secrete Ntf5 (now called Ntf4) [18]. The remaining molecule, from the tumor necrosis superfamily, is a decoy molecule that blocks the death-promoting functions of TRAIL. It is also known as CD261, Apo2, TRAIL-R1, DR4 or under the approved name of TNFRSF10A (tumor necrosis factor receptor superfamily, member 10a). Immune tissues such as spleen, thymus and blood leukocytes, particularly activated T cells express this molecule [19].

Some of the 13 molecules differentially down-regulated have actions that could compliment and work synergistically with above molecules to alter circulating immune cell behavior. CD56\textsuperscript{Bright} NK cells have receptors for chemokine ligands 10 and 12 (CXCR3 and CXCR4, respectively) [20]. These data may explain how a subset of CD56\textsuperscript{Bright} NK cells could become activated and egress from the circulation to the uterus. Activated leukocyte cell adhesion molecule (ALCAM or CD166) is likely being detected in its soluble form in the plasma samples.
Soluble ALCAM enhances endothelial cell migration, inhibits endothelial tube formation and disrupts ALCAM-ALCAM homodimer tethering of a cell’s actin cytoskeleton. Platelet-derived growth factor alpha (PDGFA) functions through a VEGF homology region [21]. It also disrupts actin filaments permitting membrane ruffling and cell migration. Cells expressing PDGFRA would be expected to be more adhesive with PDFGA reduction and endothelial cells (rather than hematopoietic cells) would be more likely affected [21]. Chemokine ligand 16 (CXCL16) mediates both chemotaxis and adhesion and is expressed by NKT cells and activated Th1 cells [22]. First trimester human trophoblast also secretes CXCL16 which, through CXCR6, recruits T cells, including gamma/delta T cells and monocytes but not NKT cells or NK cell subsets [23]. The down regulation of CD14, colony stimulating factor 1 receptor (CSF1R), sialic acid binding immunoglobulin-like lectin 5 (SIGLEC5) and inhibin beta A (INHBA) suggests attenuated functions of monocyte/macrophages [24-27]. The decrease in Fas ligand (FASLG) would reduce cell death and compliment the gain in circulating TNFRSF10A. Decreased circulating leukemia inhibitory factor (LIF) may be linked with the gains in CSF3 and OSM. SELL is the lymphocyte receptor previously identified as a key molecule involved in CD56\textsuperscript{Bright} NK cell binding to decidual endothelium in \textit{in vitro} adhesion assays under shear forces [4-7]. Like ALCAM, it is shed from the cell surface and the soluble form would be detected in this assay. SELL shedding occurs during transendothelial migration by leukocytes. A reduction in soluble SELL would mean there is less competition for binding of the SELL receptors on endothelium by circulating lymphocytes, thus increased likelihood of CD56\textsuperscript{Bright} NK cell extravasation at activated endothelial sites.

When the differentially detected molecules were used as seed nodes to create an interaction map, CD16 and SIGLEC5 were left unpartnered. Molecules ALCAM and INHBA had limited predicted interacting partners. Some of these molecules may have limited or no interaction partners, possibly because they are false positives that were included because of FDR.
The other differentially detected molecules were part of an extensive interaction relationship. Given that the statistical analysis detected differentially expressed molecules with the inclusion of false positives, future work on molecules that showed extensive interaction with other molecules that were differentially detected, such as VEGFA, may possibly be more fruitful than molecules with little relationship, such as CD14.

The array data highlight the dynamic changes of immunological consequence that occur in women over the menstrual cycle. The results identify immuno-regulatory molecules that are potentially involved in the extravasation of \( \text{CD56}^{\text{Bright}} \) NK cells for egress to the uterus. The data also highlight potential relationships between some of these molecules that may merit further investigation.

### 3.6 References


4.1 Abstract

Wingless-type mouse mammary tumor virus integration site family, member 5A (Wnt5a), is expressed in mouse decidua and is thought to play an important role in decidualization. Experiments examined expression of the receptor for WNT5A, receptor tyrosine kinase-like orphan receptor 2 (Ror2), in the uteri of cycling and pregnant mice. Immunohistochemistry revealed that in the virgin uterus, Ror2 is expressed in stromal cells and on the basal side of uterine gland and endometrial epithelial cells. During pregnancy, both the luminal and basal side of uterine gland epithelial cells expressed Ror2, stromal cell expression of Ror2 became more frequent and Ror2 expressing uNK cells and cells lining the maternal vascular space emerged. Immunofluorescence imaging and flow cytometry revealed that although uterine NK cells expressed Ror2, NK cells of the spleen were Ror2 negative. The expression of Ror2 by endometrial epithelial cells may suggest WNT signaling has roles in uterine epithelial cell polarity or implantation. Expression of Ror2 by uterine NK cells may suggest WNT signaling regulates uterine NK cell functions such angiogenesis and regulation of trophoblast migration. In summary, the results show that Ror2 expression by maternal uterine cells is influenced by pregnancy.
4.2 Introduction

Receptor tyrosine kinase-like orphan receptor 2 (Ror2) is a type 1 transmembrane protein expressed during embryonic development. Important in chondrocyte formation, Ror2 plays a role in cartilage and growth plate development [1]. Individuals with mutations in \textit{ROR2} display brachydactyly B (aplasia/hypoplasia of phalanges) [2] and Robinow syndrome [3], a syndrome characterized by a malformation of the limbs, face, head and genitalia [4]. Ror2 interacts with melanoma antigen family D, 1 (MAGED1) [5], wingless-type mouse mammary tumor virus integration site family, member 5A (WNT5A) [6] and frizzled homolog 2 (FZD2) [7]. Initially, it was reported that Ror2 participates in Wingless (WNT) signaling through the non-canonical beta-catenin (CTNNB1) independent pathway [8]; however, recent work has shown that Ror2 is also capable of canonical (CTNNB1 dependant) signaling [7].

