Immune modulation of normal and pathological human pregnancy

Ingrid Granne

St Hugh's College

Trinity Term 2011

Immune modulation in normal and pathological human pregnancy

Ingrid Granne

Trinity Term 2011, St Hugh's College

A thesis submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy at the University of Oxford

Abstract

The first half of this thesis addressed the clinical question of whether the phenotype of peripheral immune cells is affected by the normal menstrual cycle and whether there are differences in women with recurrent unexplained miscarriage. In chapter 2, a mid-luteal type 1 shift was demonstrated in NK^{dim} and cytotoxic T cells in the menstrual cycle. In addition, women with recurrent miscarriage had increased numbers of type 1 (IL-18R+) cytotoxic lymphocytes and an increased number of NK^{dim} and NK^{bright} cells in the mid-luteal phase of the cycle.

The shift to type 2 immunity seen in normal pregnancy has been previously identified from the second half of the first trimester. In chapter 3 this type 2 shift was seen in NK and T cells as early as 9 days post implantation. ST2L (the ligand for IL-33 and a marker of type 2 cells) was consistently up regulated at this very early stage post implantation in women who were pregnant.

The second half of this thesis investigated IL-33 and ST2 in normal and pathological pregnancy. In chapter 4 it was shown that circulating IL-33 did not change over the course of pregnancy but that sST2 (the soluble decoy receptor) increased significantly in the 3rd trimester of normal pregnancy. Both IL-33 and ST2 were variably expressed by the pre-implantation human blastocyst, as well as by the 1st and 3rd trimester placenta.

Finally, chapter 5 showed a potential role for ST2 in pre-eclampsia, an inflammatory disease of pregnancy. sST2 was significantly elevated in the third trimester in women who went on to develop pre-eclampsia even prior to the onset of disease. Using an in vitro model of placental perfusion, it was shown that sST2 can be secreted by the placenta, suggesting that sST2 may play a significant role in pregnancies complicated by this disease.

Table of Contents

Acknowledgements x

List of abbreviations 3

1. Introduction 1

1.1 The Immunology of pregnancy 4

1.2 Type 1 /type 2 immune responses 6

1.3 Uterine and peripheral immune cells 8

1.3.1 Uterine immune cells 8

1.3.2 Differences between peripheral and uterine cells 10

1.4 Aims of this thesis 13

2. Do peripheral NK, iNKT and T cells change in the normal menstrual cycle and in women with recurrent miscarriage? 15

2.1 Introduction 15

2.1.1 Hormonal control of the menstrual cycle 15

2.1.2 The effect of the menstrual cycle on peripheral immune cells 18

2.1.3 Peripheral immune cells and recurrent miscarriage 20

2.1.4 Markers of type 1/ type 2 cells and potential cytokine production 22

2.1.5 CD69 as a marker of cell activation 24

2.2 Aims 25

2.3 Materials and methods 26

2.3.1 Subject recruitment 26

2.3.1.1 Menstrual cycle study 26

2.3.1.2 Recurrent miscarriage menstrual cycle study 27

2.3.2 Peripheral blood sampling and preparation of peripheral blood mononuclear cells (PBMCs) 28

2.3.3 Antibody labelling of peripheral blood mononuclear cells 29
2.3.4 Flow cytometry analysis 31
2.3.4.1 Comparison of fluorescence minus one gating with and without IgG controls 31
2.3.4.2 Identification of NK cells 34
2.3.4.3 NK^{dim} cells 35
2.3.4.4 NK^{bright} cells 35
2.3.4.5 Identification of iNKT cells 36
2.3.4.6 Identification of T cell populations 37
2.3.4.7 Determining the absolute count of cells 38
2.4 Statistics 39
2.5 Results 40
2.5.1 The effect of the menstrual cycle on peripheral NK, iNKT and T cells in the women with no history of miscarriage 40
2.5.1.1 NK cells 40
2.5.1.2 iNKT cells 45
2.5.1.3 T cells 47
2.5.2 The effect of the menstrual cycle on peripheral NK, iNKT and T cells in the women with recurrent unexplained miscarriage 52
2.5.2.1 NK cells 52
2.5.2.2 iNKT cells 56
2.5.2.3 T cells 58
2.5.3 The effect of recurrent unexplained miscarriage on peripheral NK, iNKT and T cells throughout the menstrual cycle: a case-control study... 62
2.5.3.1 NK cells 62

2.5.3.2 iNKT cells 66
2.5.3.3 T cells 68
2.5.1 Does the measurement of mid-luteal NK cells discriminate between women with recurrent miscarriage and controls? 73
2.6 Discussion 75
2.6.1 Cell numbers in the normal menstrual cycle 75
2.6.2 Cell numbers in women with recurrent unexplained miscarriage 77
2.6.3 Cell activation measured by CD69 expression 81
2.6.4 Markers of type 1 and type 2 peripheral immune cells in the menstrual cycle and in recurrent miscarriage 82
2.7 Summary 85
3. The influence of IVF treatment and early pregnancy on peripheral immune cells 86
3.1 Introduction 86
3.1.1 IVF 88
3.1.2 Implantation 92
3.2 Aims 94
3.3 Materials and methods 95
3.3.1 Subject recruitment 95
3.3.2 Blood sampling, antibody labelling and flow cytometry 97
3.4 Statistics 97
3.5 Results 98
3.5.1 Cycle outcome 98
3.5.2 The effect of the IVF cycle in women who became pregnant 100
3.5.2.1 NK cells 101

3.5.2.2 iNKT cells 106
3.5.2.3 T cells 107
3.5.3 Variation across the cycle in women with a negative pregnancy test 112
3.5.4 The effect of implantation on peripheral immune cells 112
3.6 Discussion 119
3.7 Summary 123
3.8 Appendix 124
4. IL-33 and ST2 in normal human pregnancy 132
4.1 Introduction 132
4.1.1 IL-33 and ST2 132
4.2 Aims 137
4.3 Materials and Methods 138
4.3.1 Subject recruitment 138
4.3.1.1 Human embryo study 138
4.3.1.2 Embryo culture supernatants 138
4.3.1.3 First trimester placenta and decidua 139
4.3.1.4 Third trimester placenta 139
4.3.1.5 Longitudinal IVF study 139
4.3.2 Detection of IL-33 by ELISA 140
4.3.2.1 Detection of IL-33 by ELISA in plasma and serum 141
4.3.2.2 Detection of IL-33 by ELISA in supernatants 141
4.3.3 Detection of sST2 by ELISA 142
4.3.4 Detection of IL-33 and ST2 in embryos by immunofluorescence microscopy 144

4.3.5 Detection of IL-33 and ST2 by immunohistochemistry 145
4.3.6 Detection of IL-33 and sST2 by immunoblotting 146
4.3.6.1 Protein quantification 146
4.3.6.2 Sample preparation 147
4.3.6.3 SDS-polyacrylamide gel electrophoresis (-PAGE) 147
4.3.6.4 Transfer of protein to PVDF 148
4.3.6.5 Western blot analysis 148
4.4 Statistics 149
4.5 Results 150
4.5.1 IL-33 and sST2 expression by the pre-implantation human embryo ……..……150
4.5.2 IL-33 and ST2 expression by first trimester trophoblast and decidua 151
4.5.3 Circulating IL-33 and sST2 during an IVF cycle and in early pregnancy 154
4.5.4 Circulating IL-33 and sST2 in each trimester of normal pregnancy ……..……156
4.5.5 Third trimester placental expression of IL-33 and ST2 by western blotting. 159
4.5.6 IL-33 and sST2 localisation in third trimester placenta by immunohistochemistry 160
4.6 Discussion 161
4.7 Summary 166

5. IL-33 and ST2 in pre-eclampsia 167
5.1 Introduction 167
5.1.1 Pre-eclampsia 167
5.1.2 Placental theory of pre-eclampsia 169
5.1.3 Placental derived factors and the pathogenesis of pre-eclampsia 171
5.1.4 The systemic immune response in pre-eclampsia 172
5.1.5 The role of IL-33 and sST2 in disease 174
5.1.6 Aims 175
5.2 Materials and Methods 176
5.2.1 Subject recruitment 176
5.2.1.1 Matched pre-eclampsia case-control study 176
5.2.1.2 Longitudinal study 178
5.2.1.3 Placental tissue 179
5.2.2 Peripheral blood sampling 180
5.2.3 Plasma and serum preparation 180
5.2.4 Detection of IL-33 and ST2 by ELISA, western blotting and immunohistochemistry 180
5.2.5 Placental perfusion 181
5.3 Statistics 183
5.4 Results 184
5.4.1 Circulating IL-33 in pre-eclampsia 184
5.4.2 Circulating sST2 in pre-eclampsia 186
5.4.3 IL-33 and ST2 expression of pre-eclampsia placentas by western blotting 191

5.4.4 IL-33 and sST2 localisation in normal and pre-eclampsia placentas by immunohistochemistry 193
5.4.5 Placental production of IL-33 and ST2 194
5.5 Discussion 196
5.6 Summary 201
6. Summary and Conclusions 202
6.1 Circulating NK and T cells in the normal menstrual cycle and recurrent miscarriage 202
6.2 Circulating NK and T cells in early pregnancy 205
6.3 IL-33 and ST2 in normal pregnancy 206
6.4 IL-33 and ST2 in pre-eclampsia 208
References 210

Acknowledgements

There are a number of people whom I would like to thank. Firstly I am grateful to my supervisor Ian Sargent who agreed to take me on when he really didn't have to. I have appreciated immensely his help over the past three years, both as a supervisor and as a constant supporter of my ambition to pursue an academic career. I must also thank Chris Redman who is an inspiration for anyone aspiring to be a clinical academic. Thanks to Tim Child and Enda McVeigh, who employed me as a Clinical Fellow at the Oxford Fertility Unit. Without them this research would not have happened. Tim has also been both a supportive supervisor and career mentor over the years.

This research was generously funded primarily by the Oxford Fertility Unit, along with a fellowship from the Oxford Biomedical Research Centre and the Oxford Health Services Research Committee.

I am grateful to the patients from both the Oxford Fertility Unit and the John Radcliffe Hospital who participated in these studies. Thanks to all the nurses at the Oxford Fertility Unit who were fantastic at helping me recruit patients, particularly Ginny Mounce and Debbie Barber. Many of the samples used in these studies were from patients recruited to the Oxford Pregnancy Biobank. Thanks to all the research midwives: Nicola Higgins, Ali Chevassut, Tess Norris, Libby Searle, Katherine Edwards, Hazel Meacher and Linda Holden who collected samples and Jacqui Marks and Tiffany lodge for organising the samples. A particular thank you must go to Carol Simms who has always gone the extra mile to help me. The early pregnancy work was performed in collaboration with Dr Judith Cartwright and Professor Guy Whitley. Thanks to them for allowing me to spend some time working in their laboratory at St Georges' Hospital, London and to their research group for being so welcoming. James Snider of Critical Diagnostics kindly provided the Pressage sST2 assays used in this work.

Arriving in the NDOG as a laboratory rookie, Ian's lab was an exceptionally welcoming environment. Although there may have been a collective groan by Jen Southcombe, Bec Dragovic, Dionne Tannetta and Gavin Collett at the arrival of yet another medic who didn't know the difference between a FACs machine and a Fax machine, I received nothing but support for my often slow learning curve. Thank you all for your patient teaching. In addition I would like to thank Dionne for providing the placental perfusates and Janet Carver who showed me how to do the embryo experiments.

A special thanks must go to Jen. With Jen as my post doc I landed on my feet. Single handed she took me from being a woman who didn't know one end of a pipette from the other to having a real enthusiasm for science and the lab. Most invaluable of course were the Friday evenings in the social. Along with Bec, Jen also survived the ups and downs of my pregnancy, reluctant motherhood, grant writing, job applications and the write up. Thank you both for the many 'blinds down in the flow room moments'.

My only tip for anyone embarking on a DPhil is to find a friend who has recently done one. For me, that person has been Katy Vincent. Thanks for all the advice from ethics application, to how to keep sane. Perhaps more important than the advice, was the accompanying wine.

After the arrival Jack this DPhil would never have been completed without the amazing support of my parents. Not only did they care for him daily from the age of 6 weeks, which allowed me to get back in the lab, but their day in, day out involvement in our lives has been invaluable. I really could not have done it without them.

Although it would have been preferable if Jack had chosen to arrive at a more convenient time in my studies (he could have at least waited until I had submitted an ethics application) he has made the last 3 years eventful and mainly fun. I hope he will forgive my minimalist parenting style. So finally to my husband Joe who has had to live with the consequences of my career choices. Thanks for forgiving the many absent weekends and shortened holidays .It remains true that we could both do with a wife. I however, could not have a better husband.

List of abbreviations

α-gal-cer	α-Galactosylceramide
ANOVA	Analysis of Variance
APC	Allophycocyanin
APC-Cy7	Allophycocyanin-cyanine 7
AUC	Area Under Curve
BCA	Bicinchoninic Acid
BSA	Bovine Serum Albumin
CCL5	Chemokine Ligand 5
CCR	Chemokine Receptor 4
CD	Cluster of Differentiation
CTL	Cytotoxic Lymphocyte
CXCR-	Chemokine Receptor
DAB	3, 3'-Diaminobenzidine
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-Linked Immunosorbant Assay
FC receptor	Fragment-Crystallisable receptor
FCS	Fetal Calf Serum
FITC	Fluorescein Isothiocyanate
FMO	Fluorescence Minus One
FSH	Follicle Stimulating Hormone
GnRH	Gonadotrophin Releasing Hormone
HCG	Human Chorionic Gonadotrophin
HELLP	Haemolytic anaemia Elevated Liver Enzymes Low Platelets
HEPES lysis buffer	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid lysis buffer
HFEA	Human Fertilisation and Embryology Authority
HLA	Human Leukocyte Antigen
HMGB1	High Mobility Group Box 1
HPO	Hypothaliamic Ovarian Pituitary
HRP	Horseradish Peroxidase
HUVECs	Human Umbilical Vein Endothelial Cells
ICSI	Intracytoplasmic Sperm Injection
IFN-γ	Interferon-γ
IgG	Immunoglobilin
IL	Interleukin
IL-18R	Interleukin-18 Receptor
IL-1RAcP	Interleukin-1 Receptor Accessory Protein
iNKT	invariant Natural Killer T
IP-10	Interferon Inducible Protein-10
IRAK	Interleukin Receptor-Associated Kinase
IUGR	Intrauterine Growth Restriction
IVF	In Vitro Fertilisation
KIR	Killer Immunoglobulin Receptor
LH	Luteinising Hormone
MAPK	Mitogen-Activated Protein Kinase
MFI	Median Fluorescence Intensity
MHC	Major Histocompatibility Complex
MyD88	Myeloid Differentiation Primary Response Gene 99

NCAM	Neural Cell Adhesion Molecule
NF-HEV	Nuclear Factor from High Endothelial Venules
NF-κB	Nuclear Factor Kappa-light-chain-enhancer of activated B cells
NK	Natural Killer cells
NKp40	Natural Killer p40 related protein
PBMCs	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PE	Phycoerythrin
PE	Pre-eclampsia
PeCy5	Phycoerythrin Cyano-5
PHA	Phytohaemaglutinin
PLGF	Placental growth factor
PMA	Phorbol Myristate Acetate
PMT	Photo Multiplier Tube
PVDF	Polyvinylidene Fluoride
ROC	Receiver Operator Characteristics
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide gel electrophoresis
SEM	Standard Error of the Mean
SFlt-1	Fms-like tyrosene kinase-1
SIGIRR	Single Ig IL-1-related molecule
SLE	Systemic inflammatory erythematosis
SPSS	Statistics Package for Social Sciences
sST2	soluble Suppression of Tumorigenicity 2
ST2L	Suppression of Tumorigenicity 2 full length
TBST	Tris-buffered saline-Tween
TGF	Transforming Growth Factor
Th	T helper
TMB	3,3',5,5'-Tetramethylbenzidine
TNFα	Tumour Necrosis Factor-α
TRAF6	Tumour Necrosis Factor Receptor Associated Factor-6
VEGF	Vascular Endothelial Growth Factor
Vα-24	α-chain Variable Gene Segment 24
Vβ-11	β-Chain Variable Gene Segment 24

1. Introduction

At least 50% of naturally conceived human embryos do not result in a live birth. Many embryos do not implant, or do not develop for long enough to result in a recognised pregnancy while 15% of clinical pregnancies result in miscarriage (Figure 1-1) (Rai & Regan, 2006). Although chromosomal abnormality accounts for approximately half of early pregnancy losses, many chromosomally normal embryos also fail to implant, or miscarry after implantation. The cause of these pregnancy failures is uncertain.

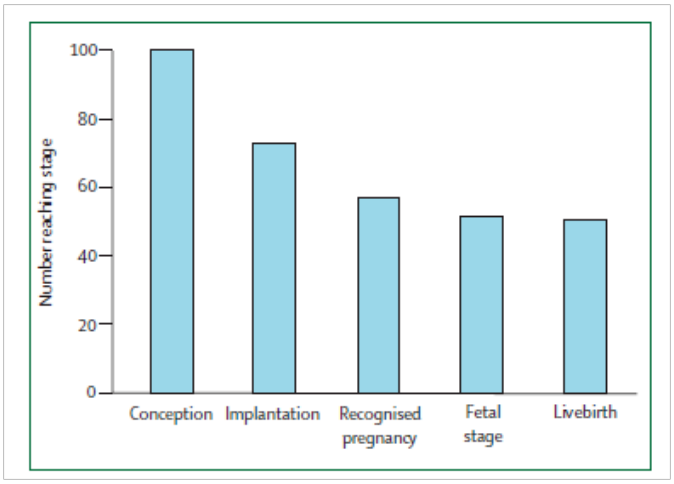


Figure 1-1 Number of conceptions expected to reach each stage of pregnancy. Reproduced from Rai et al. 2006.

Dysfunctional implantation and placentation may link a number of pregnancy pathologies (Christiansen *et al.*, 2006; Rai & Regan, 2006). At one end of the spectrum, complete failure of the embryo to implant leads to infertility. Post implantation, the clinical consequences of abnormal placentation may vary, but include recurrent miscarriage, intrauterine growth restriction (IUGR) and pre-eclampsia (Figure 1-2) (Burton & Jauniaux, 2004; Merviel *et al.*, 2004; Norwitz,

2006; Burton *et al.*, 2009). One theory is that an abnormal maternal immune response may be a cause of failed implantation or abnormal placentation (Clark, 2008).

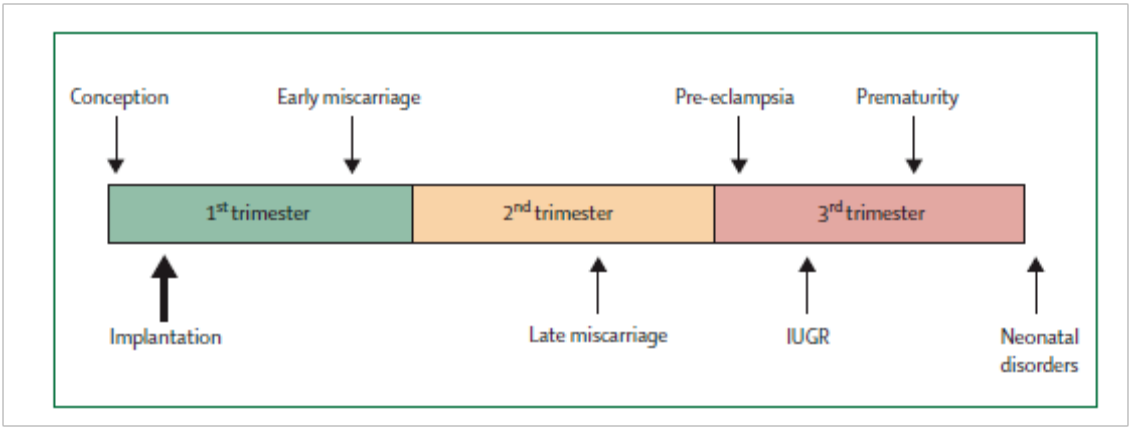


Figure 1-2 The spectrum of pregnancy pathology. Reproduced from Rai et al 2006.

Although there are instances of proven autoimmune pregnancy pathology, notably the anti-phospholipid syndrome (Rai *et al.*, 1995), good evidence of immune mediated pregnancy failure is scarce. However for many years there have been persistent reports of a possible association between 'abnormal' levels of peripheral immune cells (predominantly NK cells) or abnormal cytokine production (by NK and T cells) and poor pregnancy outcomes, such as failed implantation after in vitro fertilisation (IVF) or recurrent miscarriage (Aoki *et al.*, 1995; Coulam *et al.*, 1995; Beer *et al.*, 1996; Siu Chui *et al.*, 2002; Kwak-Kim *et al.*, 2003; Moffett *et al.*, 2004; Rai *et al.*, 2005). There are a growing number of centres that offer peripheral blood immune cell testing to women with a poor reproductive history.

The argument in support of testing for altered peripheral immune cells in women with recurrent miscarriage or implantation failure has arisen from the theory that immune interventions such as corticosteroids, intravenous immunoglobulins or biologic agents such as anti-TNF-α antibodies, may 'dampen down' the abnormal immune response, a marker of which is high peripheral immune cell numbers (Clark *et al.*, 2006; Thum *et al.*, 2008; Clark, 2009). Advocates of such testing argue that treatment increases the chance of successful implantation, ongoing pregnancy and live birth. Certainly the evidence for treatment remains controversial due to the lack of large, good quality randomised controlled trials. Despite this, many clinics continue to offer both testing and treatment. As yet, there is no definitive evidence that any of these treatments improve outcomes. Indeed two meta analyses of published studies have shown no increase in the live birth rate (Porter *et al.*, 2006; Ata *et al.*, 2011).

Published studies investigating peripheral immune cells and reproductive failure are often criticised as they have been performed by only a small number of individual research groups (i.e.: they have not been repeated by the wider research community). The Royal College of Obstetricians and Gynaecologists Scientific Advisory Committee has advised that there remains insufficient evidence for testing peripheral immune cells as a causative factor for pregnancy failure in women with recurrent miscarriage, and consequently do not advocate treatment of apparently 'abnormal' NK cells (Rai, 2008).

1.1 The Immunology of pregnancy

During pregnancy the maternal immune system (both innate and adaptive) is regulated in order to accommodate the semi-allogenic fetus. The theory of maternal tolerance to the fetus was first addressed by Sir Peter Medawar, who put forward three hypotheses: the physical separation of maternal and fetal tissues, the antigenic immaturity of fetal tissues and the immunological inertness of the mother (Medawar, 1953). The three arguments presented by Medawar have not been held up in their entirety; however they have formed the basis of much of the research in reproductive immunology in the intervening years.

Although the trophoblast acts as a barrier between the fetus and the mother, it is certainly not impenetrable. Firstly, it is known that maternal IgG antibodies are actively transferred across the placenta during fetal development (Hanson *et al.*, 2003). Secondly it has been shown that there is significant microchimerism between the mother and her fetus. Fetal cells can be detected in the maternal circulation not only during, but after pregnancy (Nelson, 2003). In addition, the surface of the placenta, the syncytiotrophoblast, itself sheds microvesicles into the maternal blood (Germain *et al.*, 2007b).

There are two distinct interfaces between maternal and fetal cells (Figure 1-3).The first is that of the extravillous cytotrophoblast. This invades the decidua eventually reaching the myometrium (interface I). The second interface is the syncytiotrophoblast that lies in direct contact with maternal blood (interface II). In line with Medawar's hypothesis of fetal antigenic

"immaturity", extravillous cytotrophoblast cells have a unique repertoire of class I Human Leukocyte Antigens (HLAs). They down regulate HLA-A and B and express HLA-C, HLA-E and HLA-G, of which only HLA-C is polymorphic with the potential to be a fetal allo-antigen (Moffett-King, 2002). In the second half of pregnancy the predominant fetal tissue in contact with the maternal immune system is the syncytiotrophoblast that does not express class I or class II Major Histocompatability (MHC) antigens (Sunderland *et al.*, 1981) and therefore cannot be recognised by maternal T cells.

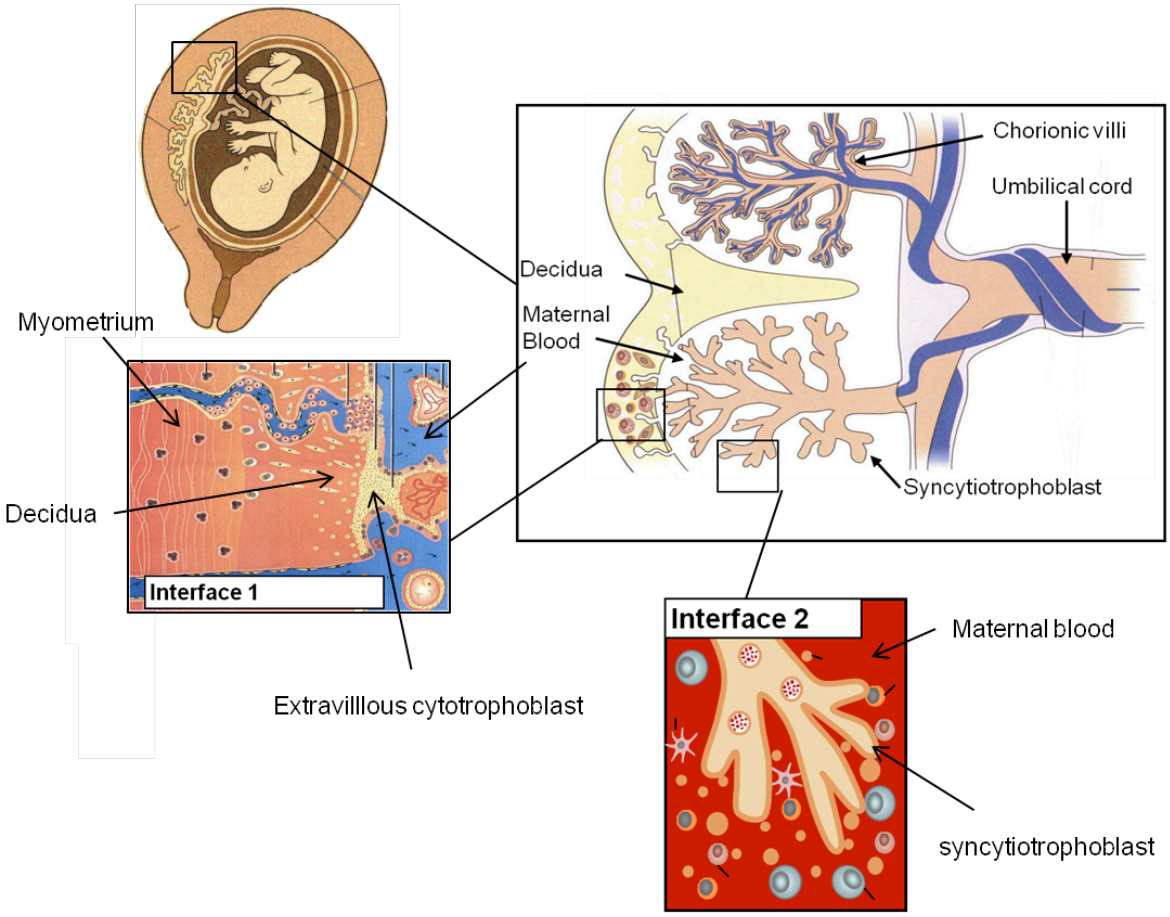


Figure 1-3 The two feto-maternal interfaces of human pregnancy. Reproduced with permission from Professor Ian Sargent

Medawar's final argument for feto-maternal tolerance was that the mother during pregnancy is immunologically inert. His hypothesis was that the uterus is an immunologically privileged site, however tissue allografts inside the uterus are rejected and extrauterine pregnancy does not fail because of a maternal immune response (Chaouat *et al.*, 2010).

1.2 Type 1 /type 2 immune responses

The concept of immune suppression during pregnancy was subsequently developed by Wegmann, who showed that normal pregnancy is associated with a bias of CD4+ T helper cells towards Th2 (antibody mediated) immune responses and away from Th1 (cell mediated) immune responses (Wegmann *et al.*, 1993). There are a number of functionally different subsets of CD4+ T cells: Th1, Th2 (Mosmann *et al.*, 1986), Th17 cells (Harrington *et al.*, 2005) and regulatory T cells (Hori *et al.*, 2003). Th1 cells are characterised by their production of IL-2, IL-12, IFN-γ and TNF and are primarily involved in cell mediated immune responses. Th2 cells are characterised by the synthesis of IL-4, IL-5, IL-10 and IL-13 and facilitate humoral responses. Th-17 cells were so called because they produce IL-17 along with IL-22. They are implicated in autoimmunity and host defence against extracellular bacteria. In contrast to other subsets of CD4+ cells, Foxp3+ regulatory T (Treg) cells confer a tolerogenic immune response and there is increasing interest in their role in human reproduction. Treg and Th17 cells are not considered in detail in this thesis.

Subsequent to the discovery of Th cell subsets, it became clear that CD8+ cytotoxic T cells could also be characterised as type 1 and type 2 in their cytokine profile (Noble *et al.*, 1995; Vukmanovic-Stejic *et al.*, 2000). This sub-division, according to cytokine profile, has since been extended to innate immune cells such as Natural Killer (NK) cells. NK cells cultured with IL-12 or IL-4 differentiate into NK1 or NK2 cells respectively (Peritt *et al.*, 1998).

Evidence of disease modification during pregnancy supports the theory of type 2 shift. For example, classical type 1 mediated conditions such as rheumatoid arthritis may improve during pregnancy, whereas type 2 associated conditions such as systemic lupus erythematosus (SLE) often worsen. However over the past decade it has been shown that this type 1/ type 2 dichotomy is too simplistic to explain the immune regulation of pregnancy. Firstly, normal pregnant women exhibit a low-grade systemic inflammatory response, including increases in pro-inflammatory cytokines (e.g. TNF-α) relative to non-pregnancy (Germain *et al.*, 2007c). Secondly, it is clear that the process of implantation is in itself inflammatory. Although it is not possible to study in vivo peri-implantation events in humans, type 1 cytokines are present in the murine uterus at the time of implantation (McMaster *et al.*, 1992; Sanford *et al.*, 1992; Croy *et al.*, 2002). In addition, recent evidence suggests that cells of the innate rather than acquired immune system, such as Natural Killer (NK) cells, may be dominant in maternal immune regulation (Borzychowski *et al.*, 2005). However, a shift towards type 2 immunity undoubtedly occurs in normal pregnancy.

1.3 Uterine and peripheral immune cells

1.3.1 Uterine immune cells

The process of transforming the uterine endometrium into the decidua of pregnancy begins before embryo implantation, in the luteal phase of the menstrual cycle. One of the most notable changes occurs post ovulation, when there is a dramatic increase in NK cells (King *et al.*, 1989). In the absence of implantation, not only does the endometrium shed and subsequently regenerate, but the population of NK cells rapidly declines and then reappears in the next menstrual cycle. If implantation and pregnancy occurs, uterine NK cells persist until mid-gestation where they account for around 70% of the decidual leukocytes (King, 2000). Uterine NK cells have a characteristic phenotype and appear to have a role in both trophoblast invasion and vascular remodelling in pregnancy (Hanna *et al.*, 2006; Lash *et al.*, 2006).

Uterine NK cells do not express CD16 (the FCγ receptor III), but express very high levels of CD56 (Neural Cell Adhesion Molecule (NCAM)) (CD16-$CD56^{superbright}$ NK cells). In addition, they express killer immunoglobulin receptors (KIRs) and contain perforin and granzymes and therefore have cytotoxic potential (King *et al.*, 1991; King *et al.*, 1993; Verma *et al.*, 1997). Uterine NK cells produce a number of mitogenic and angiogenic stimulants including vascular endothelial growth factor (VEGF) and placental growth factor (PLGF) (Li *et al.*, 2001). Cytokines and chemokines such as IL-8, interferon inducible protein 10 (IP-10) and chemokine ligand-5 (CCL5) are also expressed by uterine NK cells. Once implantation has occurred, uterine NK cells promote trophoblast invasion by producing ligands (e.g. IL-8 and IP-10)

for chemokine receptors (e.g. CXCR-1, 3 and 4 and CCR3) expressed on invasive trophoblast. Decidual stromal cells have also been shown to express ligands for the activating NK receptors NKp30 and NKp44 (Hanna *et al.*, 2006).

One immune mechanism that may modulate trophoblast invasion is the interaction between maternal uterine natural killer cells and the trophoblast cells. Extravillous cytotrophoblast cells have been shown to express paternal as well as maternal HLA-C allotypes (Apps *et al.*, 2008; Hiby *et al.*, 2010). Killer Immunoglulin-like Receptors (KIRs) on uterine NK cells interact with HLA-C influencing trophoblast invasion. The HLA-C ligands for KIRs are divided into two groups. Group 1 (C1) bind inhibitory KIRs whereas group 2 (C2) bind both inhibitory and activating KIRs. There are two major KIR haplotypes (A and B). They differ principally in that the B haplotype has additional activating receptors. Individuals who are homozygous for KIR haplotype A (KIR AA) have no activating receptor and this particular genotype has been shown to be associated with recurrent miscarriage, IUGR and pre-eclampsia (Hiby *et al.*, 2004; Hiby *et al.*, 2008). More specifically, the interaction between the maternal KIR-AA haplotype and fetal extravillous trophoblast cells expressing paternally inherited HLA-C2 ligands increases the risk of these pregnancy complications (Hiby *et al.*, 2010).

Invariant Natural Killer T (iNKT) cells are a small population of immune cells expressing the invariant T cell receptor that consists of two chains: Vα-24 and Vβ-11 (Porcelli *et al.*, 1993; Prussin & Foster, 1997). iNKT cells are activated by the presentation of glycolipid antigens by CD1d (a molecule homologous to

the major histocompatibility-1 (MHC-1) complex which presents peptide ligands to classical T cells). Activated iNKT cells produce both type 1 and type 2 cytokines, and influence further NK and T cell activation. iNKT cells have been shown to be present in first trimester decidua at ten times the frequency seen in the periphery (Boyson *et al.*, 2002). Treatment of pregnant mice with α-gal-cer (the exogenous glycolipid ligand for iNKT cells) leads to miscarriage due to IFN-γ release, suggesting a possible role for iNKT cells in miscarriage.

T cells are a relatively rare cell population in the uterus (accounting for approximately 10% of uterine immune cells) and vary little over the course of the menstrual cycle or in the early pregnancy decidua. During pregnancy, CD8+ T cells are the largest population of T cells at the maternal-fetal interface (Tilburgs *et al.*, 2009b). In HLA-C mismatched pregnancies there is an increase in CD4+CD25^{bright} regulatory T cells in the decidua (Tilburgs *et al.*, 2009a) and it has been hypothesised that in this situation these cells may suppress T cell activation so that no detrimental allogeneic response is initiated (Tilburgs *et al.*, 2010).

1.3.2 Differences between peripheral and uterine cells

There are significant differences between the immune cell populations found in the peri-implantation endometrium and those circulating in peripheral blood. In contrast to uterine NK cells, there are two distinct populations of NK cells that circulate in the peripheral blood. The majority (90%) of peripheral NK cells express CD16 and have low levels of CD56, and are therefore termed CD16+CD56^{dim} (NK^{dim}) cells. They are highly cytotoxic. The second population of peripheral NK cells have low cytotoxic potential, but produce high levels of

cytokines. These cells lack CD16 expression but have high levels of CD56 and are therefore known as CD56NK^{bright} (NK^{bright}) cells. Although this smaller NK^{bright} population is often reported as being the same as the uterine NK population, it is clear that these populations are phenotypically different to the uterine CD16-CD56^{superbright} NK cells. In addition to differences in CD56 expression, peripheral blood NK^{bright} cells (unlike uterine NK cells) do not express KIRs (Jacobs, 2001).

The relationship between uterine and peripheral NK cells is unclear. The origin of uterine NK cells is not known, however recent studies have suggested that peripheral NK cells, or even their precursors, are recruited to the endometrium, in the secretory phase of the cycle, from the peripheral blood (Chantakru, 2002; Carlino *et al.*, 2008; Male *et al.*, 2010). This theory has been questioned however, as in one study uterine NK cells were found to lack the necessary chemokine receptors in both the proliferative and secretory phase making migration from the periphery unlikely (Manaster *et al.*, 2008). In addition, recent data has suggested that uterine NK cells may arise from CD34+ haemopoietic precursors found in the endometrium that differentiate on contact with stromal cells (Vacca *et al.*, 2011).

Like NK cells, iNKT cells that reside in the decidua of pregnant women have a different phenotype from those in the peripheral blood. CD4+ uterine iNKT cells are biased towards IFN-γ/type-1 cytokine production upon antigenic stimulation, whereas CD4+ iNKT cells in the periphery produce IL-4/type-2 cytokines (Boyson *et al.*, 2002). Peripheral iNKT cells may be of particular

interest in pregnancy as they have been shown to regulate NK cell activity and appear to have a role in priming type 2 immune responses (Prussin & Foster, 1997; Carnaud *et al.*, 1999; Singh *et al.*, 1999; Eberl & MacDonald, 2000; Southcombe *et al.*, 2010).

1.4 Aims of this thesis

The introduction to this thesis has described the significant differences between peripheral and uterine immune cells. However, scientists and clinicians working in reproductive immunology still debate the relevance or significance of peripheral cells in reproductive failure. Some authors argue very firmly that differences in the two cell populations mean that measuring peripheral cells is illogical precisely because the phenotypes are so different (Moffett *et al.*, 2004). Others suggest that they may, at the very least, be a marker of immune dysfunction that could potentially be amenable to treatment (King *et al.*, 2010). There is as yet no scientific consensus in this debate.

This thesis aims to investigate two specific areas. Firstly, to determine whether there are constitutive, pre-pregnancy differences in circulating immune cells between women with recurrent miscarriage and controls, at defined points of the menstrual cycle. Secondly, by recruiting women undergoing in vitro fertilisation (IVF) treatment (in order to obtain blood samples at known time points soon after potential implantation) it aims to investigate whether there are pre-pregnancy differences in peripheral immune cells in women who have a successful cycle and those who don't. In addition, peripheral changes in the early post implantation period are investigated.

Although some authors argue that in the early stages of pregnancy the syncytiotrophoblast is in direct contact with maternal blood (Clark, 2008), prior to 8 weeks' gestation the embryo receives histiotrophic nutrition (Burton *et al.*, 2002). Therefore the hypothesis of this thesis was that peripheral immune cells

would neither affect, nor reflect immune cells changes in the uterus before this time. No differences in peripheral immune cells or their type 1/ type 2 bias were anticipated between women with a history of recurrent miscarriage and controls at any phase of the menstrual cycle. In addition, no pre-pregnancy differences were expected between women who achieved implantation after IVF and those who did not. Given the lack of contact between the embryo and maternal circulating immune cells, no changes were predicted in circulating immune cells or their type 1/ type 2 bias in the first 6 weeks of pregnancy.

Chapter 2 investigates circulating NK, iNKT and T cells and their immune bias throughout the menstrual cycle of women with recurrent miscarriage and controls.

Chapter 3 investigates NK, iNKT and T cells and their immune bias in very early pregnancy by studying women undergoing a cycle IVF.

During the course of this work, interesting data regarding ST2L (used as a marker of type 2 cells) led me to investigate this receptor, its soluble decoy receptor, sST2 and its ligand IL-33 in the context of normal pregnancy (presented in chapter 4) and pre-eclampsia (chapter 5).

2. Do peripheral NK, iNKT and T cells change in the normal menstrual cycle and in women with recurrent miscarriage?

2.1 Introduction

In order to establish if a real relationship exists between peripheral immune cells and pregnancy pathology such as implantation failure or recurrent miscarriage it is important to first establish normal parameters throughout the menstrual cycle. This chapter addresses that question by investigating peripheral NK, iNKT and T cells at various time points in normal menstrual cycles, and concludes by comparing those to cycles of women who have a history of recurrent unexplained miscarriage.

2.1.1 Hormonal control of the menstrual cycle

The cyclical changes that occur in the female reproductive system do so in preparation for implantation and pregnancy. If implantation does not occur then menstruation follows. The average menstrual cycle length is around 28 days, although healthy ovulating women may commonly have cycle lengths between 26 and 35 days. By convention, the first day of the cycle is the first day of menstruation. The first part of the cycle, when the dominant follicle develops and subsequently ovulates is known as the follicular or proliferative phase. The second phase of the cycle is known as the luteal or secretory phase.

The regulation of the menstrual cycle is complex, involving both positive and negative feedback along the hypothalamic-pituitary-ovarian (HPO) axis. Pulsitile release of gonadotrophin releasing hormone (GnRH) from the hypothalamus acts on the pituitary to stimulate pulsitile release of two gondaotrophins: follicle stimulating hormone (FSH) and luteinising hormone (LH).

The actions of gonadotrophins are to promote follicular development and ovulation and to stimulate steroid hormone production. At the beginning of each menstrual cycle a number of primordial follicles (containing immature oocytes) begin to enlarge. Around day 6, one follicle will become dominant whilst the rest become atreitic. The dominant follicle develops two layers of cells capable of steroidogenesis. The inner layer of cells is called granulosa. FSH stimulation of these cells causes the aromatisation of androgens to produce oestrogen. The outer layers of cells are called theca cells. They respond to LH and synthesise progesterone and androgens from cholesterol. A surge of LH induces final maturation of the oocyte, subsequent rupture of the follicle and ovulation. Ovulation occurs 24-36 hours after the LH surge. Detection of this surge forms the basis of commercially available ovulation kits.

After ovulation, the ruptured follicle develops into the progesterone secreting corpus luteum which persists for 10 days unless pregnancy occurs. Without the stimulus of human chorionic gonadotrophin (HCG) (secreted from the early implanting embryo), the corpus luteum degenerates into the corpus albicans that does not continue to produce progesterone. The withdrawal of

progesterone support to the endometrium causes its degeneration and the onset of menstruation. The luteal phase of the menstrual cycle is almost always 14 days in length because of the fixed lifespan of the corpus luteum. Therefore variations seen in menstrual cycle length are usually related to the differing length of the follicular phase.

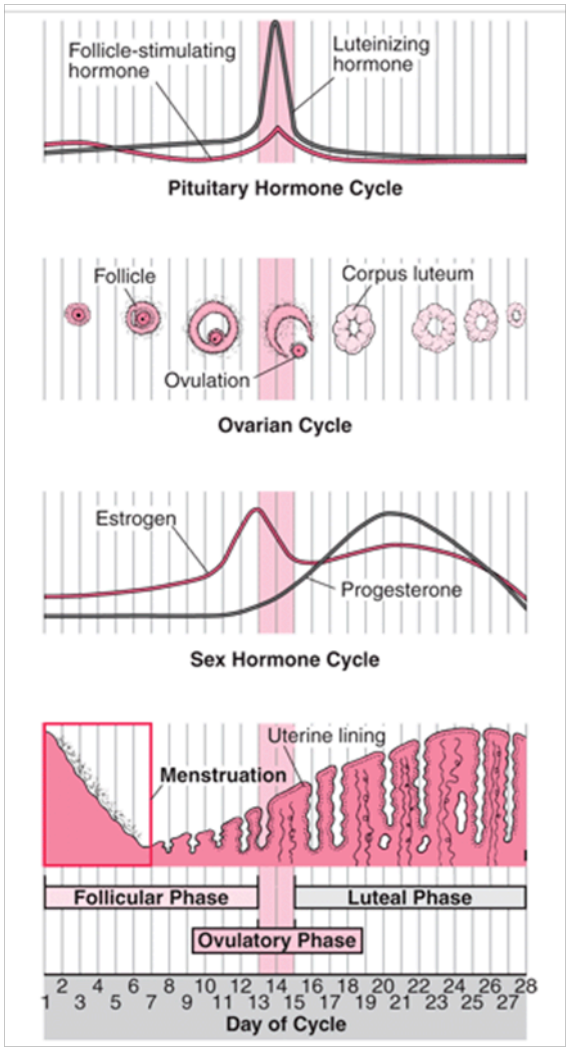


Figure 2-1 The phases and relative hormone levels of the menstrual cycle. Adapted from www.merck.com

2.1.2 The effect of the menstrual cycle on peripheral immune cells

Previous studies analysing the effect of the menstrual cycle on peripheral immune cells are conflicting. One study found an increase in NK cell numbers in the peri-ovulatory (Yovel *et al.*, 2001) phase, although this study was small (between 6 and 9 subjects in each group). Others have found an increase in the numbers of NK, T helper cells and cytotoxic T cells in the mid-luteal phase compared to the mid-follicular phase of the cycle (Faas *et al.*, 2000; Bouman *et al.*, 2001). Recently one group identified a decreased percentage of Th cells and an increase in $CD56^{dim}$ NK cells (as a proportion of the whole lymphocyte population) in the mid luteal phase (Lee *et al.*, 2010). Although the same study investigated CD3+CD56+ NKT-like cells, no published studies have addressed the effect of the menstrual cycle on circulating iNKT cells.

A number of small studies have addressed peripheral immune cell function during the menstrual cycle, either by using cytotoxicity assays (for NK cells) or by measuring cytokine production. One study found a decrease in NK cytotoxicity (Souza *et al.*, 2001) in the mid-luteal phase that did not correlate with levels of progesterone, however another study failed to confirm this finding (Yovel *et al.*, 2001). Other studies have shown a decrease in NK activity in the peri-ovulatory phase (Sulke *et al.*, 1985a, b). It is possible any changes may be driven by pregnancy related hormones, however progesterone has not been shown to affect peripheral NK activity in vitro when used at physiological concentrations (Uksila, 1985), and although high doses of 17-β oestrodiol

suppressed NK cell activity, more physiological doses of 17-β oestrodiol were found to have no effect (Ferguson & McDonald, 1985).

Several groups have investigated lymphocyte production of intracellular cytokines (IFN-γ, IL-2, IL-4 and IL-10) by stimulating cells with PMA and calcium ionophore from women in the follicular or luteal phases of the cycle. Again, conflicting reports emerged with one group finding no differences in cytokine production (Faas *et al.*, 2000) whereas another reported a decrease in IL-2 production in the mid-luteal phase (Trzonkowski *et al.*, 2001). In a study of 13 women, stimulated lymphocytes produced increased levels of intracellular IL-4 in the Th cell population, and so the authors concluded there was a type 2 shift in the mid-luteal phase of the menstrual cycle (Piccinni *et al.*, 1995). When intracellular cytokines were measured in NK cells, there was no variation between the luteal and follicular phase (Bouman *et al.*, 2001).

These inconclusive and often contradictory findings may reflect the different methods of measuring cell numbers (as a percentage or absolute count), type of stimulation, variations in the length of time cells were stimulated prior to cytokine measurement and the period of time cells were allowed to be activated post stimulation. In addition there is variation in the methods of determining the precise time points of the menstrual cycle. This study aimed to look once again at this issue; defining time points carefully and using cell surface markers of type 1 and type 2 cytokine producing cells in order to avoid in vitro stimulation of cells.

2.1.3 Peripheral immune cells and recurrent miscarriage

Miscarriage is the commonest complication of pregnancy. There are many different known causes of miscarriage; including endocrine dysfunction, structural abnormalities, autoimmune disorders and most commonly, chromosomal abnormalities. Miscarriage may be sporadic or recurrent. Most authors agree that the definition of recurrent miscarriage is three consecutive miscarriages. Approximately one percent of couples will suffer recurrent miscarriage. This observed frequency is much higher than expected by chance alone (0.34%) and in only 50% of those women will a cause be identified (Quenby & Farquharson, 1993; Rai & Regan, 2006).

There is increasingly strong evidence that high numbers of uterine NK cells are associated with recurrent unexplained miscarriage. Several studies have detected increased numbers of CD56+ cells in the mid-luteal phase of the cycle in women with recurrent miscarriage compared to controls (Clifford *et al.*, 1999; Quenby *et al.*, 1999; Quenby *et al.*, 2005; Tuckerman *et al.*, 2007). One further study showed increased numbers of CD16+CD56^{dim} cells and decreased numbers of CD16-CD56^{bright} cells in the mid-luteal endometrium of women with recurrent unexplained miscarriage (Lachapelle *et al.*, 1996).

The potential value of measuring peripheral immune cells is that they may be reflective of endometrial pathology and are (in contrast to endometrial biopsy) a simple sample to obtain. Even though there are clear phenotypic and functional differences between peripheral and decidual NK cells, several reports have associated pregnancy failure (either recurrent unexplained

miscarriage or implantation failure after IVF) with an increase in peripheral blood NK cells, either when expressed as a percentage of total lymphocytes (Beer *et al.*, 1996; King *et al.*, 2010) or as an increased absolute count of NK cells (Thum *et al.*, 2005). In addition, expression of early markers of activation, for example the glycoprotein CD69, on NK cells (Thum *et al.*, 2004) or T cells (Aramilena *et al.*, 2008) has been suggested to be related to poor pregnancy outcome.

In mice, stimulation of iNKT cells has been shown to cause miscarriage via both a perforin dependent and independent pathway, the latter being associated with increased serum type 1 cytokine levels (Boyson *et al.*, 2006). There are no data investigating the relationship between peripheral iNKT cells and recurrent miscarriage in humans, although one small non randomised controlled study suggested that intravenous immunoglobulin increased the ongoing pregnancy rate with women with 'high' CD3+CD56+ NKT cells (Marianne *et al.*, 2007).

As previously discussed, it is clear that successful pregnancy is associated with a shift towards type 2 immunity. There are a number of studies claiming an association between a type 1 phenotype and miscarriage, either in circulating cytokine levels, or in production of cytokines by stimulated lymphocytes. In the majority of these studies however, blood samples were taken at the time of the miscarriage and the results may reflect the inflammatory response to a miscarriage rather than reflect the underlying

cause (Makhseed *et al.*, 2000; Raghupathy *et al.*, 2000a; Makhseed *et al.*, 2001a; Wilson *et al.*, 2004b).

This study aims to re-investigate this question by determining whether there are constitutive, pre-pregnancy differences between women with recurrent miscarriage and controls, which in some way pre-dispose an individual to pregnancy failure. This study is unique in investigating changes at multiple time points of the cycle in women with recurrent unexplained miscarriage. Others have focused on changes at miscarriage diagnosis or in early pregnancy or by looking at unspecified pre-pregnancy time points.

2.1.4 Markers of type 1/ type 2 cells and potential cytokine production

Type 1 and type 2 shifts in the peripheral immune response have classically been identified by measuring cytokine levels secreted in plasma or from artificially stimulated isolated peripheral blood mononuclear cells (PBMCs). It is not clear, using these methods, from which cells particular cytokines are secreted. Intracellular cytokine staining using flow cytometry is an alternate method for addressing this question but it too has limitations. Intracellular cytokine staining requires in vitro stimulation of cells prior to their being fixed and permeabilised. This process may potentially alter their in vivo phenotype (Pala *et al.*, 2000; Sacks *et al.*, 2001). Although cell sorting enables the purification of individual cell lines, this method would not be practical to isolate each of the individual cell types investigated in this thesis. Therefore in order to classify the cells into type 1 or type 2 cytokine producers it would be

advantageous to have a stable cell surface marker for each. The IL-18R receptor and the ST2L receptor have been identified as such stable cell surface markers of type 1 (IL-18R) and type 2 (ST2L) deviated T and NK cells respectively (Xu *et al.*, 1998a; Xu *et al.*, 1998b; Chan *et al.*, 2001). The IL-18 receptor is structurally similar to the IL-1 receptor complex. IL-18 binds to the IL-18R and the resulting complex recruits the IL-18R accessory protein known as AcPL, forming a heterodimeric complex that initiates signalling (Bohn *et al.*, 1998; Thomassen *et al.*, 1998; Arend *et al.*, 2008). IL-18 acts on type 1 deviated cells to increase production of IFN-γ. Although IL-18 may also act on Th2 cells under certain circumstances (such as allergic inflammation) the selective expression of IL-18R on Type 1 T cells (and in very low levels in undifferentiated T cells) and NK cells makes it a useful distinguishing marker.

ST2L is also expressed by both NK and T cells. It is a transmembrane protein, similar to the IL-1 receptor. ST2L forms a heterodimer with the IL-1 receptor accessory protein (IL-1RAcP) (Palmer *et al.*, 2008). The ligand for ST2L is the cytokine IL-33. IL-33 is a potent activator of Type 2 cells and has been shown to stimulate production of IL-4, IL-5 and IL-13 (Schmitz *et al.*, 2005) .We have previously used these markers in a study of pre-eclampsia, where in normal pregnancy increased expression of ST2L compared to IL-18R was seen. This type 2 shift was not seen in pre-eclamptic pregnancies, instead a type 1 shift was identified, which was most apparent in the peripheral NK cell population (Borzychowski *et al.*, 2005).

To distinguish the phenotype of iNKT cells, CD4 and CD8 have been previously used as cell surface markers. Peripheral iNKT cells expressing CD4

have been shown to produce high levels of IFN-γ and IL-4 (i.e. a Th0 response). Double negative (CD4-/CD8-) iNKT cells express high levels of IFN-γ but low levels of IL-4 (i.e a Th1 response) whilst CD8+ iNKT cells produce only IFN-γ (Takahashi *et al.*, 2000; Gumperz *et al.*, 2002; Lee *et al.*, 2002; Takahashi *et al.*, 2002). At the time these studies were planned there was no published data as to the expression of ST2L or IL-18R on iNKT cells and no evidence as to whether in this population they would represent stable type 1/ type 2 surface markers. Hence CD4 and CD8 were used in these studies to phenotype iNKT cells and therefore the type 1/ type 2 ratio could not be calculated. Since then it has been shown that ST2L is expressed by iNKT cells, however IL-33 stimulation of iNKT cells not only increases type 2 cytokines but also IFN-γ (Smithgall *et al.*, 2008).

2.1.5 CD69 as a marker of cell activation

CD69 is a human transmembrane C-Type lectin. It appears to be the earliest inducible cell surface glycoprotein after cell activation has occurred (Ziegler *et al.*, 1993). It has also been suggested that CD69 may be a regulatory molecule modulating the inflammatory response (Sancho *et al.*, 2005). Increased CD69 expression by peripheral NK and T cells has been suggested by a number of authors to be associated with poor reproductive outcomes including sub-fertility, recurrent miscarriage and IVF failure (Thum *et al.*, 2004).

2.2 Aims

1 To establish whether the numbers of peripheral blood NK cells, iNKT cells, Th cells and cytotoxic T cells (CTLs) vary throughout normal menstrual cycles.

2. To investigate whether there are any changes to the type 1/ type 2 bias of peripheral blood NK, iNKT, Th and CTL cells throughout normal menstrual cycles.

3. To investigate the effects of the menstrual cycle on the numbers and immune bias of peripheral blood NK, iNKT, Th cells and CTLs in women with unexplained miscarriage.