Most of the literature concerning Ror2 addresses its interactions with WNT5A that occur during development. Recently however, Ror2 functions in other contexts have been described, such as its role in gut epithelial cell regeneration. In murine small intestine, Ror2 has been localized to epithelial cells with expression along the crypt-villus axis [9]. \textit{In vitro} studies demonstrated that activation of the extracellular calcium-sensing receptor (CaSR) on subepithelial myofibroblasts stimulated the synthesis and secretion of WNT5A [9]. A paracrine interaction was then revealed between myofibroblast WNT5A and epithelial Ror2, by showing that WNT5A stimulation of Ror2 increased the caudal homeodomain factor CDX2 protein expression and stimulation of sucrase-isomaltase promoter activity. WNT5A has also been shown to stimulate Ror2 on adenomatous polyposis coli (\textit{APC})-truncated colon cancer cells to inhibit defective CTNNB1 signaling by increasing E-type ubiquitin ligases [10]. This suggests in the normal adult intestine Ror2 can signal non-canonically.
Ror2 signaling appears dependent on the cell type and tissue it is expressed in. Cell proliferation occurs in many adult tissues; however, the process of decidualization, the expansion of endometrial stromal cells in the uterus under the influences of progesterone and other signals, is one of the most critical because it is essential for blastocyst implantation and pregnancy in primates and rodents [11]. In humans, decidualization is initiated during the late secretory phase of the menstrual cycle to create a lush uterine wall receptive for implantation of the embryo [12-14]. If implantation occurs, decidualization continues. If implantation does not occur, the decidualizing uterine endometrium regresses and is shed as menstrual fluid. In rodents, primary decidualization is initiated anti-mesometrially in response to blastocyst implantation. This is followed by a secondary wave of decidualization that proceeds mesometrially to form the decidua basalis [15].

WNT-related and calcium regulating molecules are expressed in the decidualizing uterus. In pregnant mice, stanniocalcin-1 (STC1), a calcium regulator, is expressed by mesometrial stromal cells [16], suggesting calcium may play a role in decidualization. In the rat uterus, extracellular CaSR is hormonally regulated and expressed by decidualizing stromal cells [17]. The canonical WNT signaling molecule CTNNB1 [18] and inhibitor of WNT signaling, Dickkopf homologue 1 (DKK1), are expressed in the human endometrium [19]. The latter promotes trophoblast invasion in mice [20] and is regulated positively by progesterone in human endometrial stromal cells [21]. In mice, Wnt5a is expressed in the decidua [22], however expression of its receptor, Ror2, has not been addressed.

The goal of this investigation was to evaluate the expression of Ror2 in the virgin and pregnant mouse uterus and to identify the cell populations expressing this receptor. Given the expression of Wnt5a in the decidua, it was hypothesized that its receptor, Ror2, would also be present. By identifying the cell populations expressing Ror2, functions for WNT signaling in
uterine biology can be postulated and advance the understanding of decidualization and pregnancy.

4.3 Materials and Methods

4.3.1 Mice and tissue collection

C57BL6/J mice aged 6-8 weeks were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and were housed under conventional conditions and used under protocols fully approved by the Queen’s University Animal Care Committee. Tissue was collected from virgin (n =15), gd6 (n =9), gd10 (n =18) and gd12 (n =9) mice. For tissues from virgin females, the estrous cycle stage was determined by visual examination of the vulva, microscopic examination of vaginal smears and histological characterization of the uterine glands, stroma and epithelium [33-35]. For gestational tissues, estrous females were paired with males and examined the following morning for a copulation plug. The day of plug detection was designated gd0. Uteri were used for RNA isolation, flow cytometry or immunohistochemistry. Spleens were collected from virgin mice and prepared for immunofluorescent wet-mount studies or flow cytometry.

Additionally, 8 wk old female Rag2<sup>−/−</sup>Il2rg<sup>−/−</sup> knock-out mice (n=3) genetically devoid of T, B and NK cells were used. These mice were treated with 5-fluorouracil (5FU; 150mg/kg intraperitoneally) to eliminate progenitor cells. 48 hrs after 5FU, the mice were given bone marrow intravenously from an adult syngeneic male wild type (WT) donor (n=3). The bone marrow recipient female mice were subsequently mated by a male Rag2<sup>−/−</sup>Il2rg<sup>−/−</sup> mouse and implantation sites were collected and used for immunohistochemistry on gd12. Bone marrow was prepared by flushing femurs and tibias. Red blood cell lysis was performed under hypotonic conditions (NH<sub>4</sub>Cl 0.15M, KHCO<sub>3</sub> 10mM, EDTA 0.1mM) and 2x10<sup>7</sup> viable white cells were used for injection.
4.3.2 Immunohistochemistry

Tissue was fixed using 4% paraformaldehyde (pH 7.4, 4°C, 16 hrs), processed automatically and embedded into paraffin blocks. Paraffin blocks were sectioned at 7µm and mounted onto charged glass slides, baked dry, de-waxed using xylene and rehydrated in graded ethanol concentrations. For peroxidase-based detection, slides were treated with 3% hydrogen peroxide for 30 min to quench endogenous peroxidase activity before being washed and blocked with 1% bovine serum albumin (BSA) for 30 min at room temperature. The sections were incubated with rabbit-anti-Ror2 antibody (1:200, Cell Signaling; Boston, MA, USA) overnight at 4°C followed by biotinylated goat-anti-rabbit secondary antibody for 2 hr at room temperature (1:200, DAKO; Mississauga, ON, Canada), ExtrAvidin Peroxidase for 30 min at room temperature (1:50, Sigma; Oakville, ON, Canada) and diaminobenzidine for detection (Liquid DAB+ Substrate, DAKO; Mississauga, ON, Canada). Slides were counterstained in Harris’ hematoxylin for 10 sec, dehydrated, mounted and coverslipped. For fluorescence based detection, hydrogen peroxide pre-treatment was omitted. The rabbit-anti-Ror2 primary antibody was followed with fluorescent Alexa 594 goat-anti-rabbit antibody (1:200, Molecular Probes; Burlington, ON, Canada) for 2 hr at room temperature. Sections were then incubated with fluorescein isothiocyanate (FITC) conjugated Dolichos biflorus agglutinin (DBA) lectin (1:200, Sigma; Oakville, ON, Canada) for 2 hrs at room temperature to detect uterine NK cells. To quench autofluorescence, slides were incubated with 20mM L-lysine (Sigma; Oakville, ON, Canada) for 30 min at room temperature before being coverslipped with 4',6-diamidino-2-phenylindole (DAPI) added mounting media (DAPI Gold with Anti-Fade Agent, Molecular Probes; Burlington, ON, Canada). Negative controls with the primary antibody replaced with 1% BSA were done on each slide.

4.3.3 Wet-mount immunofluorescence
Spleens were cut into pieces and sieved through a wire mesh to achieve single cell suspensions. Red blood cell lysis was performed using hemolytic conditions and the white cell enriched splenocytes were used for analysis. Cell suspensions were blocked using 20% bovine serum, then stained using PE conjugated anti-NK1.1 antibody (1:100, BD Pharmingen; Mississauga, ON, Canada) and rabbit-anti-Ror2 antibody (1:100) for 1hr, followed with Alexa 488 goat-anti-rabbit antibody (1:100, Molecular Probes; Burlington, ON, Canada) for 30 min and DAPI added mounting media. Negative, isotype and single stained controls were also prepared. Slides were imaged under fluorescence using Axiovision computer software (Zeiss; Toronto, ON, Canada).

4.4 Results

4.4.1 Ror2 is expressed in the pregnant mouse uterus

Using peroxidase based immunohistochemistry, it was determined that in gd10 implantation sites Ror2 expression was localized to cells that appeared to be uNK cells and to placental labyrinthine cells lining the maternal vascular space as recognized by nucleated red blood cells (Figure 5).

4.4.2 Uterine NK cells express Ror2 but splenic NK cells do not

To confirm that uNK cells expressed Ror2, a uNK cell specific marker, DBA lectin, was used. Co-localization of fluorescently tagged DBA lectin positive and Ror2 expressing cells showed that uterine NK cells did indeed express Ror2 (Figure 6A). Whether NK cells found in lymphoid organs were Ror2 positive was addressed using splenic NK cells. Co-localization of NK1.1 and Ror2 by immunofluorescent staining showed that splenic NK1.1 positive cells were Ror2 negative (Figure 6B, C). For further confirmation that uterine NK cells expressed Ror2, female $Rag2^{−/}Il2rg^{−/}$ mice genetically devoid of T, B and NK cells were used. Gestation day 12
implantation sites of these mice lacked Ror2 positive DBA lectin staining uterine NK cells (not shown). However, when mice were engrafted with syngeneic, wild type (WT), bone marrow intravenously and subsequently mated by a male \textit{Rag2\textsuperscript{-/-}Il2rg\textsuperscript{-/-}} mouse, gd12 implantation sites were populated with Ror2 expressing DBA lectin\textsuperscript{+} uNK cells (Figure 7).

**4.4.3 Ror2 is expressed in the virgin and pregnant uterus by multiple cell types**

To ask if any additional cell populations expressed Ror2, fluorescent immunohistochemistry was performed on virgin, gd6 and gd12 uteri (Figure 8). In virgin mice, the basal side of the uterine epithelium was positive for Ror2. Uterine stromal cells were rarely positive for Ror2 in virgin uterus, but were frequently positive for Ror2 at gd6 in the mesometrial decidua. Weaker Ror2 staining was found in the anti-mesometrial decidua. In the virgin uterus, uterine gland epithelial cells were positive for Ror2 on the basal side; however at gd6, they were Ror2 positive on both the basal and luminal sides. Gestation day 6 uterine NK cells expressed Ror2. At gd12, uterine NK cells in both the MLAp and decidua basalis expressed Ror2. The myometrium was also positive for Ror2.
Figure 5: Gestation day 10 mouse uterus was stained for Ror2 using immunohistochemistry.

Peroxidase-DAB detection localized Ror2 expression to uNK-like cells (arrows) in the MLAp (A) and decidua (B). Placental labyrinth cells expected but not confirmed to be trophoblast cells (arrowheads) lining the maternal circulation (C) were also positive. Size bar =50µm.
Figure 6: Fluorescent immuno-detection reveals uNK cells express Ror2 while splenic NK cells do not.

Using immunohistochemistry, gd10 implantation sites were stained for Ror2 expression and uterine NK cells (using DBA lectin) and counterstained using DAPI. Co-localization of these markers revealed uNK cells express Ror2 (A; bar =20µm). To ask if splenic NK cells also expressed Ror2, immunohistochemistry (B; bar =5µm) was done using a peripheral NK cell marker, NK1.1, on splenocytes. Splenic NK1.1+ cells were negative for Ror2.
Figure 7: Ror2 expressing uNK cells are absent in $\text{Rag}2^{-/-}\text{Il2rg}^{-/-}$ mice, but appear after engraftment of WT bone marrow.