2.3 Materials and methods

2.3.1 Subject recruitment

Ethical approval for these studies was obtained from the Oxford Research Ethics Committee C. Written informed consent was given prior to venepuncture.

2.3.1.1 Menstrual cycle study

Women aged 38 or less (n=16) who were not using hormonal contraceptives and had regular cycles (between 26 and 35 days) were recruited. Characteristics of the group are seen in Table 2-1. All were non-smokers. Blood samples were taken between 8.00 and 10.00am (as peripheral immune cells may be subject to some diurnal variation (Northern *et al.*, 1994)) and analysed immediately. Blood samples were taken at four time points over the course of a cycle:

Baseline: Day 1-5 of the menstrual cycle

Mid-follicular: This was timed depending on the length of menstrual cycle, for example, day 8-10 of a 28 day cycle or day 10-12 of a 30 day cycle.

Peri-ovulation: This was timed using urinary LH sticks (InstAlert, Acon Labs, San Diego). Once the LH surge is detected this indicates that ovulation will occur in approximately 36 hours. In each recruit a sample was taken between 36 and 60 hours of the LH surge (i.e. within 24 hours of ovulation)

Mid-luteal: 7-9 days post ovulation. Ovulation was confirmed by measuring serum progesterone. Serum progesterone of ≥16 ng/ml was considered indicative of ovulation.

	Controls
Age	28.4 (5.2)
Nulliparity	8/16
Number of miscarriages	0
Mid-luteal progesterone (ng/ml)	48 (22)

Table 2-1 Clinical characteristics of women recruited to the normal menstrual cycle study, with no history of miscarriage or subfertility. Data are shown as mean (+/- standard deviation).

2.3.1.2 Recurrent miscarriage menstrual cycle study

Women aged 38 or less (n=10) with a history of at least 3 consecutive unexplained miscarriages were recruited from the John Radcliffe Hospital Recurrent Miscarriage Clinic. All women had previously undergone a full recurrent miscarriage screen. This screen comprises a full blood count, follicle stimulating hormone (FSH), luteinising hormone (LH), testosterone, prolactin, thyroid stimulating hormone (TSH), full thrombophilia screen (anti-cardiolipin antibodies, lupus anticoagulant, protein C, protein S, anti-thrombin III, factor V leiden), karyotype from both the male and female partner and trans-vaginal ultrasound to exclude uterine anomalies. Only women who screened negative in all these investigations were recruited. Women with a diagnosis of polycystic ovarian syndrome were also excluded from the study. Samples were taken at the same four times points as in the menstrual cycle study.

Recurrent unexplained miscarriage patients were matched for age (+/- 4 years) and parity (0 or 1-2) with women (from the menstrual cycle study) who had never had a miscarriage. Both patients and controls were non-smokers as there is some evidence for altered peripheral NK cell numbers in smokers

(Hughes *et al.*, 1985; Tollerud *et al.*, 1989). Characteristics of patients and matched controls are seen in Table 2-2.

	Controls	Recurrent miscarriage	
Age	32.5 (3.10)	32.6 (3.65)	N.S.
Nulliparity	6/10	6/10	N.S.
Number of miscarriages	0	4.9 (1.59)	P< 0.001.
Mid-luteal progesterone (ng/ml)	46 (23)	42 (21)	N.S.

Table 2-2 Clinical characteristics of women with recurrent miscarriage and controls. Data are shown as mean (+/- standard deviation). N.S. = not significant.

2.3.2 Peripheral blood sampling and preparation of peripheral blood mononuclear cells (PBMCs)

15 ml venous blood was obtained and anti-coagulated with sodium heparin (10IU/mL) (Wockhardt, Wrexham, UK). Blood samples were diluted (1:1) with phosphate buffered saline (PBS) (Sigma-Aldrich, Gillingham, UK) + 0.5% fetal calf serum (FCS) (PAA, GmBH, Pasching, Austria) and underlayed with 10ml of lymphoprep (Axis Shield Diagnostics, Cambridgeshire, UK). PBMCs were separated by density gradient centrifugation and spun for 30 minutes at 300g. The buffy coat layer (between the plasma and lymphoprep) containing monocytes and lymphocytes was removed and placed in a 50ml Falcon tube. 20 ml of PBS+ FCS (0.5%) was added and the cells were washed at 825g for 10 minutes. The resulting pellet was re-suspended in 20ml PBS+FCS (0.5%) and washed for 10 minutes at 300g. The final pellet was re-suspended in 2ml of PBS+FCS (0.5%). 5µl of the cell suspension were diluted with 95µl of PBS. Cells were counted under the microscope with a haemocytometer to calculate

the total number of cells/ml. Initial experiments (n=3) assessing cell viability using TO-PRO-3 iodide (Invitrogen, Carlsbad, California, USA) showed a mean (+/- standard error of the mean (SEM)) viability of 96.3% (1.2) of CD3+ cells and 97.5 % (1.6) of the NK cell population.

2.3.3 Antibody labelling of peripheral blood mononuclear cells

NK^{bright}, NK^{dim}, iNKT cells, T helper cells and cytotoxic T cells were identified using six-colour flow cytometry. The following directly conjugated antibodies were purchased.

- CD3-PeCy5 (Biolegend, San Diego, USA)
- CD4-APC (e-Bioscience)
- CD8-PeCy7 (e-Bioscience)
- CD16-APC-Cy7 (Becton Dickinson (BD), Oxford, UK)
- CD56-PECy7 (BD)
- CD69-APC (BD)
- Vα-24-PE (Beckman Coulter, High Wycombe, UK)
- Vβ-11-FITC (Beckman Coulter)
- IL-18R-PE (Biolegend)
- ST2L-FITC (MD Biosciences, Zurich, Switzerland)

All antibodies were titrated with freshly isolated PBMCs to determine saturation concentrations (data not shown). Table 2-3 shows the three panels of six

antibodies used to identify each cell type, its type 1/ type 2 bias and early cell activation.

	Antibodies					
Fluorochromes	FITC	PE	PeCy5	APC-Cy7	PeCy-7	APC
NK cells	ST2-L	IL-18R	CD3	CD16	CD56	CD69
iNKT cells	Vβ-11	Vα-24	CD3	CD4	CD8	CD69
T lymphocytes	ST2-L	IL-18R	CD3	CD4	CD8	CD69

Table 2-3 Antibody-fluorochrome combinations used to identify NK, iNKT and T cells

iNKT cells are a relatively rare population of peripheral cells therefore increased numbers of cells (2x 10^6) cells were labelled to identify the iNKT population. 0.5x 10^6 cells were labelled to identify each of the T and NK cell populations. 'Fluorescence minus one' gating controls were used for CD69-APC in all panels and IL-18R-PE and ST2L-FITC in T and NK cell panels. Cells were stained and incubated at 4^{o}C for 30 minutes then washed twice in 1ml PBS + 0.5% FCS and spun down in a microcentrifuge at 10,000g for 5 seconds. The iNKT cell panel was re-suspended in 1ml PBS/FCS and T cell/ NK cell panels were re-suspended in 0.5ml PBS/FCS. Samples were analysed immediately.

2.3.4 Flow cytometry analysis

All samples were analysed using a Becton Dickinson LSR II flow cytometer (Oxford, UK) with a 488nm and a 633nm laser. Data were analysed using FACS DIVA software. Prior to each experiment a quality control process was performed to assess the calibration of the instrument using Rainbow Fluorescent Particles, 3.0-3.4μm (mid-range FL1 fluorescence) and Rainbow Calibration particles 3.0-3.4μm (8 peaks) (BD). In order to adjust for spectral overlap, fluorescence colour compensation was performed using single antibody-labeled compensation beads (BD). Colour compensation was performed prior to the first experiment and after any adjustment had been made to the laser alignments of the instrument over the course of the study.

In order to obtain an adequate number of each type of cell of interest, 100,000 events were acquired to identify NK cell populations, 1 $x10^6$ events were acquired to identify the iNKT cell population and 50,000 events to identify the T cell populations. Lymphocytes were distinguished from monocytes based on their forward and side scatter. Sub-populations of cells were identified within the lymphocyte population by their antigen expression.

2.3.4.1 Comparison of fluorescence minus one gating with and without IgG controls

It has been argued that the use of isotype controls is of limited value in multicolour flow cytometry (more than 4 colours) because it assumes that the isotype control has the same non-specific ‘stickiness’ as its matched antibody. In addition, when isotypes are used alone, spectral overlap is not considered

when designing gating strategies. Instead, many flow cytometry experts advise the use of fluorescence minus one (FMO) gating controls to determine where the negative staining gating lies, for all antibody-fluorochromes where the threshold may be uncertain (Herzenberg *et al.*, 2006; Mahnke & Roederer, 2007). For example, in order to determine CD69 expression in the NK panel, 100,000 cells were labelled with the six antibodies of interest and 100,000 cells were labelled with each of the antibodies apart from the CD69-APC antibody. Gating the CD69 negative cells using this second panel (without CD69-APC) ensures that the spectral overlap from all the other fluorochromes is apparent and therefore the gate and the increase in median fluorescence intensity (MFI) can be accurately determined.

Many groups still use IgG controls however. In order to establish whether significant differences could be identified between either method, each fluorescence minus one gating control was assessed with and without an IgG control for the NK population of cells (n=3). 0.5 $x10^6$ cells were labelled in each of 11 samples (see Table 2-4). For each fluorochrome the gate was set at 1% and the number of positive events was compared between the FMO gating and the equivalent IgG isotype control.

Tube	Antibody-Fluorochrome					
1	CD3-PeCy5	CD56-PeCy7	CD16-APC-Cy7	CD69-APC	ST2L-FITC	IL-18R-PE
2	CD3-PeCy5	CD56-PeCy7	CD16-APC-Cy7	-	ST2L-FITC	IL-18R-PE
2a	CD3-PeCy5	CD56-PeCy7	CD16-APC-Cy7	IgG1-APC	ST2L-FITC	IL-18R-PE
3	CD3-PeCy5	CD56-PeCy7	CD16-APC-Cy7	CD69-APC	-	IL-18R-PE
3a	CD3-PeCy5	CD56-PeCy7	CD16-APC-Cy7	CD69-APC	IgG1-FITC-	IL-18R-PE
4	CD3-PeCy5	CD56-PeCy7	CD16-APC-Cy7	CD69-APC	ST2L-FITC	-
4a	CD3-PeCy5	CD56-PeCy7	CD16-APC-Cy7	CD69-APC	ST2L-FITC	IgG1-PE
5	CD3-PeCy5	-	CD16-APC-Cy7	CD69-APC	ST2L-FITC	IL-18R-PE
5a	CD3-PeCy5	IgG-1-PeCy7	CD16-APC-Cy7	CD69-APC	ST2L-FITC	IL-18R-PE
6	CD3-PeCy5	CD56-PeCy7	-	CD69-APC	ST2L-FITC	IL-18R-PE
6a	CD3-PeCy5	CD56-PeCy7	IgG-1-APC-Cy7	CD69-APC	ST2L-FITC	IL-18R-PE
7	-	CD56-PeCy7	CD16-APC-Cy7	CD69-APC	ST2L-FITC	IL-18R-PE
7a	IgG-1-PeCy5	CD56-PeCy7	CD16-APC-Cy7	CD69-APC	ST2L-FITC	IL-18R-PE

Table 2-4 Antibody fluorochrome and IgG combinations used to compare FMO gating with the use of isotype controls.

No significant differences were identified in the expression of any fluorochome in each of the experiments carried out (data not shown).

2.3.4.2 *Identification of NK cells*

CD3 is a signaling complex associated with the T cell receptor and therefore NK cells were identified within the CD3 negative lymphocyte population. The two distinct NK populations were identified using CD16 and CD56 expression. Population gates were then drawn on NK^{bright} ($CD16^{-}CD56^{bright}$) and NK^{dim} ($CD16^{+}CD56^{+}$) cells (Figure 2-2).

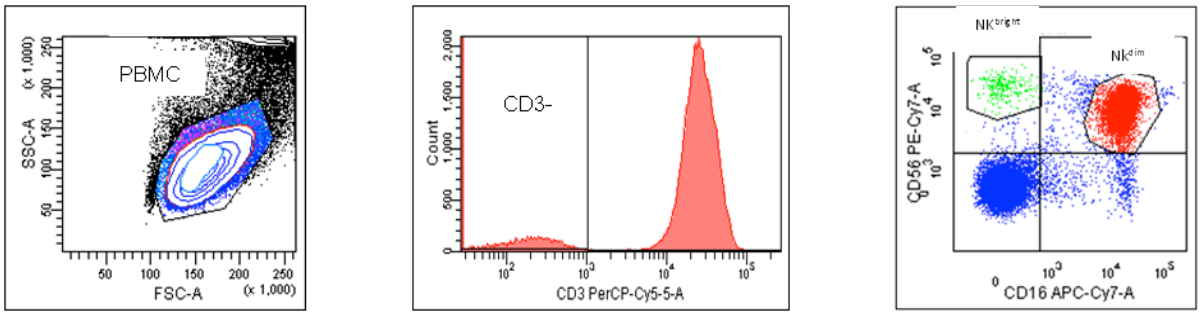


Figure 2-2 Flow cytometry identification of NK cell populations

Each population of cells was then analysed for expression of IL-18R, ST2L and CD69 in order to quantify their type 1 or type 2 bias and cell activation (Figure 2-3).

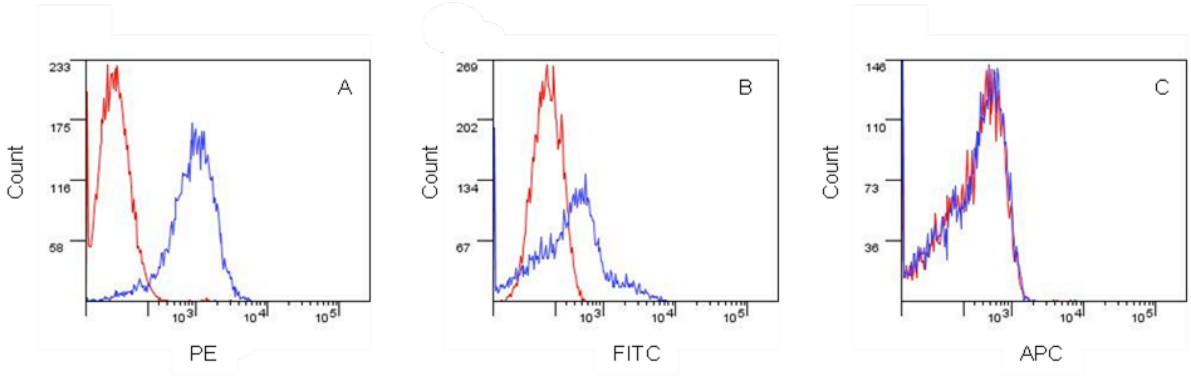


Figure 2-3 Histograms showing an example of typical expression of (A) IL-18R-PE, (B) ST2L-FITC and (C) CD69-APC (where minimal expression was seen in the majority of experiments on NK^{dim} cells. In each case the red line represents the FMO control and the blue line represents the antibody expression.

2.3.4.3 NK^{dim} cells

Plots of side scatter verses PE (IL-18R), FITC (ST2L) and APC (CD69) were gated on the NK^{dim} population and the gates were set at <1% positive cells using three separate panels of fluorescence minus one (FMO) gating controls (Table 2-5). Expression of each of IL-18R, ST2L and CD69 by NK^{dim} cells was measured in three ways:

1. The percentage of IL-18R, ST2L and CD69 positive cells was determined.
2. The absolute count/ml blood of type 1 and type 2 cells and the number of activated cells was quantified in relation to the absolute count of CD3+ cells.
3. The median fluorescence intensity of IL-18R, ST2L and CD69 was measured

The ratio of type 1 to type 2 cells was then determined using each of these methods.

2.3.4.4 NK^{bright} cells

The type 1 or type 2 bias and cell activation in the NK^{bright} population of cells was determined by quantifying the increase in median fluorescence intensity (MFI) using three separate panels of fluorescence minus one (FMO) gating controls (Table 2-5). The absolute count (see section 2.3.4.7) of CD69+, IL-18R+ or ST2L+ cells was not determined. This is because the number of NK bright cells is relatively small (e.g.200-400 cells out of 100,000 events). Therefore it was difficult to accurately set a <1% gate and so the MFI was used

to determine the level of positive staining. The ratio of type 1 to type 2 expression was also determined.

Tube	Antibody-Fluorochrome					
1	CD3-PeCy5	CD56-PeCy7	CD16-APC-Cy7	CD69-APC	ST2L-FITC	IL-18R-PE
2	CD3-PeCy5	CD56-PeCy7	CD16-APC-Cy7	-	ST2L-FITC	IL-18R-PE
3	CD3-PeCy5	CD56-PeCy7	CD16-APC-Cy7	CD69-APC	-	IL-18R-PE
4	CD3-PeCy5	CD56-PeCy7	CD16-APC-Cy7	CD69-APC	ST2L-FITC	-

Table 2-5 Three separate fluorescence minus one gating controls were used (tubes 2-4) to enable accurate gating of IL-18R, ST2L and CD69 expression

2.3.4.5 Identification of iNKT cells

iNKT cells were identified within the CD3+ lymphocyte population by gating double positive populations for Vα-24+ and Vβ-11 (Figure 2-4A). Vα-24+ and Vβ-11 are the two chains of the invariant NKT cell receptor. Sub-populations of CD4+ and CD8+ cells were identified within the iNKT population (Figure 2-4B). Although ideally an FMO gating control should be used for each antibody, this is not practical when collecting large numbers of events to investigate rare cell population. In fact, initial experiments using FMO for each antibody showed that for each of the antibodies other than CD69, there was a very clear shift from negative to positive and therefore FMO gating was only used to determine the expression of CD69 (Table 2-6).

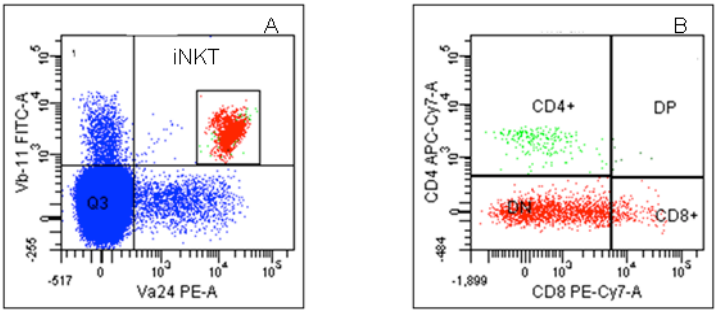


Figure 2-4 Flow cytometry identification of (A) iNKT cells and (B) CD4 and CD8 expression

Tube	Antibody-Fluorochrome					
1	CD3-PeCy5	Vβ-11-FITC	Vα-24-PE	CD4-APC-Cy7	CD8-PeCy7	CD69-APC
2	CD3-PeCy5	Vβ-11-FITC	Vα-24-PE	CD4-APC-Cy7	CD8-PeCy7	-

Table 2-6 A fluorescence minus one gating control were used (tube 2) to enable accurate gating of CD69 expression

2.3.4.6 Identification of T cell populations

CD4 is a marker of T helper (Th) cells. It binds to class II MHC molecules, restricting Th cells to peptides presented by class II MHC. CD8 is a T cell co-receptor and a marker of cytotoxic lymphocytes (CTLs), restricting these cells to peptides presented by class I MHC molecules. Quadrant gates were used to identify T helper (Th) cells (CD3+CD4+) and cytotoxic T lymphocytes (CD3+CD8+) (Figure 2-5). The percentage, absolute count and median fluorescence intensity of type 1 and type 2 cells and cells activation (CD69 expression) was determined in the same way as for the NK^{dim} population.

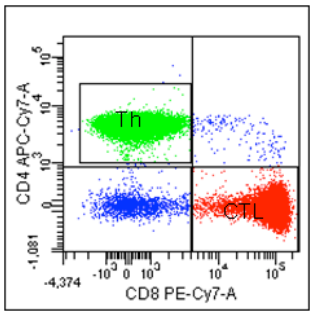


Figure 2-5 Flow cytometry identification of CD4+ T helper (Th) and CD8+ cytotoxic (CTL) T lymphocytes.

2.3.4.7 Determining the absolute count of cells

The concentrations of PBMC and CD3+ T cells were determined by flow cytometry using Cytognos Perfect Count beads (Caltag-MedSystems, Buckingham, UK). Using a reverse pipetting technique, 50µl whole blood was incubated with CD3-PeCy5 antibody (e-Biosciences) for 15 minutes at room temperature in the dark. Red blood cells were then lysed with a 1:10 dilution of 10x FACS Lysing Solution (BD) for 15 minutes at room temperature in the dark. 50µl of Perfect Count beads were added. 50,000 events were acquired by flow cytometry. The concentration of PBMC or CD3 positive T cells per ml blood was calculated using the formula:

$$\text{Absolute count (cells/ml blood)} = \frac{\text{number of the target sub-population counted x number of Perfect-Count/µl x 1000}}{\text{Total number of the microspheres counted}}$$

2.4 Statistics

The Gaussian distribution of each data set was assessed using a Kolmogorovv-Smirnov test. Clinical data was compared using a paired t-test. In the longitudinal studies, where the data was found to be normally distributed, a repeated measures ANOVA was used to identify significant variations across the cycle. Where significant differences were identified a Bonferroni post hoc test was used to establish which time points were significantly different. For non-parametric longitudinal data a Kruskal Wallace test was used and where a significant variation across the cycle was identified a Dunn's post hoc test was used to establish the time points with significant differences. In the study where women with recurrent miscarriage were matched to controls an ANOVA was used to identify significant differences between the groups and to establish the presence or absence of a significant group interaction or a group phase interaction. Where differences were identified a post hoc t-test was used to calculate at which specific time points differences were seen. Where significant differences were identified between miscarriage patients and controls a Receiver Operator Characteristics (ROC) curve was generated. Values of $p<0.05$ were considered to be significant. Graphpad Prism (San Diego, California, USA) and SPSS (Chicago, Illinois, USA) software were used to analyse the data.

2.5 Results

2.5.1 The effect of the menstrual cycle on peripheral NK, iNKT and T cells in the women with no history of miscarriage

Peripheral blood NK, iNKT and T cells were measured at four defined time points in the menstrual cycle of normal women in order to determine whether there were changes in cell numbers, expression of the IL-18R or ST2L receptors on NK and T cells, or CD4 and CD8 expression on iNKT cells and hence the type 1/ type 2 bias of these cells throughout the menstrual cycle. In addition, expression of CD69 by each of the cells populations was determined as a measure of cell activation.

2.5.1.1 NK cells

There was no effect of the menstrual cycle on the numbers of NK^{dim} cells either when measured as a proportion of the lymphocyte population or when the absolute numbers of cells/ml of blood was calculated (Figure 2-6).

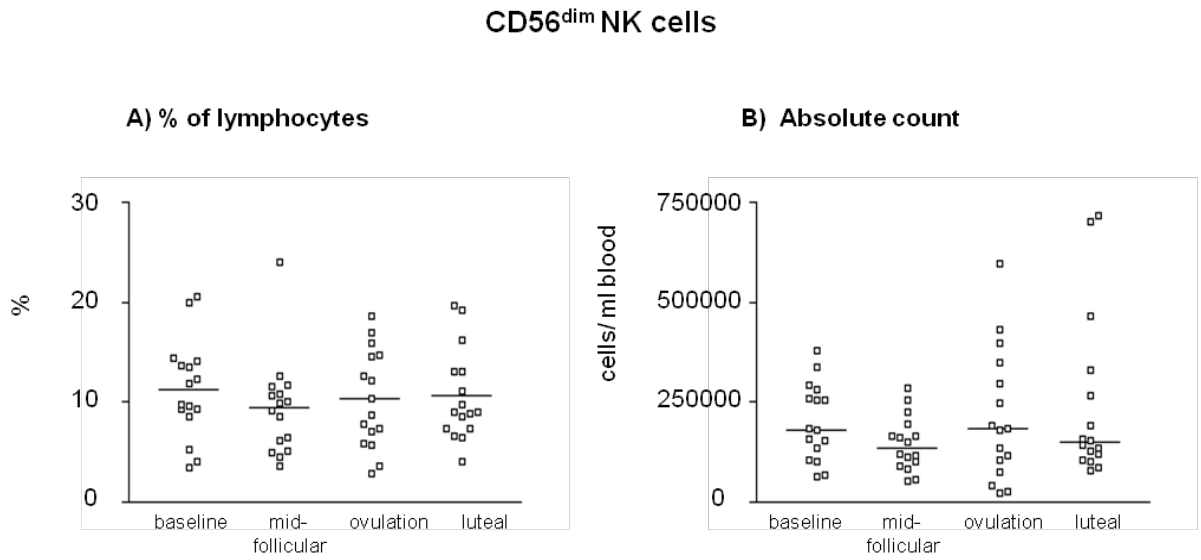


Figure 2-6 NK^{dim} cells throughout the normal menstrual cycle. (A) The percentage of lymphocytes that were NK^{dim} cells. (B) The absolute count of NK^{dim} cells. Bars represent mean values (n=16).

The percentage of cells expressing CD69 was measured together with the absolute count of CD69+ cells and the increase in the median fluorescence intensity of CD69-APC staining. There was no variation in any of these parameters across the menstrual cycle (Figure 2-7).

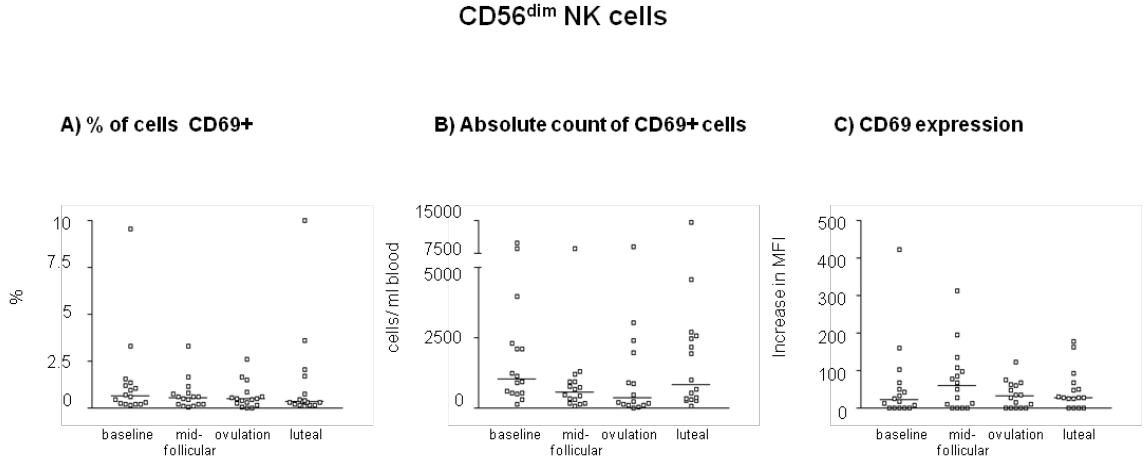


Figure 2-7 Expression of CD69 by NK^{dim} cells throughout the menstrual cycle. CD69 expression was measured as (A) the percentage of CD69+ NK^{dim} cells, (B) the absolute count of CD69+NK^{dim} cells and (C) the increase in median fluorescence intensity (MFI) of CD69-APC staining on NK^{dim} cells. Bars represent median values (n=16).

Expression of IL-18R and ST2L were evaluated in the same way as CD69 (percentage, absolute count and increase in median fluorescence intensity). Neither IL-18R nor ST2L expression varied significantly across the cycle. However there was a significant effect of the menstrual cycle on the type 1: type 2 ratio when calculated using the percentage ($F=13.79$, $p=0.0032$), the absolute count ($F=13.79$, $p=0.0032$) or the median fluorescence intensity ($F=16.81$, $p=0.0008$). A Dunn's post hoc test identified a significant type 1 shift in the mid-luteal phase of the cycle compared to baseline ($p<0.05$) in each of the ratios calculated. There was a significant type 1 shift between the mid-follicular and mid-luteal phase of the cycle ($p<0.05$) when the ratio was calculated from absolute counts or from the median fluorescence intensity. When the ratio was calculated using the median fluorescence intensity, there was also a significant type 1 shift seen at the peri-ovulation time point compared to the baseline and mid-follicular time points ($p<0.05$) (Figure 2.8).

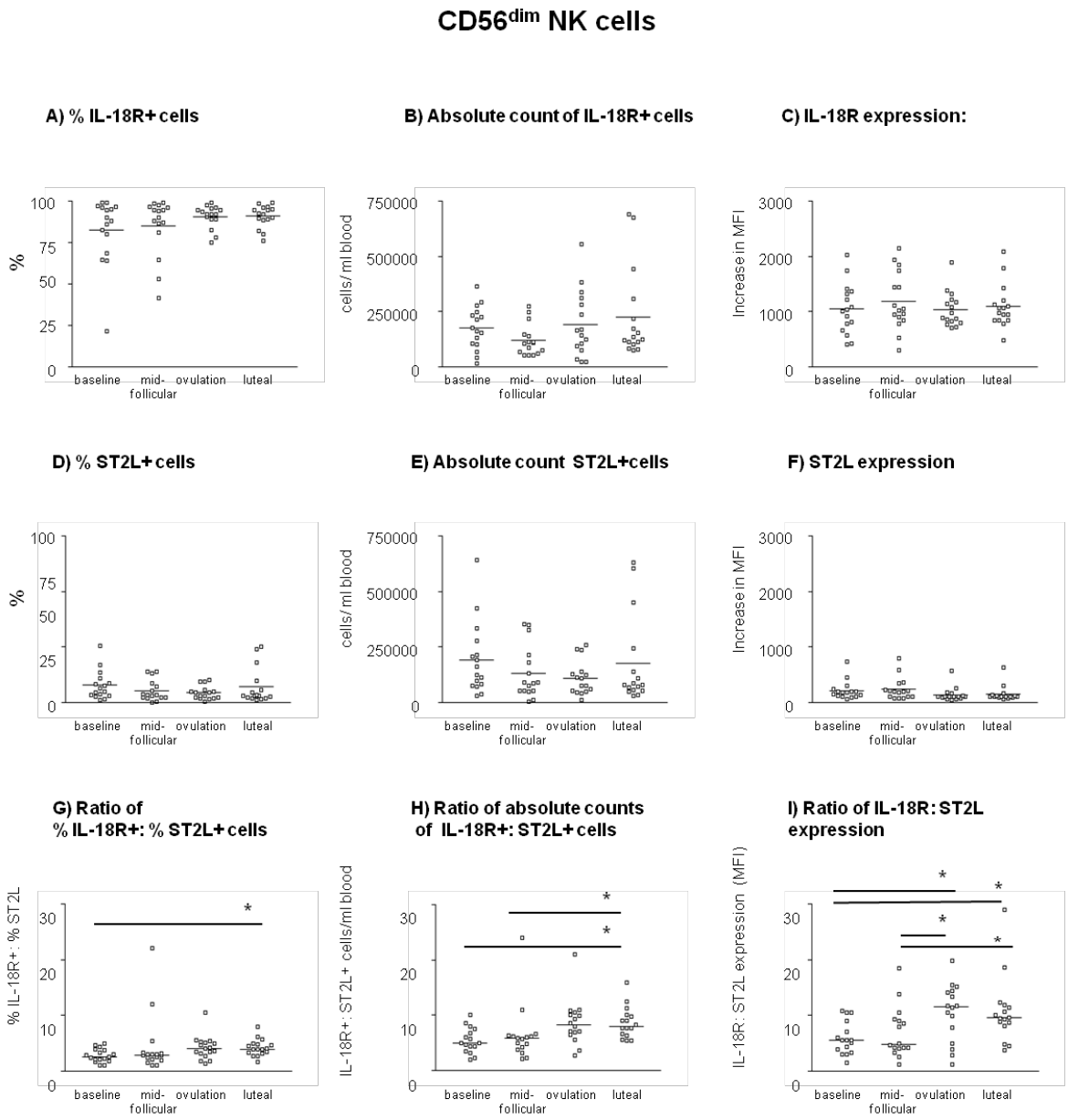


Figure 2-8 Type 1 and Type 2 NK^{dim} cell subsets throughout the menstrual cycle. IL-18R (Type 1) and ST2L (Type 2) expression was measured by (A, D) the percentage, (B, E) the absolute count and (C, F) the increase in median fluorescence intensity (MFI). Bars represent mean values. (G, H, I) Ratios of Type 1: Type 2 CD56^{dim} NK cells were then calculated for each measurement. There was a significant main effect of menstrual cycle on the ratio of type 1: type 2 cells (Ratio of percentage expression, $p<0.05$, ratio of absolute count $p<0.01$, ratio of MFI $p<0.001$). A Dunns' post hoc test showed a significant type 1 shift cells between baseline and the mid-luteal phase of the cycle in each calculation of Type 1: Type 2 NK cells (G, H, I) (*$p<0.05$,) There was a significant type 1 shift between the mid-follicular phase and mid-luteal phase when the ratio was calculated using the absolute count of cells and the MFI (H, I). There was a significant type 1 shift identified between the mid-follicular phase and the peri-ovulation sample when the ratio was calculated using the MFI (I). The ratios were not normally distributed and therefore bars represent median values (n=16).

There was no variation in the number of NK^{bright} cells, when expressed as a percentage of the whole lymphocyte population, or the absolute count of cells throughout the menstrual cycle (Figure 2-9A, B). As discussed in 2.3.4.4, due to the low numbers, expression of CD69, IL-18R and ST2L were only measured by the increase in MFI in this cell population. There was minimal CD69 expression by NK^{bright} cells and this did not vary throughout the cycle (Figure 2-9C). There was no variation in the expression of IL18R or ST2L across the cycle. The ratio of type 1 to type 2 expression was calculated and no significant changes were observed (Figure 2-9D, E, F).

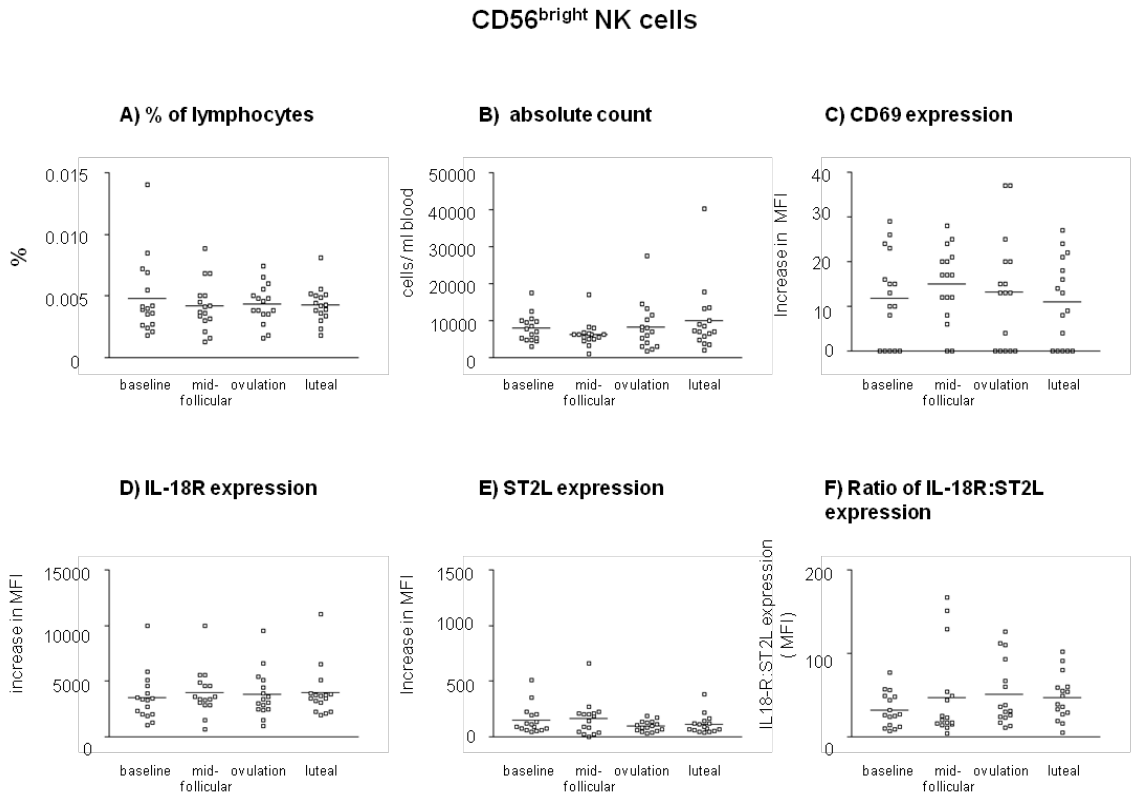


Figure 2-9 NK^{bright} cells throughout the normal menstrual cycle. (A) The percentage of peripheral blood mononuclear cells (PBMCs) that were NK^{bright} cells. (B) The absolute count of NK^{bright} cells. (C) Expression of CD69, (D) IL-18R (Type 1) or (E) ST2L (Type 2) were measured by the increase in median fluorescence intensity (MFI). (F) Ratios of Type 1 and Type 2 cells were then calculated. Bars represent mean values (n=16).

2.5.1.2 iNKT cells

Invariant NKT cells were measured across the cycle. There were no changes in either the number of iNKT cells as a percentage of the lymphocyte population or absolute count of iNKT cells. There was large range in the percentage and absolute count of circulating iNKT cells between individuals, but the number of cells was stable over the cycle in each individual. There was no variation in their expression of CD69 across the cycle (Figure 2-10).

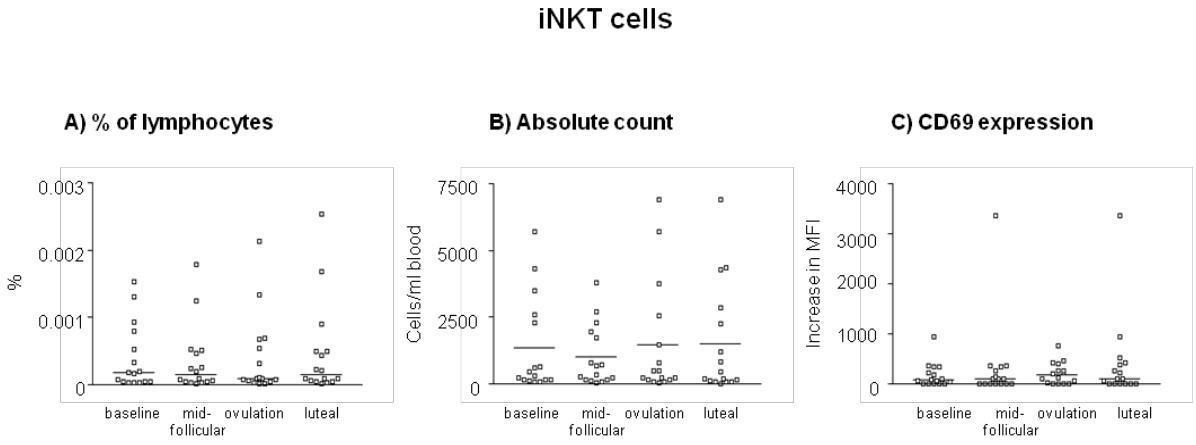


Figure 2-10 (A) The percentage of the lymphocyte population that were iNKT cells, (B) Absolute count of iNKT cells and (C) expression of CD69 by iNKT cells throughout the cycle. Bars represent median values (n=16).

The percentages of CD4+, CD8+ and CD4-/CD8- cells were measured across the cycle. Although the levels of expression varied between individuals, the phenotype was not affected by the menstrual cycle. In fact the phenotype of iNKT cells was remarkably stable over the cycle in each individual studied (Figure 2-11).

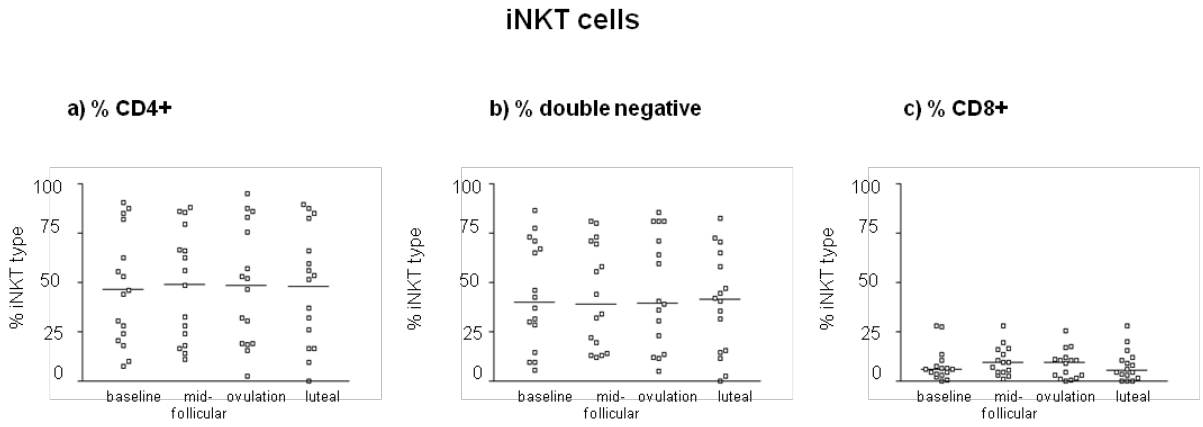


Figure 2-11 Phenotyping of iNKT cells throughout the normal menstrual cycle. (A) Antibodies to CD4 or CD8 identified CD4+, (B) CD4-CD8- and (C) CD8+ iNKT cells. Bars represent mean values (n=16).

2.5.1.3 T cells

There was no significant effect of the menstrual cycle on the percentage of Th cells or the percentage of cytotoxic T cells. Neither did the absolute count of these cell populations vary across the cycle (Figure 2-12).

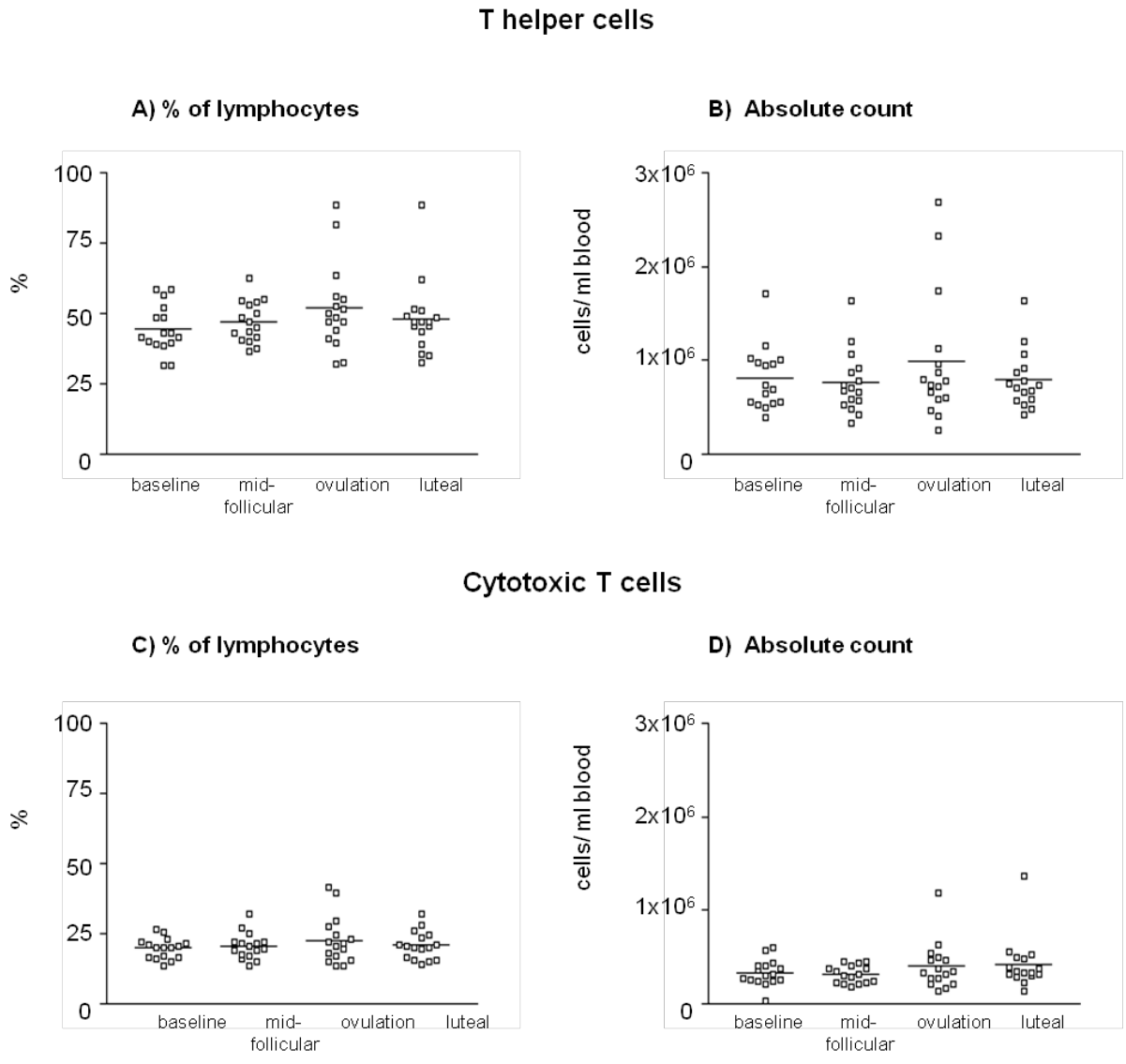


Figure 2-12 Th cells and cytotoxic T cells throughout the normal menstrual cycle. The percentage of PBMCs that were (A) Th cells and (C) cytotoxic T cells. The absolute count of (B) Th Cells and (D) cytotoxic T cells. Bars represent mean values (n=16).

There was no effect of the menstrual cycle on any measure of CD69 expression in either of the T cell populations (Figure 2-13).

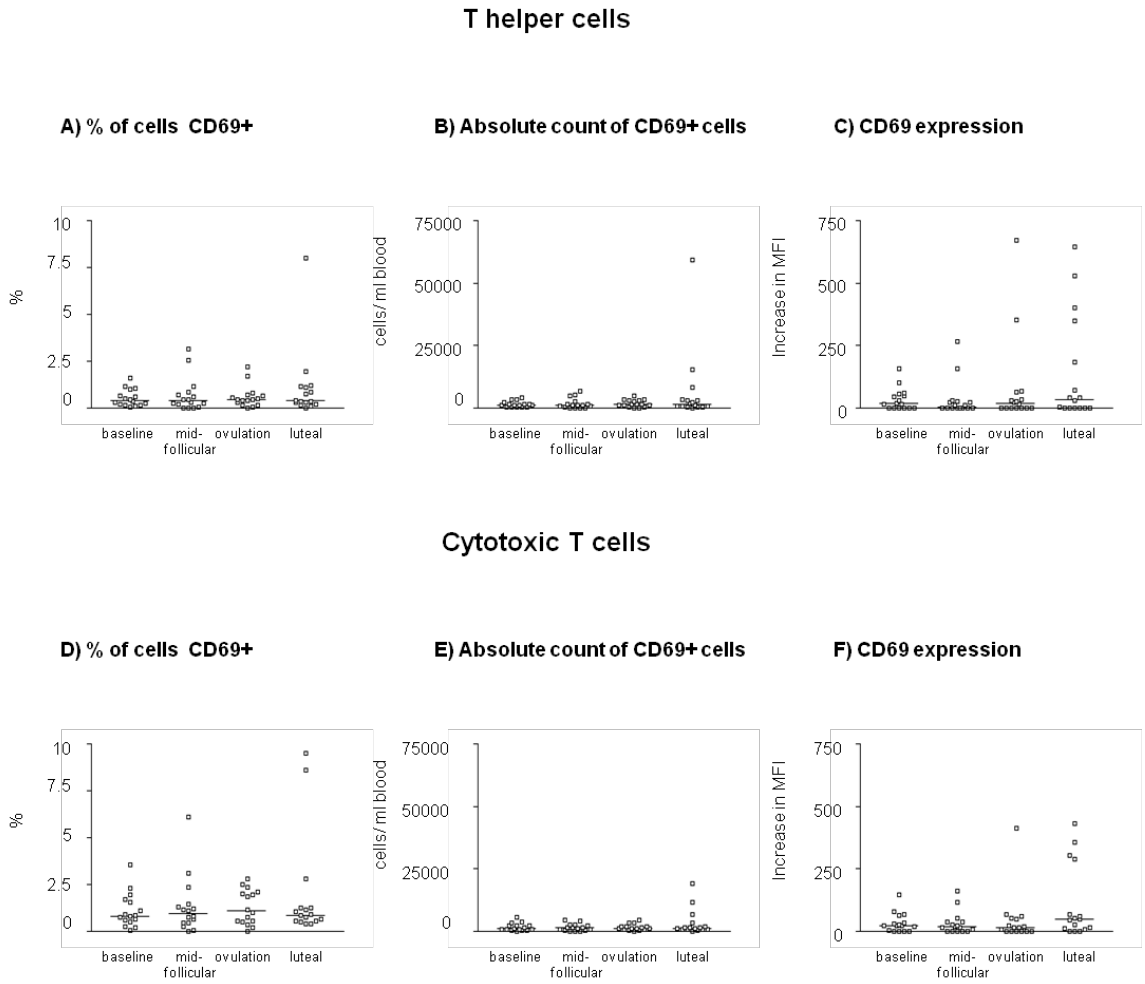


Figure 2-13 Expression of CD69 by Th and cytotoxic T lymphocytes throughout the menstrual cycle. CD69 expression was measured as the percentage of cells expressing CD69 (A, D), the absolute count of CD69+ cells (B. E) and the increase in median fluorescence intensity (MFI) of CD69-APC on the cells (C, F). Bars represent median values (n=16).

IL-18R expression and ST2L expression were measured in both populations. There was no effect of the menstrual cycle on the expression of either receptor or the ratio of type1: type 2 cells in the Th cell population (Figure 2-14).

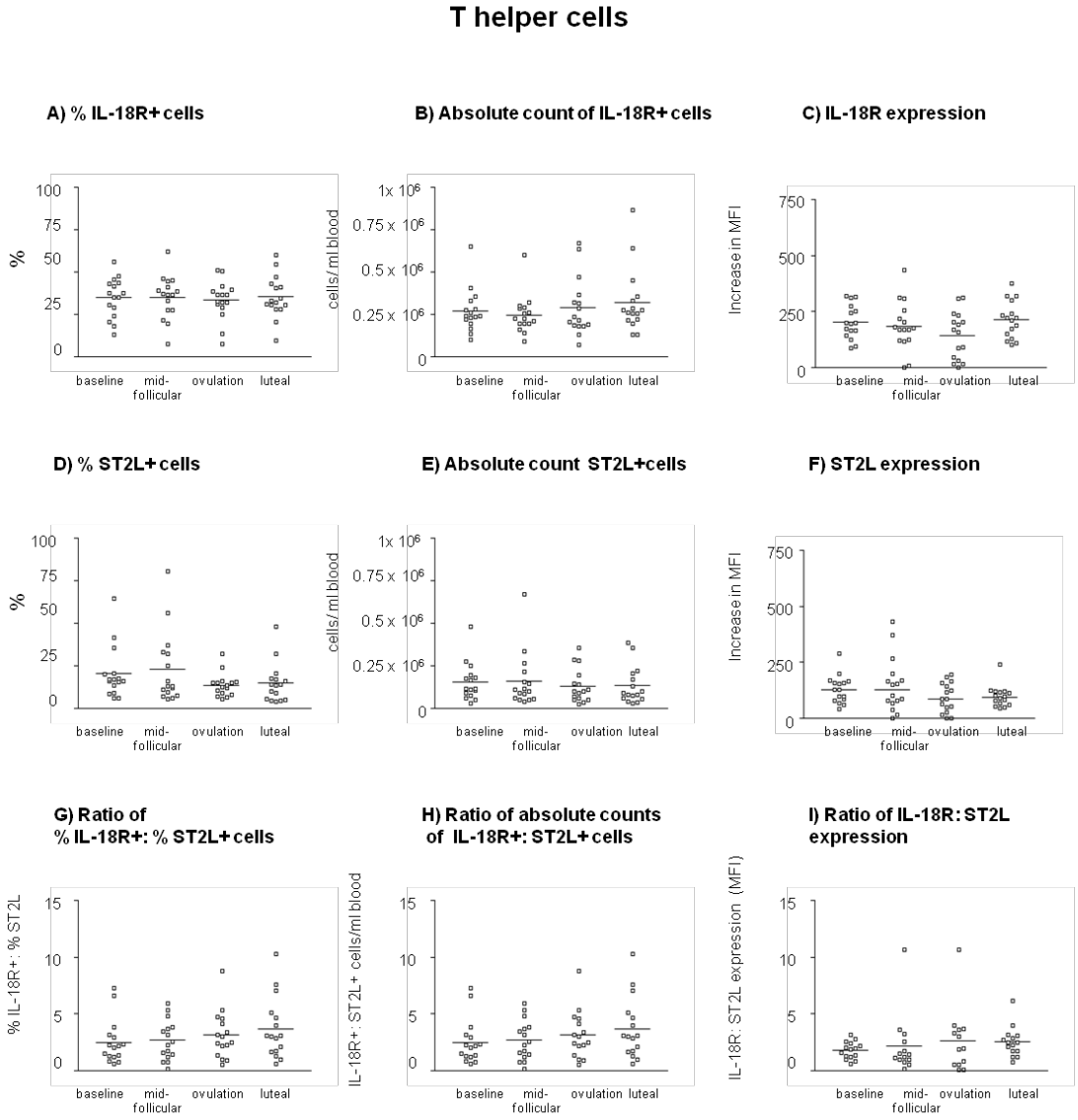


Figure 2-14 Th1 and Th2 subsets throughout the menstrual cycle. IL-18R (Th 1) and ST2L (Th2) expression was measured by (A, D) the percentage, (B, E) the absolute count and (C,F) the increase in median fluorescence intensity (MFI). (G, H, I) Ratios of Th1 and Th2 cells were then calculated. Bars represent mean values (n=16).

In the cytotoxic T cell population there was no effect of the menstrual cycle on the expression of IL-18R or ST2L. However there was a significant main effect of the menstrual cycle on the type 1/ type 2 ratio when the ratio was calculated from the percentage expression or the absolute count ($F=3.443$, $p=0.0244$). Post hoc testing identified a significant type 1 shift at mid-luteal compared to mid-follicular time points ($p<0.05$) (Figure 2-15).