$\text{Rag}2^{-/-}\text{Il2rg}^{-/-}$ mice, genetically deficient in T, B and NK cells, also lack Ror2 expressing uNK cells. Adult mice were conditioned with the chemotherapeutic agent, 5-flourouracil (5FU), and given syngeneic bone marrow intravenously 48 hrs later. These mice are then set up for pregnancy and their uteri become populated with Ror2 expressing uNK cells. The absence of Ror2 positive lymphocytes in pregnancies of $\text{Rag}2^{-/-}\text{Il2rg}^{-/-}$ mice (not shown) is further support that uNK cells express Ror2. Size bar =20 µm.
Rag2^{-/-}Il2rg^{-/-}

8 wk old

5 FU

Bone Marrow

WT

Rag2^{-/-}Il2rg^{-/-}

Mate

Gd 12

Tissue Collection

ROR2

DBA Lectin

DAPI

Merge
Figure 8: Time-course fluorescent immunohistochemistry staining of the uterus.

Virgin, gd6 and 12 uteri were stained for Ror2 (red). DBA lectin staining (green) was used to localize uterine NK cells and DAPI (blue) nuclear counterstaining was performed. In the virgin stroma, Ror2 expressing cells are rare (arrow). Ror2 expression was observed on the basal side of uterine gland and endometrial epithelial cells. Gd 6 mesometrial stroma showed positive Ror2 staining in both stromal cells (arrow) and uterine NK cells (arrowhead). At gd6, compared to the mesometrial stroma, the anti-mesometrial stroma appeared weaker in Ror2 expression. Uterine glands at gd6 expressed Ror2 on both the luminal and basal side. In gd12 implantation sites, MLAp area stromal cells (arrow) and uterine NK cells (arrowhead) expressed Ror2. Gestation day 12 uterine NK cells in the decidua also expressed Ror2. The uterine wall was also Ror2 positive. Size bar =50µm.
4.5 Discussion

This investigation reports several novel observations on the expression of Ror2. Experiments document Ror2 expression in the uterus: on uterine stromal cells, gland and endometrial epithelial cells, uNK cells, labyrinth cells lining the maternal vascular space and the myometrium. Ror2 expression is maintained throughout the estrous cycle of virgin mice, however during pregnancy the Ror2 positive endometrium at implantation sites is replaced by Ror2 expressing uNK cells and labyrinth cells lining the maternal vascular space. In this way, the expression of Ror2 by maternal uterine cells changes in response to pregnancy. This investigation is also the first to ask if Ror2 is expressed in immune cells. Although DBA lectin+ uNK cells expressed Ror2, NK1.1+ cells of the spleen were negative.

The function of Ror2 expression on the basal side of uterine endometrial epithelial cells is yet to be determined; however there are several postulates. One possibility is that Ror2 plays a role in cell polarization. Non-canonical WNT signaling via van gogh like 2 (Vangl2) regulates cell polarity in the uterine epithelium during embryonic development [23]. Perhaps Ror2-mediated, non-canonical, WNT signaling is maintained on the basal side of the uterine endometrium to preserve cell polarity as the epithelium forms, dies and regenerates. In silico analysis of Ror2 using ExPASy [24] generated an instability index of 53.24, which classifies the protein as unstable with an estimated half-life of 1 hr. Considering this, the interpretation would be that it is not the case that the protein is transcribed early and maintained, rather, Ror2 expression is dynamic, possibly as a consequence of WNT signaling events that occur continuously.

Functionally, uNK cells play important roles in angiogenesis, vascular remodeling and regulation of trophoblast invasion [25-27]. It has been reported WNT5A is involved in regulation of angiogenesis [28], trophectoderm migration [29] and in endothelial cell proliferation [30;31]
and migration [30]. These activities seen in WNT5A activated cells fit well with the observation that uNK cell express Ror2, a receptor for Wnt5a. Furthermore, the observation that splenic NK cells do not express Ror2 suggests that Ror2-related WNT signaling is responsible for uNK cell functions that phenotypically distinguish this lineage from peripheral NK cells. During embryonic development, cell migration within the cleft palate is directed by a Wnt5a gradient along the anteroposterior axis via Ror2 signaling [6]. Whether Wnt5a directs movement of Ror2 expressing uterine NK cell in the decidua is yet to be determined. Although the presence of Wnt5a in the MLAp and decidua was detectable, Wnt3a was not detected in these tissues. Since uNK cells are abundant in these tissues but rare in placenta [32], it can be postulated that non-canonical, CTNNB1-independent, WNT cell signaling is more likely to be occurring within uNK cells.

In conclusion, this chapter reports the presence of a new WNT signaling protein in the uterus; Ror2. Others have shown that the uterus is equipped for canonical WNT signaling, however the present study shows that a major player in non-canonical WNT signal transduction, Ror2, is present on the endometrial and uterine gland epithelium, uNK and stromal cells, labyrinthine cells of the placenta lining the maternal circulation and myometrium. However, the signal(s) which direct and determine how and where non-canonical WNT signaling occurs in the uterus is yet to be determined. Further investigation on the functional consequence of the presence of this receptor on many different cell types of the uterus merits further investigation.

4.6 References


Pacheco II, MacLeod RJ. CaSR stimulates secretion of Wnt5a from colonic myofibroblasts to stimulate CDX2 and sucrase-isomaltase using Ror2 on intestinal epithelia. AJP - Gastrointestinal and Liver Physiol 2008; 295: G748-G759.

MacLeod RJ, Hayes M, Pacheco I. Wnt5a secretion stimulated by the extracellular calcium-sensing receptor inhibits defective Wnt signaling in colon cancer cells. AJP - Gastrointestinal and Liver Physiol 2007; 293: G403-G411.