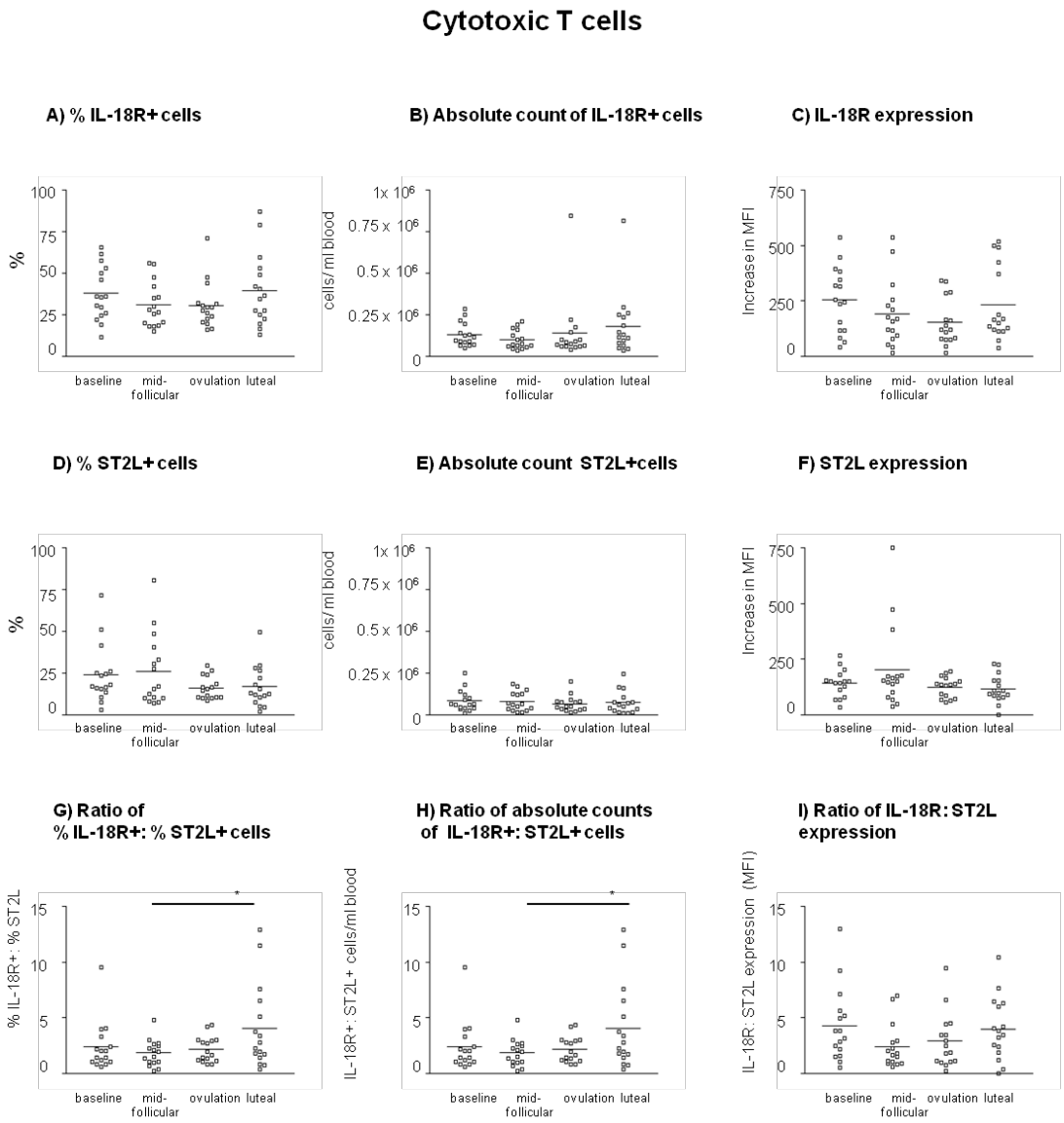


Figure 2-15 Type 1 and Type 2 subsets of cytotoxic lymphocytes throughout the menstrual cycle. Expression of IL-18R (Th1) and ST2L (Th2) was measured by (A, D) the percentage, (B, E) the absolute count and (C, F) the increase in median fluorescence intensity (MFI). (G, H, I) Ratios of Th1 and Th2 cells were then calculated. There was a significant main effect of the menstrual cycle on the ratio of type 1 and type 2 cells calculated using the percentage expression or absolute count (F=3.443, p-0.0244) with a significant type 1 shift identified between the mid-follicular and mid-luteal time points. Bars represent mean values (n=16).

2.5.2 The effect of the menstrual cycle on peripheral NK, iNKT and T cells in the women with recurrent unexplained miscarriage

NK, iNKT and T cells were next measured at the four defined time points in women with a history of recurrent unexplained miscarriage in order to determine whether there was an effect of menstrual cycle phase in this group of women.

2.5.2.1 NK cells

In the women with recurrent unexplained miscarriage the phase of the menstrual cycle had no significant effect on the number of NK^{dim} cells as a percentage of the whole lymphocyte population. However there was a significant main effect of the menstrual cycle on the absolute count of NK^{dim} cells ($F=7.887$, $p=0.0006$). A post hoc test identified a significant increase in the number of cells at the mid luteal time point compared to the baseline or peri-ovulation time points ($p<0.01$) (Figure 2-16).

Similar to the recruits with no history of miscarriage, expression of the early cell activation marker CD69 did not vary significantly in the recurrent miscarriage group at any point across the cycle (Figure 2-17).

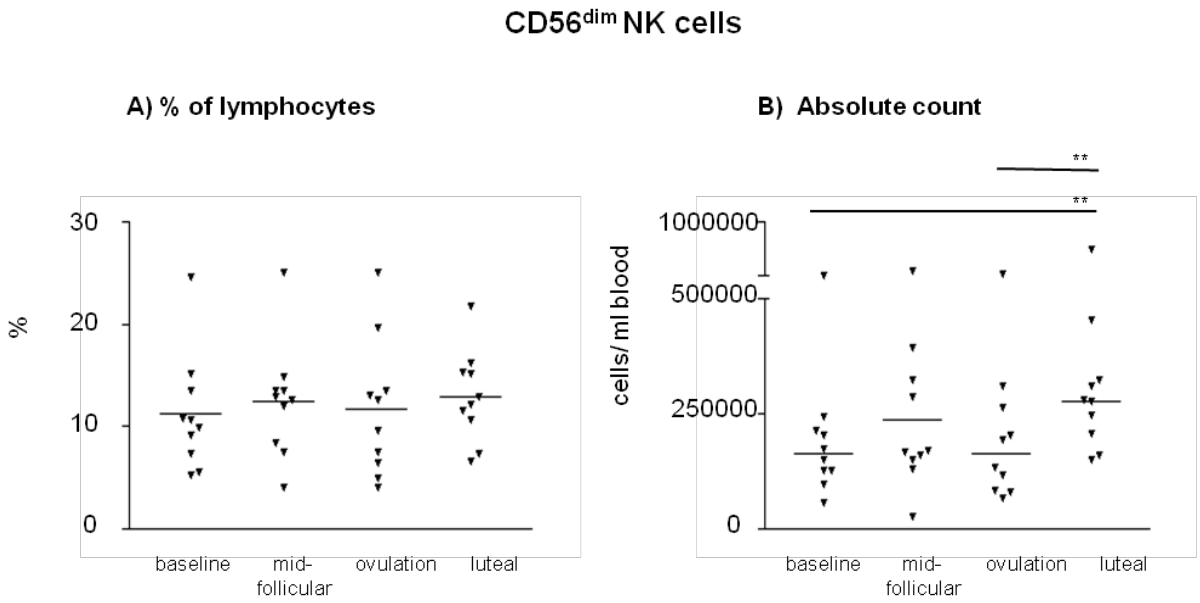


Figure 2-16 NK^{dim} cells throughout the menstrual cycle of women with recurrent unexplained miscarriage. (A) The percentage of lymphocytes that were NK^{dim} cells. (B) The absolute count of NK^{dim} cells. There was a significant increase in the number of cells at the mid luteal time point compared to baseline or the peri-ovulation sample (**p<0.01). Bars represent mean values (n=10).

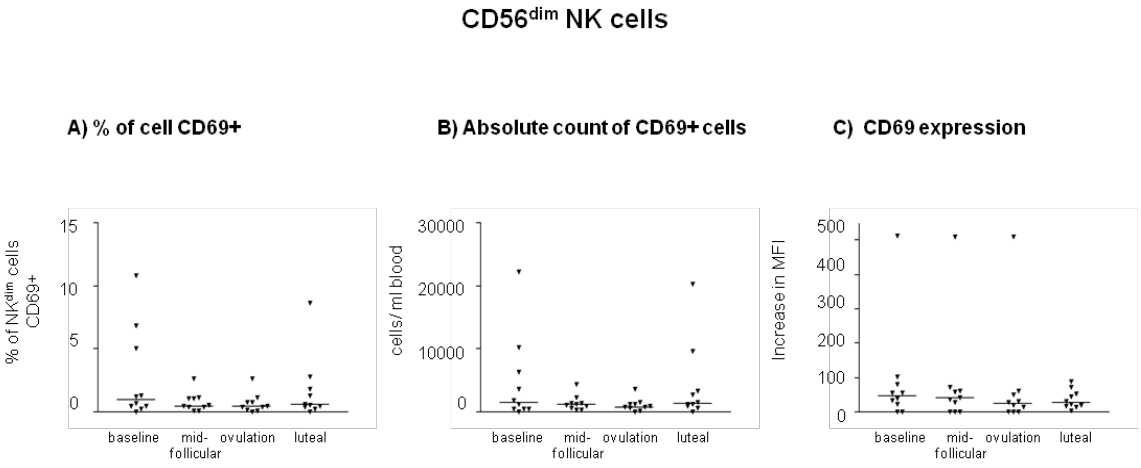


Figure 2-17 Expression of CD69 by NK^{dim} cells throughout the menstrual cycle of women with recurrent unexplained miscarriage. CD69 expression was measured as (A) the percentage of CD69+NK^{dim} cells, (B) the absolute count of CD69+ NK^{dim} cells and (C) the increase in median fluorescence intensity (MFI) of CD69-APC on NK^{dim} cells. Bars represent median values (n=10).

Once again the type 1/ type 2 bias of the NK^{dim} cells was calculated by measuring IL-18R and ST2L expression. There was no variation across the cycle and in contrast to the control group with no history of miscarriage, no mid-luteal type 1 shift was identified. (Figure 2-18)

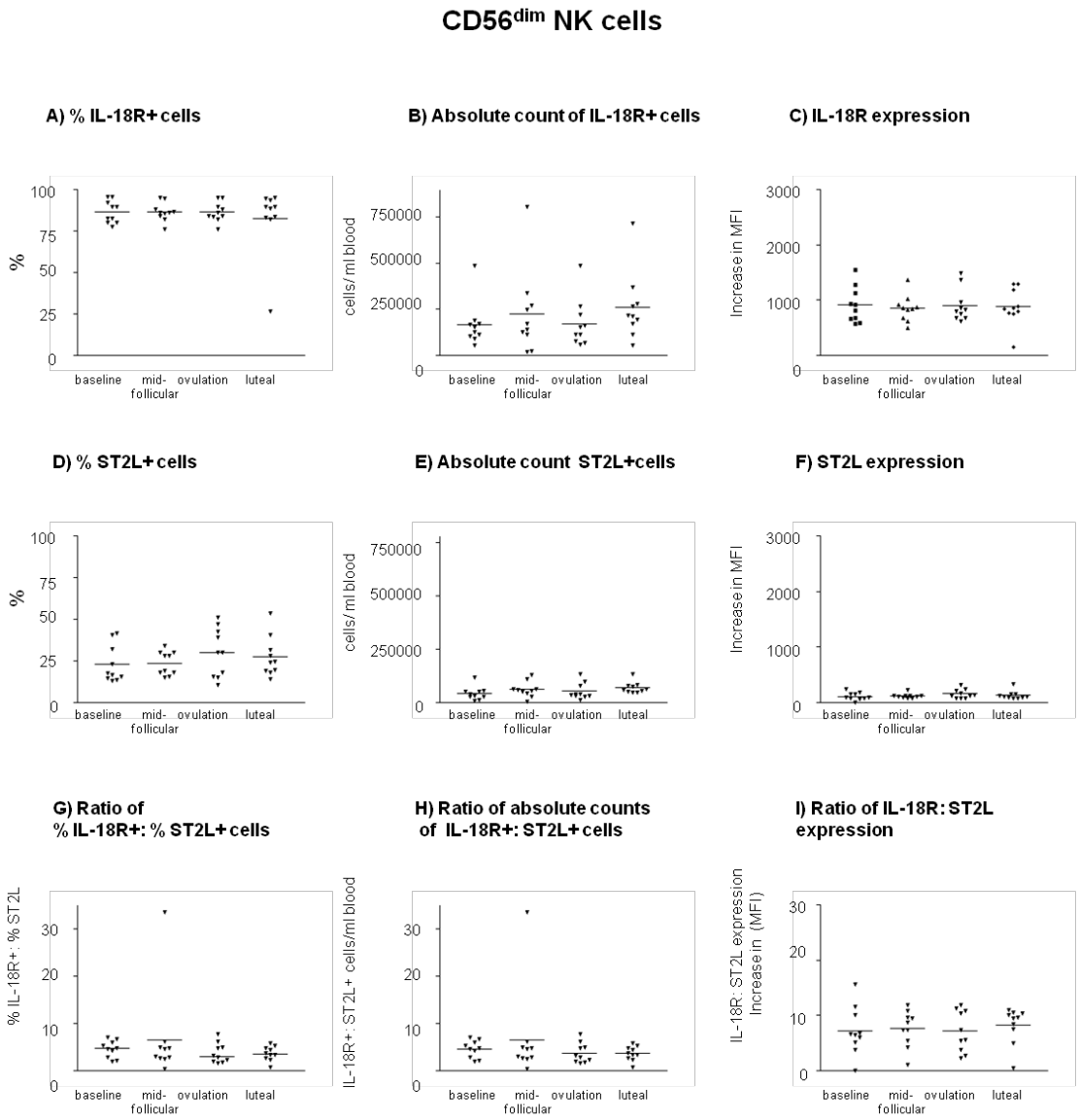


Figure 2-18 Type 1 and Type 2 $CD56^{dim}$ NK cell subsets throughout the menstrual cycle of women with recurrent unexplained miscarriage. Expression of IL-18R (Type 1) and ST2L (Type 2) was measured by (A, D) percentage, (B, E) the absolute count and (C, F) the increase in median fluorescence intensity (MFI). (G, H, I) Ratios of Type 1: Type 2 NK^{dim} cells were then calculated. Bars represent mean values (n=10).

There was no variation in the number of NK^{bright} cells as a percentage of the lymphocyte population and no variation in the absolute count of NK^{bright} cells across the menstrual cycle. Neither was there any change in CD69 expression in this population. The expression of IL-18R and ST2L was similar throughout the cycle and the type 1/type 2 ratio did not vary significantly (Figure 2-19).

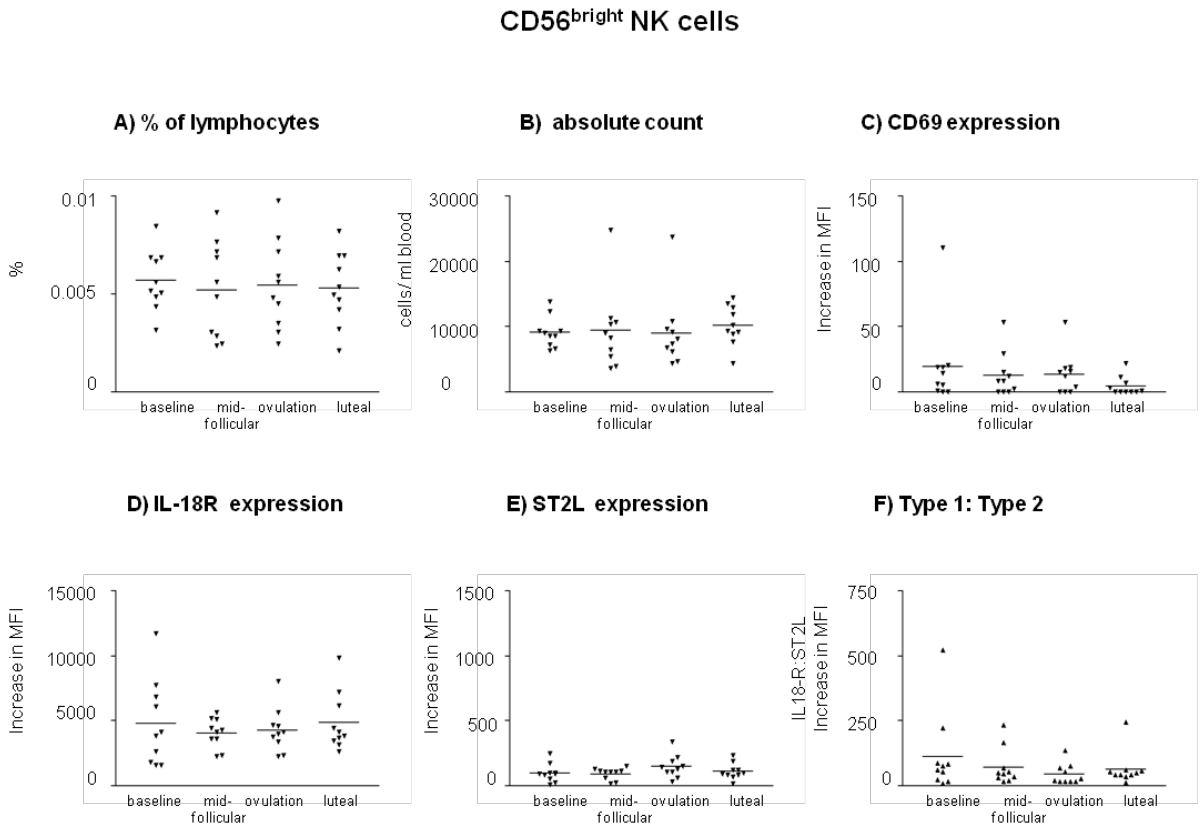


Figure 2-19 NK^{bright} cells throughout the menstrual cycle of women with recurrent unexplained miscarriage. (A) The percentage of peripheral blood mononuclear cells (PBMCs) that were NK^{bright} cells. (B) The absolute count of NK^{bright} cells. (C) Expression of CD69, (D) IL-18R (Type 1) or (E) ST2L (Type 2) was measured by the increase in median fluorescence intensity (MFI). (F) Ratios of Type 1: Type 2 cells were then calculated. Bars represent mean values (n=10).

2.5.2.2 iNKT cells

Invariant NKT cells were measured across the cycle in the recurrent unexplained miscarriage group. There was no significant effect of the menstrual cycle on the number of iNKT cells measured as a percentage of the lymphocyte population or per ml of blood and no variation in the expression of CD69 (Figure 2-20).

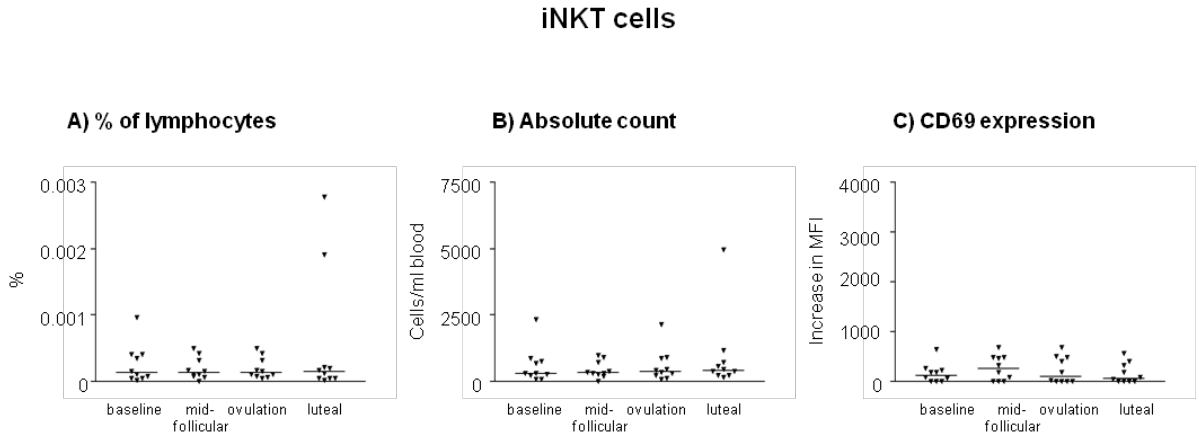


Figure 2-20 (A) The percentage of the lymphocyte population that were iNKT cells, (B) Absolute count of iNKT cells and (C) expression of CD69 by iNKT cells throughout the menstrual cycle of women with recurrent unexplained miscarriage. Bars represent median values (n=10).

No differences were identified across the cycle in the iNKT cell phenotype (CD4+, CD8+ or double negative cells) (Figure 2-21).

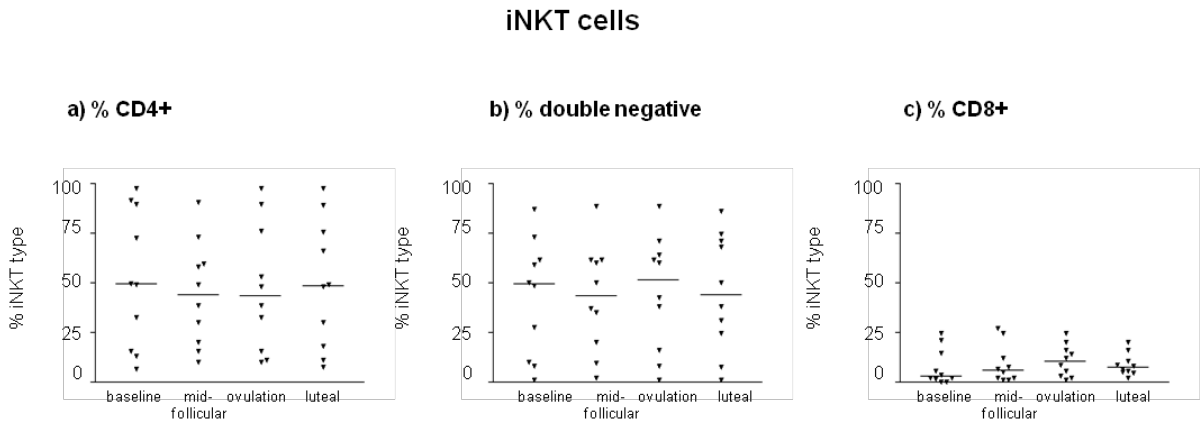


Figure 2-21 Phenotyping of iNKT cells throughout the menstrual cycle of women with recurrent unexplained miscarriage. Antibodies to CD4 or CD8 identified (A) CD4+, (B) CD4-CD8- and (C) CD8 iNKT cells. Bars represent mean values (n=10).

2.5.2.3 T cells

In women with recurrent unexplained miscarriage there was no significant effect of the menstrual cycle on the number of Th or cytotoxic T cells as a percentage of the lymphocyte population or the absolute count (Figure 2-22). Neither was there any significant variation in CD69 expression across the cycle (Figure 2-23).

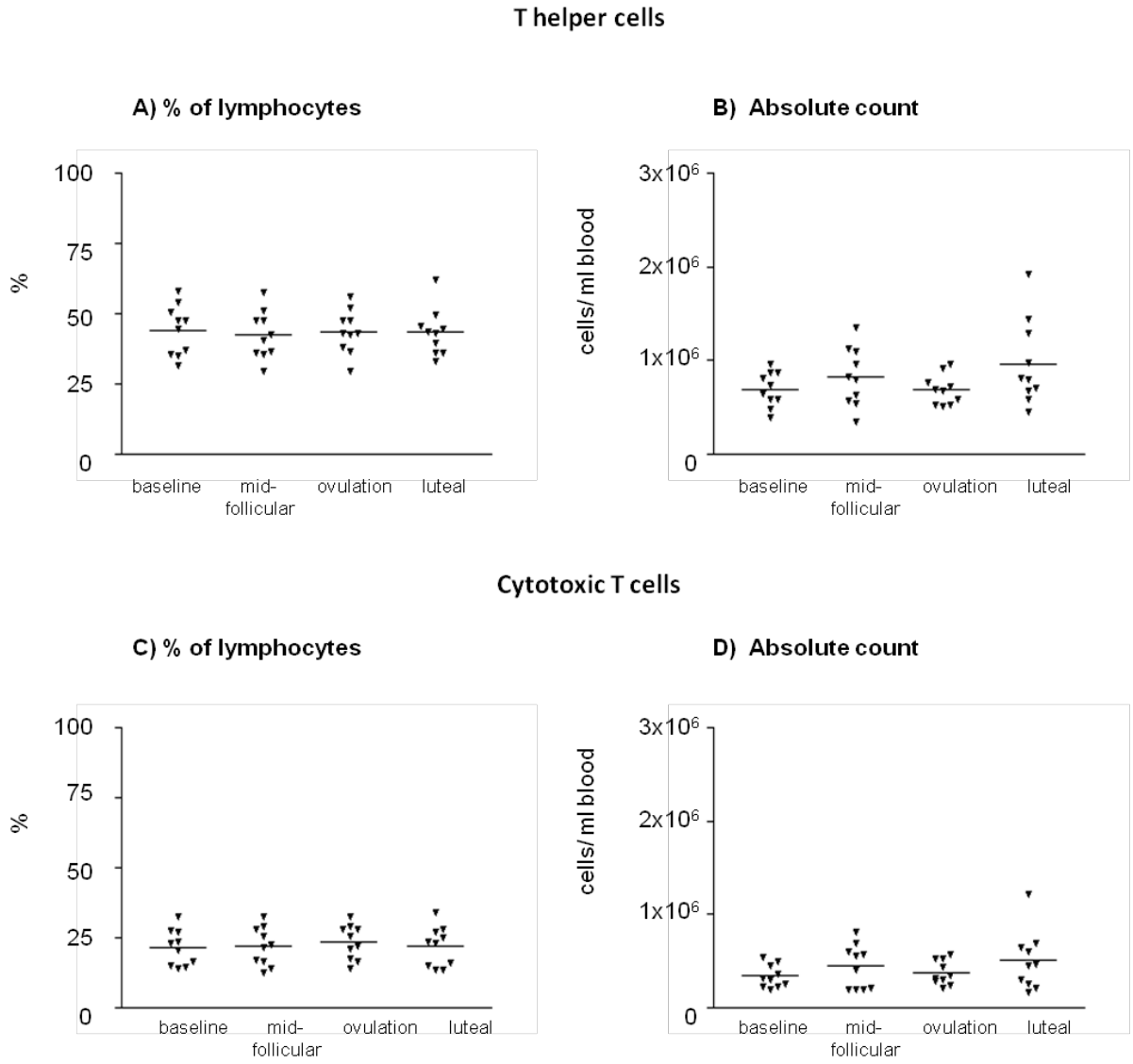


Figure 2-22 Th cells and cytotoxic T cells throughout the menstrual cycle of women with recurrent unexplained miscarriage. The percentage of lymphocytes that were (A) Th cells and (C) cytotoxic T cells. The absolute count of Th Cells (B) and cytotoxic T cells (D). Bars represent mean values (n=10).

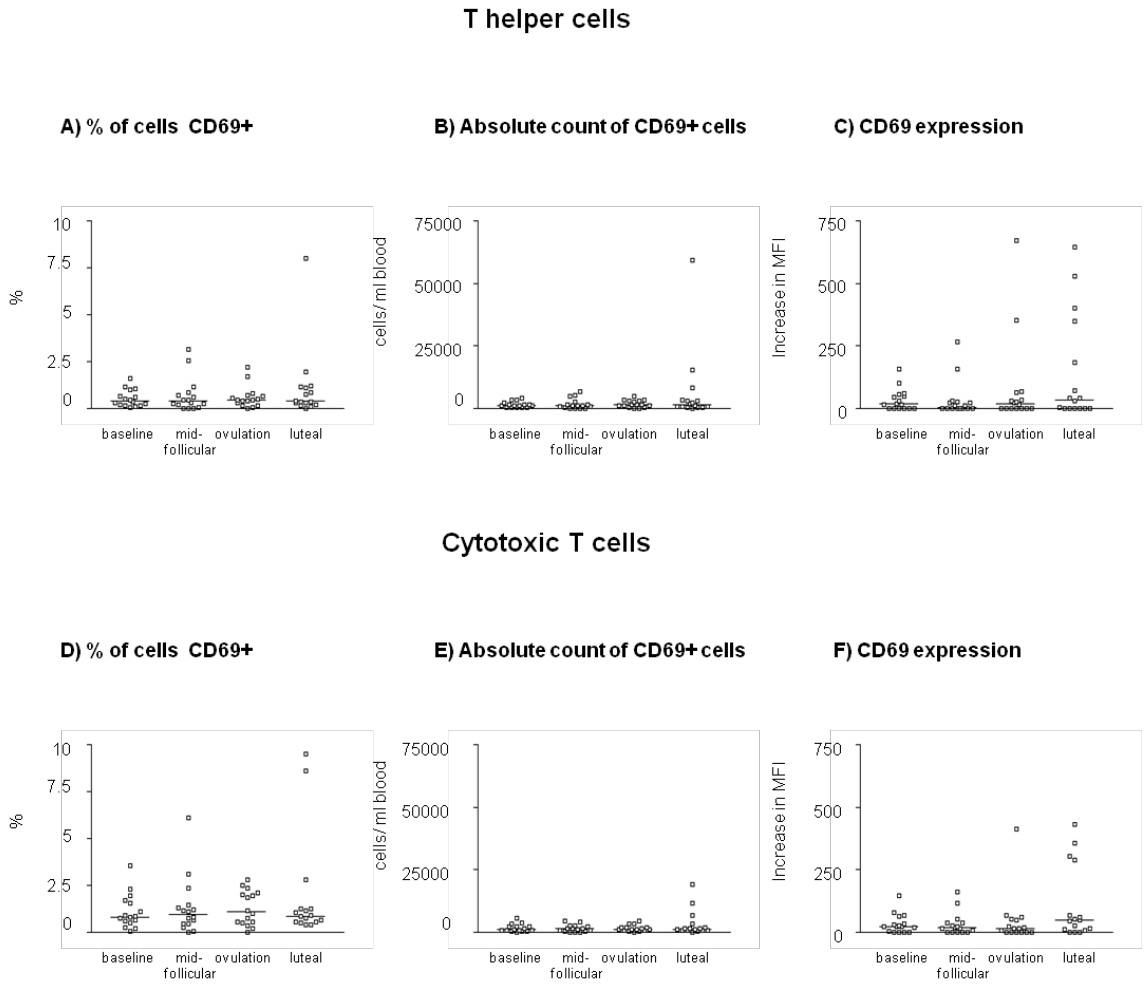


Figure 2-23 Expression of CD69 by Th and cytotoxic T lymphocytes throughout the menstrual cycle of women with recurrent unexplained miscarriage. CD69 expression was measured as (A, D) the percentage, (B. E) the absolute count and (C, F) the increase in median fluorescence intensity (MFI) of CD69-APC on the cells. Bars represent median values (n=10).

The stage of the menstrual cycle had no effect on the expression of IL-18 or ST2L and no effect on the ratio of expression in either population of T cells (Figures 2-24, 2-25).

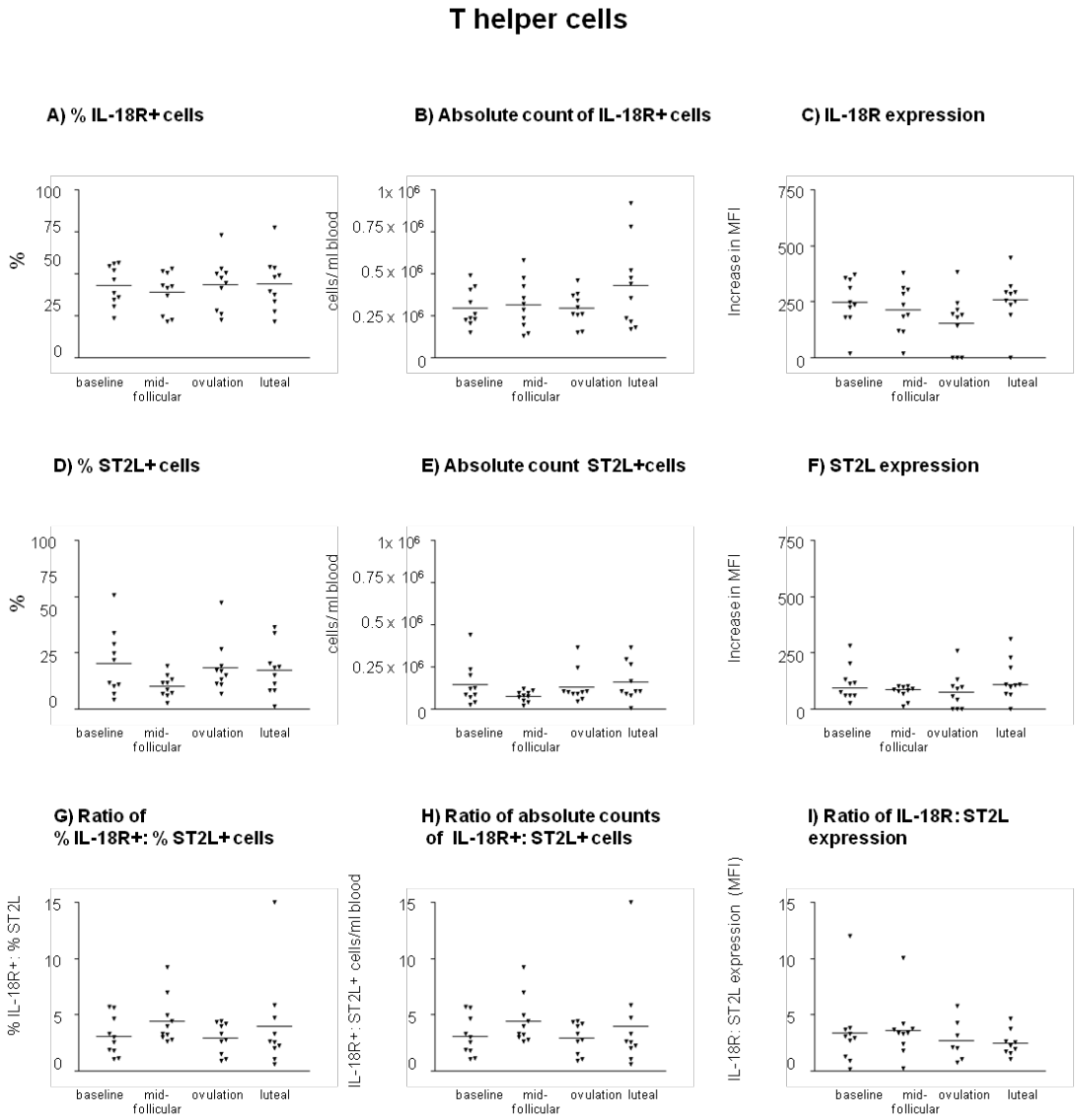


Figure 2-24. Th1 and Th2 subsets throughout the menstrual cycle of women with recurrent unexplained miscarriage. Expression of IL-18R (Th1) and ST2L (Th2) was measured by (A,D) percentage, (B,E) the absolute count and (C,F) the increase in median fluorescence intensity (MFI). Bars represent mean values. (G, H, I) Ratios of Th1 and Th2 cells were then calculated. Bars represent mean values (n=10).

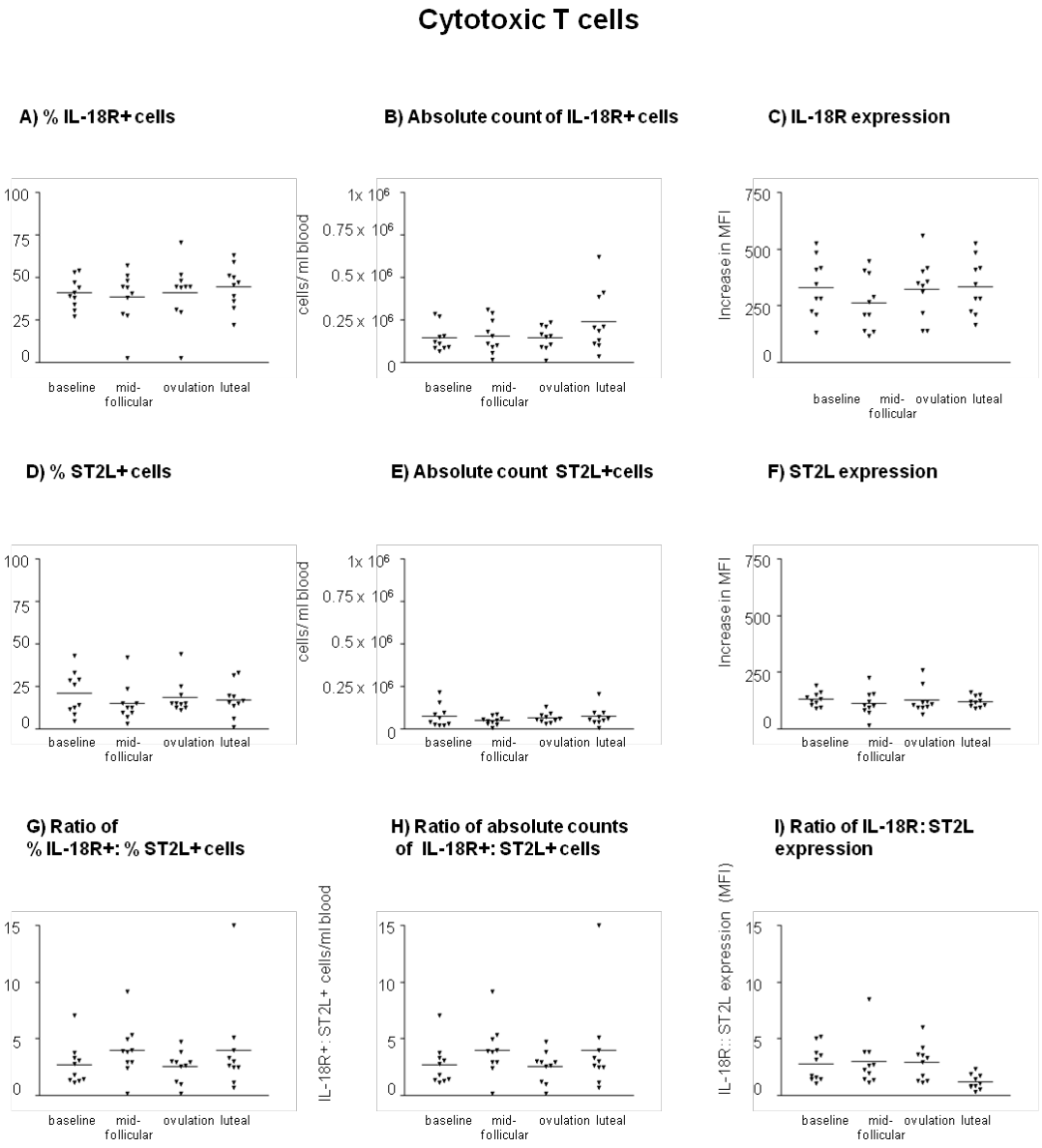


Figure 2-25 Type 1 and Type 2 subsets of cytotoxic T lymphocytes throughout the menstrual cycle of women with recurrent unexplained miscarriage. Expression of IL-18R (Type 1) and ST2L (Type 2) cells was measured by (A, D) percentage expression, (B, E) the absolute count and (C, F) the increase in median fluorescence intensity (MFI). (G, H, I) Ratios of Th1 and Th2 cells were then calculated. Bars represent mean values (n=10).

2.5.3 The effect of recurrent unexplained miscarriage on peripheral NK, iNKT and T cells throughout the menstrual cycle: a case-control study.

In the final part of this study women with recurrent unexplained miscarriage were matched for age and parity with controls in order to identify whether a history of recurrent miscarriage had an effect on the numbers or immune bias of NK, iNKT and T cells. In addition, the study aimed to identify if there is a significant interaction between the group (miscarriage or control) and the phase of the menstrual cycle on peripheral immune cells. Mid luteal progesterone levels were measured in both groups in order to confirm that ovulation had taken place. There was no difference in the mean (+/-SEM) progesterone level (28.2 (3.288) and 28.0 (2.929) nmol/L) between miscarriage patients and controls. There was no correlation between the progesterone levels and any cell type measured in either group.

2.5.3.1 NK cells

There was no main effect of group on the percentage or absolute count of NK^{dim} cells. However there was a significant interaction between the group and the phase of the menstrual cycle ($F=3.335$, $p=0.026$). This suggests that the effect of the menstrual cycle on NK^{dim} cells is different in women with recurrent miscarriage compared to normal controls. Post hoc tests showed this effect to be significant between the baseline and the mid-luteal time points ($p<0.001$) (Figure 2-26).

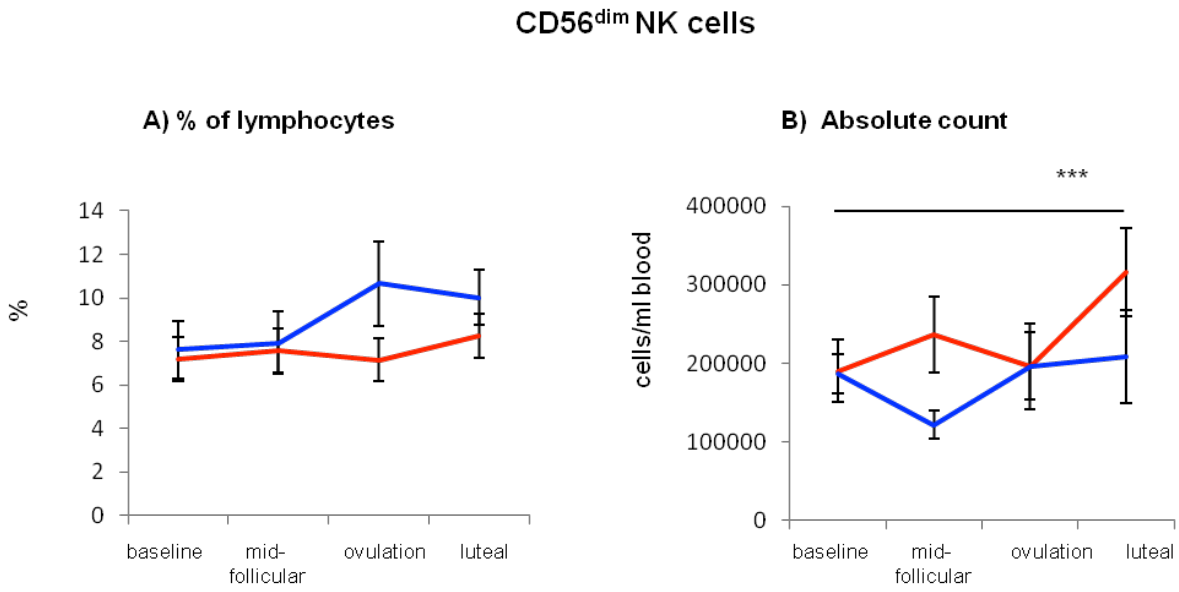


Figure 2-26 NK^{dim} cells throughout cycle of women with recurrent unexplained miscarriage (red) and matched controls (blue). (A) The percentage of lymphocytes that were NK^{dim} cells. (B) The absolute count of NK^{dim} cells. There was a significant phase group interaction in the absolute count of NK^{dim} cells between baseline and the mid-luteal phase (B) (*** $p<0.001$). Points represent mean values +/- SEM (n=10).

There was no significant effect of group or a group phase interaction on the expression of CD69 (Figure 2-27).

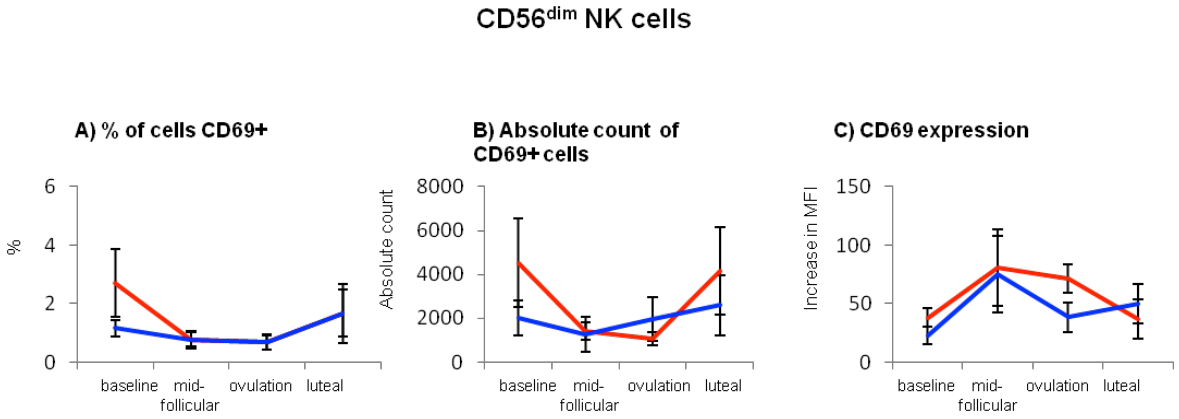


Figure 2-27 CD69 expression by NK^{dim} cells throughout cycle of women with recurrent unexplained miscarriage (red) and matched controls (blue). (A) The percentage of NK^{dim} cells that were CD69+. (B) The absolute count of CD69+ NK^{dim} cells. (C) The increase in median fluorescence intensity (MFI) of CD69-APC on NK^{dim} cells. Points represent mean values +/- SEM (n=10).

There was no significant effect of group or a group phase interaction on the expression of IL-18R, ST2L or the type 1, type 2 ratio in the NK^{dim} population. The type 1 shift seen in the mid-luteal phase of the cycle in the initial menstrual cycle study group (n=16) was not identified in this group. However similar to the larger menstrual cycle study group, there was a trend towards a type 1 shift in the control group with the progression of the menstrual cycle. The lack of significance in this control group may be due to the smaller sample size (Figure 2-28).

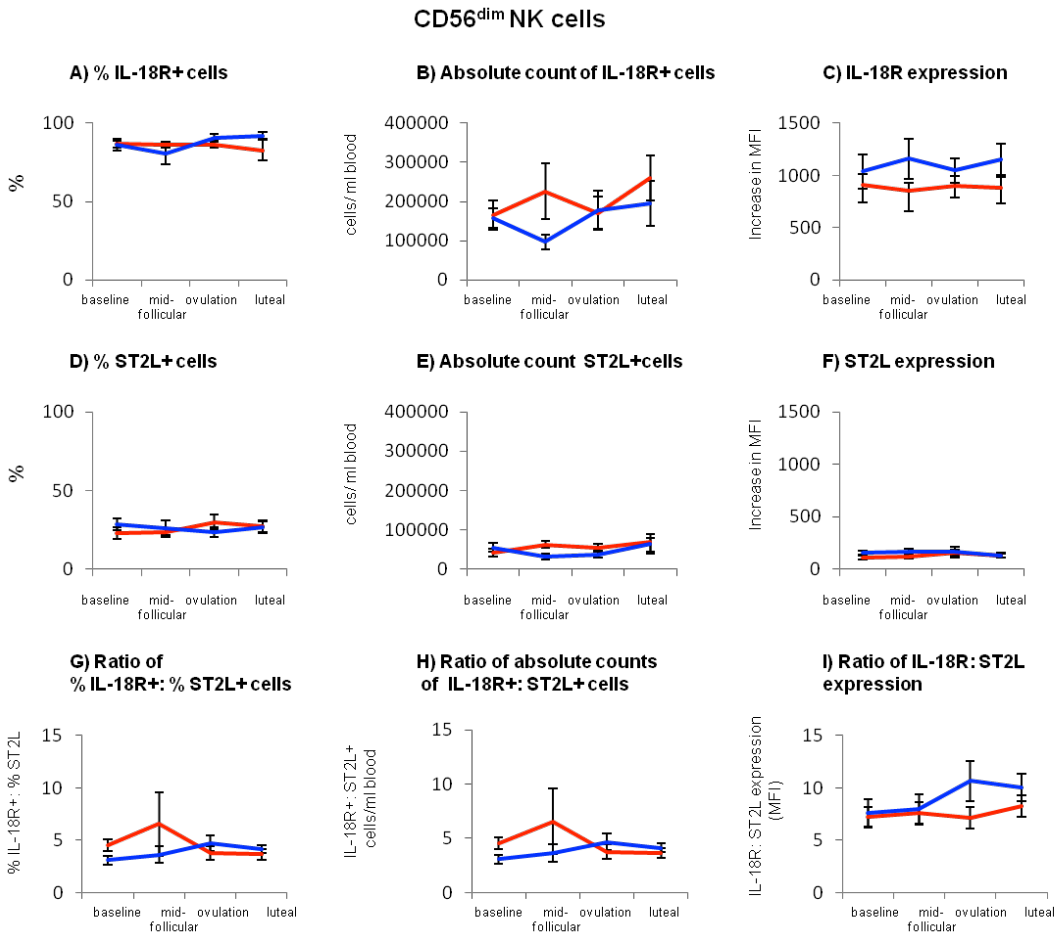


Figure 2-28 Type 1 and Type 2 NK^{dim} cell subsets throughout the menstrual cycle of women with recurrent unexplained miscarriage and matched controls. Expression of IL-18R (Type 1) and ST2L (Type 2) was measured by (A, D) percentage of the lymphocyte population, (B, E) the absolute count and (C, F) the increase in median fluorescence intensity (MFI). (G, H, I) Ratios of Type 1: Type 2 NK^{dim} cell were then calculated. Points represent mean values +/- SEM (n=10).

There was a significant main effect of group on the absolute count of NK^{bright} cells (F=4.442, p=0.044). Women with recurrent miscarriage had significantly higher numbers of NK^{bright} cells in the mid luteal phase of the menstrual cycle (Figure 2-29). A history of recurrent miscarriage did not affect the expression of IL-18R, ST2L or the type 1/ type 2 ratio.

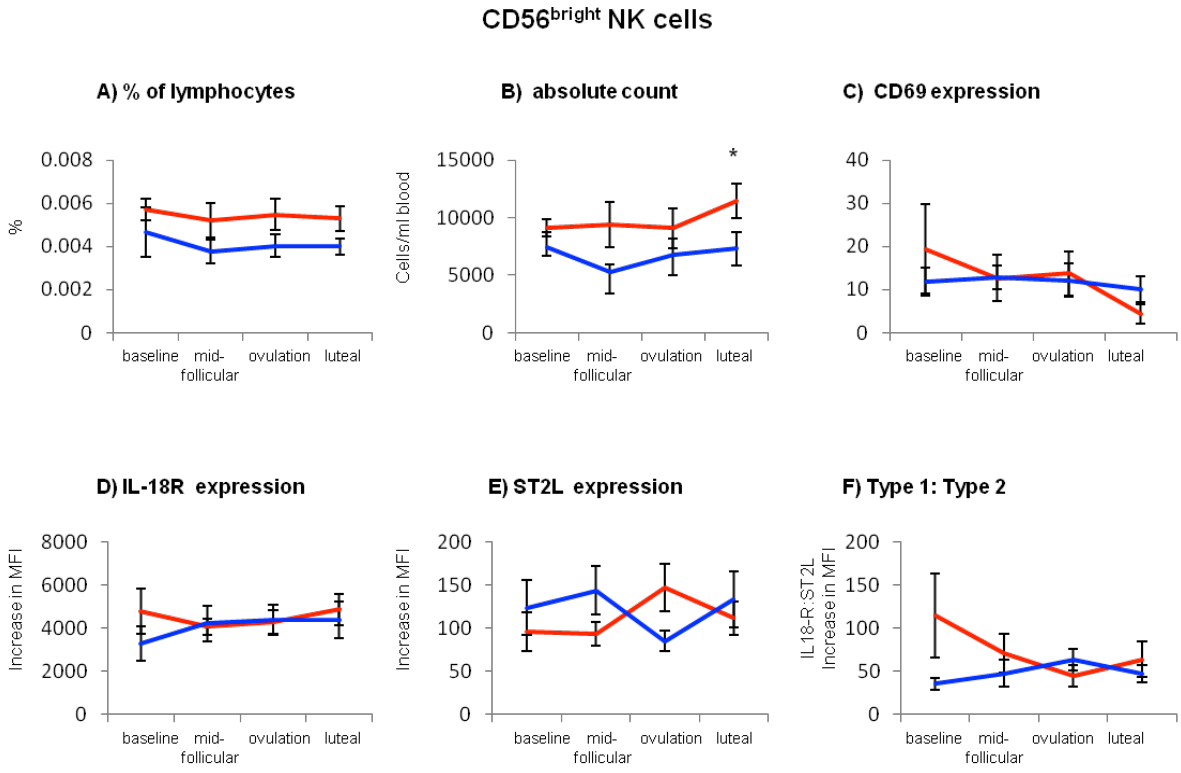


Figure 2-29 NK^{bright} cells throughout the cycle of women with recurrent unexplained miscarriage (red) and matched controls (blue). (A) The percentage of peripheral blood mononuclear cells (PBMCs) that were NK^{bright} cells. (B) The absolute count of NK^{bright} cells. Expression of (C) CD69, (D) IL-18R, (E) ST2L was measured by the increase in median fluorescence intensity (MFI). (F) Ratios of Type 1: Type 2 cells were then calculated. There was significant increase in the number of NK^{bright} cells in the mid-luteal phase of the cycle in women with a history of unexplained recurrent miscarriage (* p<0.05) (B). Points represent mean values +/- SEM (n=10).

2.5.3.2 *iNKT cells*

There was no difference in the absolute count of iNKT cells between women with recurrent unexplained miscarriage and controls. iNKT cell numbers were not normally distributed and therefore an ANOVA could not be used to identify significant differences between the groups. In both groups iNKT numbers were very stable in each individual across the menstrual cycle and therefore the baseline samples from each group were compared using a Wilcoxon matched pairs test to identify if there were differences between the two groups. There was no significant difference in cell numbers or CD69 expression between miscarriage patients and controls (Figure 2-29).

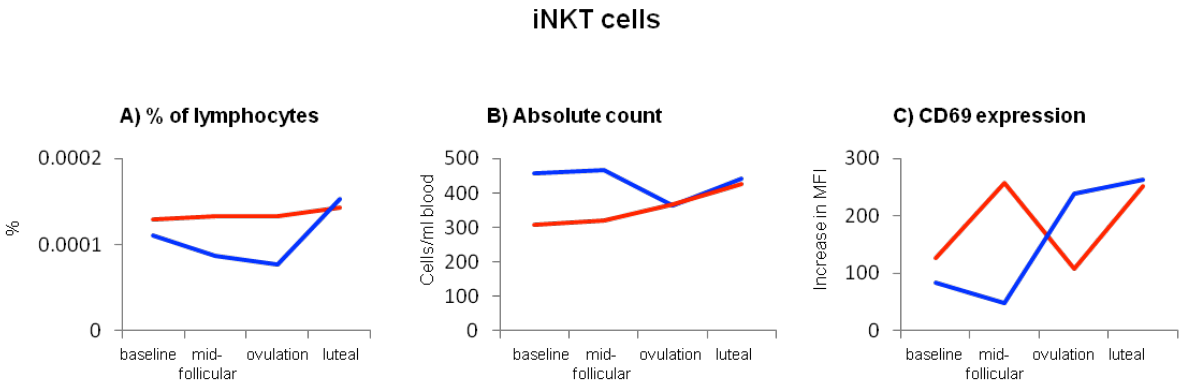


Figure 2-30 (A) The percentage lymphocytes that were iNKT cells throughout cycle of women with recurrent unexplained miscarriage (red) and matched controls (blue) (B) The absolute count (C) The increase in median fluorescence intensity (MFI) of CD69-APC on iNKT cells. Points represent median values (n=10).

No difference was identified in the percentage of CD4+, double negative or CD8+ cells between miscarriage patients and controls (Figure 2-30)

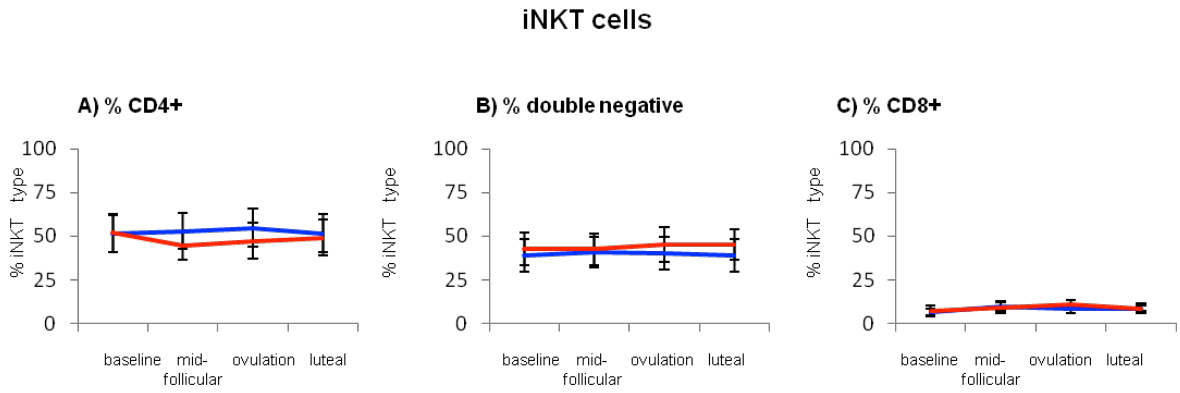


Figure 2-31 Phenotyping of iNKT cells throughout the normal menstrual cycle. Antibodies to CD4 or CD8 identified (A) CD4+, (B) CD4-CD8- and(C) CD8+ iNKT cells. Bars represent mean values (+/-SEM) (n=10).

2.5.3.3 *T cells*

There was no effect of group on the number or percentage (of the lymphocyte gate) of Th or cytotoxic T cells. There was no significant interaction between the cycle phase and the group. (Figure 2-31).

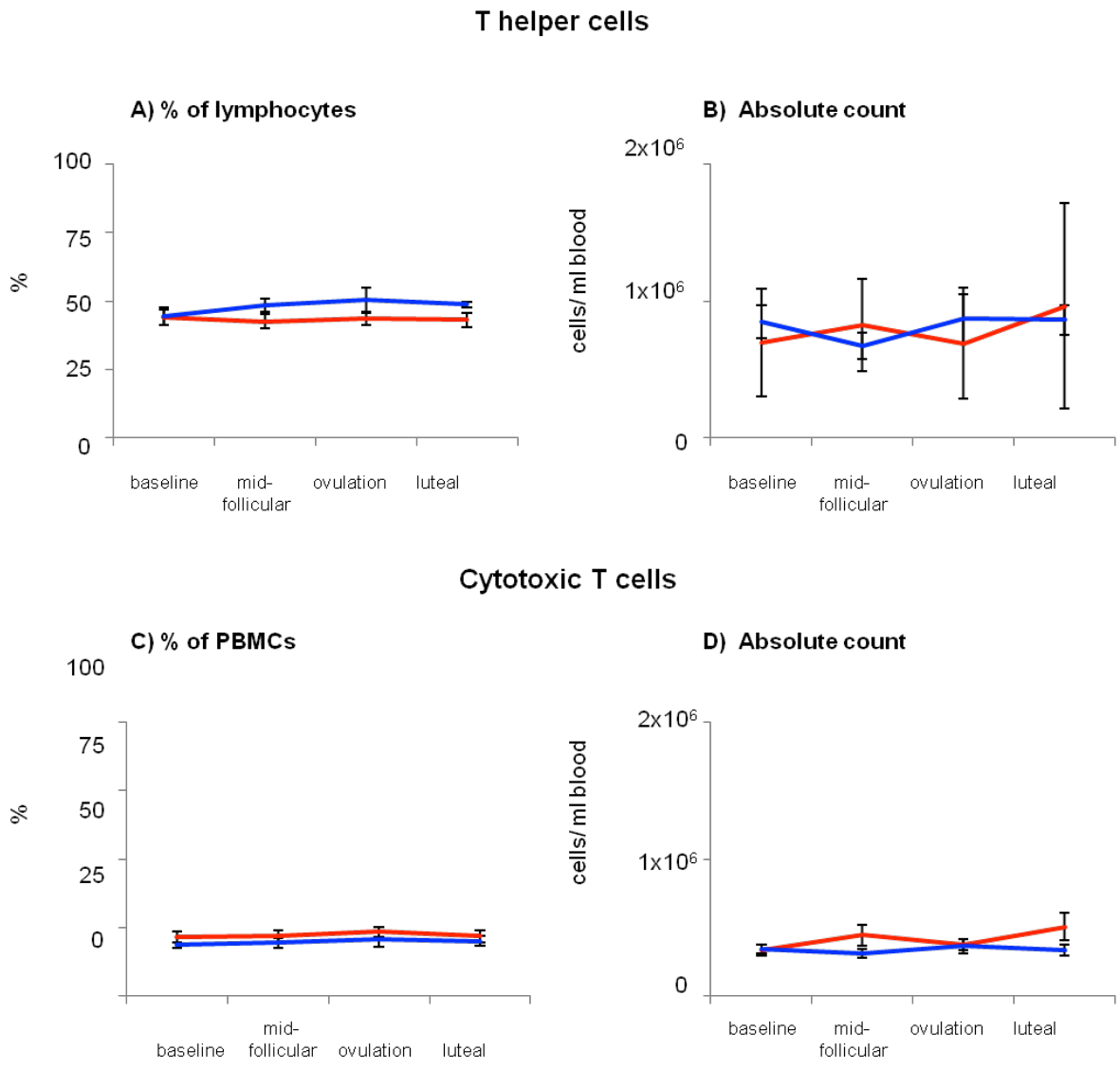


Figure 2-32 Th cells and cytotoxic T cells throughout the normal menstrual cycle of women with recurrent unexplained miscarriage and matched controls. The percentage of lymphocytes that were (A) Th cells and (C) cytotoxic T cells. (B) The absolute count of Th Cells and (D) cytotoxic T cells. Points represent mean values +/- SEM (n=10).

The expression of CD69, IL-18R and ST2L was measured on T helper cells and cytotoxic T cell populations. No differences were seen in CD69 expression in either cell population (Figure 2-33).

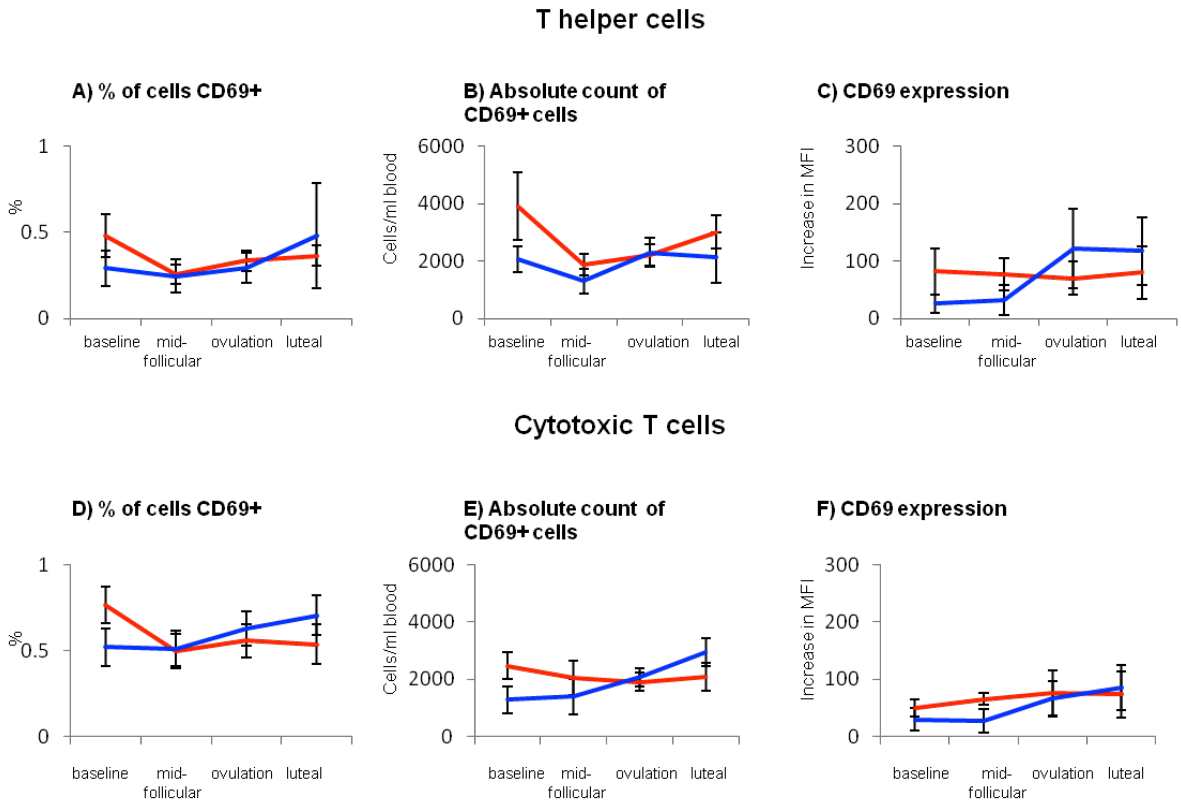


Figure 2-33 CD69 expression by T helper cells and cytotoxic T cells throughout cycle of women with recurrent unexplained miscarriage (red) and matched controls (blue). (A, D) The percentage of cells that were CD69+. (B, E) The absolute count of CD69+ cells. (C, F) The increase in median fluorescence intensity (MFI) of CD69-APC. Points represent mean values +/- SEM (n=10).

In the Th cell population there was no effect of the group or any group phase interaction in the expression of IL-18R, ST2L or on the ratio (Figure 2-34).

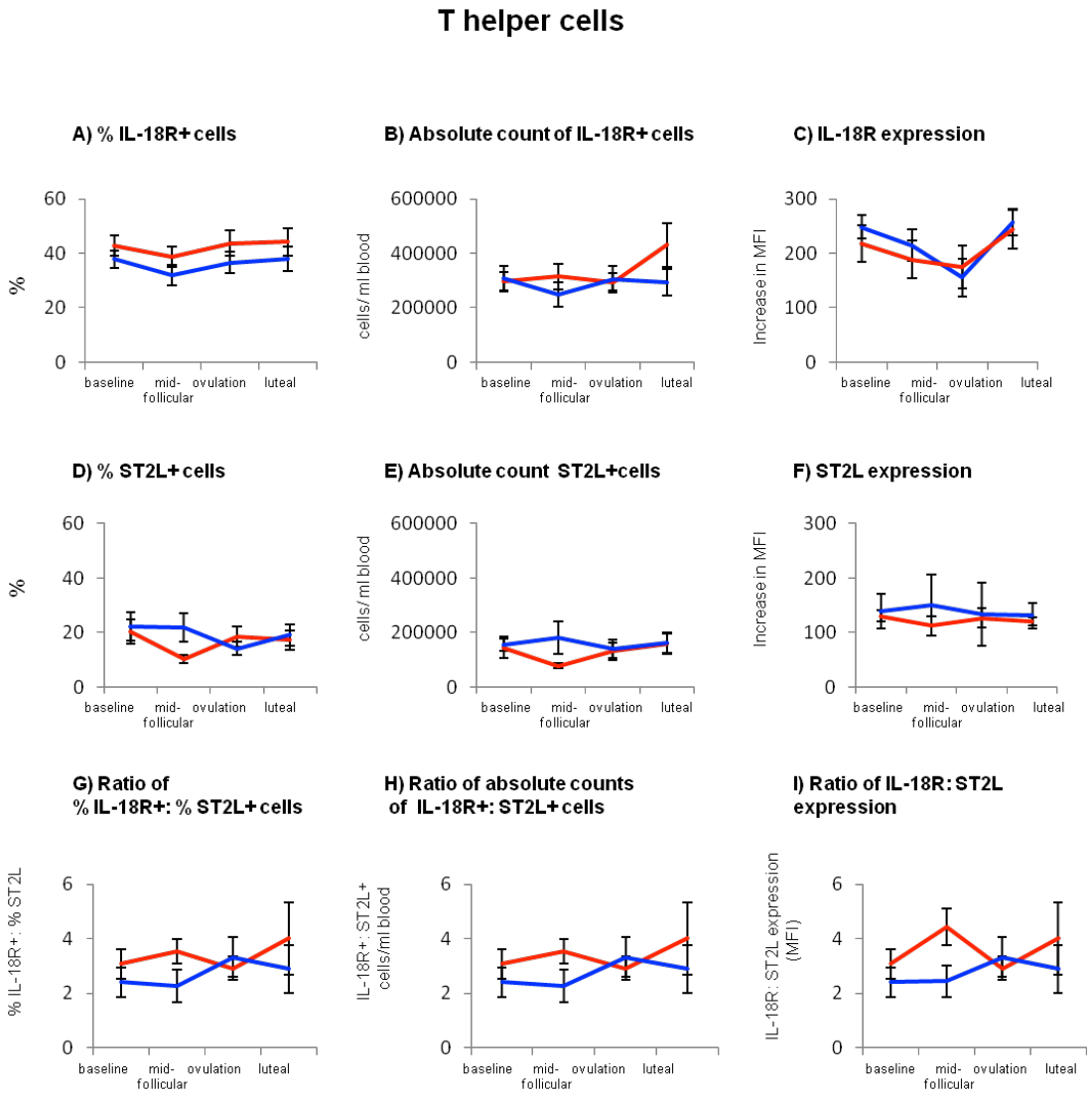


Figure 2-34 Th1 and Th2 subsets throughout the menstrual cycle of women with recurrent unexplained miscarriage and matched controls. Expression of IL-18R (Th 1) and ST2L (Th2) was measured by (A, D) percentage expression, (B, E) the absolute count and (C, F) the increase in median fluorescence intensity (MFI). (G, H, I) Ratios of Th1 and Th2 cells were then calculated. Points represent mean values (+/- SEM) (n=10).

There was a significant main effect of group on the expression of IL-18R by cytotoxic T cell populations measuring the percentage expression ($F=5.24$, $p=0.024$), the absolute count of cells ($F=4.660$, $p=0.045$) and the increase in median fluorescence intensity ($F=7.686$, $p=0.013$). There was a trend to increased IL-18R expression in women with recurrent miscarriage compared to controls at all time points. This reached significance at the mid-luteal time point when IL-18R expression was measured as a percentage, the absolute count or the median fluorescence intensity (Figure 2-35A, B, C). In addition, women with recurrent miscarriage had a significantly increased percentage of cells expressing IL-18R at ovulation and a significantly increased IL-18R expression (measuring MFI) (Figure 2-35A, C). At the mid-follicular time point the miscarriage group had a significant increase in the absolute count of IL-18R+CD8+ T cells (Figure 2-35B) ($p<0.05$). When the ratio of type 1/ type 2 cytotoxic T cells was calculated there was a relative type 1 shift in women with recurrent unexplained miscarriage compared to the control group. This effect of group reached significance only when measuring the ratio of type 1/ type 2 expression using the median fluorescence intensity ($F=6.97$ $p=0.01$). Post hoc tests showed a significant difference at the mid-follicular, ovulation and mid-luteal time points (Figure 2-35i).

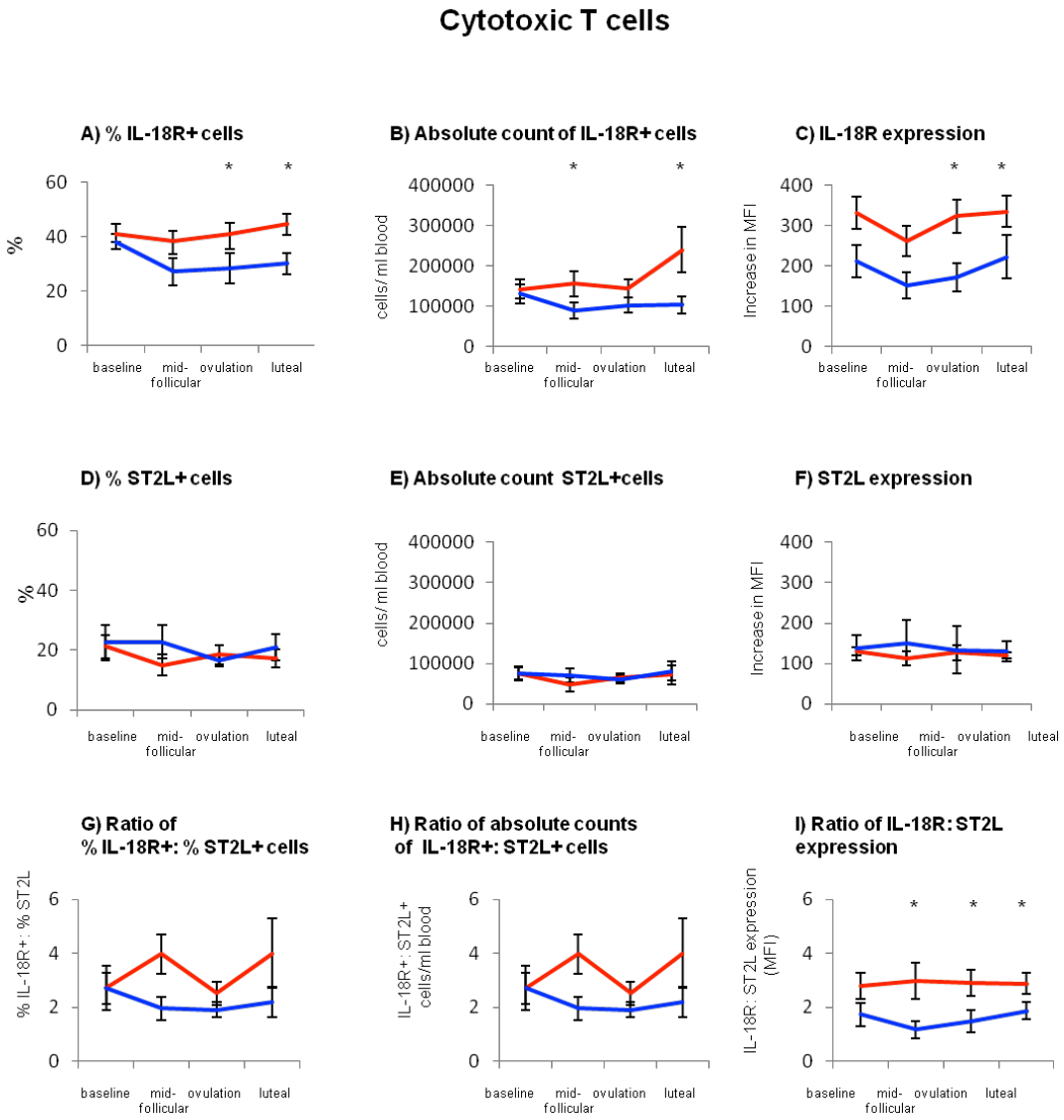


Figure 2-35 Type 1 and Type 2 subsets of cytotoxic lymphocytes throughout the menstrual cycle of women with recurrent unexplained miscarriage and matched controls. Expression of IL-18R (Th1) and ST2L and (Th2) was measured by (A, D) percentage, (B, E) the absolute count and (C, F) the increase in median fluorescence intensity (MFI). (G, H, I) Ratios of Th1 and Th2 cells were then calculated. There was a significant effect of group on the expression of IL-18R which was increased in women with recurrent unexplained miscarriage (A, B, C). This resulted in a relative type 1 ratio when calculated using the increase in MFI (I) (*=$p<0.05$) (n=10).

2.5.1 Does the measurement of mid-luteal NK cells discriminate between women with recurrent miscarriage and controls?

The absolute count of both NK^{dim} and NK^{bright} cells were increased in women with recurrent miscarriage compared to controls in the mid-luteal phase and therefore in order to assess the possible value of these measurements in discriminating women with recurrent miscarriage and controls, a receiver operator characteristic (ROC) curve was plotted. The ROC curve represents the probability of a true positive (sensitivity) as a function of the probability of a false positive (1-specificity). The area under the curve (AUC) measures how good (close to 1) or poor (close to 0.5) a test is. When the data for NK^{dim} cells were plotted, the area under the curve (AUC) was 0.750 (95 C.I. 0.519 to 0.981).

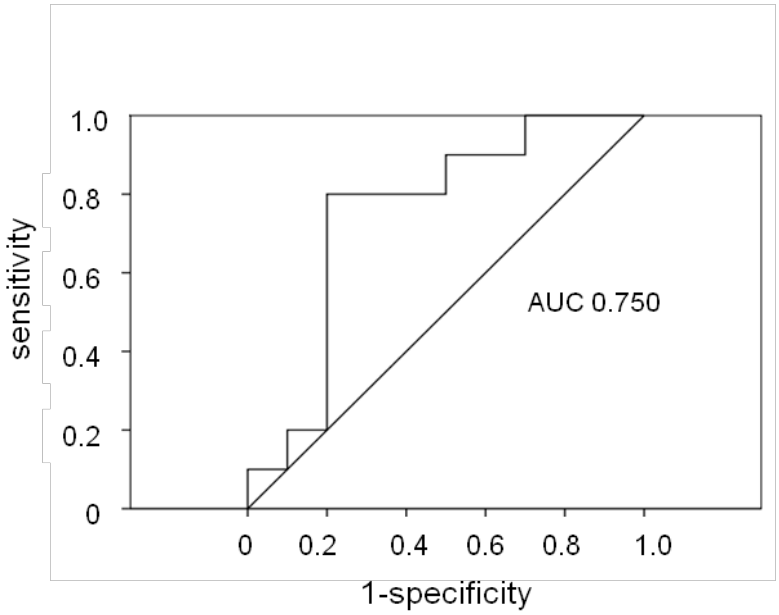


Figure 2-36 ROC curve of NK^{dim} absolute count in the recurrent miscarriage group compared to controls.

Using a cut off point of $1.98x10^5$ NK^{dim} cells/ml to identify women with recurrent miscarriage, the test has a sensitivity of 80% with a specificity of 80%. Using a cut off point of $1.46x10^5$ NK^{dim} cells/ml, the test has a sensitivity of 90% with a specificity of 50%.

When the ROC curve was calculated for the absolute count of NK^{bright} cells, the area under the curve (AUC) was 0.790 (95% C.I. 0.579 to 1.0) suggesting that the measurement of NK^{bright} cells is more predictive of recurrent miscarriage than NK^{dim}. Using a cut off point of 7437 NK^{bright} cells/ml to identify women with recurrent miscarriage, the test has a sensitivity of 90% with a specificity of 70%. Using a cut off point of 9127 NK^{bright} cells/ml, the test has a sensitivity of 70% with a specificity of 80%.

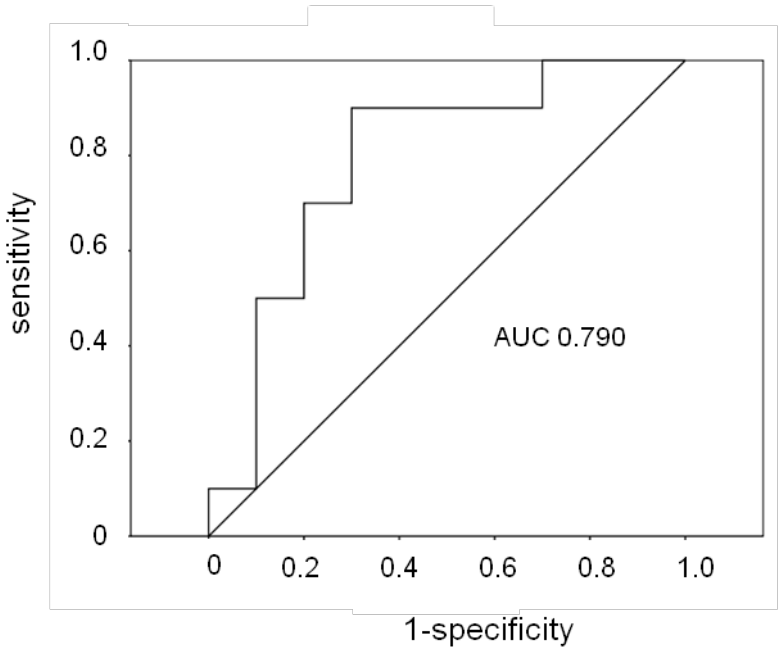


Figure 2-37 ROC curve of NK^{bright} absolute count in the recurrent miscarriage group compared to controls.

2.6 Discussion

The work presented in this chapter suggests that the menstrual cycle does affect some peripheral immune cells in both normal women and in women with a history of recurrent unexplained miscarriage. Firstly, in women with a normal menstrual cycle and no history of subfertility or miscarriage, there is a type 1 shift in the mid luteal phase, in the NK^{dim} and cytotoxic T cell populations. Secondly there is a significant main effect of the menstrual cycle on the NK^{dim} population in women with recurrent miscarriage resulting in an increased absolute count of cells in the mid-luteal phase of the cycle. There is a significant interaction between the patient group (miscarriage or controls) and the phase of the cycle which suggests that the menstrual cycle affects peripheral NK^{dim} cells differently in women with a history of recurrent miscarriage compared to controls. In the mid-luteal phase of the cycle, women with recurrent unexplained miscarriage have a significantly increased absolute count of NK^{dim} cells compared to controls. Women with recurrent miscarriage also have an increased absolute count of NK^{bright} cells, increased cell surface expression of IL-18R by NK^{bright} cells and an increased count of IL18R+ cytotoxic T cells.

2.6.1 Cell numbers in the normal menstrual cycle

In this study the phase of the menstrual cycle did not have a significant effect on the percentage or absolute count of any cell type investigated in the normal population. This is in contrast to a previous study that showed that numbers of circulating T helper cells, cytotoxic T cells as well as monocytes, and granulocytes increased in the luteal phase of the cycle compared to the mid-

follicular phase (Faas *et al.*, 2000). Lee et al recently published data looking at the numbers of CD3+CD4+, CD3+CD8+ and CD3-CD56+ cells in the menstrual cycle of normal women measuring each cell type only as a percentage of lymphocyte population (Lee *et al.*, 2010). They found a significant decrease in the percentage of CD4+ T cells in the mid and late luteal phase and no variation in the CD8+ population. Conversely, they found an increase in CD3-CD56^{bright} and CD3-CD56^{dim} cells in the mid luteal phase. The results of that study illustrate how measuring cells as a percentage of the lymphocyte population rather than the cell count makes data difficult to interpret. For example, by quantifying NK cells as a percentage of the lymphocyte population, it is impossible to know whether the increased percentage of NK cells occurs simply because the numbers of another cell type (such as the T helper cell population) has decreased. Determining the number of cells, rather than the percentage of the lymphocyte population, provides a more accurate quantification. The results presented in this chapter included cell percentages in addition to the absolute count of cells, to enable comparison with previous published work. An additional reason that may explain why the results published by Lee et al. are different from the data presented here is that the time points they used were based solely in relation to menses. Predicting cycle phase purely based on the date of the last menstrual period without the use of hormone measurements has been shown to be inaccurate (Alliende, 2002). This study aimed to define precise inclusion criteria, carefully defined time points within the menstrual cycle (confirmed by urinary LH and serum progesterone) and well phenotyped cell populations.

2.6.2 Cell numbers in women with recurrent unexplained miscarriage

Most previous studies use a wide inclusion criteria defining recurrent miscarriage as 2 or more miscarriages (Emmer *et al.*, 2000; Morikawa *et al.*, 2001; Ntrivalas *et al.*, 2001; Souza *et al.*, 2002; Michou *et al.*, 2003; Yamada *et al.*, 2003; Prado-Drayer *et al.*, 2008). Each of the patients in this study had a minimum of three consecutive miscarriages (with a mean of 4.9). An additional problem with a number of previous studies is that they have not defined the time point of the menstrual cycle when samples were taken, therefore not allowing for any potential effect of menstrual cycle phase on the results (Beer *et al.*, 1996; Yamada *et al.*, 2000; Morikawa *et al.*, 2001; Ntrivalas *et al.*, 2001; Michou *et al.*, 2003; Perricone *et al.*, 2006).Of the three studies that report luteal phase data, (Souza *et al.*, 2002; Prado-Drayer *et al.*, 2008; King *et al.*, 2010). only two studies confirmed the cycle phase by reporting luteal phase serum progesterone levels (Souza *et al.*, 2002; King *et al.*, 2010) and one of these included women with only 2 miscarriages (Souza *et al.*, 2002).

The majority of previous studies addressing the association between recurrent miscarriage and NK cells have reported the whole NK cell population rather than the NK^{bright} or NK^{dim} population despite the fact that they are phenotypically and functionally different. Those studies that have reported separate cell populations have usually looked at the percentage of each population (Beer *et al.*, 1996; Emmer *et al.*, 2000) rather than the more precise cell count. No previous studies have demonstrated a significant difference in

the absolute count of the NK^{dim} or NK^{bright} cells as a proportion of the lymphocyte population.

One recent study has addressed some of these issues, sampling in the luteal phase (although they do not report progesterone levels) and analysing the whole CD56+NK population, as well as the NK^{dim} population, both as a percentage of the lymphocyte gate and absolute count (King *et al.*, 2010). However the only measurement of the NK^{bright} population was a ratio of the count of NK^{bright} and NK^{dim} cells. In that study the only parameter that was significantly different between the recurrent miscarriage group (of women with a negative autoimmune screen) and controls was the percentage of total NK cells (11.1% v 8.80%).

No previous study has investigated NK cells in recurrent miscarriage patients across the menstrual cycle. In contrast to the normal menstrual cycle study, there was an effect of the menstrual cycle on NK^{dim} cells in women with recurrent unexplained miscarriage. Significantly increased numbers of NK^{dim} cells were present in the mid-luteal phase of the cycle compared to the baseline time point and a significant group phase interaction suggests that the menstrual cycle has a different effect on peripheral blood NK^{dim} cells in women with recurrent miscarriage compared to controls. The most obvious difference between these two points of the menstrual cycle is the high progesterone and oestrogen levels in the mid-luteal phase of the cycle compared to baseline.

It remains disputed as to whether oestrogen and progesterone can exert direct effects on peripheral NK cells. Using RT-PCR one group failed to identify mRNA for oestrogen receptors from NK^{bright} or NK^{dim} cells (van den Heuvel *et al.*, 2005; Peralta *et al.*, 2008b) However a recent report using isolated CD3-CD56+ cells identified intracellular oestrogen receptors by flow cytometry (Pierdominici *et al.*, 2010). The presence of progesterone receptors on peripheral blood NK cells is also disputed (Peralta *et al.*, 2008a), however one study found NK^{dim} but not NK^{bright} cells to have nuclear progesterone receptors (Arruvito *et al.*, 2008). That study also found that progesterone induced NK^{dim} cell apoptosis in a dose dependent fashion as well as decreasing IFN-γ secretion. The results presented in this chapter showed no differences in serum progesterone between the recurrent miscarriage group and controls (Table 2-2). The fact that there was an increase (rather than a decrease) in the number of NK^{dim} cells in the miscarriage group would suggest progesterone was not a direct underlying factor. Of course it is possible that progesterone modulates other hormonal or cytokine pathways which then have an indirect effect on peripheral immune cells.

NK cells are known to express β- adrenergic receptors (Landmann, 1992) and anxiety or stress may cause the rapid recruitment of NK cells into the peripheral circulation (Benschop *et al.*, 1996). One study took blood immediately after venous cannulation and again after 20 minutes. That study showed that women with recurrent miscarriage had significantly higher NK cells than controls in the initial blood sample but in a sample taken 20 minutes later (without the need for another needle) they had similar levels to controls.

The authors concluded that the NK cell levels seen in miscarriage patients may be secondary to a transient catecholamine release related to anxiety (Shakhar *et al.*, 2006). The findings presented in this chapter do not concur with this. Although the absolute count of NK^{bright} cells was increased in miscarriage patients at each time point, in the NK^{dim} population only the mid-luteal sample showed an increased NK^{dim} count compared to controls. There is no reason why anxiety levels would be higher at the time of luteal phase sampling relative to any other time point.

No previous study has found an increase in the percentage or number of circulating NK^{bright} cells in recurrent miscarriage patients compared to controls. In this study there was a significant main effect of group on the absolute count of NK^{bright} cells. The mean number of NK^{bright} cells was increased in the miscarriage group at each time point although post hoc tests showed the only significant time point to be the mid-luteal phase (Figure 2-25B). It is possible that a larger sample size would show a significant increase at each time point. One possible reason why this study has shown a difference in peripheral NK^{bright} cells in miscarriage patients when other studies have not, may be the study design. The majority of published studies do not distinguish NK^{bright} cells from the whole NK population. In addition, previous published studies have obtained data from no more than 20,000 events or a maximum of 2000 NK cells (which are approximately equivalent) (Beer *et al.*, 1996; Michou *et al.*, 2003; Yamada *et al.*, 2003; Prado-Drayer *et al.*, 2008; King *et al.*, 2010). In this study 100,000 events were collected by flow cytometry in each experiment. In

collecting 5 fold more events, this study has more power to detect differences in this relatively rare population of cells.

The functional significance of increased NK cells in women with a history of recurrent unexplained miscarriage is as yet unknown and requires investigation. In addition, it is as yet unclear as to whether the findings in the peripheral blood in any way reflect the cell populations in the mid-luteal endometrium. Studies looking at both peripheral and endometrial cells are required to assess if peripheral cell measurements are perhaps a surrogate marker of changes within the endometrium that may be detrimental to normal implantation and pregnancy.

2.6.3 Cell activation measured by CD69 expression

In this study no changes were identified in CD69 expression across the menstrual cycle in any cell group and no differences were identified between miscarriage patients and controls. A small number of studies have previously addressed this issue. One poorly designed study, grouped women with a history of implantation failure (two failed cycles) and women with a history of (two or more) miscarriages together as one cohort. Although a significant increase in the percentage of CD69+CD8+ T cells was identified in this combined study group when compared to controls, no differences were seen when the miscarriage group alone was compared to controls (Yang *et al.*, 2010). A further study identified an increase in the percentage of CD3+CD69+ cells in women with recurrent unexplained miscarriage. However this is a heterogeneous population of cells and the study gives no further information as to which particular cells express CD69 (Prado-Drayer *et al.*, 2008). That study

also found that 83.3% of women with recurrent unexplained miscarriage had more than 3% of NK^{dim} cells that were CD69+ whereas none of the controls had this level of CD69 expression. The level of 3% expression is arbitrary however, and the authors offer no explanation as to its significance. The data presented in this chapter showed 3/10 recurrent miscarriage patients and 2/10 controls with more than 3% CD69 expression.

2.6.4 Markers of type 1 and type 2 peripheral immune cells in the menstrual cycle and in recurrent miscarriage

In order to identify if there were differences in the type 1/ type 2 bias in women with recurrent miscarriage this study first investigated the changes over the menstrual cycle in normal women. There was a significant main effect of the menstrual cycle on the ratio in NK^{dim} cells and in cytotoxic T cells, resulting in a type 1 shift in the mid-luteal phase. This finding contradicts the current published data, where using intracellular cytokine staining or by measuring secreted cytokines from stimulated lymphocytes, other authors have found either no change (Bouman *et al.*, 2001) or possibly a type 2 shift in the mid-luteal phase (Faas *et al.*, 2000; Trzonkowski *et al.*, 2001).

Recent reports have suggested that in mice IL-33 may act on its receptor ST2L, to cause high levels of IFN-γ production (in the presence of IL-12) by NK and iNKT cells (Bourgeois *et al.*, 2009). In humans one group has shown that IL-33 can cause IFN-γ production by Th2 CD4+ cells (Smithgall *et al.*, 2008). These findings, published after this work was started, do mean that the results

of these studies need to be interpreted carefully. It is likely that cells may be only rarely purely type 1 or type 2 deviated and these recent findings in relation to IL-33 would be consistent with that notion.

In this study women with recurrent unexplained miscarriage had increased numbers of IL-18R+ cytotoxic T cells and the cells had significantly increased expression of IL-18R (measured by the increase in median fluorescence intensity). There was a trend to increased numbers of IL-18R+ cells and increased expression of IL-18R by cytotoxic lymphocytes at each time point that reached significance in the mid-luteal phase in each case. Although it is clear that successful implantation is dependent on a relatively inflammatory (type 1) state (Chaouat *et al.*, 2007), it is possible that an excess type 1 environment could be detrimental to the developing embryo. Of course it is important to remember that there remains no clear evidence of a significant relationship between markers of type 1/ type 2 cells in the periphery, pre-pregnancy and the impact that may have at the site of implantation. However these findings do support previous data suggesting a type 1 bias in women with recurrent miscarriage. Several studies have shown that women undergoing recurrent miscarriage have increased circulating type 1 cytokines (Makhseed *et al.*, 2000) or that lymphocyte stimulation (using PHA) of cells from women with recurrent miscarriage produced more type 1 cytokines than controls (Raghupathy *et al.*, 2000a; Makhseed *et al.*, 2001b). However measuring cytokines at the time of miscarriage may be misleading as a type 1 inflammatory cytokine response may be a consequence of miscarriage rather than a predisposing cause (Chaouat, 2007).

One study measured IL-12, IL-18 and IFN-γ in women with a history of recurrent miscarriage at 6 weeks gestation. Women who went on to miscarry had increased circulating type 1 cytokines, although these women were not scanned to confirm viability at the time of pregnancy and thus it is not possible to say if fetal demise had already occurred in some of the miscarriage group potentially leading to a peripheral inflammatory response (Wilson *et al.*, 2004b). There are few studies that investigate pre-pregnancy cytokine bias in recurrent unexplained miscarriage. One group found a significant decrease in intracellular IL-10 but no increase in type 1 cytokines in CD3+CD8+ cells after stimulation with PMA and ionomycin in non pregnant women with a history of recurrent miscarriage. They also found an increase in the ratio of type 1 to type 2 cytokines in T helper cells (Kwak-Kim *et al.*, 2003). A further study has shown an increase in circulating IFN-γ in non-pregnant women with a history of recurrent miscarriage compared to controls. In women with a poor reproductive history; those who went on to complete successful pregnancies had significantly lower serum IL-18 than those who did not (Wilson *et al.*, 2004a).

Aware that successful pregnancy is associated with a shift to type 2 immunity, a number of authors have suggested that the finding of increased NK cells or increased type 1 cytokines in women with recurrent miscarriage justifies treatment with immunotherapy such as steroid treatment to dampen down this response (Szereday *et al.*, 1999; Van Den Heuvel *et al.*, 2007; Heilmann *et al.*, 2010). However it is clear that inflammatory cytokines are present in the endometrium, in both the pre-implantation and early post-implantation period (McMaster *et al.*, 1992; Chaouat *et al.*, 2007). It may be that the presence of

some ‘type 1’ inflammation is required for normal implantation although an excess may be detrimental. Studies of peripheral immune cells and their type 1/type 2 bias in very early pregnancy form the basis of the following chapter.

2.7 Summary

This chapter presents evidence of a type 1 shift in the mid-luteal phase of the menstrual cycle of normal women. Women with recurrent unexplained miscarriage had in addition, increased numbers of type 1 (IL-18R+) cytotoxic lymphocytes and an increased number of NK^{dim} and NK^{bright} cells in the mid-luteal phase of the cycle. It is possible that these data reflect a necessary type 1 shift during the window of implantation and an additional, excess inflammatory response in women predisposed to recurrent miscarriage. However the fact that inflammatory cytokines are required for normal implantation to occur highlights the dangers of unproven immunotherapy used to dampen down the inflammatory response.

3. The influence of IVF treatment and early pregnancy on peripheral immune cells

3.1 Introduction

Chapter 2 presented evidence of changes in the phenotype of peripheral NK and T cells over the course of the menstrual cycle. In addition, pre-pregnancy differences in NK cell numbers were identified in women with a history of recurrent unexplained miscarriage. A number of authors who advocate the testing and treatment of changes in peripheral immune cells in cases of recurrent unexplained miscarriage have also suggested that changes in these cells can be identified pre-pregnancy in women with unexplained sub-fertility (that may potentially be the result of the failure of the early embryo to implant), or in women with recurrent implantation failure after multiple IVF cycles (Beer *et al.*, 1996; Fukui *et al.*, 1999; Ntrivalas *et al.*, 2001). These authors suggest that 'high' numbers of circulating NK cells and their expression of CD69 may be the underlying cause of subfertility or implantation failure. In addition, it has been suggested that an increased absolute count of circulating $CD56^{dim}CD69+$ NK cells at the time of oocyte collection is negatively correlated with the likelihood of IVF success (Thum *et al.*, 2004).

These findings have been questioned by authors who point to the anatomical separation of fetal cells and circulating maternal immune cells in early pregnancy (Moffett *et al.*, 2004) as previously described in section 1.1. There are two main fetal-maternal immune interfaces in human pregnancy. The first

interface occurs where there is contact between invasive extravillous cytotrophoblast and maternal immune cells in the decidua (Moffett-King, 2002). This interface is present from implantation onwards. The second interface is between the circulating immune cells and the placental syncytiotrophoblast. However this interface is not established until the onset of the uteroplacental circulation between 8 and 9 weeks' gestation (Jauniaux *et al.*, 2000). This suggests that although locally infiltrating immune cells may be altered by and/or affect implantation, peripheral immune cells do not contact the developing conceptus, and should have no affect on implantation or ongoing pregnancy.

There is good evidence of a shift towards a type 2 phenotype in peripheral NK cells in later pregnancy (Marzi *et al.*, 1996; Kruse *et al.*, 2000; Raghupathy *et al.*, 2000a; Borzychowski *et al.*, 2005), but it has not yet been established when these changes can first be detected. Studies are hampered because it is difficult to recruit women at the very earliest stages of pregnancy. The majority of studies have recruited women when they are already pregnant, often when they present for their first scan at 10-12 weeks. Our laboratory have previously studied the type 1/ type 2 bias of peripheral blood NK cells throughout pregnancy using ST2L as a type 2 marker. In that study, women were recruited at 12 weeks' gestation and a blood sample was taken in each trimester. Recruits were matched to non pregnant controls for age and parity. There was a significant increase in the percentage of ST2L+NK^{bright} cells at 12 weeks gestation compared to non pregnant controls ($p<0.01$) that persisted throughout pregnancy. There was a trend to increased expression of ST2L by

NK^{dim} cells in the first and second trimester that became significant ($p<0.05$) in the third trimester of pregnancy (unpublished data) (Borzychowski, 2005).

In this study women undergoing a cycle of in vitro fertilisation (IVF) were recruited. As well as enabling the study of peripheral immune changes before and after embryo implantation, investigating this group has the additional advantage in that we can study the effect of hormonal changes on immune cells in vivo; the patients have carefully controlled, and varying extremes of hormone levels (see below), over the course of the cycle.

Given that there is no contact between maternal circulating immune cells and early fetal trophoblast cells, this chapter tests the hypothesis that there are no pre-pregnancy differences in peripheral NK, iNKT and T cell numbers, their immune bias, nor evidence of cell activation between those women who become pregnant after an IVF cycle and those who do not. In addition no changes in these cells were anticipated in the very early stages of pregnancy. Prior to describing the study, I will describe how a cycle of in vitro fertilisation works, and discuss the process of human embryo implantation

3.1.1 IVF

The IVF cycle described here is the protocol used at the Oxford Fertility Unit, from where the patients in this study were recruited. The process of IVF, from initiating the cycle to testing to see if a pregnancy has occurred, takes around 8 weeks. In the course of the normal menstrual cycle gonadotrophin releasing hormone (GnRH) (released from the hypothalamus) stimulates release of follicle stimulating hormone (FSH) from the anterior pituitary. This hormone

causes the recruitment and development of a follicle that will become dominant, mature and subsequently ovulate (Figure 3-1). The first part of an IVF cycle, a process known as ‘down-regulation’ or pituitary desensitisation, aims to stop the normal functioning of this hypothalamic-pituitary-ovarian axis. The patient is initially treated from day 21 of their own menstrual cycle with a gonadotrophin releasing hormone analogue (GnRH analogue). This causes an initial increase in FSH and LH followed by a profound decrease in the secretion of both hormones. A low plasma oestrodiol level confirms that the patient is adequately down-regulated. Once down-regulation is confirmed the process of stimulating the ovaries begins.

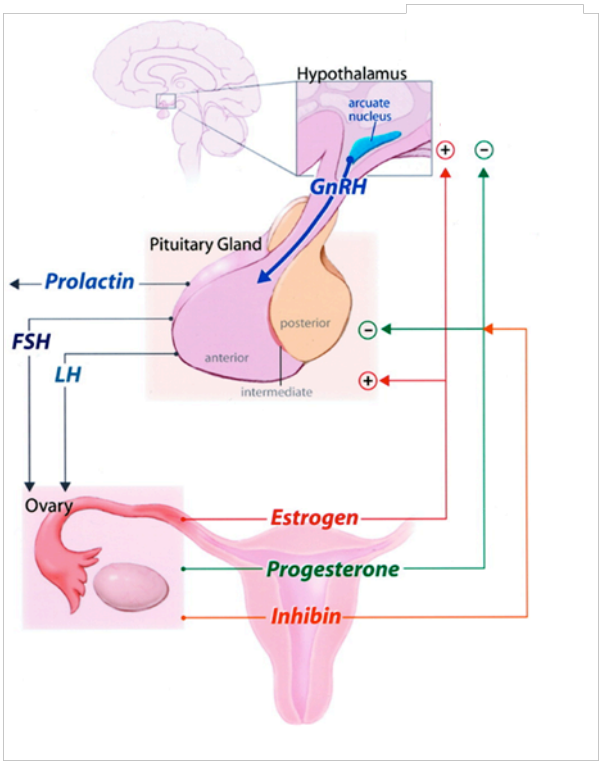


Figure 3-1 Diagram showing the hypothalamic pituitary ovarian axis. Pulsitile hypothalamic release of GnRH (gonadotrophin releasing hormone) stimulates FSH (follicle stimulating hormone) and LH (luteinising hormone) production by the pituitary. Gonadotrophins act on the ovary to stimulate follicular development and steroid hormone production. A surge of LH triggers ovulation and further oestrogen and progesterone synthesis. Oestrogen, progesterone and inhibin exert feedback on both the hypothalamus and pituitary. Reproduced from Foldvary-Shaefer & Falcone (2003)

The aim of this process is to stimulate the ovaries to produce a number of oocytes rather than the single oocyte produced by a natural cycle. Ovarian stimulation is achieved using daily injections of FSH for around 10-14 days. The dose of FSH prescribed for the first cycle of treatment is determined by a number of variables; the patient's age, body mass index and predictors of ovarian reserve (day 2-5 plasma FSH levels and ovarian antral follicle count). The developing ovarian follicles (that will each contain an oocyte) are monitored by serial ultrasound scans of the ovaries (to assess the number and the size of the follicles) in order to predict the optimal time to perform oocyte collection. Pre-ovulatory follicles are around 18mm in size although follicles of 15 mm or more are likely to contain mature oocytes. Individual follicles will develop at different rates, and therefore the timing of oocyte collection is usually dependent on a minimum number of larger follicles (for example at least 3 follicles of greater than 18 mm) identified on ultrasound. The mean number of oocytes retrieved from a single cycle is 8 although the number of oocytes retrieved can vary greatly and is largely dependent on female age.

During this process of ovarian stimulation the patient continues with GnRH analogue treatment to inhibit spontaneous ovulation. Each developing follicle produces oestrogen and therefore ovarian stimulation is associated with supra-physiological plasma concentrations of oestrodiol. Plasma oestrodiol levels are measured at the time of each ultrasound scan. In the normal ovarian cycle there is a surge of luteinising hormone (LH) around 36 hours prior to ovulation. Not only does LH stimulate ovulation, it causes resumption of meiosis within the oocytes and progression to metaphase II - the stage at which an oocyte

can successfully be fertilised. The continuation of the GnRH analogue during the stimulation phase of the cycle not only prevents premature ovulation but also prevents this final oocyte maturation. Therefore 35 hours prior to the timed oocyte collection an injection of Human Chorionic Gonadotrophin (HCG) is given. HCG is structurally similar to LH and therefore mimics the LH surge, thus maximising the number of mature oocytes obtained at the oocyte collection. Oocytes are retrieved trans-vaginally under ultrasound guidance whilst the patient is given conscious sedation (a combination of analgesia and anaesthetic agents).

Fertilisation is achieved by either incubating each oocyte overnight with approximately 100,000 motile sperm or by a process known as intracytoplasmic sperm injection (ICSI). ICSI is primarily used in cases where the cause of sub-fertility has been shown to be a moderately or severely low sperm count, or in cases where sperm have reduced motility. ICSI involves firstly stripping the oocyte of its surrounding cells (known as cumulus cells) and then injecting a single sperm into each oocyte. Fertilisation and pregnancy rates are similar whether fertilisation occurs through conventional IVF or through ICSI. In this study only couples requiring ICSI were recruited. Recruitment of couples with a diagnosis of severe male factor subfertility requiring ICSI and no female factor resulted in a cohort of ‘normal’ female population who had no underlying sub-fertility themselves.

Approximately 24 hours after the oocyte collection, an embryologist examines each of the oocytes for the presence of two pro-nuclei, the indication that

normal fertilisation has occurred. Around 75% of oocytes will fertilise normally after ICSI. Embryos can be replaced into the uterus at any point after fertilisation until day 5 of embryonic development (known as the blastocyst stage). In practice they are replaced on day 2, day 3 or day 5. The decision as to which day to replace the embryos depends on the number and the quality of the embryos obtained.

36 hours after the HCG injection is given and the oocytes have been removed, the collapsed follicles (now known as corpus lutea) begin to produce endogenous progesterone. However during an IVF cycle, exogenous progesterone is also given from the day of oocyte collection until the day of pregnancy test. Additional progesterone has been shown to increase the pregnancy rate (Soliman *et al.*, 1994). A pregnancy test is performed using a urine HCG test sixteen days after oocyte collection and if the pregnancy test is positive an ultrasound scan of the developing embryo occurs two weeks after the positive result.

3.1.2 Implantation

In order to implant, the embryo must attach to the inner lumen of the uterus which is lined by an epithelial cell layer associated with glands. The embryo must then invade through this epithelial layer to establish a pregnancy. This process of embryo implantation involves a complex sequence of events and signal interactions between the embryo and the endometrium. In humans there is a narrow time frame of endometrial receptivity of around 2-4 days in which the embryo may implant (Wilcox *et al.*, 1999; Quinn & Casper, 2009). This “window of implantation” is controlled by ovarian hormones. Oestrogen levels,

which increase prior to ovulation, induce proliferation of epithelial cells. Once ovulation has occurred, progesterone produced by the corpus luteum acts with oestrogen to induce decidualisation. This process is characterised by the influx of uterine NK cells and macrophages along with the transformation of endometrial stromal fibroblasts into secretory decidual cells (Gellersen *et al.*, 2007). In addition, progesterone inhibits the proliferative effect of oestrogen on the epithelium. These processes must be timed to occur with the arrival of the embryo into the uterus.

The embryo reaches the uterus 3-4 days after conception (Croxatto *et al.*, 1978). At this stage the 8-16 cell conceptus will compact to form a morula and on day 5, differentiation into two cell types occurs. Firstly, an outer cell mass of trophectoderm forms. These cells give rise to the extra-embryonic tissues, the developing trophoblast, and ultimately the placenta. The trophectoderm surround a fluid filled cavity known as the blastocoele. Eccentrically placed within that cavity is the inner cell mass that will give rise to the embryo. Throughout this process the embryo remains enclosed in the zona pellucida, a protective glycoprotein membrane.

The next stage of implantation is embryo attachment. In order for this to occur the embryo must first be in close apposition to the luminal epithelium. Prior to attachment, the zona pellucida is shed and the trophectoderm cells come into direct contact with the epithelium. Within hours after contact between trophectoderm and epithelium, the underlying stromal tissue develops and vascular permeability increases. Ultimately the trophoblast penetrates the

epithelium, and the underlying basement membrane, allowing the embryo to invade the stromal cells (Diedrich *et al.*, 2007).

The earliest systemic sign of pregnancy is the presence of the pregnancy hormone HCG. HCG binds to LH receptors on luteal cells of the corpus luteum and stimulates production of progesterone until adequate placental progesterone synthesis is established. HCG is synthesized by the trophoblast of the implanting embryo as early as 6-7 days post fertilisation and is detectable with commercial tests in blood and urine from around 10 days post implantation.

3.2 Aims

1. To investigate whether there are pre-IVF treatment differences in peripheral immune cells in women who become pregnant after IVF compared to those in whom implantation does not occur

2. To investigate whether implantation affects the numbers of peripheral NK cells, iNKT cells, T helper cells and cytotoxic T cells, their immune bias or activation.

3. To investigate whether the hormonal extremes resulting from an IVF treatment cycle affect the numbers of peripheral NK cells, iNKT cells, T helper cells and cytotoxic T cells, their immune bias or activation.