Chapter 5

Uterine Natural Killer cells dynamically express the vasoregulatory proteins Agtr1, Agtr2 and Nppa

5.1 Abstract

Human peripheral blood CD3+ T and CD56+ Natural Killer (NK) cells, and mouse CD3+ T cells are reported to express angiotensin II receptor type 1 (Agtr1) and type 2 (Agtr2). Engagement of these receptors leads to the effector functions of angiotensin II, which primarily is regulation of blood pressure. Agtr1 activation leads to vasoconstriction, while that of Agtr2 leads to vasodilation. Natriuretic peptide precursor A (Nppa) is a soluble protein secreted in response to high blood pressure. Nppa antagonizes Agtr1-mediated vasoconstriction. Early in pregnancy in humans and mice, uNK cells are in close contact with decidual blood vessels. In this investigation, the expression of Agtr1, Agtr2 and Nppa by mouse uNK cells was addressed. Implantation sites were examined at gestation day (gd) d6, 8, 10 and 12 using reverse-transcription polymerase chain reaction and immunohistochemistry. Expression of Agtr1 and Agtr2 was heterogeneous; the percentage of Agtr1 or Agtr2 expressing uNK cells increased between gd6 and gd10. Throughout the time course studied, uNK cells expressed both Agtr1 and Agtr2. Uterine NK cells do not express Nppa at gd6 and 8, but all uNK cells expressed Nppa at gd10 and 12. Splenic NK cells were also investigated and it was found that they expressed Agtr1, Agtr2 but not Nppa. In summary, uNK cells dynamically express vasoregulatory molecules Agtr1, Agtr2 and Nppa and thus may contribute to hemodynamic control during normal pregnancy.
5.2 Introduction

The Renin-angiotensin system (RAS) is a hormone system that acts as the master regulator of blood pressure and water balance. When blood pressure is low, the kidney responds to decreased perfusion and secretes renin, which converts the zymogen angiotensinogen (made mostly by the liver) to angiotensin I, which is further processed to angiotensin II (AngII) by angiotensin-converting enzyme (Ace), an enzyme secreted by renal and pulmonary endothelial cells [1-3]. AngII increases blood pressure by: acting as a vasoconstrictor of arterioles; acting on intraglomerular mesangial cells and associated blood vessels causing them to contract; acting on zona glomerulosa in the adrenal cortex to stimulate release of aldosterone; and by stimulating the pituitary to secrete anti-diuretic hormone (ADH, also known as vasopressin) which leads to increased water absorption [1;3;4]. These effects are mediated by signal transduction of the G-protein coupled receptor (GPCR) angiotensin II receptor type 1 (Agtr1) [1;5-8]. When blood pressure is high, the kidney stops its secretion of renin. In addition to this feedback mechanism, an inhibitor of renin and aldosterone secretion, natriuretic peptide precursor A (Nppa) [9;10], is produced by cardiac myocytes in response to increased afterload [11;12]. Nppa also dilates the afferent glomerular arteriole, constricts the efferent glomerular arteriole, relaxes mesangial and smooth muscle cells and has an overall effect of water excretion and reduces blood pressure to counter AngII [9;10;13]. Additionally, AngII mediated increases in blood pressure can also be countered by angiotensin II receptor type 2 (Agtr2). Agtr2, like Agtr1, shares AngII as its ligand. However Agtr2 signaling leads to vasodilation [5;7;8]. In this way, the physiology concerning blood pressure regulation is governed by the expression and action of several key molecules.

Although RAS pathway and related molecules have been associated with renal and cardiovascular physiology, recent work by others has shown that other biological systems such as the nervous system [14-16] and immune system produce and respond to RAS-related molecules.
For example, human peripheral blood T cells and Natural Killer (NK) cells respond to AngII via AGTR1 and AGTR2 [17-20]. AngII effects on immune cells have been described in the context of cell differentiation, activation and proliferation [21]. The concept of immune cells directly or indirectly contributing to blood pressure regulation has been suggested; however, there is limited primary literature on this emerging theme. Although peripheral blood immune cells have been characterized on the expression of vasoregulatory receptors, to date there have been no reports on the expression of these molecules in tissue-associated immune cells.

Lymphocytes in the pregnant uterus are a unique population. In early gestation this population lacks B cells, is composed of a small number of T cells and is dominated by NK cells. Uterine lymphocytes numerically increase with decidualization, the expansion of the endometrium, and therefore most abundant in the luteal phase of the menstrual cycle and first trimester of pregnancy in humans [22]. Although NK cells in the peripheral blood make up ~8-12% of lymphocytes, in the uterus uNK cells represent ~70-80% of lymphocytes when they are most abundant [22]. Human decidual histopathology and cell explant cultures [21], in vivo adoptive cell transplanted mouse models [23;24] and molecular studies in other species [25] have characterized uNK cells as highly pro-angiogenic cells functionally different from peripheral blood NK cells. In humans and mice, uNK cells physically associate with and surround decidual spiral arteries and are exclusively responsible for physiological modification of these vessels during early pregnancy [26;27]. Mouse models show that uNK cell-derived interferon gamma (Ifng) causes spiral arteries to lose their vascular smooth muscle and expand. This transformation changes these blood vessels from elastic tubes with narrow lumens into large, dilated structures delivering an increased volume of blood to the placenta [28]. In this way, uNK cells play roles in angiogenesis and establishing the proper vascular architecture in the pregnant uterus. Spiral artery modification is believed to be critical for proper placental perfusion as lack of spiral artery modification is seen in the human gestational complications of fetal growth retardation,
preeclampsia and in some cases of recurrent spontaneous abortions [29].

Because uNK cells promote placental perfusion and vasoregulatory proteins are expressed by peripheral blood NK cells, it was hypothesized that uNK cells also express vasoregulatory proteins. Since the final effector pathway of RAS is via receptor engagement, these experiments have addressed whether uNK cells express Agtr1 and Agtr2. Because Nppa is an antagonist to AngII-mediated signaling, its expression in mouse implantation sites was also investigated.