3.3 Materials and methods

3.3.1 Subject recruitment

Ethical approval for these studies was obtained from the Oxford Research Ethics Committee C. Written informed consent was given prior to venepuncture. Women aged 38 or less (n=15) who were undergoing a first cycle of IVF were recruited. All the women recruited had been investigated for sub-fertility and no female factor (poor ovarian reserve, tubal damage, anovulation) was found. Women with polycystic ovarian syndrome were also excluded from the study. In each case, a diagnosis of severe male factor sub-fertility (requiring fertilisation at IVF using ICSI) was made. Each recruit underwent a standard 'long protocol' cycle of IVF as described above. Down regulation was achieved using naferalin acetate (400µg twice daily) (Syneral, Pfizer, New York, USA). Daily subcutaneous recombinant FSH (Gonal-F, Merck Serono, Geneva, Switzerland) along with a reduced dose of naferalin acetate (200µg twice daily) until the decision for oocyte collection was made. A single injection of HCG (Ovitrelle, 6500 i.u., Merck Serono) was given 35 hours prior to the oocyte collection. Progesterone pessaries (Cyclogest, 200 mg twice daily, Activis, Zug, Switzerland) were prescribed from the day of oocyte recovery for 16 days, until the day of the pregnancy test.

Each recruit had a maximum of five blood samples taken at:

Baseline: Day 1-5 of the menstrual cycle in which they commenced IVF.

Down regulation: After two to three weeks of GnRH analogue treatment.

Oocyte Collection: 30 minutes prior to the procedure.

Pregnancy Test: 24 hours before the pregnancy test.

Six weeks gestation: If the recruit was pregnant.

Blood samples were taken between 8 and 10 am. Plasma oestrodiol levels were measured at down regulation and with each ovarian follicular monitoring ultrasound scan. The clinical characteristics of the patients and the cycle are seen in Table 3.1. There were no significant differences between the groups.

	Positive Pregnancy Test Mean (SEM)	**Negative Pregnancy Test Mean (SEM)**
Age	35.2 (1.221)	34.2 (0.931)
FSH (i.u./L)	7.083 (0.813)	8.052 (1.221)
Gondotrophin stimulation (days)	11 (0.365)	11.4 (0.422)
Total gonadotrophin dose (i.u)	2362.25 (307.4)	2246.54 (296.2)
Oocytes collected	12 (3.109)	13.4 (4.224)
2PN embryos	8.6 (1.022)	8.2 (1.366)
Day 3 A grade embryos	1.4(0.516)	1.5 (0.428)
Day 3 B grade embryos	3.333 (0.4216)	3.287 (0.307)
Day 3 C grade embryos	1.87 (0.654)	1.833 (0.543)
Day 3 D/E grade embryos	2 (1.033)	1.533 (1.033)
Embryos transferred	2 (0)	1.957 (0.142)

Table 3-1 Clinical characteristics of recruits and the IVF cycle.

3.3.2 Blood sampling, antibody labelling and flow cytometry

15 ml venous blood was obtained and anti-coagulated with sodium heparin (10IU/mL). The process of PBMC isolation, antibody labeling of cells and flow cytometry was performed as described in chapter 2.

3.4 Statistics

The Gaussian distribution of each data set was assessed using a Kolmogorovv-Smirnov test to determine the appropriate statistical test. Clinical data was compared using a paired t-test. In order to establish whether the pre-pregnancy IVF data (baseline, down-regulation and oocyte collection) could be analysed as a single group (including those who became pregnant and those who did not) the data from these three time points was analysed using a generalised linear model repeated measures ANOVA to look for significant differences between the groups. A repeated measures ANOVA was used to analyse longitudinal data (including pregnancy test and 6 weeks gestation) in both groups. Where significant differences were identified a Bonferroni post hoc test was used to establish which time points were significantly different. For non-parametric longitudinal data a Kruskal Wallace test was used and where a significant variation across the cycle was identified a Dunn's post hoc test was used to establish the time points with significant differences. Where paired data were analysed a paired t-test was used. An unpaired t-test was used to analyse the change between the baseline samples and the pregnancy test samples in the pregnant and non pregnant groups. Values of $p<0.05$ were considered to be significant. Graphpad Prism and SPSS software were used to analyse the data.

3.5 Results

3.5.1 Cycle outcome

2 of the 15 recruits had their cycles abandoned for medical reasons. One cycle was cancelled prior to oocyte collection due to a poor ovarian response and in the second case the embryos were all cryopreserved because of a complication of IVF treatment known as ovarian hyperstimulation syndrome. The following data pertains to the 13 remaining recruits.

8 of the 13 recruits had a positive pregnancy test and in each resulted in a live birth (a live birth rate of 61.5%). 3 of the 8 were twin pregnancies. The group was divided post hoc into recruits with a positive pregnancy test and those with a negative test. One aim of this study was to evaluate whether the hormonal extremes of down regulation (when oestrogen levels are very low) and oocyte collection (when there are supra-physiological oestrogen levels) had an effect on NK, iNKT and T cells in vivo. In order to exclude the possibility that there were pre-pregnancy differences in immune cells in women who achieve pregnancy after IVF and those who do not, the two groups were divided post hoc and compared to look for a main effect of group. Women who did not become pregnant had increased expression of IL-18R by NK^{bright} and NK^{dim} cells. In addition, there was increased expression of both IL-18R and ST2L on cytotoxic T cells (Table 3-2). Therefore the groups were analysed separately.

Cell Type			ANOVA	
NKbright	% of PBMC		p=0.559	F=0.362
	Absolute count		p=0.339	F=0.572
	IL-18 expression	**(increase in MFI)**	**p=0.046**	**F=5.081**
	ST2L expression	(increase in MFI)	p=0.665	F=0.145
	CD69 expression	(increase in MFI)	p=0.214	F=1.741
NKdim	% of PBMC		p=0.250	F=1.475
	Absolute count		p=0.452	F=0.608
	IL-18 expression	**(%)**	**p=0.023**	**F=6.946**
		(absolute count)	p=0.126	F=2.734
		Increase in MFI	p=0.110	F=51.021
	ST2L expression	(%)	p=0.516	F=0.451
		(absolute count)	p=0.580	F=0.324
		(increase in MFI)	p=0.594	F=0.301
	CD69 expression	(%)	p=0.426	F=0.684
		(absolute count)	p=0.470	F=0.560
		(increase in MFI)	p=0.235	F=1.442
iNKT cells	% of PBMC		p=0.992	F=0.342
	Absolute count		p=0.445	F=0.442
	% CD4+		p=0.645	F=0.664
	% double negative		p=0.445	F=0.487
	% CD8+		p=0.646	F=0.979
	CD69 expression	(increase in MFI)	p=0.255	F=.998
T helper cells	% of PBMC		p=0.552	F=0.446
	Absolute count		p=0.442	F=0.121
	IL-18 expression	**(%)**	**p=0.023**	**F=6.965**
		(absolute count)	p=0.529	F=0.423
		(increase in MFI)	p=0.179	F=2.063
	ST2L expression	(%)	p=0.376	F=0.852
		(absolute count)	p=0.939	F=0.006
		(increase in MFI)	p=0.848	F=0.038
	CD69 expression	(%)	p=0.110	F=3.015
		(absolute count)	p=0.167	F=2.191
		(increase in MFI)	p=0.177	F=2.255

Cell Type			ANOVA	
Cytotoxic T cells	% of PBMC			
	Absolute count		p=0.445	F=0.112
	IL-18 expression	**(%)**	**p=0.010**	**F=9.815**
		(absolute count)	**p=0.046**	**F=5.040**
		(increase in MFI)	**p=0.006**	**F=11.482**
	ST2L expression	**(%)**	**p=0.014**	**F=8.560**
		(absolute count)	p=0.690	F=0.168
		(increase in MFI)	**p=0.037**	**F=5.652**
	CD69 expression	(%)	p=0.371	F=0.871
		(absolute count)	p=0.679	F=0.181
		(increase in MFI)	p=0.241	F=1.518

Table 3-2 Recruits were divided post hoc into pregnant and non pregnant groups and the baseline, down regulation and oocyte collection time points were analysed. There was a significant main effect of group on each of the parameters shown in red.

3.5.2 The effect of the IVF cycle in women who became pregnant

A repeated measures ANOVA identified a significant variation in a number of the immune cell parameters across the 5 time points measured in women who became pregnant after IVF. However, post hoc tests showed that none of the significant differences occurred during the treatment cycle itself, either at the point of low oestrogen (down regulation) or oocyte collection (high oestrogen) when compared to baseline. All the significant differences related to implantation, either the pre-pregnancy test time point, or at 6 weeks' gestation. In describing the following results, 'the cycle' will refer to all 5 time points.

3.5.2.1 NK cells

NK cells were identified as previously described in chapter 2. The number of NK^{dim} cells did not vary across the cycle nor did the expression of CD69 (Figure 3-2, 3-3).

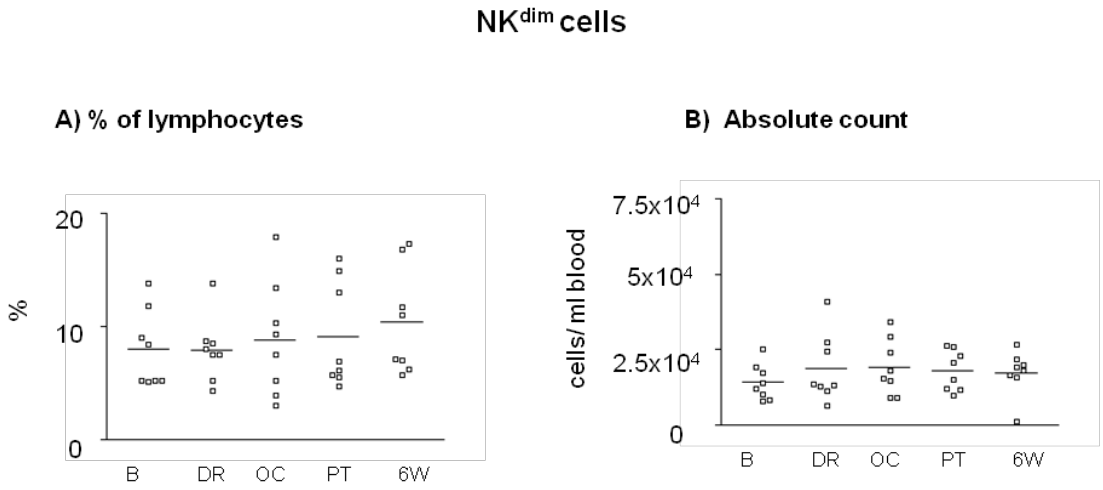


Figure 3-2 Peripheral NK^{dim} cells throughout the cycle. Samples were taken at baseline (B), down regulation (DR), oocyte collection (OC), 24 hours prior to the pregnancy test (PT) and at 6 weeks' gestation (6W). (A) The percentage of peripheral blood mononuclear cells (PBMCs) that were NK^{dim} cells. (B) The absolute count of NK^{dim} cells (n=8).

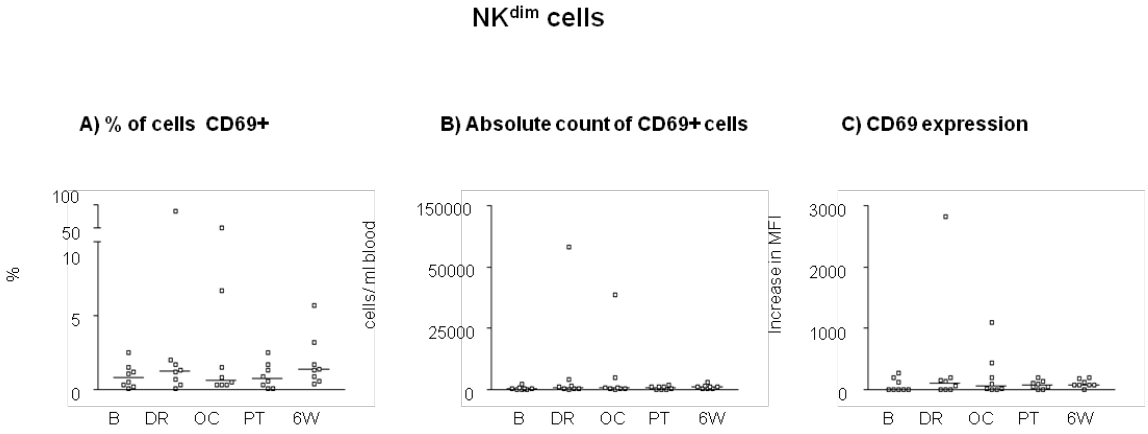


Figure 3-3 Expression of CD69 by NK^{dim} cells throughout the cycle. Samples were taken at baseline (B), down regulation (DR), oocyte collection (OC), 24 hours prior to the pregnancy test (PT) and at 6 weeks' gestation (6W). CD69 expression was measured as (A) the percentage of NK^{dim} cells expressing CD69, (B) the absolute count of CD69+NK^{dim} cells and (C) the increase in median fluorescence intensity (MFI) of CD69-APC on NK^{dim} cells. Bars represent median values (n=8).

Expression of IL-18R by NK^{dim} cells did not vary, however there was a significant main effect of cycle phase on the percentage of ST2L+ NK^{dim} cells ($p=0.0038$, $F=4.944$) and the absolute count of ST2L+ NK^{dim} cells ($p=0.0019$, $F=5.638$). There was a significant increase in ST2L expression between baseline and the pregnancy test time point ($p<0.001$) in both the percentage expression and the absolute count (Figure 3-4E-F) and a significant increase in ST2L expression between baseline and the six week gestation time point measuring the percentage of ST2L+NK^{dim} cells ($p<0.05$) and the absolute count of ST2L+NK^{dim} cells ($p<0.01$). This resulted in a significant main effect of the cycle phase when the ratio was calculated by either the ratio of percentages ($p=0.0138$ $F=3.792$) or absolute counts ($p=0.0015$ $F=1.592$). Post hoc tests identified a type 2 shift in NK^{dim} cells between baseline and both the pregnancy test sample ($p<0.05$) and the sample taken at 6 weeks' gestation ($p<0.05$) (Figure 3-4G-I).

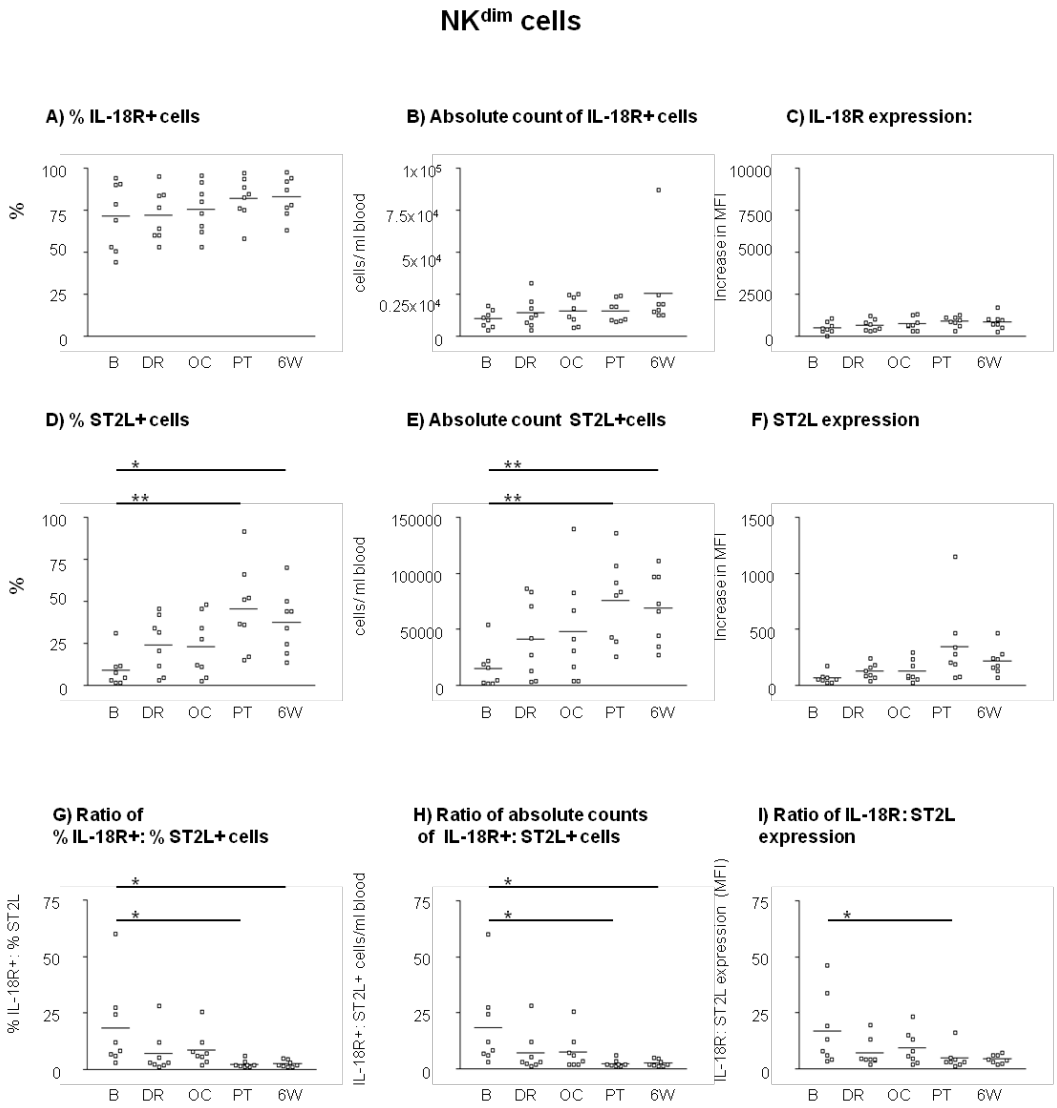


Figure 3-4 Type 1 and Type 2 NK^{dim} cell subsets throughout the cycle. Samples were taken at baseline (B), down regulation (DR), oocyte collection (OC), 24 hours prior to the pregnancy test (PT) and at 6 weeks' gestation (6W). Expression of IL-18R (Type 1) and ST2L (Type 2) cells were measured by (A, D) percentage expression, (B, E) the absolute count and (C, F) the increase in median fluorescence intensity (MFI) (G, H, I). Ratios of Type 1: Type 2 CD56^{dim} NK cell were then calculated. There was significant up regulation of ST2 on NK^{dim} cells. There was a significant main effect of the cycle on (D) the percentage and (E) absolute count of ST2L+ cells. Post hoc tests showed significant differences between baseline and both of the post implantation time points. There was a significant main effect of the cycle on the type 1/ type 2 ratio resulting in a type 2 shift at the pregnancy tests sample (G,H, I) and at 6 weeks' gestation (H,I,) (*=p<0.05, **=p<0.01). Bars represent mean values (n=8).

There was no variation in either the number of NK^{bright} cells as a percentage of the whole lymphocyte population, the absolute count of cells, or the expression of CD69 throughout the IVF cycle or in early pregnancy (Figure 3-5A-C). IL-18R expression varied significantly across the cycle ($p=0.030$ $F=3.124$). A post hoc test showed that there was a significant difference between baseline and 6 weeks gestation ($p<0.05$) (Figure 3-5D). Although there was a significant main effect of the cycle on the expression of ST2L in the NK^{bright} population ($p=0.0489$), post hoc tests did not show significant differences between any two time points (Figure 3-5E). There was a trend to increased ST2L expression at 6 weeks' gestation. There was no effect of the cycle or in the ratio of type 1/ type 2 cells (Figure 3-5F).

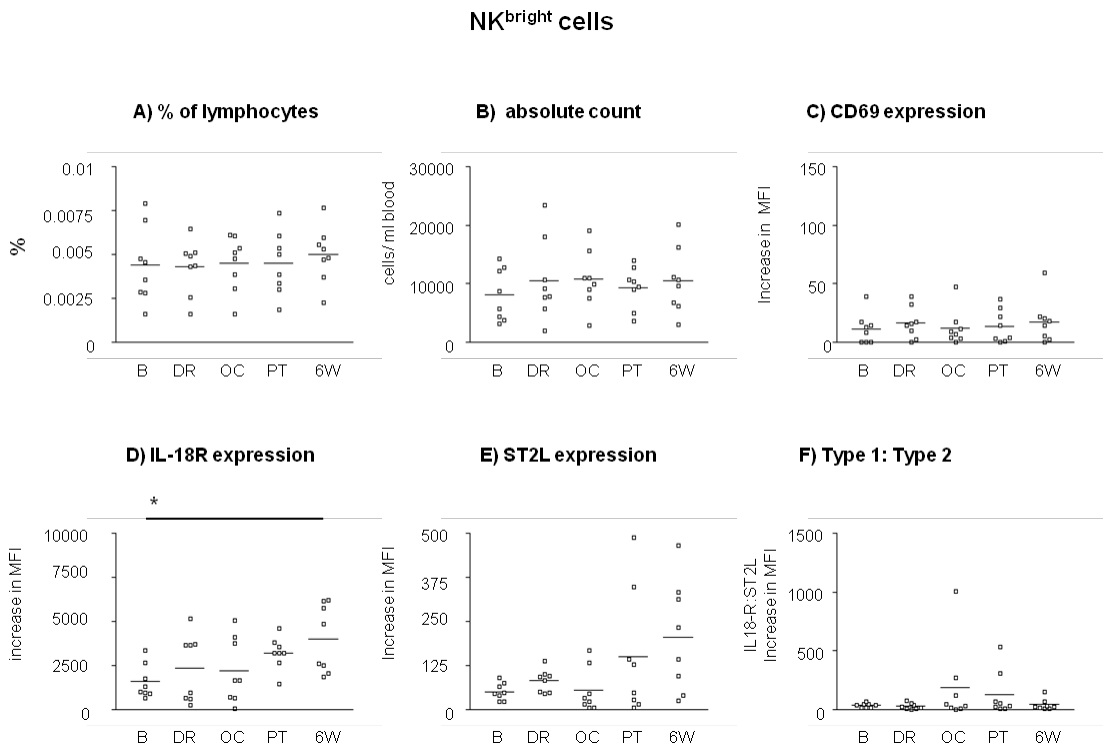


Figure 3-5 Peripheral NK^{bright} cells throughout the cycle. Samples were taken at baseline (B), down regulation (DR), oocyte collection (OC), 24 hours prior to the pregnancy test (PT) and at 6 weeks' gestation (6W). (A) The percentage of peripheral blood mononuclear cells (PBMCs) that were NK^{bright} cells. (B) The absolute count of NK^{bright} cells. (C) Expression of CD69, (D) IL-18R (Type 1) or (E) ST2L (Type 2) cells was measured by the increase in median fluorescence intensity (MFI). (F) Ratios of Type 1 and Type 2 cells were then calculated. Bars represent mean values. There was a significant increase in IL-18R expression at 6 weeks gestation compared to baseline (*$p<0.05$) (n=8).

3.5.2.2 iNKT cells

The numbers of iNKT cells and their expression of CD69 remained constant throughout the IVF cycle (Figure 3-6) and did not change in early pregnancy.

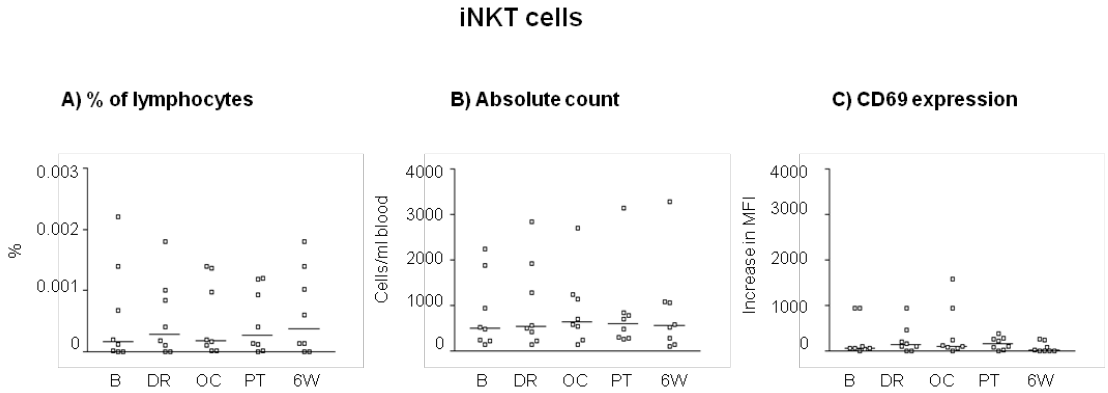


Figure 3-6 (A) The percentage of the lymphocyte population that were iNKT cells, (B) Absolute count of iNKT cells and (C) expression of CD69 by iNKT cells throughout the cycle. Samples were taken at baseline (B), down regulation (DR), oocyte collection (OC), 24 hours prior to the pregnancy test (PT) and at 6 weeks' gestation (6W). Bars represent median values (n=8).

In addition the percentage of cells that were CD4+, CD8+ or double negative and their expression of CD69 did not vary significantly (Figure 3-7).

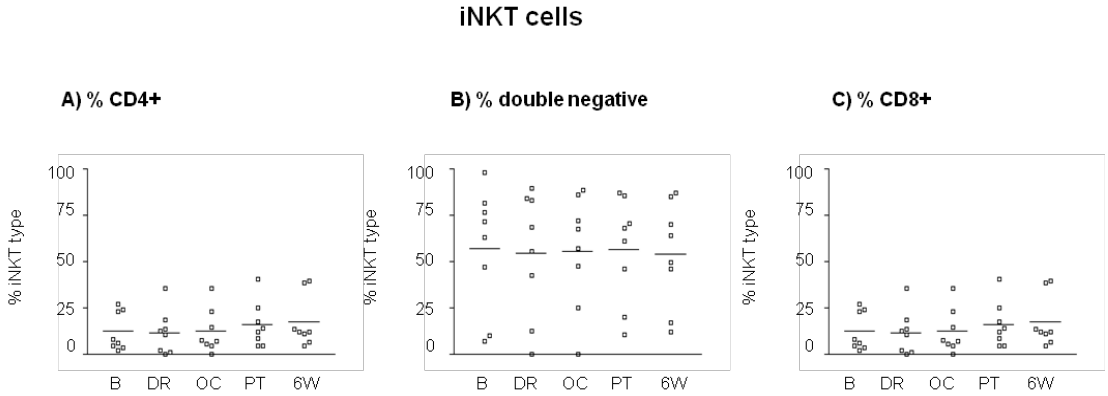


Figure 3-7 Phenotyping of iNKT cells throughout the cycle. Samples were taken at baseline (B), down regulation (DR), oocyte collection (OC), 24 hours prior to the pregnancy test (PT) and at 6 weeks' gestation (6W). Antibodies to CD4 or CD8 identified (A) CD4+, (B) CD4-CD8- and (C) CD8+ iNKT cells. Bars represent mean values (n=8).

3.5.2.3 T cells

There was no significant variation in the numbers of either T helper or cytotoxic T cells and there was no significant effect of either the cycle on their expression of CD69 (Figure 3-8, Figure 3-9).

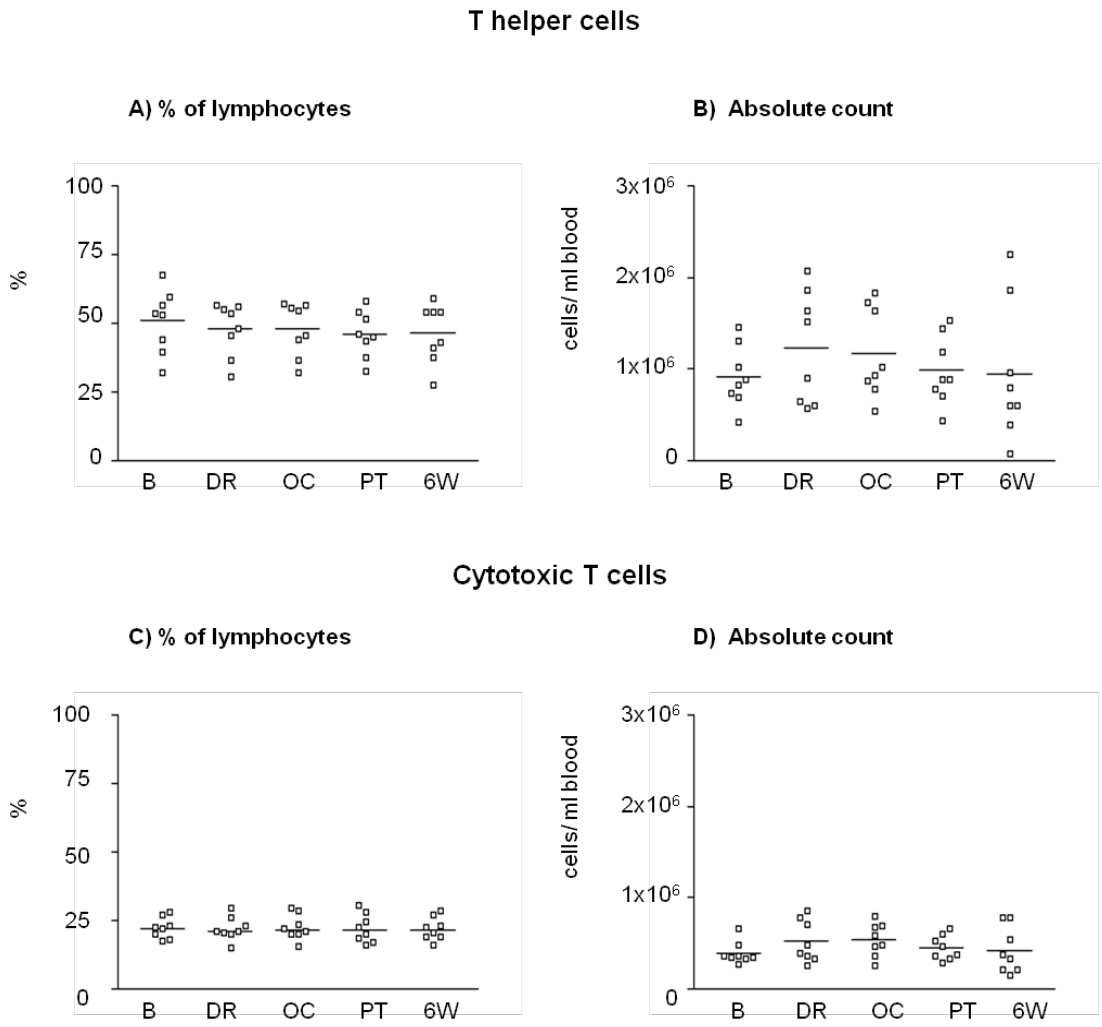


Figure 3-8 Th cells and cytotoxic T cells throughout the cycle. Samples were taken at baseline (B), down regulation (DR), oocyte collection (OC), 24 hours prior to the pregnancy test (PT) and at 6 weeks' gestation (6W). Data shown as the percentage of PBMCs that were (A) Th cells and (C) cytotoxic T cells, or the absolute count of (B) Th Cells and (D) cytotoxic T cells. Bars represent mean values (n=8).

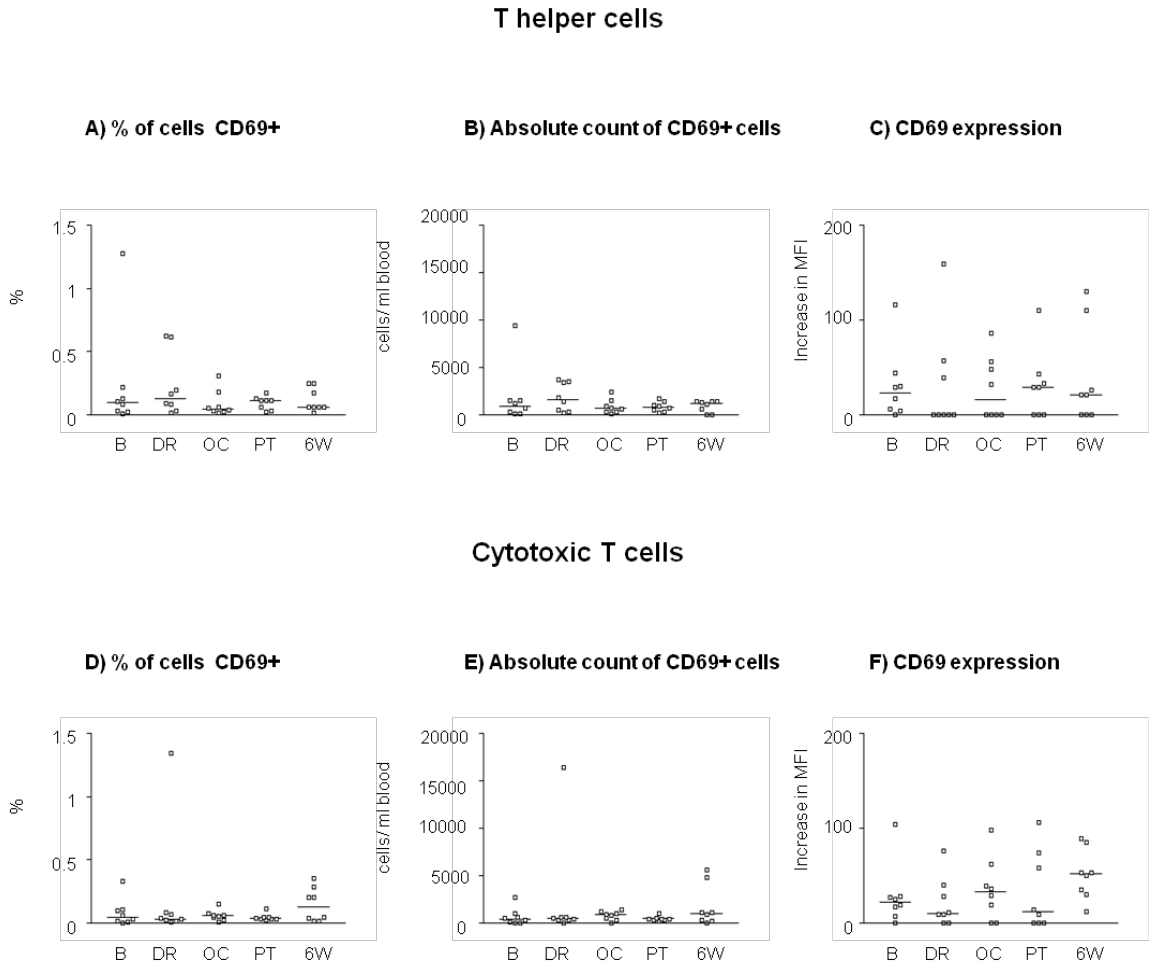


Figure 3-9 Expression of CD69 by Th and cytotoxic lymphocytes throughout the cycle. Expression was measured as (A,D) the percentage of cells expressing CD69 (B,E) the absolute count of CD69+ cells and (C, F) the increase in median fluorescence intensity (MFI) of CD69-APC on the cells. Bars represent median values (n=8).

In the T helper population there was no change in IL-18R expression, however there was a significant main effect of the cycle stage on the percentage of T helper cells that were ST2L positive ($p=0.0155$, $F=3.692$). Post hoc tests showed a significant increase in the percentage of ST2L positive cells between the baseline and pregnancy test, as well at 6 weeks' gestation ($p<0.05$) (Figure 3-10D). There was no variation in the type 1/ type 2 ratio across the cycle.

Similar findings were observed in the cytotoxic T cell population with no change in ST2L expression but a significant main effect of the cycle on the expression of ST2L. There was a variation across the cycle in both the percentage of CD8+ST2L+ cells ($p=0.0016$, $F=5.777$) (Figure 3-11D) and the absolute count of ST2L+ cells ($p=0.0066$, $F=4.447$) (Figure 3-11E). Post hoc tests revealed an increase in ST2L+ cells at the pregnancy test time point compared to baseline ($p<0.05$). When the type 1/ type 2 ratio was calculated from the percentage of cells expressing each marker, there was a significant main effect of the cycle on the ratio of cytotoxic T cells ($p=0.0102$, $F=4.059$) (Figure 3-11G). Post hoc tests identified a significant type 2 shift at both down-regulation ($p<0.05$) and at the time of the pregnancy test ($p<0.05$).

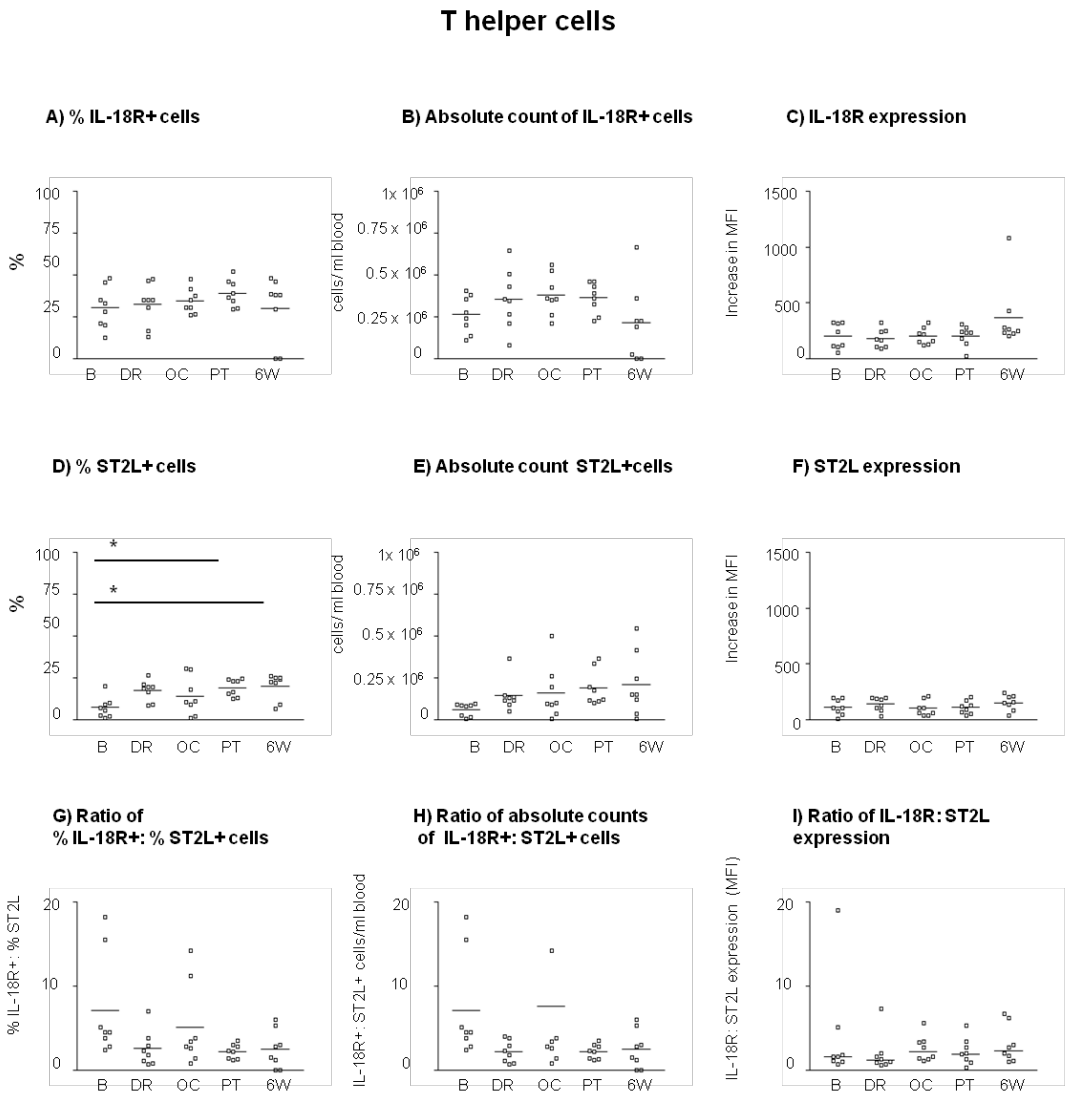


Figure 3-10 Th1 and Th2 subsets throughout the cycle. Samples were taken at baseline (B), down regulation (DR), oocyte collection (OC), 24 hours prior to the pregnancy test (PT) and at 6 weeks' gestation (6W). Expression of IL-18R (Type 1) and ST2L (Type 2) cells were measured by (A, D) percentage expression, (B, E) the absolute count and (C, F) the increase in median fluorescence intensity (MFI) (G, H, I). Ratios of Type 1: Type 2 cells were then calculated. There was a significant main effect of the cycle on (D) the percentage of ST2L+ cells. Post hoc tests showed a significant increase between baseline and both of the post implantation time points (*=$p<0.05$). Bars represent mean values (n=8).

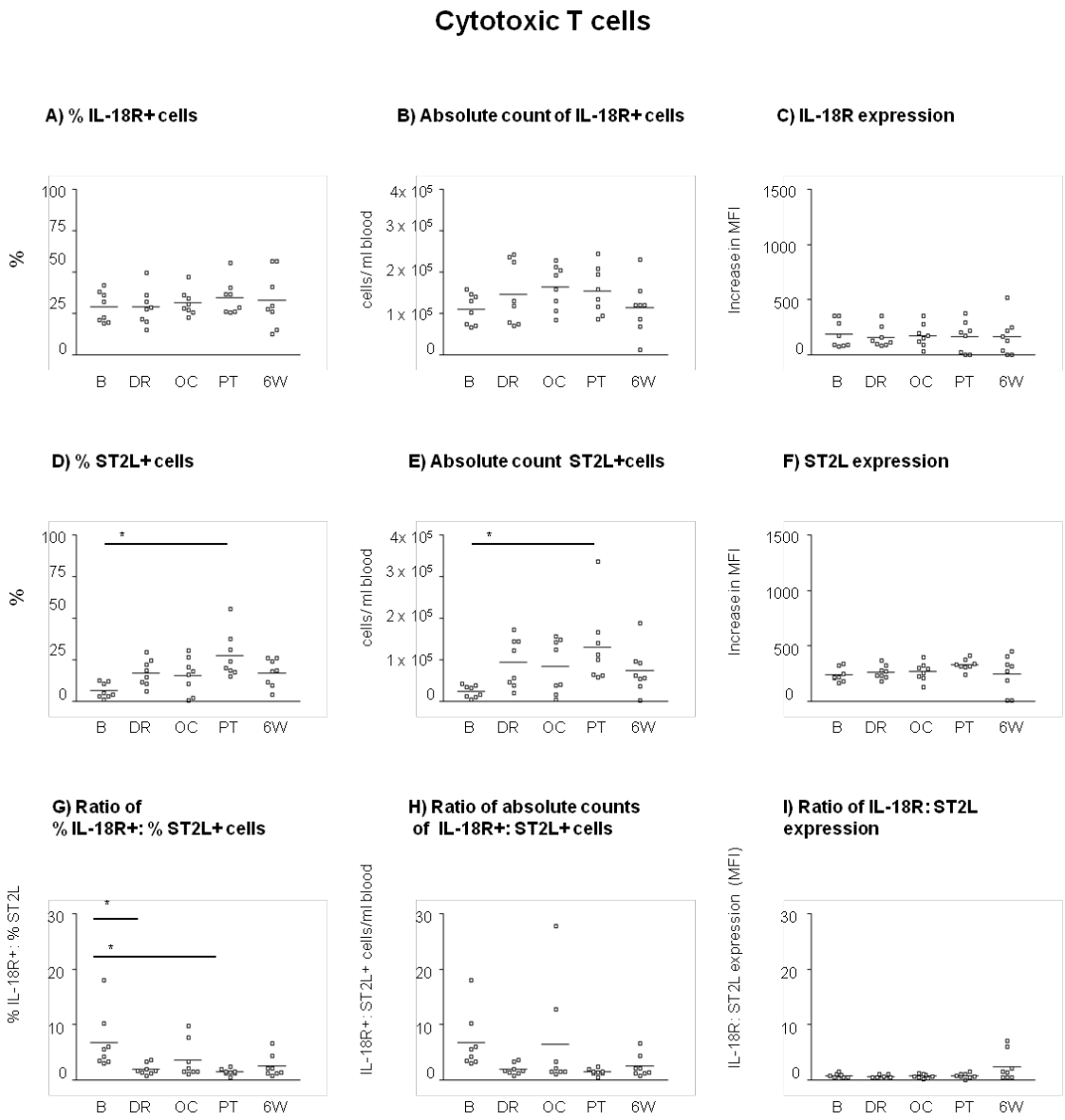


Figure 3-11 Type 1 and Type 2 subsets throughout the cycle. Samples were taken at baseline (B), down regulation (DR), oocyte collection (OC), 24 hours prior to the pregnancy test (PT) and at 6 weeks' gestation (6W). Expression of IL-18R (Type 1) and ST2L (Type 2) cells were measured by (A, D) percentage expression, (B, E) the absolute count and (C, F) the increase in median fluorescence intensity (MFI) (G, H, I). Ratios of Type 1: Type 2 cells were then calculated. There was a significant main effect of the cycle on (D) the percentage and (E) the absolute count of ST2L+ cells. Post hoc tests showed a significant increase between baseline the pregnancy test sample (*=p>0.05). There was a significant main effect of the cycle on the type 1/ type 2 ratio calculated from (G) the percentage of positive cells resulting in a type 2 shift at down regulation and at the pregnancy test sample. Bars represent mean values (n=8).

3.5.3 Variation across the cycle in women with a negative pregnancy test

Although the numbers are too small to prove conclusive, and therefore these results must be interpreted with caution, there was no significant main effect of the cycle on any of the cells investigated in women who went on to have a negative pregnancy test. The repeated finding of up regulation of ST2L seen in the women with a positive test did not occur in any of the women who had a negative test (See Appendix 1).

3.5.4 The effect of implantation on peripheral immune cells

In order to investigate the effect of implantation (resulting in a positive pregnancy test) on the peripheral NK and T cell populations, the change in cell numbers between baseline and pregnancy test was first calculated in pregnant and non pregnant women. In addition, the change in expression (the percentage of positive cells, the absolute count and the change in median fluorescence intensity) of IL-18R and ST2L between the baseline samples and the pregnancy test were compared between those recruits who became pregnant and those who did not. There was no difference in the change of any cell numbers in any of the populations investigated between the pregnant and non pregnant groups (data not shown).

In the NK^{bright} population there was no significant difference in the change of expression of either ST2L or IL-18R in those women who became pregnant

compared to these who did not (Figure 3-12). There was no difference in the change of CD69 expression (data not shown).

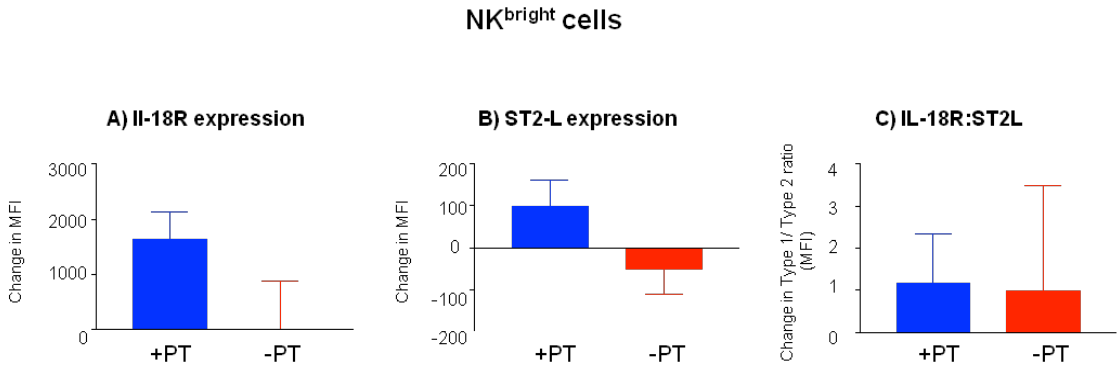


Figure 3-12 The change in expression of (A) IL-18R, (B) ST2L and (C) the ratio of change in median fluorescence intensity (MFI) between baseline and the pregnancy test sample in women who had a positive pregnancy test (+PT (blue)) and those who had a negative pregnancy test (-PT (red)). Bars represent mean +SEM.

In the NK^{dim} population there was up regulation of ST2L (either measuring the percentage of ST2L positive cells (p=0.0081) or the absolute count (p=0.0016) of the cells) in women who became pregnant compared to those who did not (Figure 3-13D, E). When the type 1/type 2 ratio was calculated using either the percentage of positive cells (p=0.030), the absolute count (p=0.029) or the change in median fluorescence intensity (p=0.0018), there was a significant type 2 shift in the women who went on to have a positive pregnancy test compared to those who did not (Figure 3-13G, H, I). There was no difference in the change of CD69 expression (data not shown).

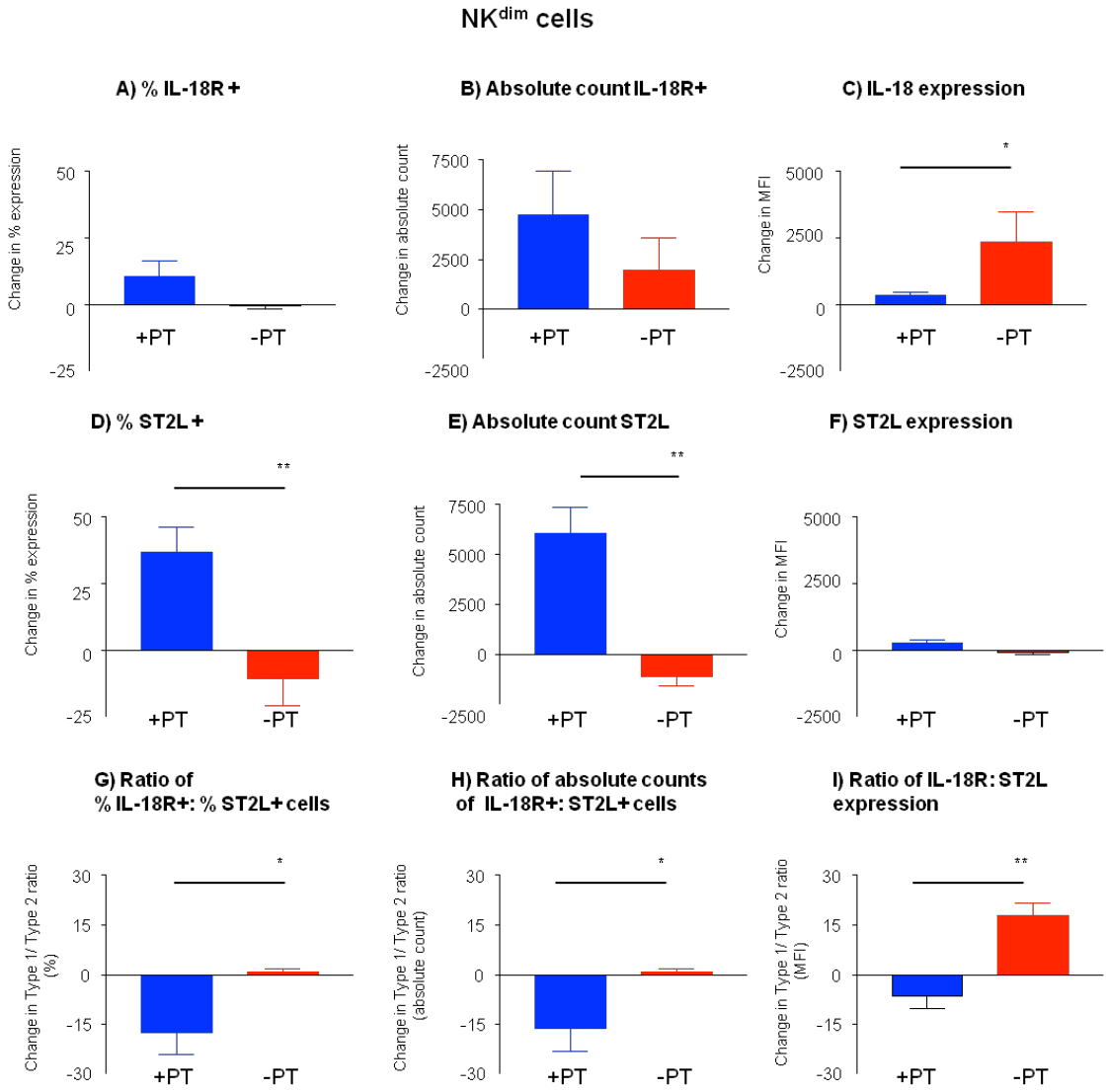


Figure 3-13 The change in expression between baseline and the pregnancy test (PT) sample of Type 1 (IL-18R+) and Type 2 (ST2L) cells were measured by (A, D) the percentage of positive cells, (B, E) the absolute count and (C, F) the increase in median fluorescence intensity (MFI) (G, H, I) in women who had a positive pregnancy test (+PT (blue)) and those who had a negative pregnancy test(-PT (red)). Ratios of Type 1: Type 2 cells were then calculated. There was a significant increase in the expression (MFI) of (C) IL-18R in women between baseline and PT in women with a -PT (*=p<0.05) compared to those with a +PT. There was a significant increase in the change of (D) the percentage of ST2L positive cells and (E) the absolute count of ST2L+ cells (**=p<0.05) between baseline and PT in women who were pregnant. (G,H,I) There was a shift towards a type 2 phenotype between baseline and the pregnancy test sample in women who were pregnant compared to those with a negative PT(*=p<0.05). Bars represent mean +SEM.

In the iNKT cell population there was no significant difference in the change of any of the phenotypic markers (CD4+, double negative or CD8+) between baseline and the pregnancy test sample between women who became pregnant and those who did not (data not shown).

In the T helper cell population there was a significant difference in the change in the percentage of cells expressing IL-18R ($p=0.0155$) (Figure 3-14A) and a corresponding difference in the change in the percentage of cells expressing ST2L ($p=0.0008$) (Figure 3-14D, E) between baseline and the pregnancy test in women who became pregnant compared to those who did not. The change in ST2L expression was greater than that of IL-18R. This resulted in a relative type 2 shift calculated by the percentage expression ($p=0.0452$) or the absolute count ($p=0.0491$) in women who were pregnant compared to those who were not (Figure 3-14G, H). There was no difference in the change of CD69 expression (data not shown).

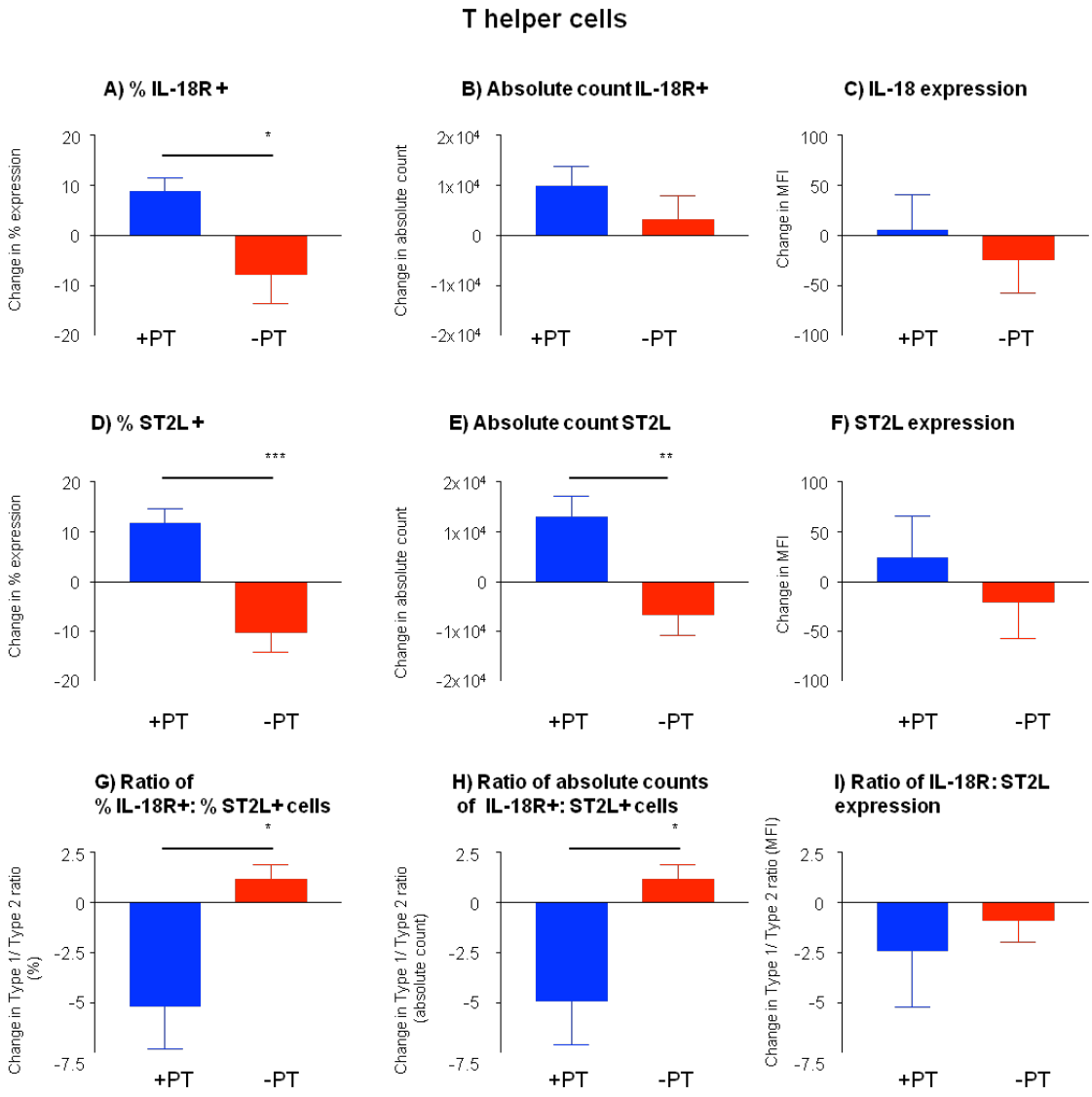


Figure 3-14 The change in expression between baseline and the pregnancy test (PT) sample of Type 1 (IL-18R+) and Type 2 (ST2L) cells were measured by (A, D) the percentage of positive cells, (B, E) the absolute count and (C, F) the increase in median fluorescence intensity (MFI) (G, H, I) in women who had a positive pregnancy test (+PT (blue)) and those who had a negative pregnancy test(-PT (red)) (G, H, I). Ratios of Type 1: Type 2 cells were then calculated. There was a significant increase in (A) the percentage of IL-18R+ cells in women with between baseline and PT in women with a +PT (*=p<0.05) relative to those with a -PT. There was a significant increase in the change (D) the percentage of ST2L positive cells (***=p<0.001) and (E) the absolute count of ST2L+ cells (**=p<0.01) between baseline and PT in women who were pregnant. (G, H). There was a shift towards a type 2 phenotype between baseline and the pregnancy test sample in women who were pregnant compared to those with a negative PT(*=p<0.05). Bars represent mean +SEM.

Similar results were seen in the cytotoxic T cell population where there was a significant change between baseline and pregnancy test in the percentage ($p=0.0074$) and absolute count ($p=0.0491$) of cells expressing IL-18R in those women who has a successful implantation (Figure 3-15A, B). There was also a significant up regulation of ST2L in both the percentage ($p=0.0015$) and count ($p=0.0384$) of the cells (Figure 3-15D, E). Neither of these measurement resulted in a significant shift towards a type 2 phenotype in this population although the ratio of change of the median fluorescence intensity of IL-18R and ST2L did result in a type 2 shift in the women who were pregnant ($p=0.0311$) (Figure 3-15I). There was no difference in the change of CD69 expression (data not shown).

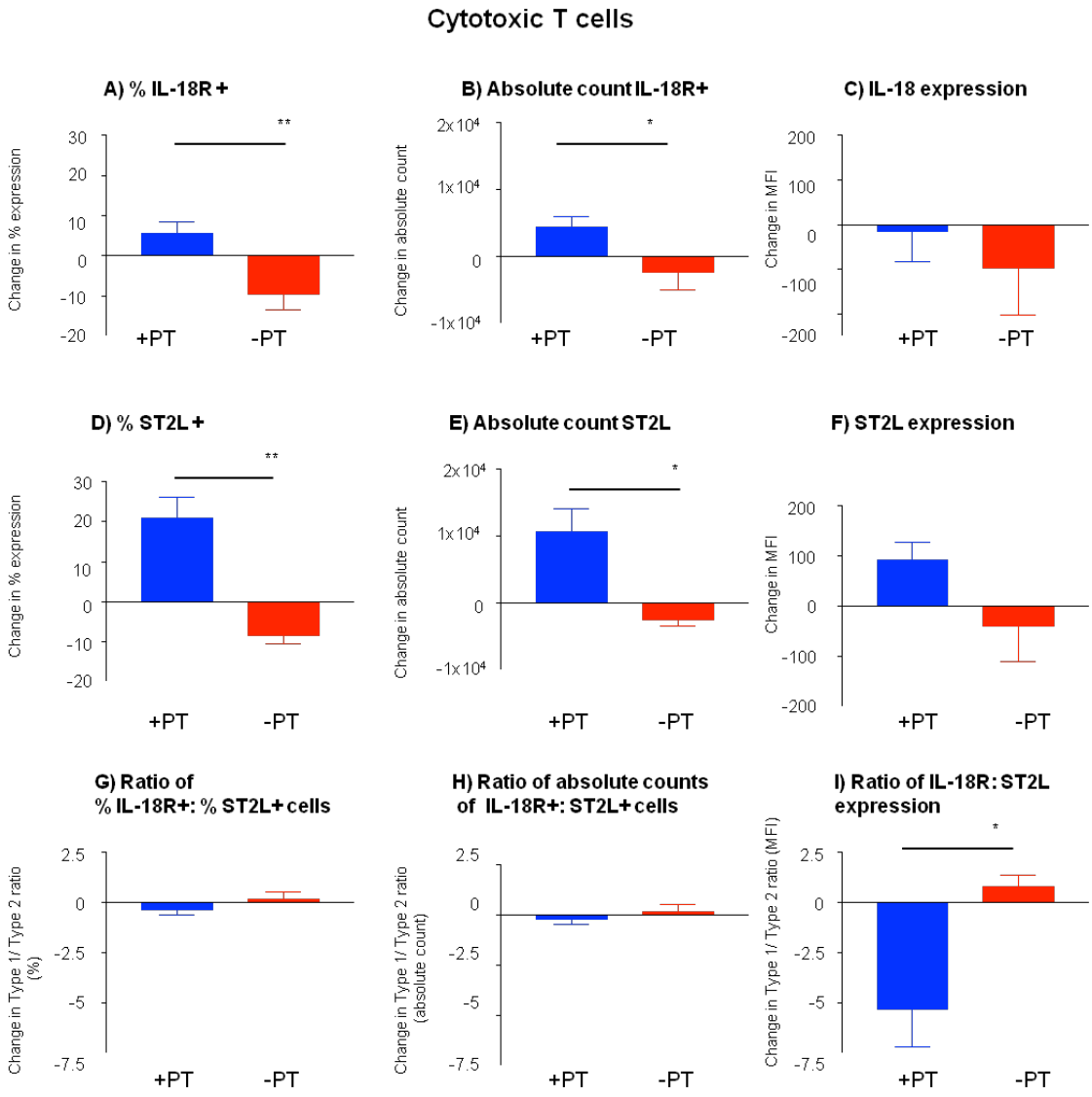


Figure 3-15 The change in expression between baseline and the pregnancy test (PT) sample of Type 1 (IL-18R+) and Type 2 (ST2L) cells were measured by (A, D) the percentage of positive cells, (B, E) the absolute count and (C, F) the increase in median fluorescence intensity (MFI) (G, H, I) in women who had a positive pregnancy test (+PT (blue)) and those who had a negative pregnancy test(-PT (red)) (G, H, I). Ratios of Type 1: Type 2 cells were then calculated. There was a significant increase in (A) the percentage (**=p<0.05) and (B) the absolute count (*=p<0.05) of IL-18R+ cells in women with between baseline and PT in women with a +PT relative to those with a -PT. There was a significant increase in the change of (D) the percentage of ST2L positive cells (**=p<0.01) and (E) the absolute count of ST2L+ cells. (*=p<0.05) between baseline and PT in women who were pregnant. (I) There was a shift towards a type 2 phenotype between baseline and the pregnancy test sample in women who were pregnant compared to those with a negative PT(*=p<0.05). Bars represent mean +SEM.

3.6 Discussion

In this study there was no variation across the IVF cycle in the numbers of NK, iNKT, T helper or cytotoxic T cell populations either in those women who became pregnant or in those in whom implantation did not occur. The striking finding in this study is the consistent up regulation of ST2L on peripheral immune cells in women who become pregnant. Surprisingly, this occurs as early as 24 hours prior to taking a pregnancy test; a maximum of 9 days post implantation. Similar findings were not seen in women who did not become pregnant. When the change in ST2L expression between the baseline sample and the pregnancy test sample was compared in women who did, and did not become pregnant, there was a statistically significant up regulation of ST2L on NK^{dim} cells, T helper cells and cytotoxic T cells in the pregnant group. Although there was also up regulation of IL-18R on T helper and cytotoxic T cells, there remained a trend to a type 2 shift at this time in each of these three cell types and the shift was significant in both NK^{dim} and T helper cells.

The association between pregnancy and a type 2 phenotype is well documented and a number of authors have looked for peripheral changes in the first trimester. Ragupathy et al (2000) stimulated peripheral blood mononuclear cells (PBMCs) from women in the first trimester (mean gestation age of 12 weeks) of normal pregnancy with phytohaemaglutanin (PHA) and found they produced increased levels of type 2 cytokines (IL-4, IL-6, IL-10) and decreased type 1 cytokines (IL-2 and TNF-α) compared to women undergoing miscarriage. However in that study it is possible that the relative type 1 cytokine production identified in the miscarriage group was simply secondary

to the inflammatory process of miscarriage (Raghupathy *et al.*, 2000b). Another group found a decrease in IFN-γ production by PHA stimulated PBMCs in first trimester pregnancy (mean age 8 weeks) compared to non pregnant controls (Marzi *et al.*, 1996). Measuring cytokine mRNA from lysed blood preparations, Kruse et al found decreased IL-18, IFN-γ and IL-2 in the first trimester compared the non-pregnant state (Kruse *et al.*, 2000). This finding was supported by a study that measured intracellular cytokines by flow cytometry and showed a decrease in IFN-γ in CD3-CD56+NK cells and CD3+CD56+ NKT like cells in women between 8 and 10 weeks' gestation compared to non-pregnant women (Shi *et al.*, 2007). Higuma-Myojo and colleagues showed an increase in the proportion of both NK^{bright} and NK^{dim} cells that were IL-10 positive in women between 6 and 12 weeks gestation (Higuma-Myojo *et al.*, 2005). One further study showed a decrease in intracellular IL-4 production by stimulated CD4+ T cells in the first trimester (mean age 9.7 weeks) compared to non pregnant women (Saito *et al.*, 1999). All these studies point to a type 2 phenotype in the second half of the first trimester.

The unique aspect of this study was the ability to investigate possible peripheral immune modulation in very early pregnancy by recruiting patients undergoing IVF treatment. The women recruited were undergoing IVF because their partner had male factor subfertility requiring ICSI and therefore spontaneous conception would be highly unlikely. However each of the recruits had also been investigated for any female cause of subfertility and none had been found. Therefore these women represent a relatively normal (with no evidence of subfertility themselves) population in whom peripheral immune

cells could be investigated at a known point of embryo implantation. In addition, in vivo effects of hormones could be assessed. As no changes were identified at the three measured time points during treatment, this indicates that very low levels of oestrogen and supraphysiologically high doses of oestrogen have no effect in vivo on the numbers or immune bias of any of the cells investigated. In addition, all women receive exogenous progesterone from the day of oocyte collection until the pregnancy test and significant changes were identified in pregnancy test sample in the non pregnant group. The significant change in surface expression of ST2L therefore appears to be related to the presence of an implanted embryo. This change also resulted in a type 2 shift in the peripheral NK^{dim} and cytotoxic T cell populations that has not previously been identified at this very early stage post implantation.

There was no significant change in the number of IL-18R+ cells or change in expression of IL-18R at the pregnancy test sample, compared to previous time points, in women who became pregnant. However there was a significant positive change (between baseline and pregnancy test) in the percentage of T helper and cytotoxic T cells that were IL-18R positive in those women who became pregnant compared to those who did not. Despite this, there remained a type 2 shift in the T helper cell population and no significant change in type 1/ type 2 bias in the cytotoxic T cell population. It may be that very early pregnancy results in a generalised inflammatory response although the overall bias remains towards type 2.

Most previous published data showing peripheral immune changes investigate women in the latter half of the first trimester. The surprising finding in this study is that peripheral immune changes are present before the onset of the utero-placental circulation that does not occur until 8-9 weeks (Jauniaux *et al.*, 2000). The only known embryo derived factor that is circulating at the time of the pregnancy test is HCG and therefore one may speculate that HCG could perhaps be the stimulus for ST2L up regulation. In this study, recombinant HCG was given to women undergoing IVF 35 hours prior to oocyte collection and no changes were noted in any of the cells investigated at that time point. It is possible however that recombinant HCG does not have the same biological effects as endogenous HCG. Endogenous HCG has been shown to be present in both a variant hyperglycosolated as well as regular form, and the biological properties of these forms are known to be different (Cole, 2010). There is evidence for differences in the biological effect of recombinant and endogenous HCG. In a study in which IFN-γ and IL-10 production by stimulated CD4+T cells were measured after the addition of recombinant LH and HCG or recombinant FSH and HCG a significant decrease in IFN-γ production and a significant increase in IL-10 was found. However similar results were not recorded using urinary derived hormones (Carbone *et al.*, 2010).

Although the finding of ST2L up-regulation in the group who became pregnant is consistent, and the results highly significant, the negative findings in the group who did not become pregnant may be reflective of the small numbers Thus these results need to be interpreted with caution and further recruitment

of patients is underway in order to strengthen these findings. The primary reason to use the IL-18 receptor and ST2L was to differentiate between type 1 and type 2 cells, however the notable changes in ST2L raised the question as to the significance of this receptor and its ligand IL-33 in human pregnancy. The following chapter begins to address this question.

3.7 Summary

It is known that pregnancy is associated with a shift towards type 2 immunity. This study has shown for the first time that this occurs very early post implantation. ST2L was significantly up-regulated on both NK and T cells as early as 9 days post implantation.

3.8 Appendix

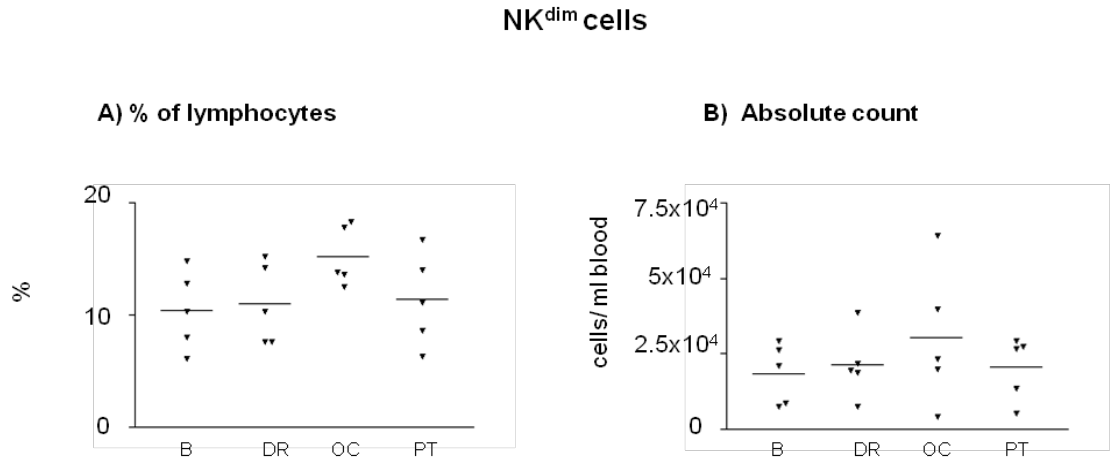


Figure 3-16 Peripheral NK^{dim} cells throughout the cycle when implantation does not occur. Samples were taken at baseline (B), down regulation (DR), oocyte collection (OC) and 24 hours prior to the pregnancy test (PT). (A) The percentage of PBMCs that were NK^{dim} cells. (B) The absolute count of NK^{dim} cells. Although there is a trend towards increased NK^{dim} cells at oocyte retrieval, this did not reach statistically significant levels, possibly due to small sample size. Bars represent mean values.

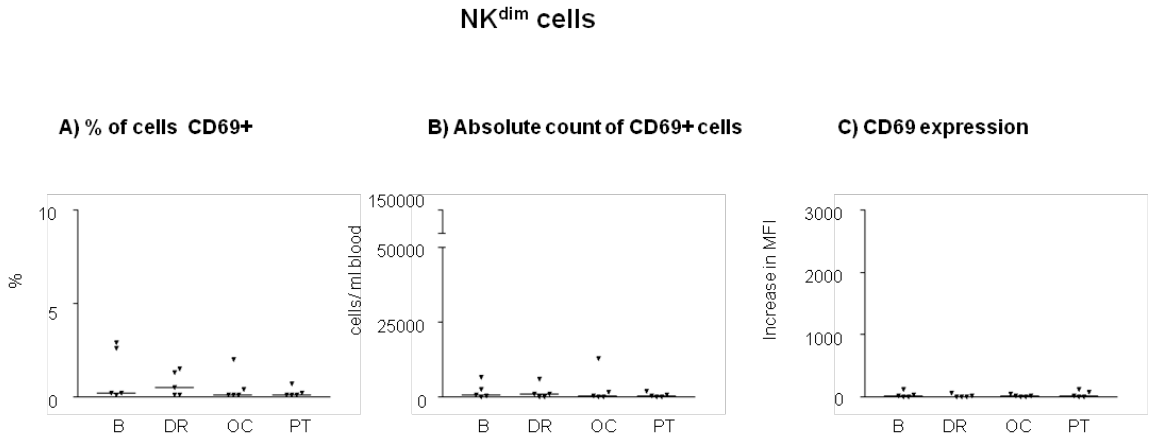


Figure 3-17 Expression of CD69 by NK^{dim} cells does not change throughout the cycle. Samples were taken at baseline (B), down regulation (DR), oocyte collection (OC) and 24 hours prior to the pregnancy test (PT). CD69 expression was measured as (A) the percentage of NK^{dim} cells expressing CD69, (B) the absolute count of CD69+NK^{dim} cells and (C) the increase in median fluorescence intensity (MFI) of CD69 antibody staining on NK^{dim} cells. Bars represent median values.

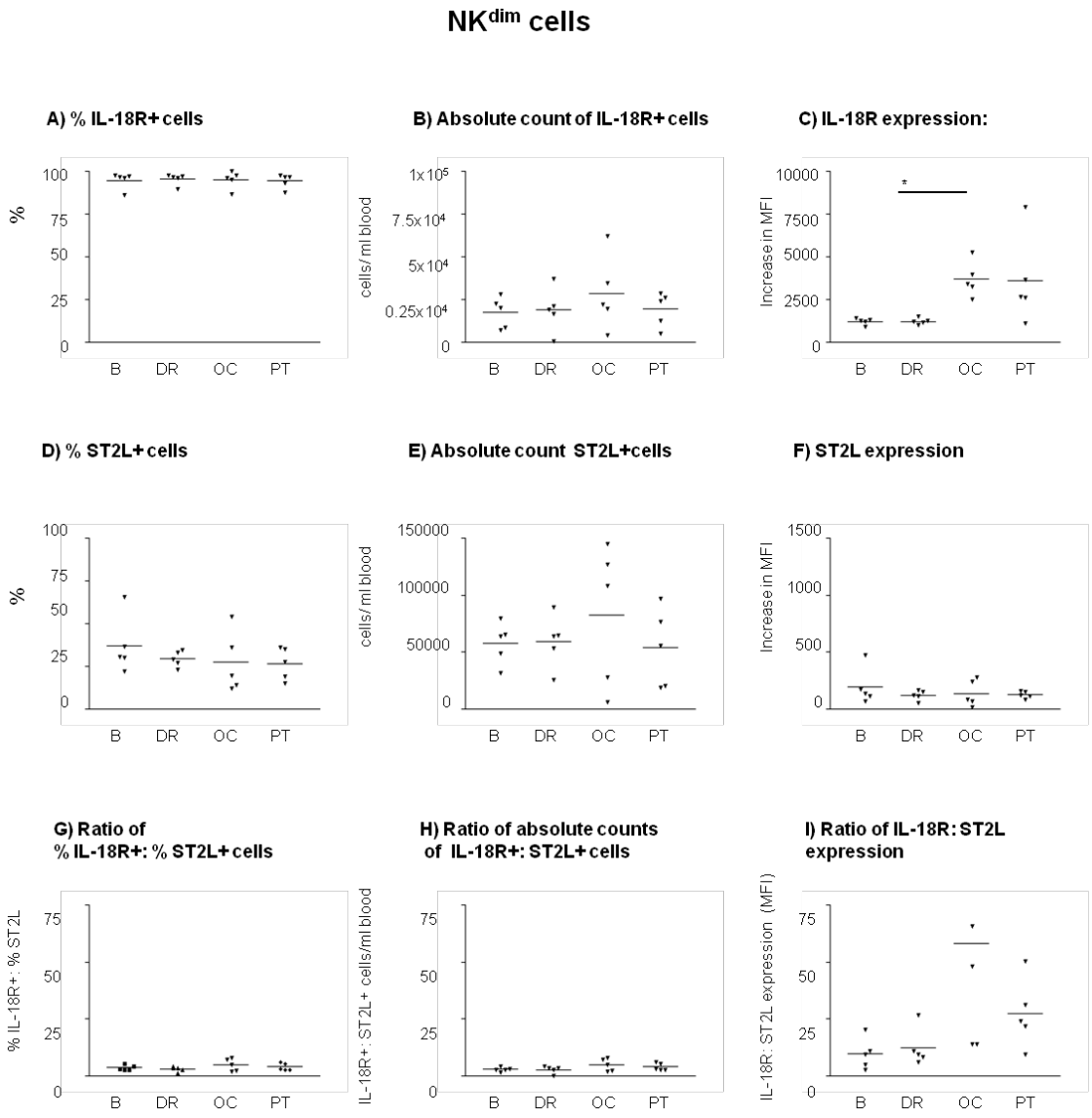


Figure 3-18 Type 1 and Type 2 NK^{dim} cell subsets throughout the cycle. Samples were taken at baseline (B), down regulation (DR), oocyte collection (OC) and 24 hours prior to the pregnancy test (PT). Expression of IL-18R (Type 1) and ST2L (Type 2) cells were measured by (A, D) percentage expression, (B, E) the absolute count and (C, F) the increase in median fluorescence intensity (MFI) (G, H, I). Ratios of Type 1: Type 2 $CD56^{dim}$ NK cell were then calculated. A significant increase in IL-18R expression was detected at oocyte collection on the NK^{dim} cells compared to baseline. Bars represent mean values.

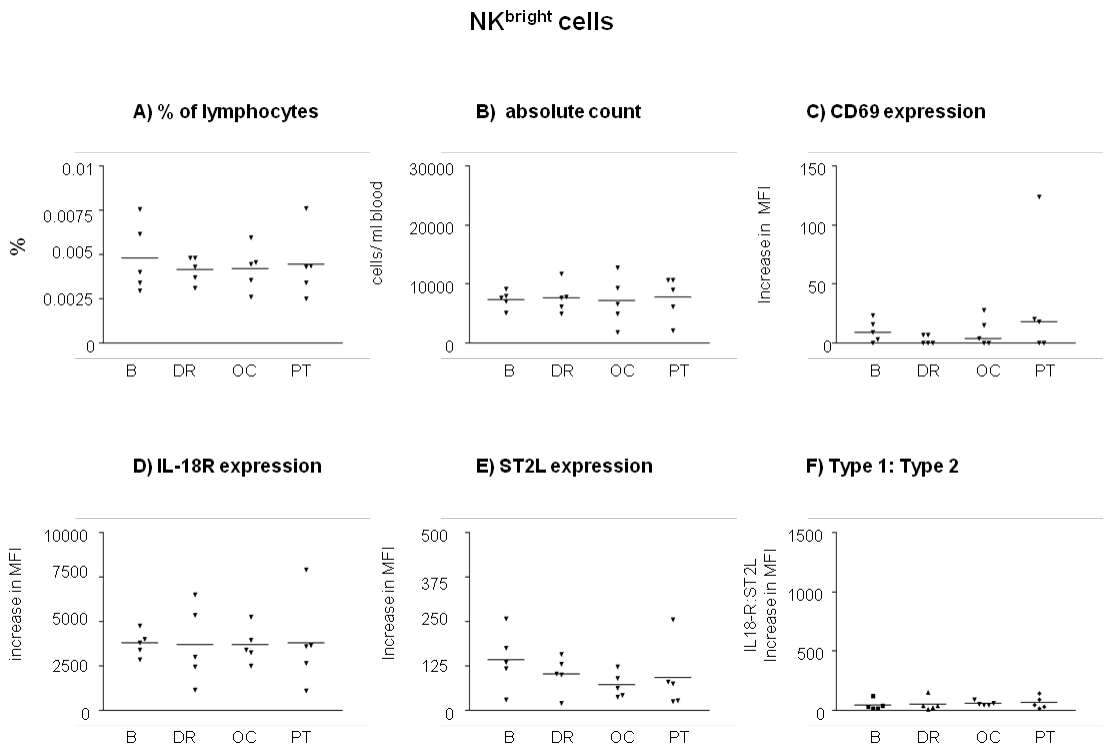


Figure 3-19 Peripheral NK^{bright} cells do not change throughout the IVF cycle when implantation does not occur. Samples were taken at baseline (B), down regulation (DR), oocyte collection (OC) and 24 hours prior to the pregnancy test (PT). (A) The percentage of PBMCs that were NK^{bright} cells. (B) The absolute count of NK^{bright} cells/ ml of blood (C) Expression of CD69, (D) IL-18R (Type 1) or (E) ST2L (Type 2) cells was measured by the increase in median fluorescence intensity (MFI). (F) Ratios of Type 1 and Type 2 cells were then calculated. Bars represent mean values.

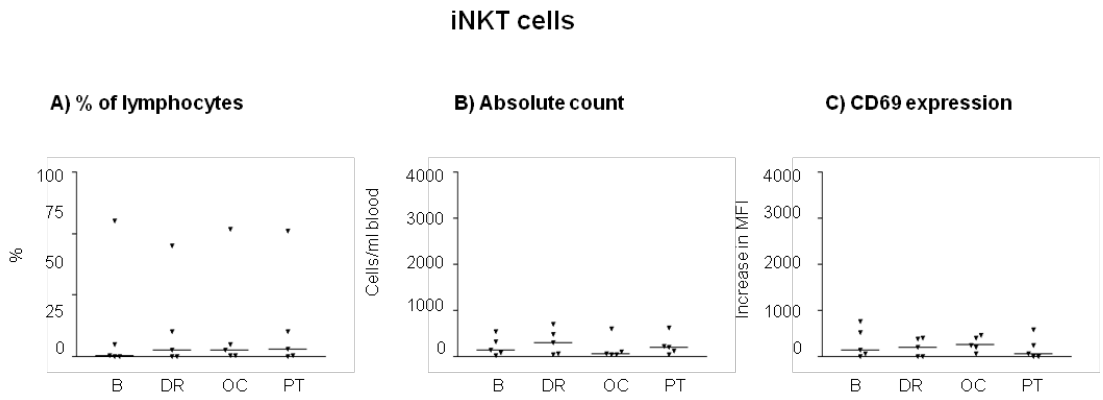


Figure 3-20 The absolute count and activation status of iNKT cells is unchanged throughout an IVF cycle, when the women do not become pregnant. (A) The percentage of the lymphocyte population that were iNKT cells, (B) Absolute count of iNKT cells and (C) expression of CD69 by iNKT cells throughout the cycle. Samples were taken at baseline (B), down regulation (DR), oocyte collection (OC), 24 hours prior to the pregnancy test (PT). Bars represent median values.

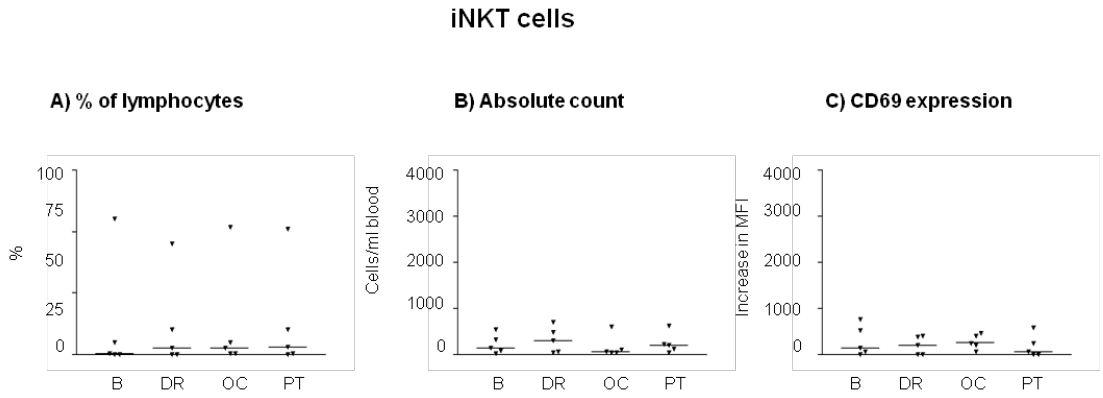


Figure 3-21 Phenotyping of iNKT cells throughout the cycle. Samples were taken at baseline (B), down regulation (DR), oocyte collection (OC) and 24 hours prior to the pregnancy test (PT). (A) Antibodies to CD4 or CD8 identified CD4+, (B) CD4-CD8- and (C) CD8+ iNKT cells. Bars represent mean values.

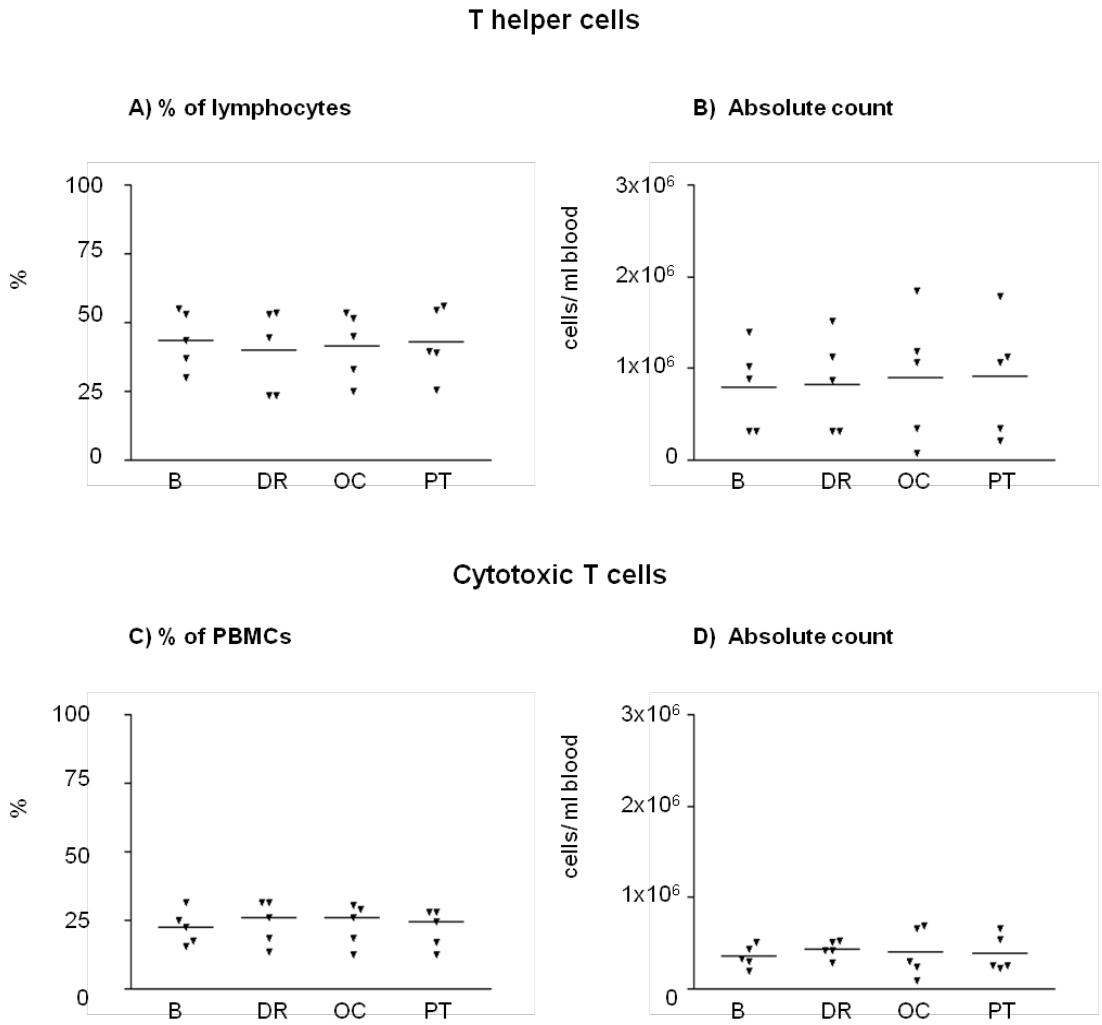


Figure 3-22 Th cells and cytotoxic T cells throughout IVF cycle. Samples were taken at baseline (B), down regulation (DR), oocyte collection (OC) and 24 hours prior to the pregnancy test (PT). The percentage of PBMCs that were (A) Th cells and (C) cytotoxic T cells. The absolute count of (B) Th Cells and (D) cytotoxic T cells. No changes were detected. Bars represent mean values.

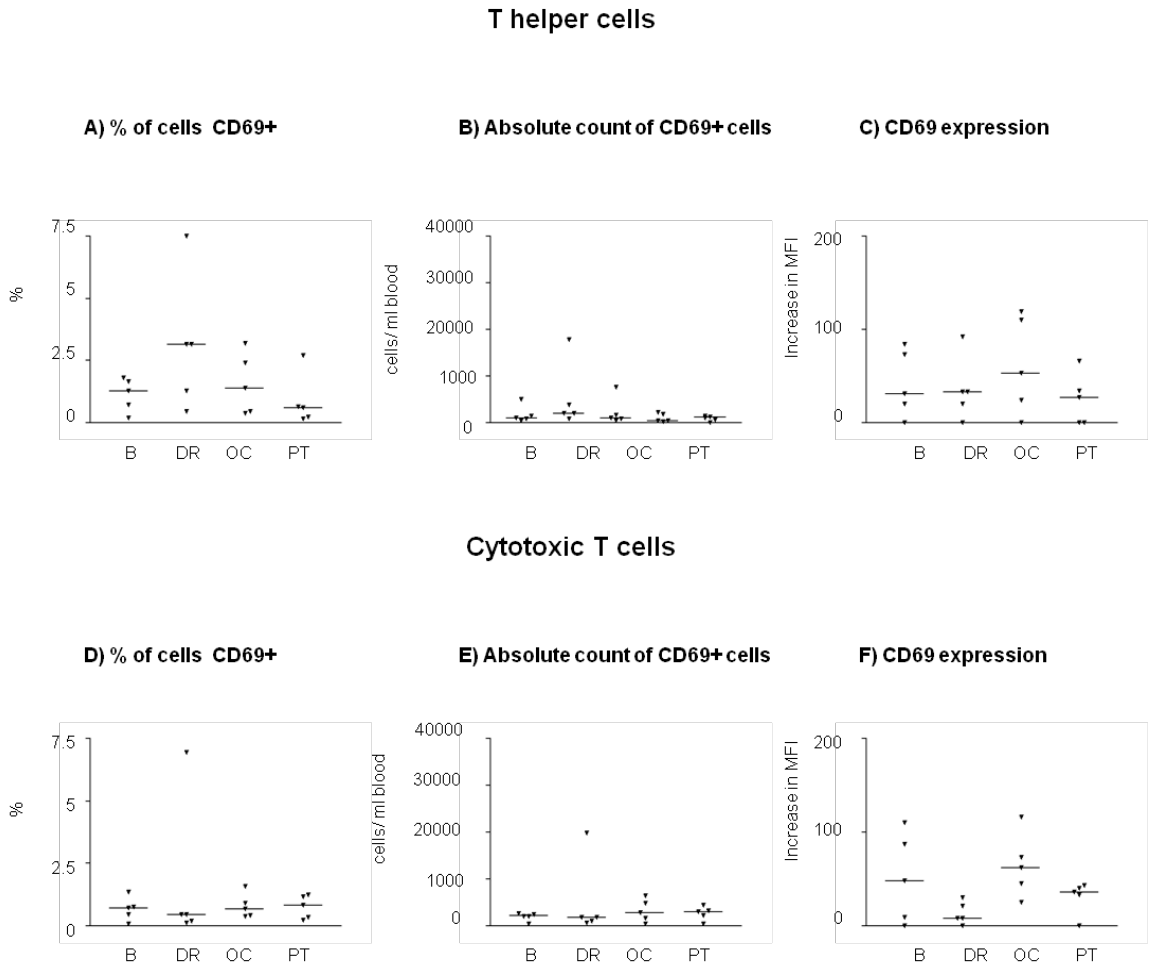


Figure 3-23 Expression of CD69 by Th and cytotoxic lymphocytes. Is unchanged throughout the IVF cycle. Expression was measured as (A,D) the percentage of cells expressing CD69 (B,E) the absolute count of CD69+ cells and (C, F) the increase in median fluorescence intensity (MFI) of CD69-APC on the cells. Bars represent median values.

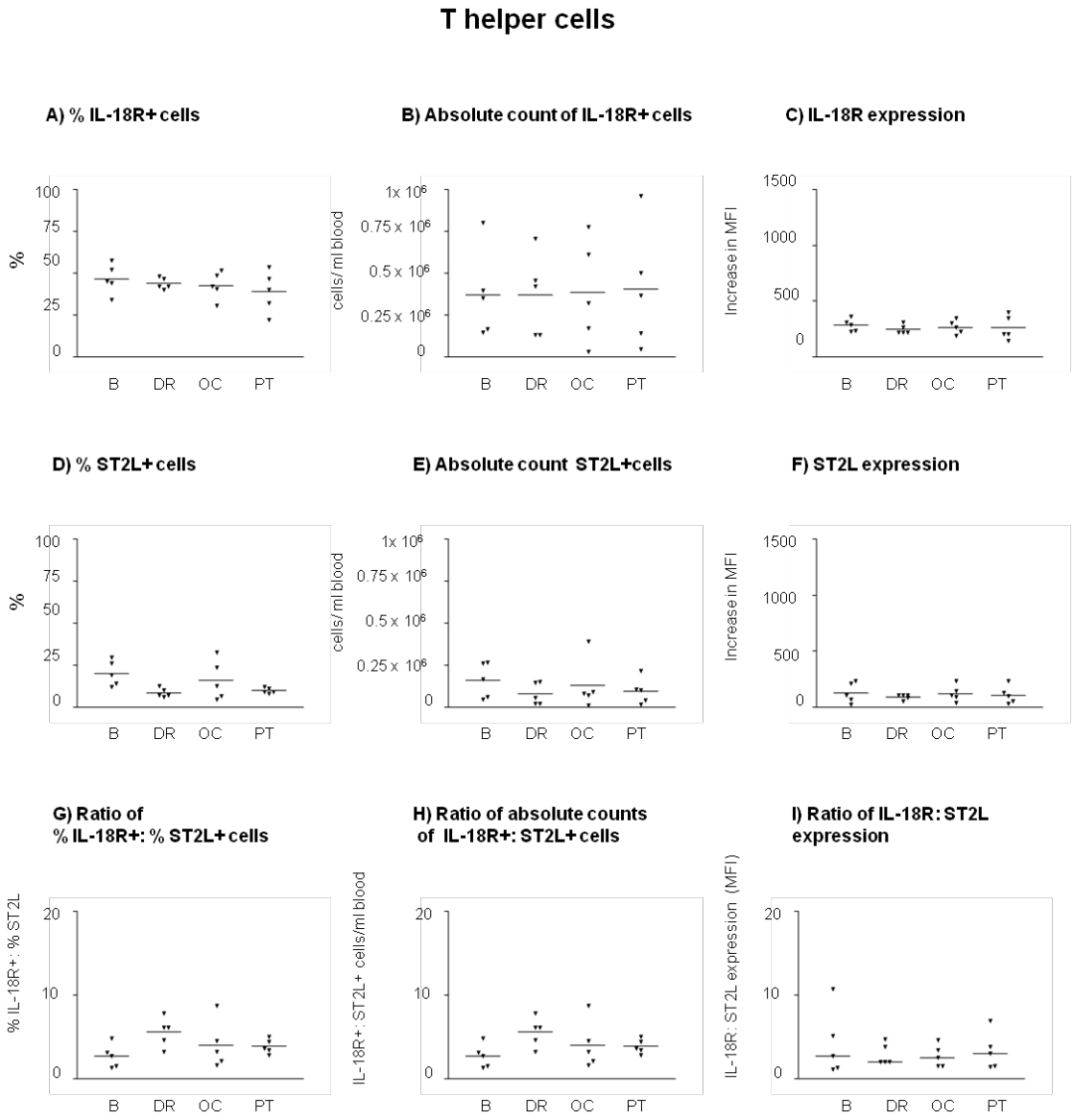


Figure 3-24 Th1 and Th2 subsets throughout the cycle. Samples were taken at baseline (B), down regulation (DR), oocyte collection (OC) and 24 hours prior to the pregnancy test (PT). Expression of IL-18R (Type 1) and ST2L (Type 2) cells were measured by (A, D) percentage expression, (B, E) the absolute count and (C, F) the increase in median fluorescence intensity (MFI) (G, H, I). Ratios of Type 1: Type 2 cells were then calculated. Bars represent mean values.

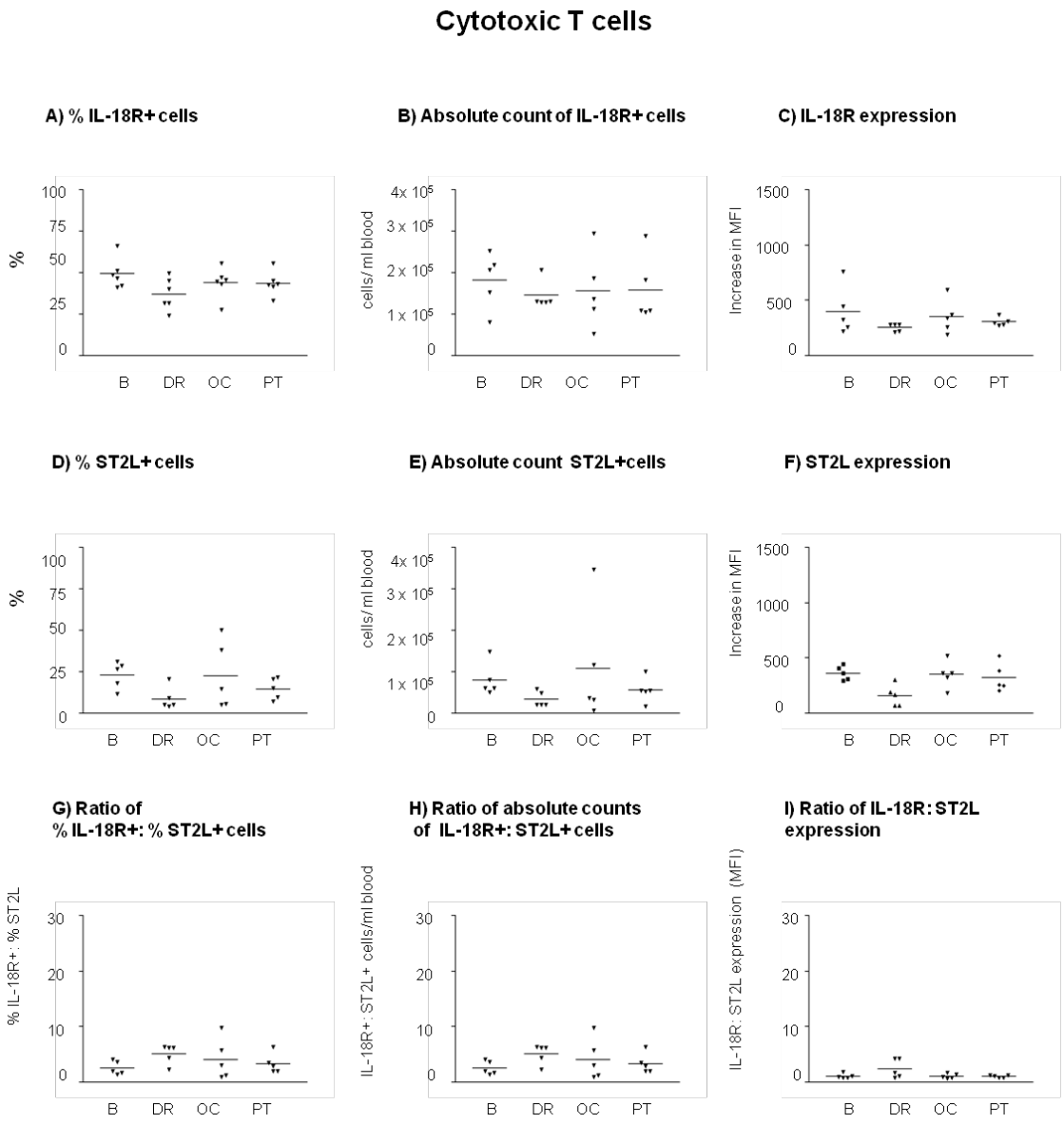


Figure 3-25 Type 1 and Type 2 subsets throughout the cycle. Samples were taken at baseline (B), down regulation (DR), oocyte collection (OC) and 24 hours prior to the pregnancy test (PT). Expression of IL-18R (Type 1) and ST2L (Type 2) cells were measured by (A, D) percentage expression, (B, E) the absolute count and (C, F) the increase in median fluorescence intensity (MFI) (G, H, I). Ratios of Type 1: Type 2 cells were then calculated. Bars represent mean values.

4. IL-33 and ST2 in normal human pregnancy

4.1 Introduction

In chapter 3 an increase in ST2L expression by circulating NK and T cells within days of successful implantation was demonstrated. This finding together with the increase in ST2L expression on circulating NK and NKT like cells shown previously by our laboratory at 12 weeks gestation, persisting in each trimester of normal pregnancy (Borzychowski, 2005), suggests a possible role for IL-33 in implantation and ongoing pregnancy.

4.1.1 IL-33 and ST2

IL-33 was originally described as a nuclear factor (NF) present in high endothelial venules (HEV) and was therefore initially named NF-HEV (Baekkevold *et al.*, 2003). In 2005 it was established that NF-HEV was a member of the IL-1 cytokine superfamily (that includes IL-1α, IL-1-β and IL-18) and the cytokine was renamed IL-33. IL-33 was shown to signal via the then orphan receptor, ST2 (Schmitz *et al.*, 2005). IL-33 is constitutively expressed in a wide variety of human tissues including small and large vessel endothelium (Moussion *et al.*, 2008). It is found in epithelial surfaces that contact with the environment, such as gut mucosa and alveolar epithelium, where it is localised in the cytoplasm as well as in the nucleus (Pastorelli *et al.*, 2010). IL-33 appears to act both as a chromatin associated nuclear factor and as a secreted cytokine. The role of IL-33 as a nuclear factor has not yet been well characterised although it may have a role as a transcriptional repressor (Carriere *et al.*, 2007; Moussion *et al.*, 2008).

The role of IL-33 as an extracellular cytokine is better established. IL-33 induces polarisation of T cells towards a Th2 phenotype (Lohning *et al.*, 1998; Meisel *et al.*, 2001; Schmitz *et al.*, 2005; Arend *et al.*, 2008; Suzukawa *et al.*, 2008). It drives the production of Th2 associated cytokines (IL-4, IL-5 and IL-13) from in vitro polarised Th2 cells and induces mucosal and pulmonary Th2 inflammation in vivo (Schmitz *et al.*, 2005). Under certain circumstances (such as in the presence of IL-12), IL-33 may also induce type 1 cytokines such as IFN-γ (Smithgall *et al.*, 2008; Bourgeois *et al.*, 2009). IL-33 activates mast cells (Allakhverdi *et al.*, 2007; Moulin *et al.*, 2007; Smithgall *et al.*, 2008), basophils (Kroeger *et al.*, 2009; Pecaric-Petkovic *et al.*, 2009; Schneider *et al.*, 2009) eosinophils (Cherry *et al.*, 2008; Suzukawa *et al.*, 2008; Pecaric-Petkovic *et al.*, 2009), natural killer cells and invariant NKT cells (Smithgall *et al.*, 2008; Bourgeois *et al.*, 2009). In addition, IL-33 has been shown to be a crucial amplifier of systemic and mucosal innate immune responses (Oboki *et al.*, 2010).

IL-33 can be identified in vivo as 33kDa pro-IL-33 and a shorter 18kDa form. Although pro-IL-33 was thought to be cleaved by caspase 1 to produce biologically active IL-33, this has been questioned. Recent studies have shown that that IL-33 lacks the cleavage site for caspase 1 (Talabot-Ayer *et al.*, 2009). It appears to be processed by the apoptosis associated caspases 3 and 7, along with calpain. It is now thought that cleavage of IL-33 renders the cytokine biologically inactive (Cayrol & Girard, 2009; Lamkanfi & Dixit, 2009; Luthi *et al.*, 2009).

The mechanism of IL-33 release into the extracellular space remains uncertain as it lacks the usual signal sequence necessary for secretion via the endoplasmic reticulum/ golgi pathway. It is possible that the major source of extracellular IL-33 is release from damaged cells undergoing necrotic cell death in a similar fashion to other molecules known as 'alarmins', such as HMGB1 and IL-1α, which also have dual nuclear and cytokine functions (Lamkanfi & Dixit, 2009; Luthi *et al.*, 2009).

As discussed previously, IL-33 is the ligand for ST2L, a member of the IL-1 receptor superfamily. ST2L is expressed on mast cells, basophils, eosinophils, macrophages, NK cells and Th2 lineage cells (Stolarski *et al.*; Xu *et al.*, 1998a; Allakhverdi *et al.*, 2007; Smithgall *et al.*, 2008).In addition to ST2L, there are three other ST2 isoforms:, ST2V, ST2LV and sST2. ST2V and ST2LV are splice variants of ST2 (Tominaga *et al.*, 1999; Iwahana *et al.*, 2004), and are as yet not well characterised in humans. Soluble ST2 (sST2) has been more widely investigated. sST2 lacks the transmembrane and cytoplasmic domains (Kakkar & Lee, 2008) of ST2L. High levels of sST2 mRNA are found in alveolar epithelium and cardiac myocytes and low levels of sST2 mRNA are found in peripheral blood mononuclear cells and endothelium (Mildner *et al.*, 2010). In addition, sST2 has been shown to be produced by venous and arterial endothelial cells stimulated with IL-1β, phorbol esther or TNF-α (Bartunek *et al.*, 2008) and by activated Th2 cells (Lecart *et al.*, 2002). Whereas IL-33 acts on the receptor ST2L and increases the production of predominantly type 2 cytokines, sST2 appears to negatively regulate the actions of IL-33. sST2 binds to IL-33 and renders it biologically inactive acting as a decoy receptor

(Trajkovic *et al.*, 2004). The effects of IL-33 are also negatively regulated by single Ig IL-1-related molecule (SIGIRR) (Bulek *et al.*, 2009).

IL-33 signals through the heterodimer complex of ST2L and the IL1-R accessory protein (IL1-RAcP) (Figure 4-1). Binding of the cytokine to the receptor complex results in the recruitment of MyD88 and the downstream activation of TNF receptor associated factor 6 (TRAF6). This leads to subsequent activation of the inhibitor of nuclear factor-κB (NF- κB) and Mitogen Activated Protein Kinase (MAPK) (Schmitz *et al.*, 2005; Chackerian *et al.*, 2007; Funakoshi-Tago *et al.*, 2008; Kurowska-Stolarska *et al.*, 2008).

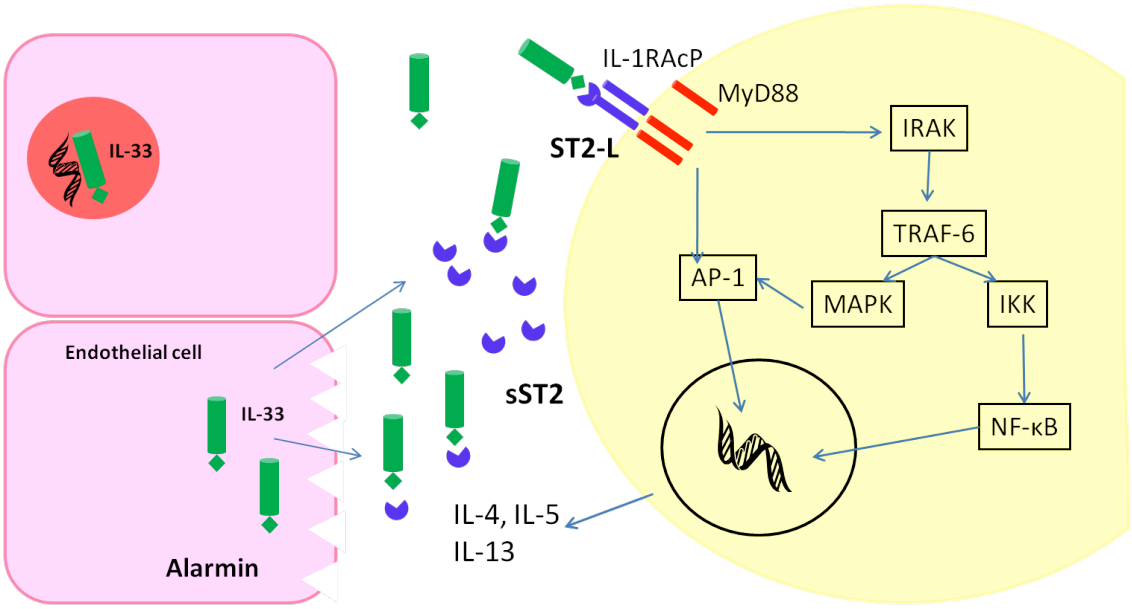


Figure 4-1. IL-33 signalling. Il-33 acts as both a transcription factor and an extracellular cytokine signalling via the heterodimeric complex of ST2L and IL-1RAcP. Binding leads to recruitment of Myd88 and TRAF-6 (via IRAK) leading to NF-κB and MAPK activation and ultimately to increased cytokine production.