5.3 Materials and Methods

5.3.1 Mice and tissue collection

Use of mice was approved by the Queen’s University Animal Care Committee. C57BL6/J mice aged 6-8 weeks were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and mated. The day of vaginal plug detection was designated gestation day (gd)0. Implantation sites, spleens and hearts were collected from female virgin (n = 3), gd6 (n = 3), gd8 (n = 3), gd10 (n = 6) and gd12 (n = 3) pregnant mice.

5.3.2 Laser capture microdissection

Gestation day 10 (n = 3) mice were used. Implantation sites were embedded in Shandon Cryomatrix (Thermo Fisher; Mississauga, ON, Canada) and snap frozen in liquid nitrogen-cooled isopentane. Seven µm sections were cut using a cryostat and melted onto charged glass slides. Slides were treated with 70% ethanol for 1 min, nuclease-free water for 30 sec, and blocked with 1% bovine serum albumin (BSA) for 5 min. Sections were subsequently incubated with fluorescein isothiocyanate (FITC) conjugated Dolichos biflorus agglutinin (DBA) lectin (1:100, Sigma; Oakville, ON, Canada) for 5 min, washed three times with nuclease free tris buffered saline (TBS), and rapidly dehydrated with increasing concentrations of ethanol (two changes each 1 min of 70%, 95% and 100% ethanol) ending with xylene for 5 min. Slides were allowed to air
dry and 1000 uNK cells were isolated using a Cell Pix II fitted with CapSure HS caps (Molecular Devices; Toronto, ON, Canada). RNA was isolated using a PicoPure isolation kit (Molecular Devices; Toronto, ON, Canada) following manufacturer’s instructions.

5.3.3 Polymerase chain reaction

The RNA isolated from LCM was reverse transcribed, ligated and amplified by QuantiTect Whole Transcriptome Kit (Qiagen, Mississauga, ON, Canada). Primers targeting Nppa (202bp; forward 5’TGAAAAGCAACACTGAGGGCT3’, reverse 5’ACACACCACAAGGGCTTAGG3’) and Gapdh (size =234bp; forward 5’GGTCGGTGTGAACGGATTTGGC3’, 5’GTGGGGTCTCGCTCCTGGAAGA3’) were used. PCR was performed using a Taq DNA polymerase PCR kit (Qiagen; Mississauga, ON, Canada) under the following conditions: 94°C for 3 minutes (1 cycle); 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds (35 cycles); and 72°C for 10 minutes (1 cycle). PCR products were separated on a 1.0% agarose gel and visualized by ethidium bromide staining.

5.3.4 Immunohistochemistry

Gestation day 6, 8, 10 and 12 mouse implantation sites and spleens, as well as virgin spleens (n =3) were dissected and fixed in 4% paraformaldehyde overnight at 4°C. Tissue was dehydrated in increasing concentrations of ethanol followed with xylene and infiltrated with hot paraffin wax in an automated tissue processor. Tissue was subsequently embedded and 6 µm sections were cut, collected on glass slides (Fisher Scientific; Mississauga, ON, Canada) and used for immunohistochemistry. Briefly, slides were de-waxed in xylene and rehydrated in an ethanol gradient to water before being blocked with 1% BSA for 30 min. Tissue sections were circled with a hydrophobic pen (DAKO; Mississauga, ON, Canada) and incubated with primary antibody overnight at 4°C. Following incubation, slides were washed with TBS, incubated with fluorescently labeled secondary antibody (or fluorescent lectin or avidin) for 2 hr at room
temperature, washed, incubated in 20mM L-lysine (Sigma; Oakville, ON, Canada) in TBS for 30 min to quench autofluorescence, mounted with 4',6-diamidino-2-phenylindole (DAPI) supplemented mounting media (ProLong Gold Antifade Reagent with DAPI, Invitrogen; Burlington, ON, Canada) and imaged using a Zeiss AxioCam microscope system with Axiovision software (Zeiss; Toronto, ON, Canada). The following primary antibodies were used: rabbit anti-mouse Nppa (1:200, in house); rabbit anti-mouse Agtr1 (1:200, Abcam; Cambridge, MA, USA); rabbit anti-mouse Agtr2 (1:200, Abcam; Cambridge, MA, USA); and biotinylated rat anti-mouse killer cell lectin-like receptor subfamily K, member 1 (Klrk1, also known as Nkg2d) (1:25, R&D Systems; Minneapolis, MN, USA). FITC-DBA lectin (Sigma; Oakville, ON, Canada) was also used. Secondary antibodies used were Alex 488 and Alexa 594 conjugated goat anti-rabbit secondary antibody as well as Alexa 488 conjugated ExtrAvidin (Invitrogen; Burlington, ON, Canada). Implantation sites were stained with anti-Nppa, anti-Agrtr1 or anti-Agrtr2 with Alexa 594 secondary antibody, FITC-DBA lectin and DAPI. Spleens were stained with anti-Nppa, anti-Agrtr1 or Agrtr2 with Alexa 594 secondary antibody, anti-Klrk1 with Alexa 488 ExtrAvidin and DAPI. Each slide contained a negative control in which the primary antibody was replaced with 1% BSA.

5.3.5 Cell counting and scoring

Gestation day 6 and 10 implantation sites stained for Agrtr1 or Agrtr2 with DBA lectin and DAPI were scored for the percentage of DBA lectin and DAPI reactive cells that also expressed Agrtr1 or Agrtr2. Twenty-five random high power fields of view per pregnancy in the decidua or MLAp were photographed at 400x magnification and the cells were enumerated based on the criteria above. N = 3 pregnancies were examined per gd, and an average was determined. Using Microsoft Excel software (Microsoft; Toronto, ON, Canada), paired one-tailed student’s t-tests were performed with p < 0.05 considered significant.
The reactivity of each unique primary antibody with Klrk1 or DBA lectin reactive NK cells was scored “−” if the antibody in question did not co-localize with the NK cell marker and “+” if there was co-localized staining.

5.4 Results

Uterine NK cells expressed Agtr1 and Agtr2 (Figure 9). Expression of these receptors by uNK cells was observed in all of the time points examined. However, uNK cells appeared heterogeneous; the frequency of Agtr1 or Agtr2 expressing uNK cells appeared proportional to the numerical population of uNK cells at the time points examined. Therefore, the percentage of Agtr1 or Agtr2 expressing uNK cells at gd6 and 10, time points when uNK cells are rarest and most abundant, was enumerated. The percentage of Agtr1 or Agtr2 expressing uNK cells increased between gd6 and 10 (Figure 10). The expression of Agtr1 or Agtr2 expressing uNK cells was not related to the size or location of uNK cells.

To determine if uNK cells expressed Nppa transcript, gd10 was chosen for initial study because the abundance of uNK cells at this time point would allow a sufficient number of uNK cells to be isolated by laser capture microdissection (LCM). Uterine NK cells were identified by FITC-DBA lectin staining and isolated by LCM (Figure 11A). RNA was isolated and RT-PCR was performed to conclude gd10 uNK cells express Nppa (Figure 11B). Experiments proceeded to confirm the expression of Nppa protein using tri-colour immunohistochemistry. Nppa targeting antibodies co-localized with gd10 uNK cells identified by FITC-DBA lectin reactivity, indicating that uNK cells express Nppa (Figure 11C). To determine if Nppa expression was stable in uNK cells over pregnancy, immunohistochemistry was repeated using gd6, 8 and 12 implantation site tissue. Gestation day 6 and 8 uNK cells had no detectable Nppa expression, while gd12 uNK cells were positive for Nppa. Therefore, uNK cells do not acquire Nppa expression until mid-pregnancy (Figure 12). Nppa expression in uNK cells was binary; all uNK were not detectable for Nppa
Figure 9: Uterine NK cells express angiotensin II receptors Agtr1 and Agtr2

Using fluorescence immunohistochemistry, Agtr1 (red) was co-localized to DBA lectin reactive uNK cells (green) (A). Likewise, uNK cells (green) also expressed Agtr2 (red) (B). Nuclei were stained with DAPI (blue). Size bar =20µm.
Figure 10: The proportion of Agtr1 and Agtr2 expressing uNK cells increases with their numerical increase

Uterine NK cells first appear in the uterus at gd6 and are few in number. They are most numerically abundant at gd10. During this expansion of uNK cells, the percentage of Agtr1 (A) and Agtr2 (B) expressing uNK cells increases.
Figure 11: Uterine NK cells express Nppa transcript and protein

Frozen sections of gd10 implantation sites were stained with FITC conjugated DBA lectin (green) to detect uNK cells. One thousand DBA lectin staining uNK cells were collected and pooled by laser capture microdissection for RNA isolation (A). *Nppa* transcript was identified by PCR amplification of cDNA (B). Using immunohistochemistry, Nppa expression (Alexa 594; red) was co-localized to FITC-DBA lectin staining uNK cells (green) (C). DAPI was used to stain nuclei (blue). Size bar =10µm.
Figure 12: Detection of Nppa expression by uNK cells is restricted to advanced stages of pregnancy.

Gestation day 8 (A, C) and 12 (B, D) implantation sites were stained for Nppa (red), DBA lectin reactive uNK cells (green) and DAPI (nuclei; blue). Uterine NK cells present before the formation of a perfused placenta did not express Nppa (A, C). Uterine NK cells after the formation of the placenta had detectable Nppa expression (B, D). Legend: c = eco-placental cone; d = decidua; f = fetus; m = Mesometrial Lymphoid Aggregate of pregnancy, MLAp; p = placenta. Size bar A, B = 500µm; C, D = 50µm.
Figure 13: Splenic NK cells stain positively for Agtr1, Agtr2 and Nppa.

Paraffin embedded spleens were sectioned and stained for Klrk1 (also known as Nkg2d; green) and Agtr1 (A), Agtr2 (B) or Nppa (C) co-localization (red). Nuclei were stained using DAPI (blue). Size bar =5µm.
Table 3: Summary of splenic and uterine NK cells expression of vasoregulatory proteins.

<table>
<thead>
<tr>
<th></th>
<th>Spleen NK</th>
<th>gd6</th>
<th>gd8</th>
<th>gd10</th>
<th>gd12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nppa</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Agtr1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Agtr2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Legend: no detection (−), positive detection (+)
expression at gd6 and 8, however all uNK cells expressed Nppa at gd10 and 12 regardless of size or location of the cell.

To determine if peripheral NK cells expressed Agtr1, Agtr2 and Nppa, tri-colour immunohistochemistry was performed on virgin or pregnant spleen tissue using Klrk1 to identify splenic NK cells. The majority of Klrk1 positive splenic NK cells expressed Agtr1, Agtr2 and Nppa (Figure 13). Table 3 summarizes the expression of these markers on spleen NK and uNK cells.