The findings in chapter 3 of up regulation of ST2L on NK^{dim} and T cells as early as 9 days post implantation suggest that IL-33 and ST2 may have a role in the regulation of maternal immune responses during implantation and early pregnancy. As previously described, IL-33 is predominantly a type 2 cytokine and the hypothesis of this chapter is that IL-33 is increased in pregnancy and stimulates type 2 deviated cells via the ST2L receptor resulting in a cytokine environment beneficial to pregnancy.

Given that ST2L is up regulated on peripheral immune cells so early after successful implantation, it is possible that the embryo (in very early pregnancy) and/ or the developing trophoblast (later in the first trimester) could be a source of IL-33. The decidua could also potentially express IL-33 in response to embryonic or placental derived factors. IL-33 may act systemically on lymphocytes expressing ST2L or could additionally act by a paracrine mechanism in the developing embryo or placenta. As the potential functional effects of IL-33 will depend on associated levels of sST2 (acting as a decoy receptor), this chapter also aimed to establish whether circulating IL-33 or sST2 change in early pregnancy.

The previous findings by our group that ST2L expression by peripheral NK and NKT like cells remains up-regulated throughout pregnancy (Borzychowski, 2005), suggests that IL-33 may also play a role in immunomodulation in later pregnancy and therefore this chapter also investigates placental expression of these proteins by third trimester placenta along with circulating levels of IL-33 and sST2 in each trimester.

4.2 Aims

1. To establish whether IL-33 and ST2 are expressed or secreted by the pre-implantation embryo.
2. To establish whether IL-33 and ST2 are expressed by the first trimester placenta or decidua.
3. To establish whether circulating IL-33 and sST2 change in early pregnancy after successful implantation.
4. To investigate whether the term placenta expresses IL-33 and ST2 and if so, where placental IL-33 and ST2 are localised.
5. To establish the levels of circulating IL-33 and sST2 in each trimester of normal pregnancy.

4.3 Materials and Methods

4.3.1 Subject recruitment

4.3.1.1 Human embryo study

For the investigation of IL-33 and ST2 expression by human embryos, ethical approval was obtained from the Human Embryology and Fertilisation Authority (HFEA ROIII) and the Oxford Research Ethics Committee B. Women undergoing IVF at the Oxford Fertility Unit gave written informed consent to donate 'spare' embryos that were unsuitable for cryopreservation (embryo freezing). 12 embryos were labelled for IL-33, ST2 or the appropriate IgG negative control.

4.3.1.2 Embryo culture supernatants

Ethical approval for this study was obtained from the Oxford Research Ethics Committee C. 30 women undergoing IVF consented for the media used to culture their embryos to be investigated once embryo transfer had taken place. Written informed consent from each patient was given prior to oocyte retrieval. The Oxford Fertility Unit cultures individual embryos from day 3 until day 5 in 50µl of blastocyst culture media. As the ELISA kits require 100µl of sample, supernatants from 3 embryos were pooled for each measurement, yielding 30 pooled supernatants for examination. Pooled supernatants were investigated by ELISA for either IL-33 or sST2.

4.3.1.3 First trimester placenta and decidua

This pilot study investigating IL-33 and ST2 expression in first trimester placental tissue was performed in collaboration with Dr Judith Cartwright, St. Georges Hospital, University of London. Samples of first trimester placenta (n=5) and first trimester decidua (n=3) were obtained from women undergoing termination of pregnancy at St. Georges Hospital. Ethical approval for these studies was obtained from Wandsworth Research Ethics Committee. Written informed consent was obtained. The gestational age varied from 6+1 weeks' to 11+4 weeks'.

4.3.1.4 Third trimester placenta

Women undergoing elective caesarean section agreed to donate placental tissue for analysis (n=9). Ethical approval for this study was obtained from Oxford Research Ethics Committee B. Written informed consent was obtained. The mean gestational age at the time of caesarean section was 38+5 weeks'.

4.3.1.5 Longitudinal IVF study

Ethical approval for these studies was obtained from the Oxford Research Ethics Committee B. Written informed consent was given prior to venepuncture. Women aged 38 or less who were undergoing a first cycle of IVF were recruited. All the women recruited had been investigated for sub-fertility and no female factor (poor ovarian reserve, tubal damage or anovulation) was found. Nine women who became pregnant in their first IVF cycle were identified and matched for age (+/- 4 years) with women who had a negative pregnancy test. To determine circulating concentrations of IL-33 and sST2 over the IVF cycle and in the early first trimester, blood samples were

taken at baseline, down regulation, oocyte retrieval and at 6 weeks gestation if pregnant.

4.3.2 Detection of IL-33 by ELISA

Circulating IL-33 in normal pregnancy was measured initially using a commercially available ELISA (R&D Systems, Minneapolis, USA). The results using this ELISA were surprising as they did not appear to be consistent with serum or plasma IL-33 levels commonly reported in the literature in either health or disease. Most reports are of levels between 0 and 1000pg/ml in contrast to up to 4000 pg/ml measured in some of the samples from both pregnant and non pregnant women. The assays were repeated several times, each time consistently high IL-33 levels were found and therefore an alternative (Biolegend) assay was purchased to compare samples using the two assays. The results shown are shown in (Table 4-1).

Sample number	R&D assay (pg/ml)	Biolegend assay (pg/ml)
1	23.4	0
2	42640	1108
3	17120	145.7
4	592	47.3
5	12280	63.41

Table 4-1 IL-33 levels measured using 2 different commercially available assays.

These results confirmed a problem with the initial (R&D) assay in the measurement of serum or plasma IL-33 in which some of the values were consistently high.

4.3.2.1 Detection of IL-33 by ELISA in plasma and serum

An IL-33 ELISA (Biolegend (San Diego, California)) was therefore used to determine serum and plasma IL-33 concentrations. All reagents used in this ELISA are provided with the assay. 50µl of standard or sample was added in duplicate to an anti-human IL-33 pre-coated 96-well Strip Microplate. The plate was covered and incubated overnight at 4ºC. The following day the plate was washed 4 times with wash buffer using an automatic plate washer. 100µl of Human IL-33 detection antibody solution was added to each well. The plate was sealed and then incubated at room temperature for 1 hour on a plate shaker at 750rpm. The plate was then washed 4 times. 100µl of Avadin-HRP B solution was added to each well and incubated on a plate shaker for 30 minutes. The plate was then washed 5 times with the plate soaked in wash buffer for 30 seconds prior to the final wash. 100µl of substrate solution was added to each well. Once the colour had developed, 100µl of STOP solution was added to each well and the plate read at 450nm

4.3.2.2 Detection of IL-33 by ELISA in supernatants

An IL-33 ELISA (R&D Systems, Minneapolis, USA) was used to determine supernatant IL-33 concentrations. A 96 well Maxisorp plate (Nunc, Denmark) was coated with 100µl per well of capture antibody (goat anti-human IL-33 diluted in reagent diluent (1% BSA (Sigma-Aldrich, Gillingham, UK) in PBS) at a concentration of 144µg/ml. The plate was covered and incubated overnight at room temperature. The following day the plate was washed 4 times with wash buffer (0.05% Tween (Sigma) in PBS) using an automatic plate washer (Skanwasher, Skantron, Sunnyvale, USA). 300µl of blocking buffer (5% BSA in

PBS) was added to each well and incubated at room temperature for 1 hour to eliminate additional protein binding. The plate was washed 4 times in preparation for the standards and samples. Standards were prepared using seven serial dilutions (in reagent diluent) of recombinant IL-33 from a high standard of 1500pg/ml. 100µl of standard or sample was added to each well. Standards and samples were tested in duplicate. Samples were tested both neat and diluted either 5- or 10-fold in PBS in order to ensure the samples measured fell within the standard curve. The plate was covered and incubated for 2 hours at room temperature and then washed 4 times in wash buffer. The detection antibody (biotinylated goat anti-human IL-33) was diluted in reagent diluent to a concentration of 200ng/ml and 100µl added to each well. The plate was covered, incubated for 2 hours at room temperature and washed. 100µl of streptavidin-HRP was added to each well and the plate was incubated at room temperature for 20 minutes before washing the plate four times. Finally, 100µl of substrate solution (2.2'-Azini-bis (3-ethylbenzothiazoline-6-sulfonic acid)) (Sigma) was added and incubated for 20 minutes. The plate was read using a FLUOstar OPTIMA plate reader (Offenburg, Germany) at 450nm.

4.3.3 Detection of sST2 by ELISA

An ELISA (Presage® ST2 Assay (Critical Diagnostics, San Diego, USA)) was used to determine plasma sST2 concentrations. All diluents and reagents used in this ELISA are provided with the assay. Standards were obtained using seven serial dilutions (in standard diluent) of sST2 from a high standard of 2000ng/ml. Samples and calibrators were diluted fifty-fold in sample diluents. 100µl of standards, samples and calibrators were added in duplicate to pre-coated anti-ST2 96 well plates. The plate was incubated on a plate shaker at

750rpm for 1 hour. The plate was then washed 5 times with wash buffer and 100µl of biotinylated antibody reagent was added to each well and incubated on a plate shaker at 750rpm at room temperature for 1 hour. After a further 5 washes with wash buffer 100µl of streptavidin-HRP conjugate reagent was added to each well and incubated for 30 minutes. After 5 washes with wash buffer 100µl of TMB reagent was added to each well and incubated at room temperature on a plate shaker at 750rpm for 20 minutes. In order to prevent further colour development 100µl of STOP solution was added to each well and the plate read at 450nm.

4.3.4 Detection of IL-33 and ST2 in embryos by immunofluorescence microscopy

Embryos were washed for 1 minute in 500µl of 1% bovine serum albumin (BSA) (Sigma-Aldrich, Gillingham, UK) in PBS (Sigma) and then washed for 1 minute in 500µl PBS. Embryos were fixed in 500µl 3% paraformaldehyde for 10 minutes and then washed twice in 500µl 1% BSA/PBS. Fixed embryos were then stored for a maximum of 48 hours at 4°C prior to primary antibody staining. Before staining, embryos were immersed for 10 minutes in 500µl permeabilisation buffer and then washed 3 times in 50µl of 1% BSA/PBS for 5 minutes at room temperature. Embryos were blocked in 50µl 3% BSA/PBS for 1 hour at room temperature. Embryos were then incubated overnight in a humidity chamber at 4ºC with anti-IL-33 antibody clone Nessy-1 (Enzo Life Sciences, New York, USA) at a concentration of 2µg/ml, anti-ST2 antibody (R&D Systems, Minneapolis, USA) at a concentration of 2µg/ml or the corresponding mouse or goat IgG antibody (Dako, Denmark) at the same concentration.

The following morning the embryos were washed 3 times in 50µL drops of 1% BSA/PBS for 5 minutes each and then incubated in the dark at room temperature with the appropriate Alexa Fluor 488 conjugated secondary antibody (Invitrogen, Carlsbad, California, USA). After 1 hour, the embryos were washed in 50µl drops of 1% BSA/PBS 3 times for 5 minutes. Embryos were mounted into individual drops (20µl) of Vectashield mounting media (containing Dapi nuclear stain) (Vector Laboratories, Burlinghame, California,

USA) on a glass slide with a Secure-Seal imaging spacer (Sigma) that prevents the embryo being compressed by the coverslip. Embryos were visualized using a Leica DMIRE2 fluorescence microscope (Leica, Solms, Germany) and photographed using a Hamamatsu Orca monochrome camera (Beijing, China) and Simple PCI software (C Imaging, Sewickley, Pennsylvania, USA).

4.3.5 Detection of IL-33 and ST2 by immunohistochemistry

Tissue samples from first trimester trophoblast (n=5) and decidua (n=3) and third trimester placentas (n=3) were fixed in 10% formalin and processed into paraffin blocks. Serial sections (5μm) were cut on coated slides and dried. Paraffin embedded placental sections pre-mounted on glass slides were deparaffinised and rehydrated by placing sequentially in the following solutions for 1 minute each;

- Previously used Histo-Clear (National Diagnostics, MERCK, Erulab S.A)
- New Histo-Clear
- 100% ethanol (Fisher Scientific, Loughborough, UK)
- 100% ethanol
- 95% ethanol
- 90% ethanol
- 80% ethanol
- 70% ethanol
- 50% ethanol
- Slides were then rinsed in water for 1 minute them PBS for 1 minute.

Antigen retrieval was then performed. Slides were immersed in 0.01M sodium citrate buffer (pH 6.0) in a water bath for 10 minutes at 95ºC and then allowed to cool to room temperature. Slides were then washed in water, then PBS, for 1 minute each. Sections were pre-blocked with 10% fetal calf serum in PBS for one hour at room temperature. The sections were incubated overnight at 4ºC

with anti-IL-33 antibody clone Nessy-1 (Enzo Life Sciences, New York, USA) at a concentration of 1µg/ml, anti-ST2 antibody (clone 9F8, Critical Diagnostics, San Diego, USA) at a concentration of 1µg/ml or a negative control mouse IgG antibody (Dako, Denmark) at the same concentration. A cytokeratin 7 antibody (Dako, Denmark) was used as a positive control to label the syncytiotrophoblast. A CSA II biotin-free, tyramide signal amplification system was used (Dako, Denmark), following the manufacturer's instructions. Briefly, endogenous peroxidase activity was quenched with hydrogen peroxide then sections incubated with antibodies towards mouse IgG conjugated with fluorescein, then further incubated with anti-fluorescein antibodies conjugated to horseradish peroxidase. Incubation times were 15 minutes and slides were washed in PBS x3 between steps and then incubated with liquid 3, 3'-Diaminobenzidine (DAB) substrate-chromogen until colour developed. The sections were finally counterstained with haematoxylin. Slides were examined using a Leitz GMBH microscope (Leica, Solms, Germany) and photographed using a Leica DFC 300FX camera and Leica IM50 image manager software.

4.3.6 Detection of IL-33 and sST2 by immunoblotting

4.3.6.1 Protein quantification

1mm^3 pieces of placenta were incubated individually at room temperature for 30 minutes with 100µl of HEPES lysis solution supplemented with Complete Mini protease inhibitor cocktail (Roche, Germany). The resulting lysate was microcentrifuged at 13,000g for 5 minutes to separate any non-lysed tissue. The amount of protein in each lysate was quantified to ensure equal loading in western blot gels. This was determined using a BCA protein assay kit (Pierce,

Thermo Scientific, Illinois, USA) using a standard curve dilution series of BSA (maximum value 2mg/ml). The placental lysates were prepared as 1:5 dilutions in HEPES lysis buffer. 12.5µl of BSA standard or sample was added to each well. Standards and samples were tested in duplicate. 100µl of BCA protein assay reagent was added to each well and the plate was incubated at room temperature until colour developed. The plate was read at 450nm.

4.3.6.2 Sample preparation

30µg of each sample was made up to a volume of 10µl in HEPES lysis buffer. 5µl of 3x reducing buffer (300mM TRIS pH 6.8, 12%SDS, 0.6% Bromophenol Blue, 60% glycerol and 10% mercaptoethanol) was added to each sample. Samples were placed in a water bath at 95ºC for 5 minutes and then left to cool to room temperature.

4.3.6.3 SDS-polyacrylamide gel electrophoresis (-PAGE)

A pre-cast NuPage 10% Bis-Tris Gel was mounted into a XCell Sure-Lock Mini Cell (Invitrogen) filled with 1000ml of 1x NuPage SDS running buffer (Invitrogen). 30µg of protein was loaded onto the gel along with molecular weight markers (Precision Plus Protein Standard, BioRad, California, USA). Proteins (30µg) were resolved by electrophoresis at 150V (constant) until the dye had run to the bottom of the gel (approximately 1 hour).

4.3.6.4 Transfer of protein to PVDF

Four 10 x 7 cm sheets of blotting paper were soaked in Anode 1 solution (0.3M Tris base, pH10.4, 20% methanol), three in anode 2 solution (25mM Tris base pH 10.4 20% methanol) and three sheets in cathode solution (25mM Tris base pH 9.4, 20% methanol, 40nM ε-aminohexanoic acid, 20% methanol). PVDF was soaked sequentially in methanol, water and then anode 2 buffer. The PVDF membrane was placed on top of the anode 1 and 2 blotting sheets. The resolved gel was placed on top of the PVDF membrane and covered with the cathode blotting papers. The gel was transferred in a semi-dry western blotting apparatus for 45 minutes at 25 volts.

4.3.6.5 Western blot analysis

Membranes were blocked for 45 minutes in blocking buffer (PBS with 5% BLOTTO (Santa Cruz, California, USA) and 0.1% Tween (Sigma) at room temperature. The membranes were then incubated overnight with the appropriate primary antibody at 4ºC and then washed x3 with PBS and 0.1% Tween (PBS-T) for 5 minutes. Primary antibodies used were anti-IL-33 (0.1µg/ml) clone Nessy-1 (Enzo Life Sciences, New York, USA), anti-ST2 (0.5µg/ml) (R&D Systems, Minneapolis, USA) or anti-β-actin (0.155µg/ml) (Abcam, Massachusetts, USA). The membranes were then incubated with the appropriate secondary antibody, either anti-mouse (IL-33 and β-actin) or anti-goat (ST2) conjugated to horseradish peroxidase (Dako, Denmark) in blocking buffer, for one hour. The membranes were then washed 3 times in PBS-T for 5 minutes. The membranes were then placed on a plastic film and 1ml of western blot enhanced chemiluminescence system substrate (Pierce, Thermo

Scientific, Illinois, USA) was added. After 1 minute the excess substrate was removed and the PVDF was covered with a plastic film. In the dark room, imaging film (GE Healthcare, Buckinghamshire, UK) was placed on top of the membrane and the cassette was closed. The film was developed after 1 minute and a second film after 20 minutes exposure. Films were developed using an x-ograph compact x2 developer with develex M developing reagents (Champion Photochemicals, Essex, UK).

4.4 Statistics

The Gaussian distribution of each data set was assessed using a Kolmogorovv-Smirnov test. Clinical data was compared using a paired t-test. In the longitudinal study, where the data was normally distributed, a repeated measures ANOVA was used to determine if there was variation across the trimesters. Where a significant variation was identified, a Bonferroni post hoc test was used to determine the significant time points. For non-parametric longitudinal data a Kruskal Wallace test was used to determine if there was variation across the trimesters. Where a significant variation was identified, a Dunn's post hoc test was used to determine the significant time points. Values of $p<0.05$ were considered to be significant. GraphPad Prism software (San Diego, California) was used to analyse the data.

4.5 Results

4.5.1 IL-33 and sST2 expression by the pre-implantation human embryo

Day 5-7 human blastocysts were labelled with IL-33, ST2 or an IgG antibody control and then a secondary fluorescently conjugated antibody. The proteins were variably expressed. 3 of the 5 embryos labelled were positive for IL-33 and 2 of the 4 embryos labelled were positive for ST2. Both proteins were identified within the trophectoderm (Figure 4-2).

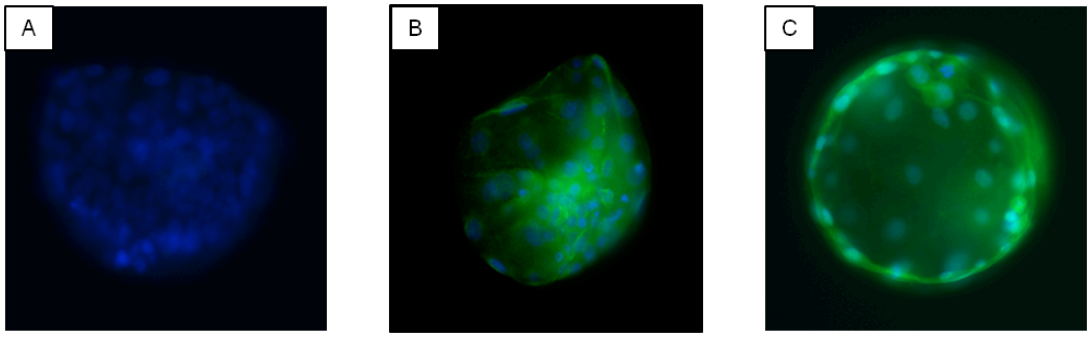


Figure 4-2 Day 5 human blastocysts were labelled with (A) IgG negative control, (B) anti-IL-33 antibody, (C) anti- ST2 antibody. Embryos variably expressed both IL-33 and ST2 (green). Embryos were counterstained with a DAPI nuclear stain (blue). A-C are representative of the 12 blastocysts labelled.

Media used to culture human embryos between day 3 and day 5 of development were pooled into 100µl volumes in order to investigate whether IL-33 or sST2 were secreted by the developing embryos. Neither IL-33 nor sST2 were detectable in the pooled culture supernatants (data not shown).

4.5.2 IL-33 and ST2 expression by first trimester trophoblast and decidua

Immunohistochemistry of first trimester trophoblast (n=5) and decidua (n=3) was carried out to establish if IL-33 and ST2 were expressed. In the placental samples, IL-33 and ST2 were variably expressed by both the syncytiotrophoblast and villus stroma (

Figure **4-3**). Samples ranged from a gestational age of 6+6 to 11+4 weeks. Expression of both proteins was identified in the later gestational samples (9-11 weeks) whereas the earlier samples (6-8 weeks) had minimal or no expression of either protein. Cytokeratin 7 was used as a positive control and strongly labelled the syncytiotrophoblast.

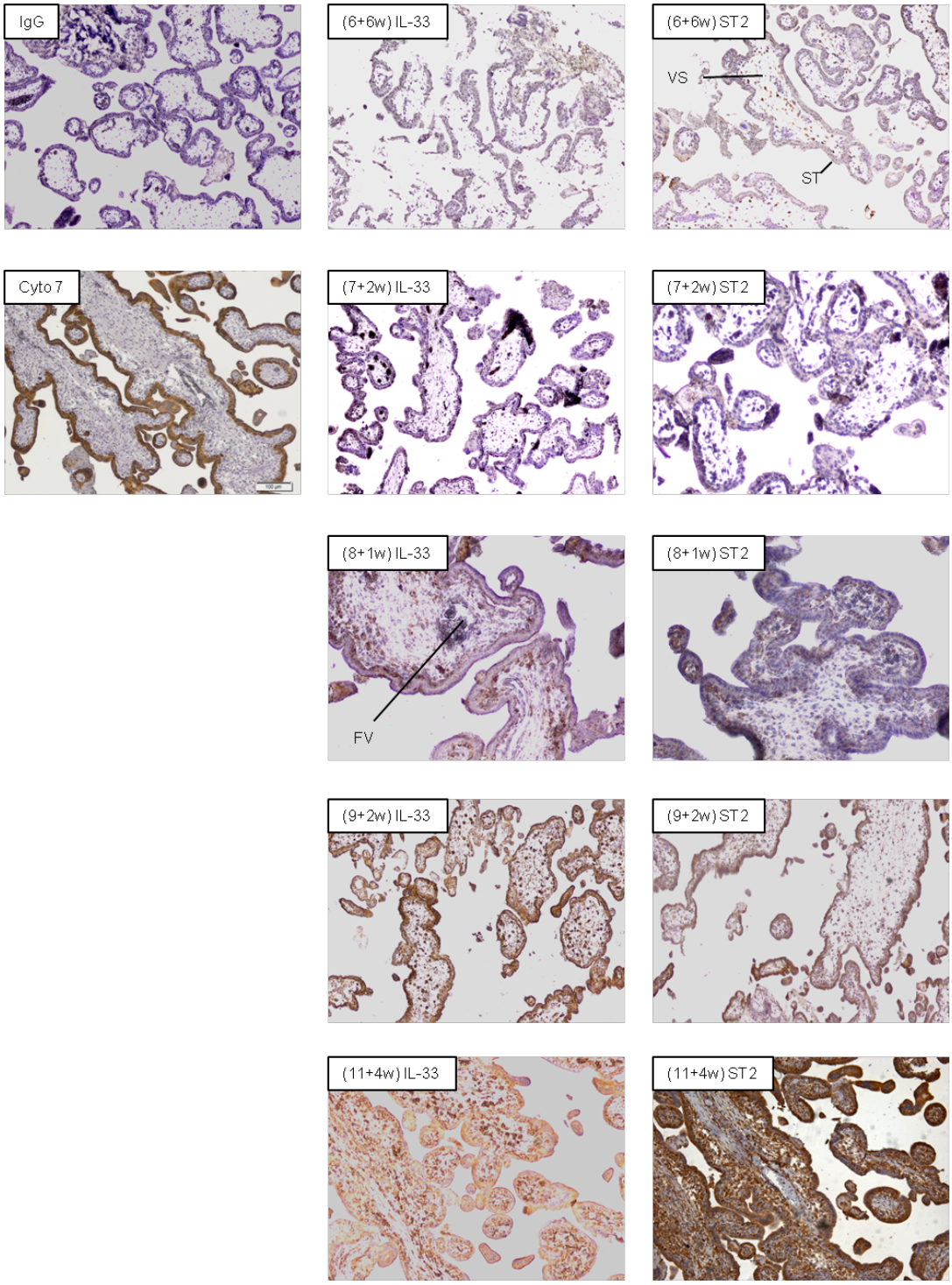


Figure 4-3 Variable expression of IL-33 and ST2 can be seen in immunohistochemistry of 1st trimester trophoblast. Expression of both proteins was increased later in the 1st trimester. Gestation is shown in weeks (w). The syncytiotrophoblast (ST), villous stroma (VS) and fetal vessels (FV) are identified

Sections of decidua ranging from 6+6 to 8 weeks gestation (n=3) were labelled with antibodies to ST2 and IL-33 (Figure 4-4). Both IL-33 and ST2 were strongly expressed by the decidual stroma. Cytokeratin-7 was used as a positive control and labelled the stromal glands.

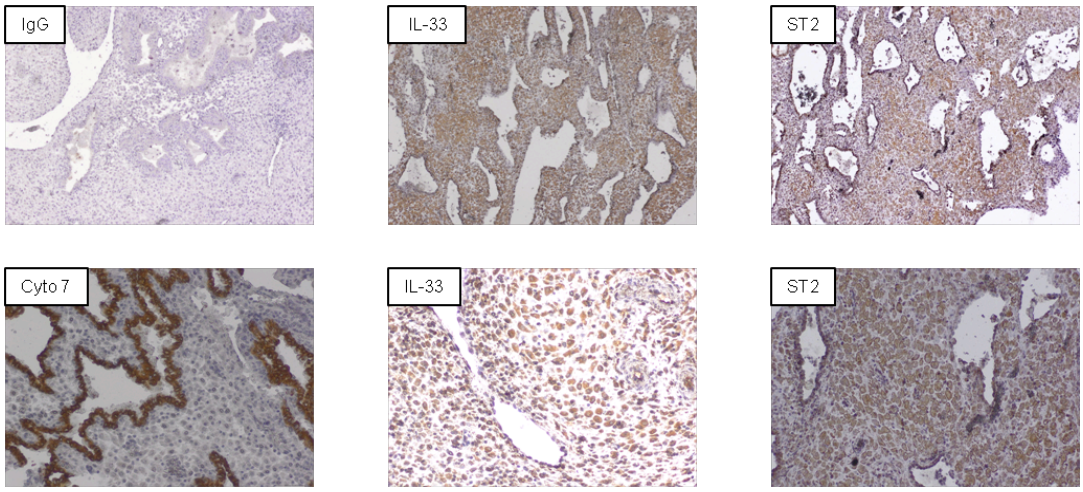


Figure 4-4 Expression of IL-33 and ST2 by first trimester decidua. Both proteins were strongly expressed within the stroma. Cytokeratin-7 was used as a positive control labelling the glands.

In addition to the immunohistochemistry, western blots of first trimester placental lysates were also carried out at St Georges, but the quality of the blots was poor and the experiments need repeating. Further studies (western blotting, and immunofluorescence double labelling of serial sections) are planned but are dependent on a material transfer agreement that has not yet been accepted by the two universities.

4.5.3 Circulating IL-33 and sST2 during an IVF cycle and in early pregnancy

IL-33 and sST2 were measured in plasma at three time points (baseline, down regulation and oocyte retrieval) from 18 women undergoing a cycle of IVF. In the nine women who became pregnant a further sample was measured at 6 weeks' gestation. Circulating IL-33 levels were not normally distributed and therefore IL-33 levels are presented as the median and the range. There was no significant variation in circulating IL-33 across the 3 time points measured during the IVF cycle and no change at 6 weeks gestation in the pregnant group (Figure 4-5). In the non-pregnant group median serum IL-33 across the three time points were 29.99 (0-147.5) pg/ml, 29.1 (0-0-135.5) pg/ml and 3.534 (0-103.4) pg/ml. In the pregnant group median serum IL-33 levels were 27.34 (0-581.9) pg/ml, 66.94 (0-1706) pg/ml, 27.40 (0-601.9) pg/ml and 16.5 (0-2100) pg/ml.

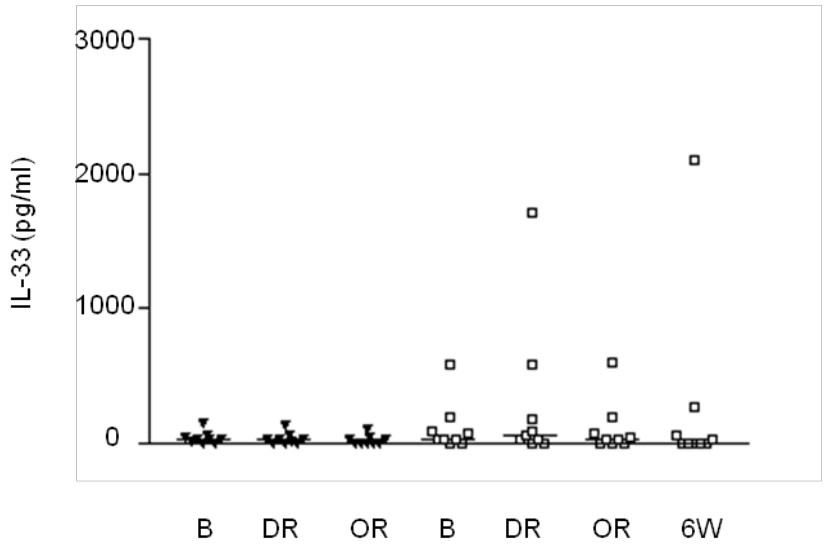


Figure 4-5 IL-33 was detected by ELISA in plasma from women (n=18) undergoing a first cycle of IVF of whom 9 became pregnant (open squares) and 9 who did not (closed triangles). Samples were taken at baseline (B), down regulation (DR), oocyte retrieval (OR) and at 6 weeks gestation if pregnant (6W). Bars represent median values.

sST2 levels were normally distributed and are therefore presented as the mean (+/- SEM). In the pregnant group, mean sST2 levels across the four time points were 30.18 (1.963) ng/ml, 29.80 (2.037) ng/ml, 29.44 (2.182) ng/ml and 39.16 (5.023) ng/ml at the four time points. A repeated measures ANOVA showed a significant effect of the cycle on circulating sST2 levels (F=3.570, p=0.0289). Although there appears to be a trend to increased circulating sST2 at the 6 week sample, Bonferroni's multiple comparison test did not show a significant difference between this and any other time point. In the non pregnant group the levels of circulating sST2 were 32.67 (2.940) ng/ml, 29.89 (2.860) ng/ml, and 35.04(4.154) ng/ml (Figure 4-6).

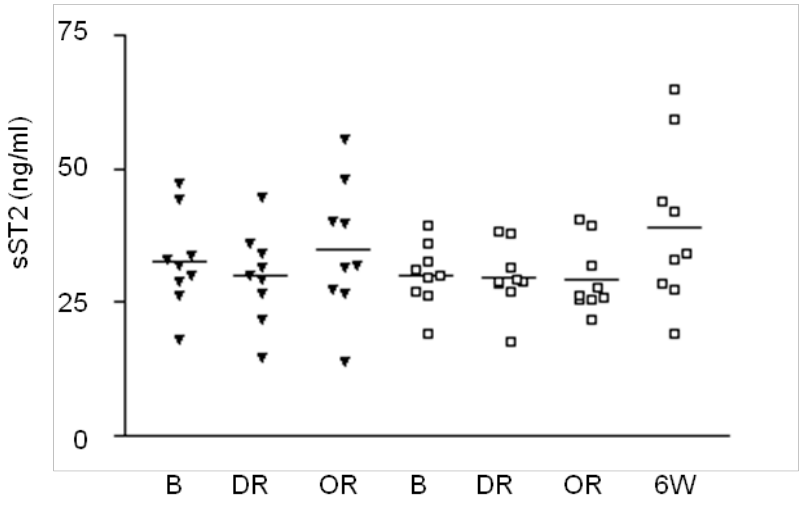


Figure 4-6 sST2 was detected by ELISA in plasma from women (n=18) undergoing a first cycle of IVF of whom 9 became pregnant (open squares) and 9 who did not (closed triangles). Samples were taken at baseline (B), down regulation (DR), oocyte retrieval (OR) and at 6 weeks gestation if pregnant (6W). There was a significant effect of the cycle in the women who became pregnant (F=3.570, p=0.0289). Bars represent mean values.

4.5.4 Circulating IL-33 and sST2 in each trimester of normal pregnancy

IL-33 and sST2 were measured in serum from non-pregnant women (n=23) and normal pregnant women (n=30) in each trimester in order to determine first, whether levels were altered in pregnancy, and secondly, if there were any changes with gestational age. Levels of circulating IL-33 did not vary significantly through the three trimesters of pregnancy (median and range: 21 (0-2674) pg/ml, 18 (0-2924) pg/ml and 19 (0-3148) pg/ml respectively) and were similar to levels seen in non pregnant women (22 (0-4264) pg/ml) (Figure 4-7**)**.

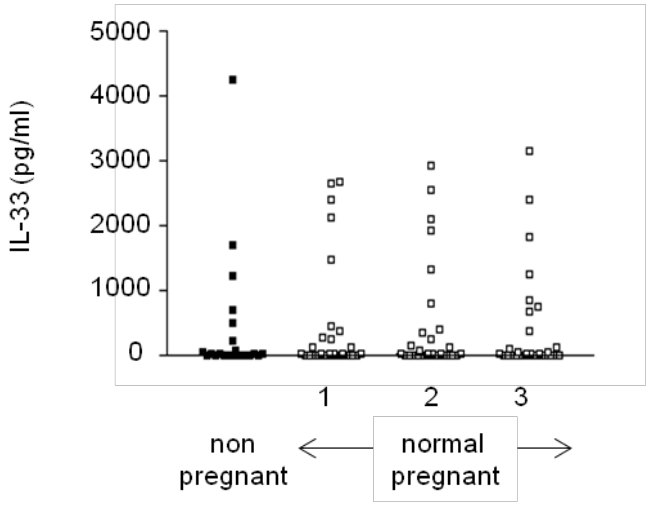


Figure 4-7 IL-33 was detected in serum by ELISA during each trimester of pregnancy (1, 2, 3) from 12 weeks gestation from women (n=30) with an uncomplicated normal pregnancy and from non-pregnant women (n=23). Bars represent median values.

As discussed in section 4.3.2 the assay used in this experiment (R&D) was shown to be inaccurate in the measurement of serum IL-33. No further aliquots were available to repeat the analysis and therefore a further 15 samples were

acquired and the results are shown below. IL-33 did not vary significantly in each trimester of normal pregnancy. The median (range) values in each trimester were 17.99 (0-187.1) pg/ml, 22.61 (0-217.6) pg/ml and 17.47 (01-193.7) pg/ml.

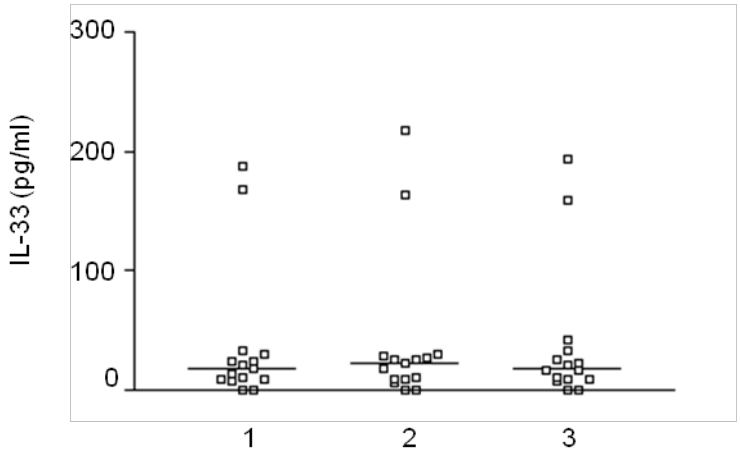


Figure 4-8 IL-33 was detected in serum by ELISA during each trimester of pregnancy (1, 2, 3) from 12 weeks gestation from women (n=15) with an uncomplicated normal pregnancy

When sST2 was measured in each trimester from 12 weeks gestation, there was a significant increase ($p<0.001$) in sST2 in the third trimester of normal pregnancy (29.17 (1.920) (mean (+/- SEM)) compared to the first (17.71 (0.763)) and second (19.64 (2.033)) trimesters. No differences between non-pregnant samples (mean 17.85 (1.193)) and normal pregnant first and second trimester samples were noted, however levels were significantly increased in the third trimester ($p<0.001$) (Figure 4-9).

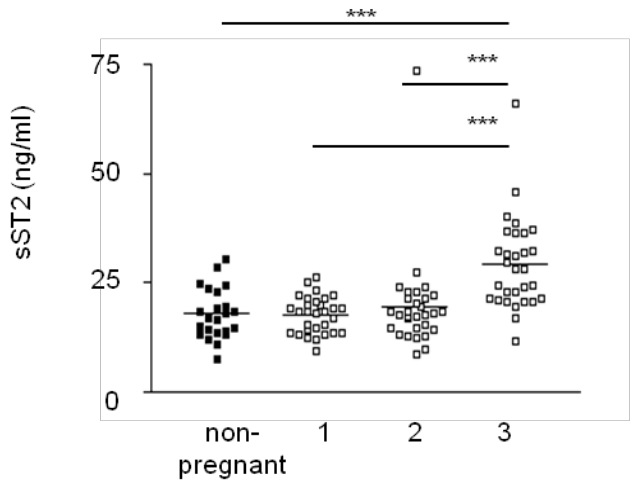


Figure 4-9 sST2 was detected in serum by ELISA during each trimester of pregnancy (1, 2, 3) from 12 weeks gestation from women (n=30) with an uncomplicated normal pregnancy and from non-pregnant women (n=23). Bars represent mean values.

4.5.5 Third trimester placental expression of IL-33 and ST2 by western blotting.

Immunoblotting showed the presence of IL-33 and ST2 in lysates of all the third trimester placental tissues studied (Figure 4-10). Two forms of IL-33 were detected. A band corresponding to full length IL-33 (closed arrow) was identified in each of 9 placentas. In addition, a band corresponding to the shorter cleaved form of IL-33 could be identified in the majority of placentas (open arrow). Antibody to ST2 identified ST2 in all of the placental lysates. Although the protein load (30µg) was determined using a BCA protein assay, the actin signal is variable. This is because placental tissue is heterogeneous, containing a number of different cell types and consequently different levels of actin.

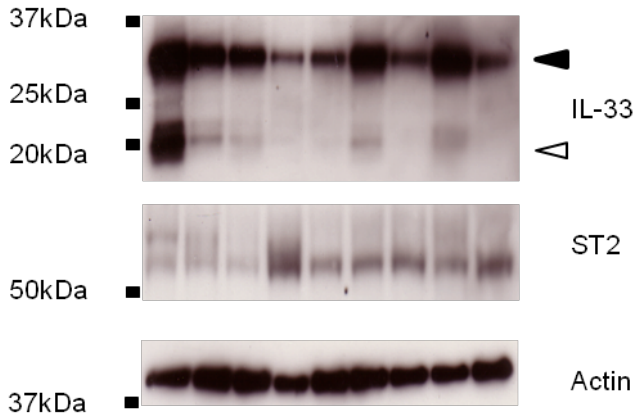


Figure 4-10 IL-33 was detected by western blotting of placental lysates from 3rd trimester placentas (n=9). Both the full length (closed arrow) and cleaved forms (open arrow) were detected. ST2 was detected from each of the placental lysates.

4.5.6 IL-33 and sST2 localisation in third trimester placenta by immunohistochemistry

Immunohistochemistry of placental sections was then carried out to determine the cellular localisation of the IL-33 and ST2 in the third trimester. Labelling for both molecules was variable (Figure 4-11). Labelling for both molecules was variable. IL-33 localised predominantly to perivascular tissue of the chorionic villi. ST2 localised to some cells in the villous stroma with staining of the syncytiotrophoblast. No specific staining was seen in IgG controls and the positive control (Cytokeratin-7) strongly stained the syncytiotrophoblast (data not shown).

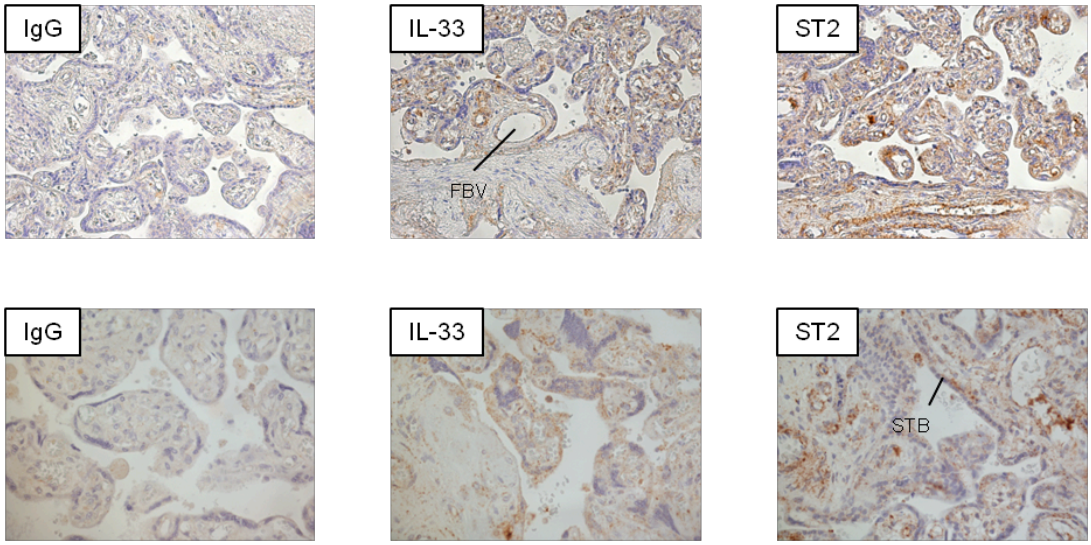


Figure 4-11 Variable expression of IL-33 and ST2 can be seen in immunohistochemistry of normal 3rd trimester placentas (n=3) The syncytiotrophoblast (STB) and fetal blood vessels (FBV) are identified.

4.6 Discussion

In chapter 3, ST2L was shown to be up regulated on peripheral blood NK and T cells within 9 days of successful implantation. It is possible that signals from the early developing embryo may induce ST2L expression. One such signal could be its ligand IL-33 and therefore expression of IL-33 by the blastocyst was investigated. There are no published reports regarding IL-33 expression in human or animal embryos. This study has shown for the first time that IL-33 is expressed by the human embryo, as early as 5 days post fertilisation. Having identified that IL-33 is expressed by the embryo, the possibility that it may act locally on embryonic ST2 was also investigated. Data regarding ST2 expression during embryogenesis is limited, with no human studies in the literature. ST2L transcripts have been shown to be weakly detectable (by northern blotting) from 10-14 days of embryogenesis in murine embryos with increasing expression after 14 days. Although in that study no transcripts for ST2 could be identified by northern blotting, low levels of sST2 mRNA could be detected from day 10 of embryogenisis until parturition using RT-PCR (Rößler *et al.*, 1995). ST2 transcripts were identified in the chicken embryo as early as day 5 of development (Iwahana *et al.*, 2004). It does not appear that ST2 is essential for murine reproduction however, as homozygous ST2 knockout mice are fertile (Hoshino *et al.*, 1999). In this study, expression of ST2 protein was identified in human embryos between day 5 and day 7 of development.

Several cytokines and factors are released by the human embryo which may aid implantation and pregnancy success and there is much interest in trying to identify factors secreted into embryo culture supernatants that may be related

to embryo potential (Tabiasco *et al.*, 2009). Therefore the presence of sST2 and IL-33 in embryo culture supernatants was investigated. Neither proteins were detectable by ELISA. However, levels of protein expression at this early time point in embryo development are extremely low. Each embryo is cultured in a 50µl droplet between day 3 and day 5 of embryo development and it is possible that even if either protein were secreted into the media, this relatively large volume (considering there are only around 8 cells on day 3 to a maximum of 250 on day 5) dilutes the sample and makes detection problematic (Sargent *et al.*, 2007). The embryos used in this study for immunofluorescence labelling were between day 5 and day 7 of development. It is possible that the genes for ST2 and IL-33 are only just switched on around this time, and therefore if the embryos were cultured for longer, the culture supernatants may contain the molecules. Further work is required to identify the exact point in development these proteins are expressed. A limiting factor of human embryo work is that most of the embryos obtained are those of relatively poor quality that are not suitable for cryopreservation. Thus the results may not reflect the expression of proteins seen in the best quality embryos. However some patients who decide to stop treatment do donate good quality cryopreserved embryos, and further studies are planned with these embryos in order to identify whether expression is related to the quality or developmental potential of the embryo.

Previous work done in our laboratory demonstrated increased expression of ST2L on peripheral blood NK and NKT-like cells from the end of the first trimester and throughout each trimester. These findings suggested that these cells might be a target for IL-33, which would contribute to the type 2 bias

associated with normal pregnancy. Therefore placental expression of IL-33 (as a potential source) and circulating levels of IL-33 in early pregnancy were investigated. First trimester placental expression of ST2 was also investigated along with circulating sST2 because the bioavailablilty of IL-33 is dependent on the circulating sST2 levels.

IL-33 and ST2 were variably expressed by first trimester placental tissue. Sections from 5 first trimester placentas were examined. There was no expression of the proteins in the earliest gestational specimens (between 6 and 7 weeks) and extensive expression in the latest gestation specimens (between 9 and 10 weeks). In the 8 week gestation specimen there was some stromal expression of both proteins but minimal syncytiotrophoblast expression. These experiments need to be repeated using a larger number of samples in order to establish if this is a consistent pattern of expression. In addition, double labelling using immunofluorescence would determine which particular cell types within the villous stroma express these proteins.

A recent published abstract has suggested that ST2 expression is found predominantly in extravillous cytotrophoblast (Pollheimer J. *et al.*, 2010) although in this study, distinct staining was identified in the syncytiotrophoblast in the later gestational samples The 3 decidual specimens examined in this pilot study also showed high levels of IL-33 expression as well as ST2. Polheimer *et al*. found minimal ST2 expression in the decidua but significant IL-33 staining and suggested that decidual IL-33 may be a target for ST2, being

expressed by stromal/mesenchymal cells, endothelial cells and smooth muscle cells.

The function of IL-33 in the process of implantation and early placentation requires investigation. It is increasingly clear that the process of implantation is inflammatory, for example decidual NK cell activation by IFN-γ is necessary for successful murine implantation (Xie *et al.*, 2005). IL-33 may stimulate the pro-inflammatory environment necessary for implantation. It has been shown that in some circumstances IL-33 can increase inflammatory cytokines such as IFN-γ (Smithgall *et al.*, 2008). Angiogenesis is also a key feature of implantation and placentation and it is possible that IL-33 may play a role in this. IL-33 has been shown to induce migration and capillary-like network formation of endothelial cells (HUVECs) (Choi *et al.*, 2009). In addition, there is preliminary data suggesting that recombinant IL-33 increases trophoblast migration in in vitro explant cultures (Pollheimer J. *et al.*, 2010).

In order to identify whether IL-33 and ST2 placental expression persists throughout pregnancy, third trimester placental samples were investigated. Both proteins were identified by western blotting in all of the placental lysates investigated. Immunohistochemical staining of the placentas was very variable. IL-33 was most apparent on fetal villous endothelium but none was identified on the syncytiotrophoblast which is the maternal-fetal interface. ST2 staining was identified on the syncytiotrophoblast in places. With respect to ST2L and sST2, these findings confirm and extend a previous report of placental ST2 by Kumar *et al* who identified three different messages using northern blotting of

human placenta. They suggested that the three transcripts would probably encode ST2 variants (Kumar *et al.*, 1997).

If IL-33 were to contribute to the type 2 dominance of normal pregnancy, increased circulating levels may have been anticipated throughout pregnancy. However, no differences in circulating IL-33 between normal pregnant and non-pregnant women were seen at any stage of gestation. Approximately two-thirds of women in each trimester had low or no detectable IL-33 and the remaining third of recruits had variable levels. A similar range was seen in the non-pregnant recruits. The hypothesis that circulating IL-33 may contribute to the type 2 dominance seen in normal pregnancy does not appear to be supported by this data. It is currently not known whether the commercial ELISAs detect both free IL-33 and sST2 bound IL-33. If only free IL-33 is detected, then possible increases in IL-33 in the third trimester may not be apparent because of the corresponding increase in sST2. In contrast to IL-33, sST2 levels changed over the course of normal pregnancy.

Even though cell surface ST2L is up regulated in early pregnancy, significantly increased circulating sST2 levels were not detected at this stage. However in the normal longitudinal cohort, circulating sST2 increased significantly in the third trimester of pregnancy. This third trimester sample was taken between 34 and 36 weeks. sST2 is normally produced by alveolar epithelial cells, cardiomyocytes and, to a lesser extent, by brain, small bowel and prostate cells (Mildner *et al.*, 2010). Endothelial cells and epithelial cells constitute major sources *in vivo* (Moussion *et al.*, 2008; Yagami *et al.*, 2010).The finding of

syncytiotrophoblast ST2 expression in the third trimester placentas suggests that sST2 could potentially be secreted into the maternal circulation leading to the increased circulating ST2 seen in the third trimester. The potential for placental secretion of these proteins is investigated further in the following chapter.

4.7 Summary

This study has shown for the first time that IL-33 and ST2 are expressed by the human pre-implantation blastocyst. In addition, both proteins can be identified in decidual and placental tissue. Although no differences were seen in circulating IL-33 in the pregnant or non-pregnant state, circulating sST2 was found to increase in the third trimester of normal pregnancy. Currently very little is known about the functional role of these proteins in human implantation and pregnancy and further investigation is planned.

5. IL-33 and ST2 in pre-eclampsia

5.1 Introduction

In chapter 4 it was shown that circulating IL-33 levels did not vary across the three trimesters of normal pregnancy; however circulating sST2 increased significantly in the third trimester. Both IL-33 and ST2 were expressed by the placenta. The increase in sST2 in the third trimester gives rise to the possibility that some of the circulating ST2 in pregnancy could originate from the placenta. Previous work in our laboratory has shown that the increase in ST2L expression on circulating NK and NKT like cells seen in normal pregnancy does not occur in pre-eclampsia, a hypertensive disorder of pregnancy (Borzychowski *et al.*, 2005), suggesting that IL-33 and ST2 may have a role in this disease. The aim of this chapter was to investigate circulating IL-33 and sST2 in pre-eclampsia and to identify whether the placenta could be the source of these circulating proteins.

5.1.1 Pre-eclampsia

Pre-eclampsia is a disease unique to human pregnancy that may be life threatening for both the baby and the mother. Worldwide, approximately 50,000 women die each year from eclampsia or complications of pre-eclampsia (W.H.O., 2005). Even in developed countries, mothers die as a direct consequence of this disease (Lewis, 2007). Pre-eclampsia affects 2.5-3% of pregnant women and is characterised by the new onset of hypertension and proteinuria

in the second half of pregnancy that resolves after delivery. Maternal complications of pre-eclampsia include pulmonary oedema, renal and liver failure, cerebral oedema and stroke, HELLP syndrome (haemolysis, elevated liver enzymes and low platelet count) and disseminated intravascular coagulation (Sibai *et al.*, 2005). Eclampsia is the end stage of the disease and is characterised by generalised seizures. Pre-eclampsia cannot be prevented and the only effective treatment is the delivery of the baby and placenta. Therefore the fetal complications of pre-eclampsia that arise most commonly are from pre-term delivery. In addition, fetal growth restriction may occur as a complication of poor placentation. In severe, early onset disease, the fetus may suffer nutritional insufficiency that may lead to intrauterine or peri-natal death.

Risk factors for pre-eclampsia include nulliparity, previous or family history of the disease, multiple pregnancy, raised body mass index and increased maternal age. Pre-existing medical conditions such as insulin dependent diabetes, autoimmune disease, anti-phospholipid antibodies, hypertension or renal disease also increase the likelihood of developing pre-eclampsia (Duckitt & Harrington, 2005).

5.1.2 Placental theory of pre-eclampsia

Although the precise aetiology remains uncertain, it is clear that pre-eclampsia results from the presence of a placenta (Redman, 1991). Pre-eclampsia is often described as a 'two stage disease'. The first stage, which is asymptomatic, occurs during placentation. In normal pregnancy maternal blood flow to the placenta is increased by spiral artery re-modelling. Cytotrophoblast cells invade the maternal decidua and, with the assistance of various immune cells, mediate a widening of the spiral arteries which increases the blood flow to the placenta (Red-Horse *et al.*, 2004). In pre-eclampsia there is insufficient invasion of the maternal decidua and spiral arteries by cytotrophoblast, consequently remodelling is poor and blood flow remains restricted. The resulting inadequate uteroplacental circulation causes intermittent ischaemia and reperfusion that may lead to placental oxidative stress in the second half of pregnancy (Hung & Burton, 2006; Burton *et al.*, 2009).