5.5 Discussion

Vasodilating Agtr1 and vasoconstricting Agtr2 were both expressed by uNK cells. This is the first report of uNK cells expressing these receptors and this observation is consistent with reports by others that peripheral NK cells in humans express AGTR1 and AGTR2. The increased frequency of these receptors at gd10 compared to gd6 may mean that uNK cells make greater contributions to vasoregulation at mid-pregnancy than early pregnancy. Of interest, this interval of time is reported as an interval of declining blood pressure systematically in pregnant mice [30;31]. Mean arterial pressures begin to drop at gd5 and this trend is reversed at gd10 when mean arterial pressure begins to rise towards its pre-pregnancy baseline in mice [30;31]. Agtr2, in addition to being vasodilating, is known to inhibit cell proliferation and induce apoptosis [32;33]. Given the data presented here showing an increase in the percentage of uNK cells expressing Agtr2 at gd10 (when uNK cells numerically reach their peak in numbers), it may be postulated that Agtr2 expression leads to uNK cell apoptosis, which begins in some uNK cells at gd8 and becomes widespread at gd11 [34;35]. Further studies will be required to determine the functional importance of these findings.

This investigation is the first report of the expression of Nppa by any lymphocyte. The uNK cell-derived Nppa is likely to have different physiological target cells than cardiomyocyte-
derived Nppa. Lymphocytes are mobile and traffic to different tissues by chemoattractant and other signals, while cardiomyocytes are restricted to the tissue structure they support. Therefore, unlike cardiac myocytes which secrete Nppa into the circulation, uNK-derived Nppa may deliver highly concentrated Nppa to specific blood vessels. Further work is necessary to determine if Nppa produced by uNK cells embedded in arterial walls could act on smooth muscle cells and antagonize Agtr1 mediated vasoconstriction in the second half of pregnancy.

In summary, uNK cells dynamically express vasoregulatory proteins Agtr1, Agtr2 and Nppa. Uterine NK cells may participate in the regulation of blood pressure or these molecules may participate in the activation of pathways related to decidualization, placentation, angiogenesis, regulation of trophoblast invasion, or other uNK cell-related functions.

5.6 References


Chapter 6
Synopsis and Conclusions

6.1 Summary

Until recently, hypotheses asserting that uNK cells have functions other than cytolysis have been uncommon. This is because of the etymology and connotation of NK cell nomenclature being named “killer cells”, because the study of uNK cells is difficult (requirement for access to first-trimester pregnancy decidua in humans) and because of classical assumptions that the expected outcome from the engagement of maternal uNK cell KIR by paternal HLA leads to killing. Recent work has shown however that genetic pairings of maternal KIR and parentally-derived HLA that lead to inhibition of KIR are associated with gestational complications of poor placentation, while genetic pairings that lead to activation of KIR are associated healthy pregnancies [1-3]. Therefore, in the case of pregnancy, uNK KIR can still be thought of as a non-self surveillance detector, however activation signals do not lead to killing, but rather to angiogenesis. This “welcome non-self” concept was groundbreaking because a large body of the literature was swayed towards the cytotoxicity of uNK cells based on observations that they store many lytic proteins in their granules. Today, the major questions regarding uNK cells include their origin and differentiation; their angiogenic regulation; and if previously unexplored functions remain to be characterized.

Regarding their origin and differentiation, a classical question regarding uNK cells has been if they are different from peripheral uNK cells. Microarrays comparing the two cell types [4;5], explants studies [5] and mouse models [6;7] are all in agreement that uNK cells differ from
blood NK cells. Based on these studies, the hallmark feature of uNK cells is their pro-angiogenic phenotype, a phenotype not shared with blood NK cells. Despite the differences that exist between blood NK and uNK cells, it is unknown if uNK cells are derived from blood NK cells. It has been postulated that blood NK cells or earlier progenitor cells traffic from the circulation and differentiate into uNK cells, or that the uterus has resident progenitor cells that differentiate into uNK cells, or both [8-10]. Experiments using mice suggest that a trafficking model is plausible. In mice genetically devoid of uNK cells, syngeneic grafts from any lymphoid organ of a wild-type mouse restore uNK cells [11]. Furthermore, a uterine graft from a wild-type mouse received by a knock-out mouse genetically devoid of uNK cells fails to populate the uterus with uNK cells [12]. In humans, the trafficking potential of blood NK cells to migrate to the uterus fluctuates over the menstrual cycle and peaks at ovulation [13]. In this thesis, progressively increased trafficking potential of human blood CD56+CD3-CD16- NK cells was observed through use of adhesion substrate decidual tissue at progressively later time points in pregnancy. Other types of blood NK cells did not show this response, suggesting that the uterus recruits specific subsets of the NK lineage. Also presented was data showing that the increased trafficking potential was dependant on adaptive immune changes that occurred in the decidua during pregnancy. The potential for priming of NK cell trafficking to the uterus during the menstrual cycle was addressed using plasma. At ovulation, the gene annotation and interactome mapping of significantly regulated cytokines and chemokines in the blood predicted increased immune cell trafficking at ovulation. Collectively, this thesis strengthens the hypothesis that at least a subset of uNK cells originates from peripheral blood.

This thesis also presents data on vasoactive and other proteins not yet reported to be expressed by uNK cells. These include Agtr1, Agtr2, Nppa and Ror2. Regarding Nppa and Ror2, this thesis is the first description of these molecules to be expressed in lymphocytes. Ror2 is expressed on uNK but not peripheral NK cells and therefore it is a new cell marker of uNK cells.
The discovery of Agtr1, Agtr2 and Nppa expression by uNK cells potentially defines a new uNK cell phenotype with an ability to regulate blood pressure. The discovery of Ror2 expression by uNK cells suggest uNK cells may additionally have functions in WNT related biological processes such as regulating cell or tissue polarity, decidualization or chemotaxis. The description of the expression of these molecules in this thesis is further support for the role of uNK cells in placentation and in arguments against the previous thinking that uNK cells simply particpate in fetal rejection.

6.2 References