This results in both fetal and maternal sequelae (summarised in Figure 5-1). Firstly, reduced placental perfusion can lead to intrauterine growth restriction. Secondly, the oxidatively stressed placenta releases factors into the maternal circulation that induce a maternal systemic inflammatory response (Redman & Sargent, 2003) and endothelial cell dysfunction (Roberts *et al.*, 1989) both of which contribute to the maternal clinical symptoms and signs of pre-eclampsia (Redman & Sargent, 2009). Thirdly, the oxidative stress itself can alter molecules, for example it may change the conformational structure of angiotensinogen in the maternal circulation, resulting in a form of the molecule

that will more effectively release angiotensin. This in turn can modulate maternal blood pressure (Zhou *et al.*, 2010).

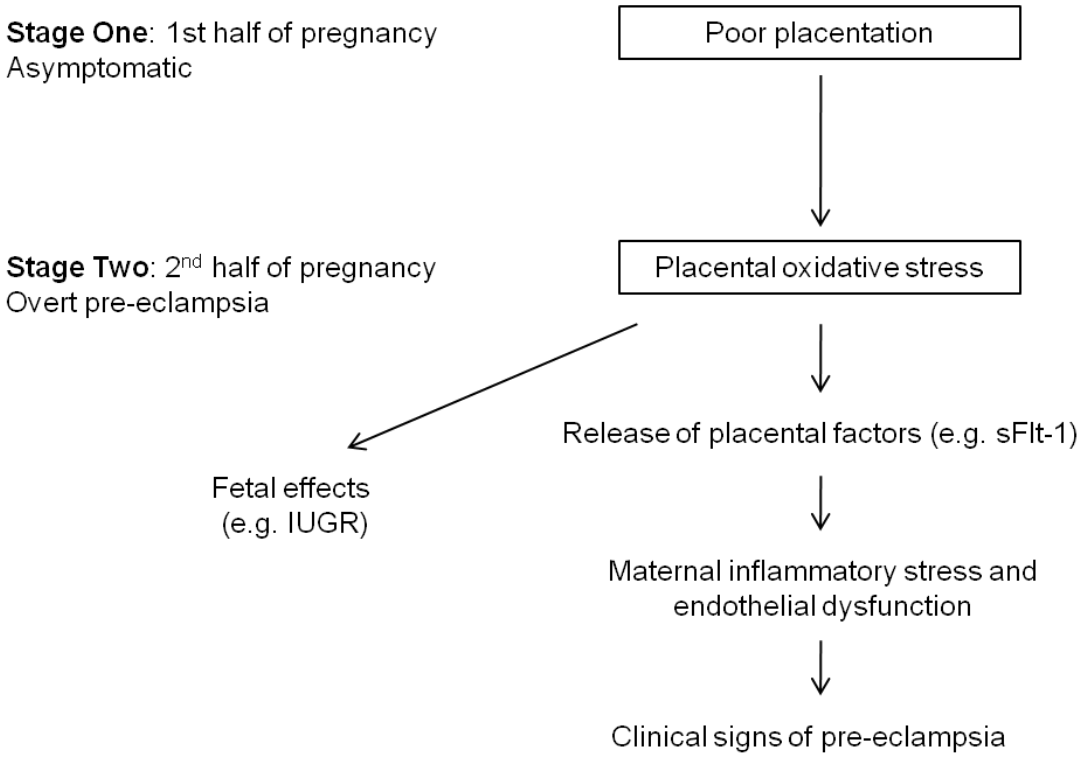


Figure 5-1 The two stage development of pre-eclampsia. In the first stage, insufficient trophoblast invasion of the decidua leads to poor placentation, placental hypoxia and oxidative stress. In the second stage the oxidatively stressed placenta releases factors, for example fms-like tyrosine kinase 1 (sFlt-1), leading to maternal inflammatory stress and endothelial cell dysfunction. This leads to the onset of clinical symptoms. Adapted from Redman et al. 2005

Although poor placentation is a significant predisposing factor to the development of the disease, it does not inevitably lead to pre-eclampsia. The disease aetiology is multi-factorial and there is evidence that in some cases of pre-eclampsia the disease is caused by an abnormal maternal inflammatory response to the pregnancy and placenta rather than abnormal placentation (Ness & Roberts, 1996).

5.1.3 Placental derived factors and the pathogenesis of pre-eclampsia

A number of placentally derived proteins are known to be involved in the pathogenesis of pre-eclampsia. Fms-like tyrosine kinase-1 (sFlt-1) is a soluble anti-angiogenic protein that acts as a decoy receptor for vascular endothelial growth factor (VEGF) and placental growth factor (PLGF). Levels of sFlt-1 are raised in pre-eclampsia resulting in a decrease in free serum VEGF and PLGF (Chappell *et al.*, 2002; Polliotti *et al.*, 2003; Levine *et al.*, 2004). When these proteins bind to sFlt-1 the balance of pro and anti-angiogenic factors shifts, and VEGF is unable to associate with endothelial cell receptors. This imbalance may, in part, result in the endothelial cell dysfunction and maternal syndrome of pre-eclampsia (Bdolah *et al.*, 2004; Karumanchi & Bdolah, 2004). Another placenta derived factor that is increased in pre-eclampsia is endoglin, which is a co-receptor for transforming growth factor β (TGF-β). More endoglin is released from the placenta in pre-eclamptic pregnancies compared to normal pregnancy. Soluble endoglin had been shown to induce hypertension and increase vascular permeability (Venkatesha *et al.*, 2006).

In addition to placentally derived proteins, microvesicles from the syncytiotrophoblast are shed into the maternal circulation (Redman & Sargent, 2008). Microvesicles are small (<200nm) membrane bound vesicles shed during membrane turnover and remodelling. Syncytiotrophoblast microvesicles have been shown to circulate in normal pregnancy and their numbers are significantly elevated in pre-eclampsia (Knight *et al.*, 1998; Germain *et al.*, 2007c). Syncytiotrophoblast microvesicles have been shown to inhibit the growth of cultures of human umbilical vein epithelial cells (HUVECs) (Smarason *et al.*, 1993) as well as stimulate inflammatory cytokine production from primed in vitro derived peripheral blood mononuclear cells (PBMCs) (Germain *et al.*, 2007a; Southcombe *et al.*, 2011). Therefore it is thought that these vesicles may be a source of inflammatory stimulus and endothelial disturbance in pregnancy, and more so in pre-eclampsia. It is clear that pre-eclampsia is a multi-factorial disease, whereby these factors and others contribute to its pathogenesis.

5.1.4 The systemic immune response in pre-eclampsia

As previously discussed, in pregnancy-related complications such as pre-eclampsia, the systemic Th2 bias associated with healthy pregnancy either fails to develop or is reversed, such that Th1 responses predominate (Saito *et al.*, 2007). However, the finding that inflammation is important in early implantation events (particularly at the decidua for trophoblast invasion) (Chaouat, 2007), systemic inflammation occurs in normal pregnancy (Sacks *et al.*, 1998) and cells of the innate immune system (e.g. dendritic, NK and invariant NKT cells) can also be biased towards Type 1 or Type 2 cytokine production (Peritt *et al.*, 1998; Gumperz *et al.*, 2002), suggests that the

Th1/Th2 hypothesis is too simplistic. However a shift towards type 2 immunity in normal pregnancy undoubtedly occurs.

As described briefly in section 5.1, our laboratory has shown that the Type 2 shift in normal pregnancy and Type 1 dominance in pre-eclampsia are predominantly expressed in peripheral blood $CD56^{bright}$ NK, $CD56^{dim}$ NK and NKT-like (CD3+CD56+) cells, rather than in Th and cytotoxic T cell populations (Borzychowski *et al.*, 2005). In that study cell surface IL-18 receptor was used as a stable cell surface marker for type 1 cells (Xu *et al.*, 1998b) and ST2L as a marker for type 2 cells (Xu *et al.*, 1998a) to avoid artefacts caused by the in vitro stimulation that is needed to measure intracellular or secreted cytokines. A significant increase in ST2L expression was found in NK cell populations in normal pregnant compared to non-pregnant women but not in samples from women with pre-eclampsia (Borzychowski *et al.*, 2005). At the time of that study the ligand for ST2L was unknown but soon after was identified as IL-33 (Schmitz *et al.*, 2005).

5.1.5 The role of IL-33 and sST2 in disease

Changes to circulating levels of IL-33 and sST2 are associated with a number of diseases. Circulating IL-33 is elevated in asthma, anaphylaxis (Pushparaj *et al.*, 2009; Liew *et al.*, 2010), rheumatoid arthritis, inflammatory bowel disease and sepsis (Brunner *et al.*, 2004; Beltran *et al.*, 2009; Seidelin *et al.*, 2009). sST2 is increased in asthma, sepsis, autoimmune diseases such as systemic lupus erythematosus, dyspnoea and acute myocardial infarction (Kuroiwa *et al.*, 2001; Oshikawa *et al.*, 2001; Weinberg *et al.*, 2003; Brunner *et al.*, 2004; Hoogerwerf *et al.*, 2010; Socrates *et al.*, 2010). sST2 binds IL-33 as a decoy receptor in the circulation, abrogating its function, therefore increased sST2 may have a negative impact on disease outcome. For example, sST2 reduces IL-33 mediated neutrophil recruitment to the site of infection in sepsis (Alves-Filho *et al.*, 2010), and is associated with poor survival rates post acute myocardial infarction (Shimpo *et al.*, 2004; Sabatine *et al.*, 2008).

Maternal blood levels of IL-33 and sST2 have not previously been investigated in pre-eclampsia. The previous finding that ST2L expression is increased on NK and NKT-like cells in normal pregnancy, but not in pre-eclampsia, suggests that IL-33 and sST2 may have a role in this disease This may be as either as a primary cause or a downstream player in the regulation of the systemic inflammatory response.

5.1.6 Aims

1. To establish whether IL-33 and sST2 are altered in pre-eclamptic pregnancy compared to normal pregnancy.
2. To establish whether IL-33 and sST2 are altered in pre-eclamptic pregnancy compared to normal pregnancy prior to the onset of clinical symptoms.
3. To investigate whether the pre-eclampsia placenta expresses IL-33 and ST2 and if so, where placental IL-33 and ST2 are localised.
4. To investigate whether IL-33 and sST2 are released into the maternal circulation by using an in vitro model of placental perfusion.

5.2 Materials and Methods

5.2.1 Subject recruitment

Ethical approval for these studies was obtained from the Oxford Research Ethics Committee C and written informed consent was obtained from each subject prior to venepuncture. All pregnant recruits had a singleton pregnancy with no known fetal abnormality and were not in labour at the time of blood sampling. None of the women had any significant medical history and were not taking any medications (with the exception of contraceptives in the non pregnant group). Normal pregnant controls were matched to pre-eclamptic patients for age (+/- 4 years), parity (0, 1-3, 4+), and gestational age (+/- 13 days). Non-pregnant controls were matched for age (+/- 4 years) and parity.

5.2.1.1 Matched pre-eclampsia case-control study

Pre-eclamptic women who were admitted to the high risk maternal medicine unit at the John Radcliffe Hospital, Oxford were recruited (n=20). Pre-eclampsia was defined as the onset of new hypertension and proteinuria in the second half of a previously normal pregnancy. Hypertension was defined as a diastolic blood pressure (BP) ≥90mmHg on at least two occasions within a 24 hour period. Proteinuria was defined as ≥300mg protein in a 24 hour urine collection, 50mg/mmol protein/creatinine ratio or at least 2+ on dipstick testing on two consecutive measurements. A control group of healthy pregnant women was recruited from the John Radcliffe Hospital antenatal clinic and local G.P. ante-natal clinics. Non-pregnant women were recruited from hospital

and university staff. Clinical characteristics of each group are presented in Table 5-1.

In order to gain further information as to how circulating IL-33 and sST2 change with gestational age in both normal and pre-eclamptic pregnancy, an additional 30 women with pre-eclampsia were matched to normal pregnant women, therefore totalling 50 cases of pre-eclampsia matched to normal pregnant controls. Clinical characteristics of this group are seen in Table 5-2. There were no significant differences in age or parity between the three study groups and no significant difference in gestation between the pre-eclamptic and normal pregnant women. Of note women with pre-eclampsia had a significantly higher systolic blood pressure at their first ante-natal clinic visit (the booking visit) ($p<0.01$). The difference seen at booking is unsurprising because women with higher blood pressure at booking are known to be at increased risk of developing the disease (Poon *et al.*, 2010)

	Non-pregnant	Normal pregnant	Pre-eclamptic	
Age (years)	30.85 (5.38)	30.7 (5.79)	29.45 (5.53)	N.S.
Nulliparity	14/20	14/20	14/20	N.S
Gestation (days)		238.12 (35.63)	241.45 (32.45)	N.S.
Booking systolic BP (mmHg)		109.45 (13.04)	122.50 (14.36)	p<0.05
Booking diastolic BP (mmHg)		65.90 (9.96)	77.15 (14.83)	p<0.05
Max systolic BP (mmHg)		124.05 (9.64)	176.30 (17.54)	p<0.0001
Max diastolic BP (mmHg)		75.21 (8.53)	113.65 (12.42)	p<0.0001
Birthweight (g)		3408.45 (402.18)	2176.25(1022.97)	P<0.001

Table 5-1 Clinical characteristics of women with pre-eclampsia, normal pregnant women and non-pregnant women (n=20). Data are shown as mean (+/-standard deviation). N.S = not significant

	Normal pregnant	Pre-eclampsia	
Age	31.43 (5.58)	30.80 (5.61)	N.S.
Nulliparity	39/50	39/50	N.S.
Gestation (days)	231.6 (34.87)	223.1 (34.22)	N.S.
Booking systolic BP (mmHg)	105.7 (11.09)	120.9 (14.28)	p<0.001
Booking diastolic BP (mmHg)	64.53 (9.817)	73.68 (12.59)	P<0.01
Max systolic BP (mmHg)	125.8 (11.62)	175.8 (18.13)	p<0.001
Max diastolic BP (mmHg)	77.56 (7.917)	125.3 (33.46)	p<0.001
Birthweight (g)	3397 (387)	1967 (1011)	p<0.001

Table 5-2 Clinical characteristics of women with pre-eclampsia and normal pregnant women (n=50). Data are shown as mean (+/- standard deviation). N.S. = not significant

5.2.1.2 Longitudinal study

To determine whether changes in IL-33 and sST2 occur prior to the onset of pre-eclampsia, longitudinal blood samples were taken (in each trimester and if possible at diagnosis) from healthy pregnant women who developed pre-eclampsia (n=15). The samples were obtained from the Oxford Pregnancy Biobank. Biobank samples were taken at the first trimester ultrasound scan (11-13 weeks gestation), the second trimester scan (20-22 weeks) and at approximately 34 weeks. Pre-eclampsia recruits were matched for age, parity and gestation with normal pregnant women who had a blood sample taken in each trimester stored in the biobank. Clinical characteristics of the patients in this study are presented in Table 5-3.

	Non-pregnant	Normal pregnant	Pre-eclamptic	
Age (years)	28.60 (4.93)	28.00 (5.50)	27.93 (5.75)	N.S.
Nulliparity	14/15	14/15	14/15	N.S
Gestation at delivery (days)		282.00 (6.11)	250.50 (33.59)	p<0.001
Booking systolic BP (mmHg)		109.80 (13.68)	115.67 (13.64)	N.S
Booking diastolic BP (mmHg)		65.53 (8.59)	70.07 (11.18)	N.S.
Max systolic BP (mmHg)		125.89 (7.57)	160.33 (13.22)	p<0.001
Max diastolic BP (mmHg)		78.93 (7.25)	107.13 (8.67)	p<0.001
Birthweight (g)		3439.93 (495.84)	2792.79 (1107.13)	N.S

Table 5-3 Clinical characteristics of women with pre-eclampsia and normal pregnant women (n=15) in the longitudinal study. Data shown are mean (+/-S.D). N.S. = not significant

5.2.1.3 Placental tissue

Nine women with pre-eclampsia undergoing caesarean section agreed to donate placental tissue for analysis. Written informed consent was obtained. The samples could not be matched with normal pregnancies because women with pre-eclampsia are likely to require delivery prior to term, whereas women with a low risk pregnancy requiring caesarean section are normally delivered between 38 and 39 weeks. However for reference, the data is presented with the data from normal placentas already shown in chapter 4. The mean gestational age of the pre-eclampsia recruits at caesarean section was 34 weeks verses 38 weeks in the normal pregnant women.

5.2.2 Peripheral blood sampling

Peripheral venous blood samples were taken using a vacutainer system and a 21-gauge needle.

5.2.3 Plasma and serum preparation

8 ml of blood for plasma samples was taken in an EDTA vacutainer (NHS Supplies). Blood was centrifuged at 2000g for 10 minutes at 20º C. Plasma was then transferred into a 1.5ml microcentrifuge tube and centrifuged at 13,000g for 2 minutes at 20ºC. Blood (8ml) for serum sample were collected in a silica clot activator (SST) vacutainer (NHS supplies). Samples were left for 30 minutes to clot and then centrifuged at 1000g for 15 minutes at 4ºC. Aliquots of plasma and serum were stored at -80ºC until analysed.

5.2.4 Detection of IL-33 and ST2 by ELISA, western blotting and immunohistochemistry

The methods for ELISA, western blotting and immunohistochemistry detection of IL-33 and ST2 are described in chapter 4.2.

5.2.5 Placental perfusion

To determine whether IL-33 or sST2 are released from the villous surface into the maternal circulation a modified dual placental perfusion system was used, as described by Eaton and Oakey (Eaton & Oakey, 1994) (Figure 5-2). Placentas were obtained with informed consent from normal pregnant, healthy women (n=8) or women with pre-eclampsia (n=5) who were not in labour and were undergoing caesarean section. Placentas were processed immediately after delivery. An individual lobule was isolated and firstly the fetal circulation perfused with 'fetal perfusion media' at a rate of 5mL/min. The media used is 0.1µm filtered modified M-199 tissue culture medium (Medium 199 with L-glutamine and Earle's salts, containing 0.8% Dextran 20, 0.5% BSA, 5000 U/L sodium heparin, and 2.75 g/L sodium bicarbonate, pH 7.4) containing a bolus of 100,000IU streptokinase to promote clot removal. The whole placenta was turned upside down and laid inside a Perspex water jacket maintained at 37°C. The maternal circulation was then established by perfusion with 'maternal perfusion medium' (Medium 199 with L-glutamine and Earle's salts, containing 0.5% BSA, 5000 U/L sodium heparin, and 2.75 g/L sodium bicarbonate, pH 7.4) through eight 1.7mm fetal feeding tubes at a controlled rate of 20 ml/min. Perfusion media were warmed in a 37°C water bath and the maternal perfusion medium was oxygenated with 95% O2, 5% CO2. The lobule was perfused for 20min to equilibrate the system, after which time the maternal circuit was closed with a total volume of perfusion medium of 600mL. The volume of fetal effluent was measured every 2 minutes and the oxygen concentration of the maternal side perfusate monitored to ensure the stability of the preparation. Pressure monitors were used to ensure no significant deviations from baseline

during the experimental period. At the end of the 3 hour perfusion period, the maternal perfusate was centrifuged in a Beckman J6-M centrifuge at 600 g for 10 minutes at 4°C. IL-33 and sST2 were detected in the perfusate by ELISA as described above.

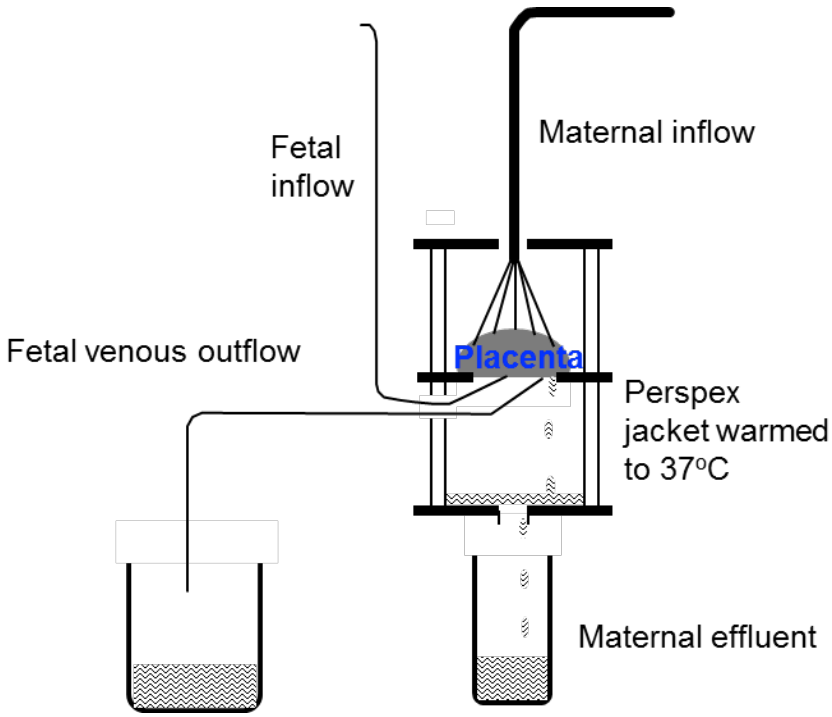


Figure 5-2 Diagram showing dual placental perfusion system whereby both the maternal and fetal circulation are perfused and the resulting effluent collected for analysis

5.3 Statistics

The Gaussian distribution of each data set was assessed using a Kolmogorovv-Smirnov test. Clinical data was compared using a paired t-test. In the longitudinal study, where the data was normally distributed, a repeated measures ANOVA was used to determine if there was variation across the trimesters. Where a significant variation was identified, a Bonferroni post hoc test was used to determine the significant time points. For non-parametric longitudinal data a Kruskal Wallace test was used to determine if there was variation across the trimesters. Where a significant variation was identified, a Dunn's post hoc test was used to determine the significant time points. Non-parametric paired data was analysed using a Wilcoxon matched pairs test. Non matched data comparing non-pregnant to longitudinal data was compared with an unpaired t test. To assess the predictive value of sST2 for pre-eclampsia a ROC curve was generated. Values of $p<0.05$ were considered to be significant. Graphpad Prism software (San Diego, California) and SPSS (Chicago, Illinois) were used to analyse the data.

5.4 Results

5.4.1 Circulating IL-33 in pre-eclampsia

In order to establish whether IL-33 was altered in pre-eclamptic pregnancy, serum levels were measured from 20 matched trios of women who were pre-eclamptic, women with a normal pregnancy and non-pregnant women (Figure 5-3A). IL-33 levels were not normally distributed and therefore the data is presented as the median and range. Although there was a trend to increased circulating IL-33 in the pre-eclamptic women (41.38 (0-656.4) pg/ml) compared to normal pregnant (0 (0-1222.9) pg/ml) or non-pregnant women (0 (0-193.0) pg/ml)), this did not reach statistical significance.

IL-33 was then measured in serum samples taken in the first, second and third trimester from 15 women who subsequently developed pre-eclampsia and 15 matched normal pregnant controls (Figure 5-3B). An additional sample, taken at the time of pre-eclampsia diagnosis was available for 13 of the women. There were no differences in IL-33 levels throughout pregnancy in women who went on to develop pre-eclampsia compared to the normal pregnant controls at any of the three time points analysed.

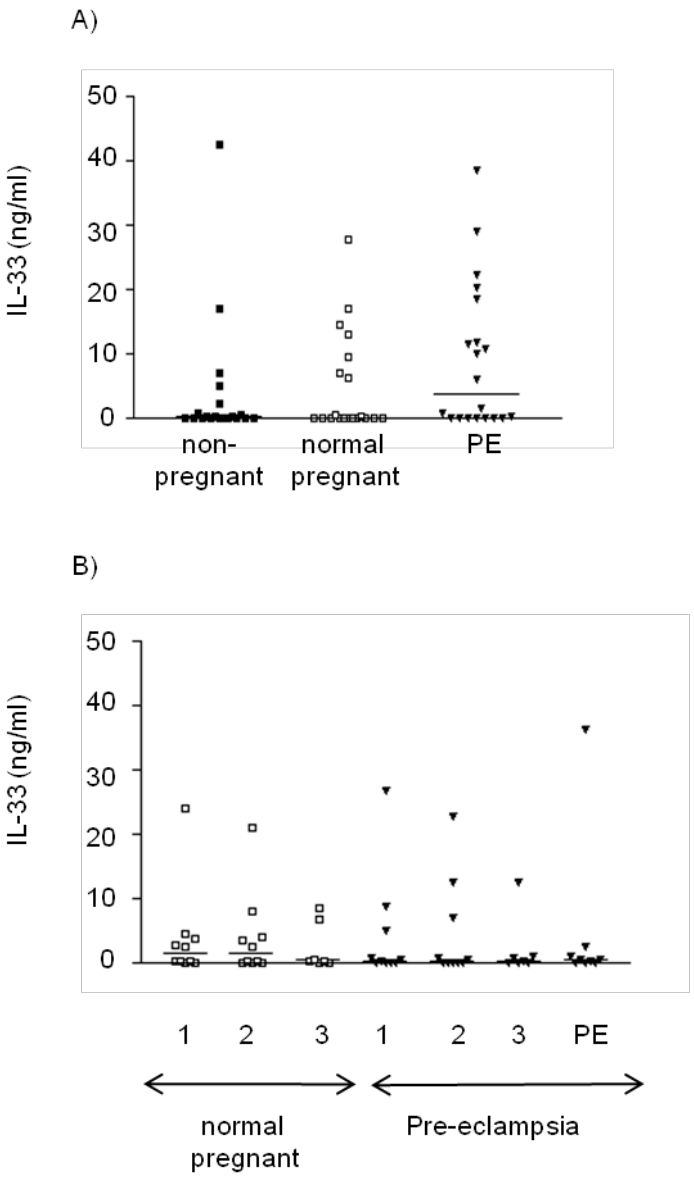


Figure 5-3 IL-33 was detected by ELISA in the serum from women with pre-eclampsia, matched controls who had a normal pregnancy and non-pregnant women (A) (n=20). IL-33 was detected in serum by ELISA in women who developed pre-eclampsia (PE) (n=15), matched controls who had an uncomplicated pregnancy and non-pregnant women (B) (n=15). Blood samples were taken in each trimester of pregnancy (1, 2, 3) and at the diagnosis of pre-eclampsia (PE). There were no significant differences throughout pregnancy or between normal and pre-eclampsia pregnancies. Bars represent median values.

5.4.2 Circulating sST2 in pre-eclampsia

Circulating sST2 was next measured in the same 20 matched trios of women with pre-eclampsia, normal pregnant women and non-pregnant controls (Figure 5-4). This data was normally distributed and is presented as the mean (+/- SEM). sST2 levels were significantly increased ($p<0.001$) in women with pre-eclampsia (mean 85.89 (11.27) ng/ml) compared to normal pregnant (mean 25.20 (3.052) ng/ml) or non-pregnant controls (mean 20.37 (1.563) ng/ml). Although the sST2 levels in the normal pregnant controls were higher than in non-pregnant women they were not significantly different, in contrast to the normal pregnant longitudinal cohort in chapter 4 (Figure 4.8). This is possibly due to the smaller sample size (n=20) than in the normal longitudinal study (n=30).

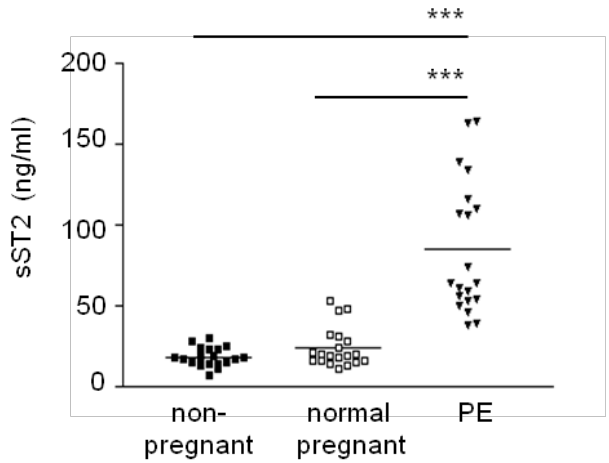


Figure 5-4 sST2 was detected by ELISA in plasma samples from women with pre-eclampsia, matched controls who had a normal pregnancy and matched non-pregnant women (n=20 for each group). sST2 was significantly raised in pre-eclampsia compared to normal pregnancy and non pregnant women ($p<0.001$***). Bars represent mean values.

This study was then extended to a larger group of 50 women with pre-eclampsia and matched normal pregnant women in order to look at the effect of gestational age on sST2 levels in both patients and controls. The statistically significant increase in circulating levels of sST2 in the pre-eclampsia group was confirmed ($p<0.001$) (Figure 5-5A). Within the pre-eclamptic patient group the diagnosis was made between 22 weeks and 41 weeks gestation. Gestational age was therefore compared to sST2 levels in both groups. In normal pregnancy, circulating sST2 levels remained similar to non-pregnant levels (mean 17.85 (1.193)) until 35 weeks then levels rose slowly toward the end of pregnancy (Figure 5-5B). In pre-eclampsia, sST2 levels were generally higher than the controls at all gestational ages, with the largest differences in cases of early onset pre-eclampsia (less than 34 weeks' gestation). In normal pregnancy circulating sST2 levels were strongly correlated with gestational age ($r=0.659$, $p<0.001$) (Figure 5-5B). In pre-eclampsia there was a poor correlation with gestational age ($r=0.079$, $p>0.05$) as even in early onset pre-eclampsia sST2 levels were high.

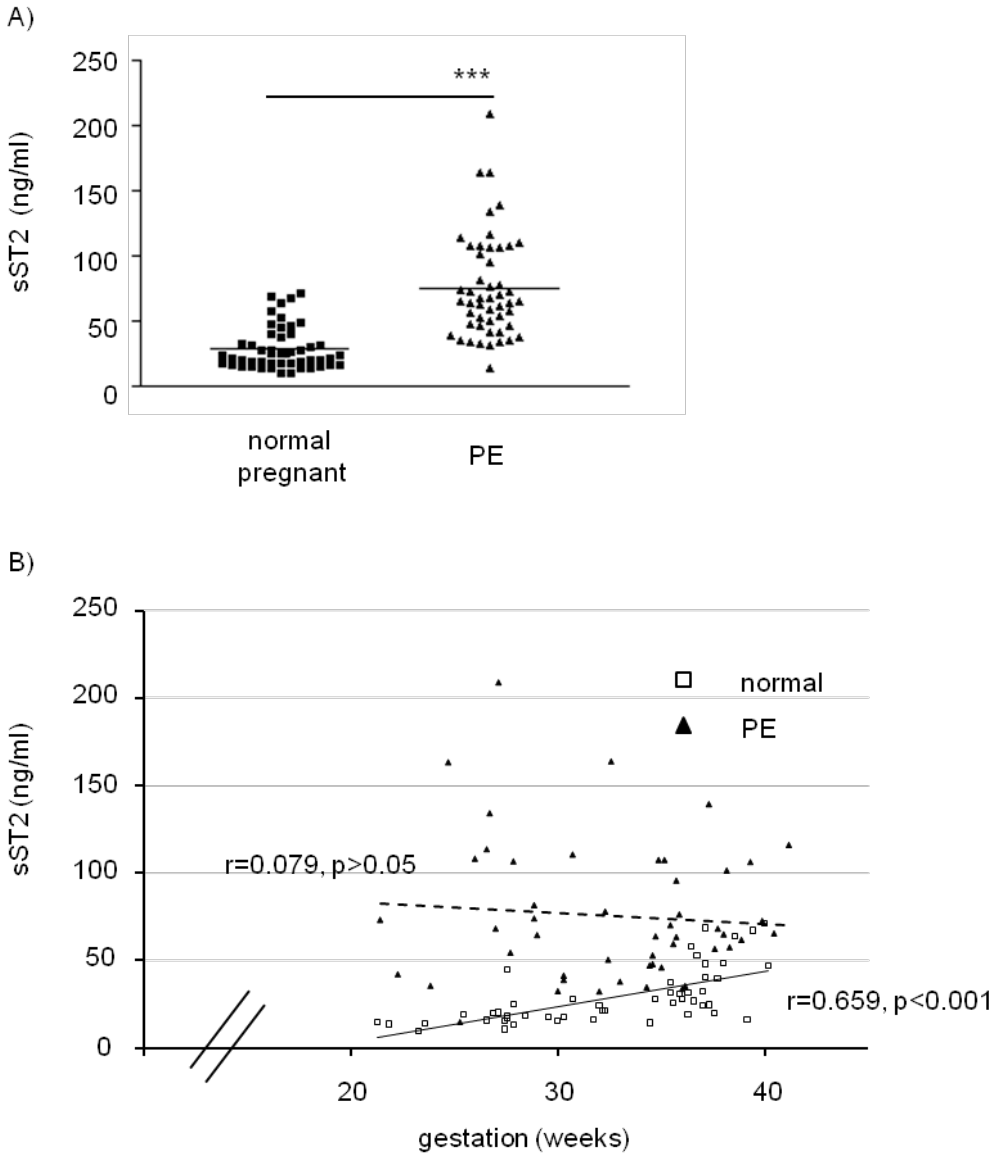


Figure 5-5 (A) sST2 was detected by ELISA in plasma from women with pre-eclampsia (PE) and matched controls who had a normal pregnancy (n=50 for each group). The significant increase in sST2 in pre-eclampsia compared to normal controls was confirmed in this larger group ($p < 0.001$). (B) Circulating sST2 levels were plotted against gestation. In normal pregnancy sST2 levels increased towards term. In pre-eclampsia sST2 levels were significantly increased compared to controls. Differences in sST2 levels in normal and pre-eclampsia pregnancies were more apparent in early onset disease.

Serial blood samples taken in each trimester from 15 women who developed pre-eclampsia and 15 matched controls were then analysed (Figure 5-6).

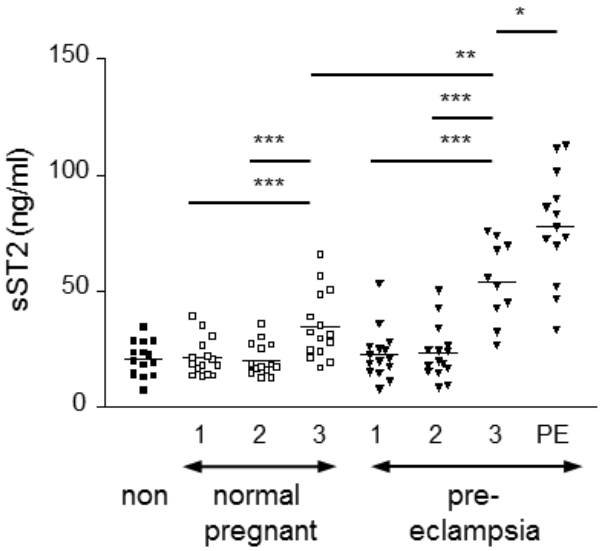


Figure 5-6 sST2 was detected by ELISA in plasma in women who developed pre-eclampsia and matched controls who had an uncomplicated pregnancy (n=15 for each group). Blood samples were taken in each trimester of pregnancy (1, 2, and 3) and at the diagnosis of pre-eclampsia (PE). There was a significant increase in sST2 in the third trimester compared to the first or second trimester in both normal and pre-eclamptic pregnancies ($p<0.001$***). sST2 was significantly elevated in the third trimester prior to the onset of disease in women who went on to develop pre-eclampsia compared to controls ($p<0.001$**). Bars represent mean values.

sST2 levels were similar in both the first and second trimester in the women who developed pre-eclampsia and the normal pregnant control group. Mean (SEM) sST2 levels in the first trimester of normal and pre-eclampsia pregnancies were 21.48 (2.086) ng/ml and 22.87 (2.992) ng/ml respectively. Mean sST2 levels in the second trimester were 20.60 (1.789) ng/ml and 23.38 (2.992) ng/ml respectively. In both groups there was a significant increase in the plasma sST2 in the third trimester compared to both the first trimester ($p<0.05$ in control group, $p<0.01$ in the pre-eclampsia group) and second

trimester ($p<0.01$). Interestingly, woman who develop pre-eclampsia had significantly increased sST2 in the third trimester 54.28 (5.501) ng/ml prior to the onset of clinical symptoms, when compared to normal pregnant controls 34.87 (26.85) ng/ml ($p<0.05$). In addition, sST2 levels were even higher once clinical symptoms of pre-eclampsia were present 77.82 (6.635). Five women developed pre-eclampsia prior to the third trimester sample, therefore only ten samples are shown in the pre-eclampsia third trimester time point.

The predictive value of sST2 at the third trimester sample time was assessed by generating a ROC curve, giving an area under the curve (AUC) of 0.813, the 95% confidence interval had a lower bound of 0.642 and upper bound of 0.984 (Figure 5-7). Using a cut off point of 32.27ng/ml the sensitivity of the test in the third trimester to predict subsequent pre-eclampsia is 90% with a specificity of 60%. Using a cut off of 40.88ng/ml the sensitivity is 80% with a specificity of 73.3%. On average the women (n=10) developed pre-eclampsia 20 days after this sample was taken. No differences between non-pregnant samples and normal pregnant first and second trimester samples were noted, however, as expected, there was a significant increase in the third trimester ($p<0.001$).

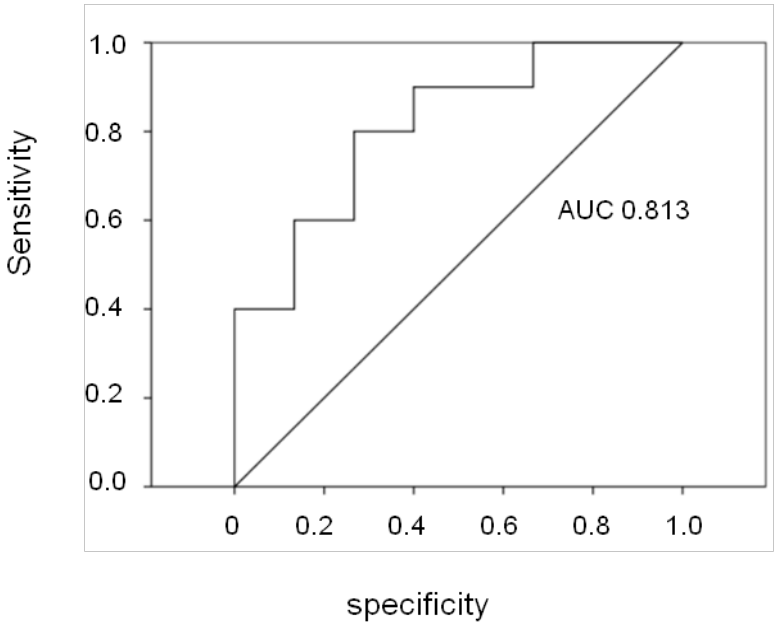


Figure 5-7 ROC curve analysis indicates sST2 levels in the third trimester sample (n=10) are likely to predict pre-eclampsia (AUC=0.813). Bars represent mean values, * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.

5.4.3 IL-33 and ST2 expression of pre-eclampsia placentas by western blotting

Having established in chapter 4 that IL-33 and sST2 are present in normal third trimester placentas, the possibility that differences may be seen in pre-eclampsia placentas or that the placenta may be the source of the increased circulating sST2 in pre-eclampsia was investigated. Similar to the normal placentas, western blotting showed the presence of IL-33 and ST2 in lysates of all the pre-eclampsia placental tissues studied (Figure 5-8). Once again, both forms of IL-33 were detected, although the shorter form of IL-33 was only present in 2 of the analysed pre-eclampsia placentas. Densitometry was performed to standardise protein expression to actin quantity, no differences between normal and pre-eclamptic placentas were detected

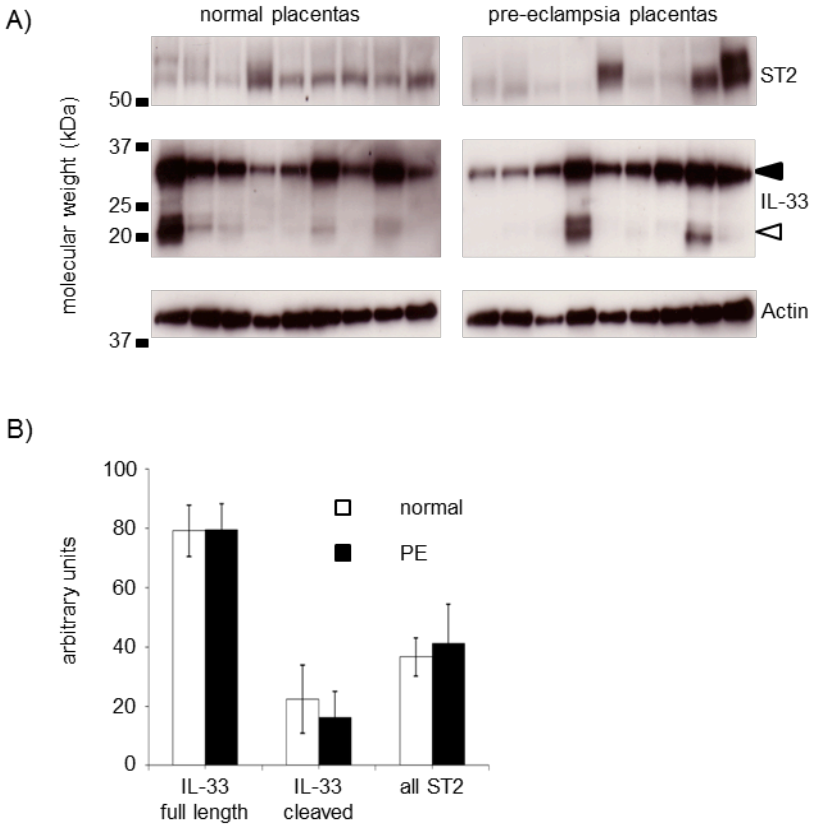


Figure 5-8 (A) IL-33 was detected by western blotting of placental lysates from normal and pre-eclampsia placentas (n=9). Both the full length (closed arrow) and cleaved forms (open arrow) were detected. ST2 was detected from each of the placental lysates. (B) Densitometry showed similar expression of both IL-33 and ST2 in normal and pre-eclampsia placentas. Bars show the mean (SEM).

5.4.4 IL-33 and sST2 localisation in normal and pre-eclampsia placentas by immunohistochemistry

Immunohistochemistry of placental sections was then carried out to determine whether the cellular localisation of the IL-33 and ST2 was similar in pre-eclampsia to normal pregnancy. Once again, labelling for both molecules was variable (Figure 5-9). IL-33 staining was largely perivascular, around chorionic villus stromal vessels as well as some syncytiotrophoblast staining. ST2 showed some villous stromal staining and some strong staining of the syncytiotrophoblast. Overall, no differences were detected between normal and pre-eclampsia placentas. No staining was seen in IgG controls and the positive control. Cytokeratin-7 (data not shown) strongly stained the syncytiotrophoblast

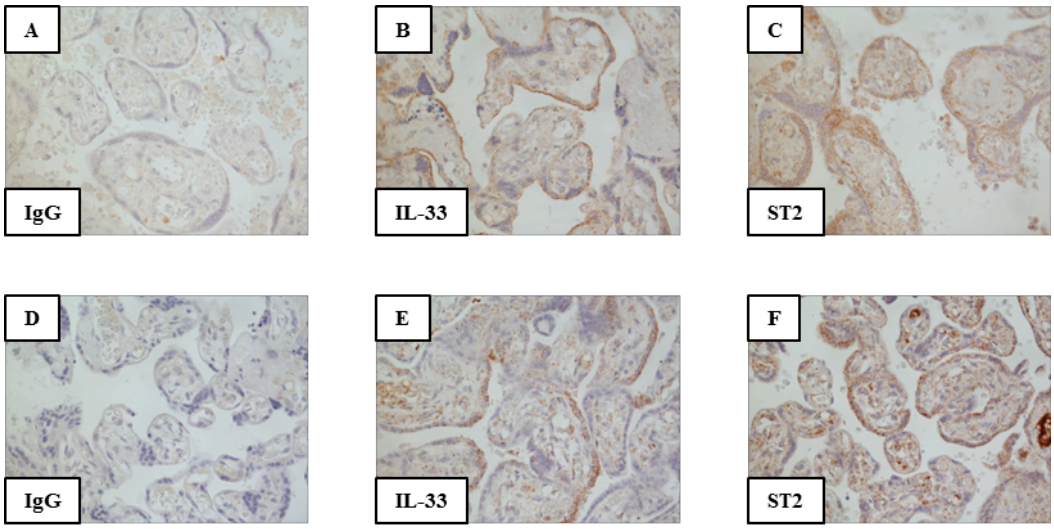


Figure 5-9 Variable expression of IL-33 and ST2 can be seen in immunohistochemistry of pre-eclampsia (n=3) placentas (A-C). A similar pattern of staining was seen in the normal placentas (D-F).

5.4.5 Placental production of IL-33 and ST2

The presence of IL-33 and sST2 in the placenta does not necessarily mean that the placenta is the source of the molecules detected in the circulation of pregnant women or in pre-eclampsia. To determine whether IL-33 and sST2 can be released from the placenta into the maternal circulation, supernatants from the maternal side of placental perfusions (whereby the maternal blood flow across the syncytiotrophoblast is mimicked and maintained ex vivo) from normal (n=8) and pre-eclampsia (n=5) placentas were assayed for these molecules. IL-33 was detected by ELISA in the perfusate (Figure 5-10A) from one normal placenta. High levels of sST2 could be detected in all of the perfusates analysed from both normal and pre-eclampsia placentas (Figure 5-10B). No IL-33 or sST2 was detected in culture media used for the perfusion.

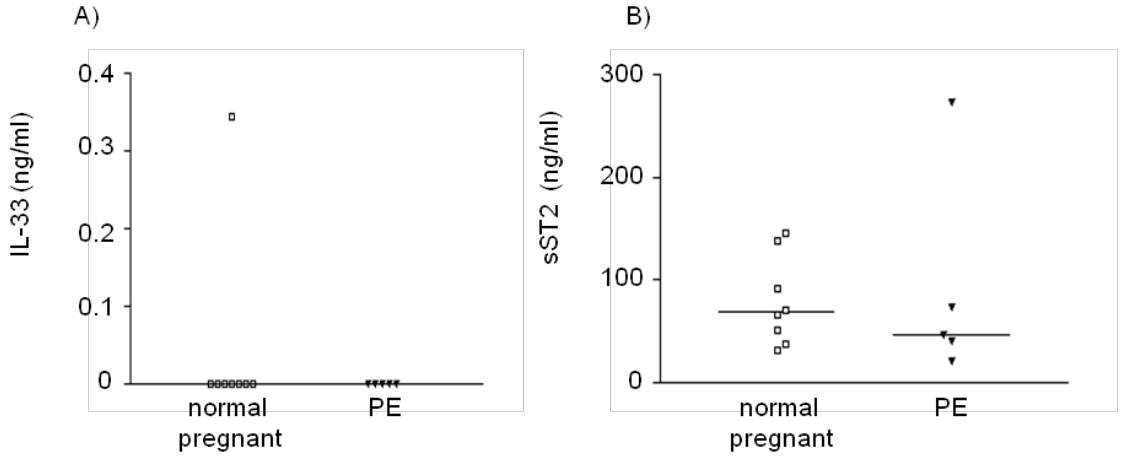


Figure 5-10 IL-33 and sST2 were detected by ELISA from media obtained from an *in vitro* model of placenta perfusion. Normal (n=8) and pre-eclampsia (PE) (n=5) placentas were perfused. (A) IL-33 could be identified in one sample. (B) sST2 could be identified in media from each of the normal and pre-eclampsia placental perfusions. Bars represent median values.

To determine whether the disparity between IL-33 and sST2 levels was due to the IL-33 ELISA being unable to detect IL-33 in the presence of high levels of circulating sST2, 250 pg/ml IL-33 (from the ELISA standards) was pre-incubated with culture medium alone or two maternal perfusion samples known to contain sST2 for 16 hours prior to ELISA. IL-33 could still be detected under each of these conditions (202.7pg/ml was detected in the culture media, 122.8pg/ml and 103.8pg/ml from the two perfusates), suggesting that neither the media nor sST2 prevent detection by ELISA.

5.5 Discussion

Data in the previous chapter showed that circulating sST2 levels change significantly over the course of normal pregnancy. Data from the controls matched to pre-eclampsia patients (Figure 5-5B), suggests that in normal pregnancy circulating sST2 levels remain fairly constant until around 34 weeks and rise slowly towards term. This is the first study to show that sST2 is not only significantly increased in the third trimester of normal pregnancy but that it is further increased in pre-eclamptic women with active disease. sST2 acts as a decoy receptor for IL-33, competing with membrane bound ST2L. Therefore, the very high levels of circulating sST2 seen in pre-eclampsia would be predicted to decrease IL-33 binding to its receptor thereby contributing to the type 1 bias seen in this disease.

Both proteins were identified by western blotting in pre-eclampsia placental lysates. The measurements on normal and pre-eclampsia placentas are not directly comparable, as the latter were, on average, delivered at an earlier gestational age (mean 34 weeks versus 38 weeks for normal placentas). There was no apparent difference between the immunohistochemical staining of pre-eclampsia and normal placentas. Placental production of sST2 was confirmed using an in vitro model of placental perfusion. Perfusates from all of the normal and pre-eclampsia placentas examined contained sST2, although it was not possible to directly compare the concentrations of sST2 in each of the perfusates because different volumes of medium were perfused through each placenta. In addition the volume and surface area of each placental lobe perfused inevitably varied between placentas.

The source of the increased circulating sST2 in pre-eclampsia is not known. In cardiac conditions associated with ventricular hypertrophy and remodelling, also a feature of pre-eclampsia (Melchiorre *et al.*, 2011), increased circulating levels of sST2 have been considered to derive from cardiomyocytes, activated by mechanical stress or pro-inflammatory stimuli (Weinberg *et al.*, 2003). However when plasma sST2 was measured from both the coronary sinus (venous blood) and coronary artery in patients with chronic heart failure there was no difference in sST2 levels suggesting that the source of sST2 was not the myocardium, rather it was likely to be the endothelium (Bartunek *et al.*, 2008). ST2 is produced by proliferating but not resting endothelium (Aoki *et al.*, 2010). Therefore it is possible that endothelial dysfunction (Roberts *et al.*, 1989), could contribute to raised levels of sST2 in pre-eclampsia. Circulating sST2 is also increased in other conditions involving the endothelium such as dengue fever (Houghton-Trivino *et al.*, 2010) or sepsis (Brunner *et al.*, 2004). sST2 may also be secreted by activated Th2 cells (Lecart *et al.*, 2002). This study raises the possibility of the placenta as yet another source.

sST2 secretion is stimulated by stress, particularly inflammatory stresses, such as by IL-1β or TNF-α (Kumar *et al.*, 1997), and has anti-inflammatory actions (Yagami et al.). Its time course of production in humans after LPS challenge peaks at 8 hours and is relatively sustained at 24 hours (van 't Veer *et al.*, 2007). It is interesting that LPS does not stimulate sST2 production from whole blood *in vitro* but does so *in vivo*, which is consistent with derivation from endothelium (van 't Veer *et al.*, 2007). Although it does not contribute to LPS

tolerance (van 't Veer *et al.*, 2007), it is likely that it down-regulates the activity of IL-33 that may also be increased in these circumstances.

As discussed in chapter 4, IL-33 does not have a signal peptide, so must be secreted by one or more non-classical pathways. These are thought to include release after cell necrosis as an alarmin (Haraldsen *et al.*, 2009). In addition, as are IL-1α and HMGB1, IL-33 is a dual function protein that serves not only as a proinflammatory cytokine but as an intracellular nuclear factor with transcriptional properties that include repression (Carriere *et al.*, 2007). As already indicated it is localised in endothelial cells and promotes Type 2 immune functions. Its general role is as an amplifier of innate immune responses (Oboki *et al.*, 2010). To what extent these functions are relevant to the pathogenesis of pre-eclampsia remains to be elucidated.

There is some evidence to suggest that sST2 may itself have immunoregulatory properties. The ST2 protein may inhibit inflammatory pathways by inhibiting IL-1R type I and TLR-4 signalling (Brint *et al.*, 2004). In LPS stimulated mice sST2 has been shown to down-regulate pro-inflammatory cytokines such as TNF, IFN-γ, IL-12 and IL-6 by direct interaction with macrophages (Sweet *et al.*, 2001). In addition sST2 down regulated TLR-4 in murine bone marrow derived macrophages and has also been shown to bind to a human monocyte line (THP-1) suppressing LPS induced production of IL-6, IL-1β and TNF-α (Takezako *et al.*, 2006). This gives rise to the possibility that sST2 may have other direct mechanisms of action in pre-eclampsia in addition to decreasing the activity of IL-33.

Although there are known risk factors for pre-eclampsia it remains difficult to predict which women will develop pre-eclampsia. Several molecules produced by the placenta have been suggested as potential biomarkers that may indicate those women at high risk. Ideally a biomarker should be detectable as early as possible so that patient care can be adjusted accordingly. sFlt-1 has been shown to be increased around five weeks prior to the onset of pre-eclampsia and endoglin somewhat earlier at around 8-12 weeks (Levine *et al.*, 2004; Levine *et al.*, 2006). PLGF levels are lower in the second trimester in women who go on to develop pre-eclampsia (Tidwell *et al.*, 2001). In addition, another molecule, placental protein 13 (PP13) also known as galectin-13, has been shown to be decreased in the first trimester in women who develop pre-eclampsia (Than *et al.*, 2008). PP13 binds to extracellular matrix sugar molecules involved in placental invasion as well as increasing prostaglandin release (Burger *et al.*, 2004; Than *et al.*, 2004). Low levels of PP13 may therefore impair normal placentation and vascular remodelling. Inhibin A and Activin A (both members of the transforming growth factor-β superfamily) are also produced by the placenta. Both proteins are involved in cell proliferation and differentiation They are higher in women with pre-eclamptic compared to normal pregnancy, and may be raised in women who go on to develop pre-eclampsia in the late first or early second trimester (Muttukrishna *et al.*, 2000) None of these biomarkers, when tested individually have, as yet, been shown to have a sufficiently high sensitivity or specificity to use in the clinical setting (Levine *et al.*, 2006; Widmer *et al.*, 2007).

Recently sST2 has emerged as a potential biomarker in a number of disease processes including acute dyspnoea, congestive heart failure and in patients with acute myocardial infarction (Weinberg *et al.*, 2003; Sabatine *et al.*, 2008). In cardiovascular disease, sST2 levels have been shown to predict increased risk of mortality at one year after an acute ischaemic event. The “Presage” assay used in these studies (licensed in Europe as a clinical tool for risk stratification in cardiac disease) is highly sensitive and sST2 levels cannot be compared directly with other research assays. However published data using this assay shows the levels seen in pre-eclampsia are similar to those seen in heart failure or chronic obstructive pulmonary disease and higher than levels seen after presentation of acute coronary syndrome (Dieplinger *et al.*, 2009; Eggers *et al.*, 2010).

The finding that sST2 is significantly raised in the circulation of women prior to the onset of pre-eclampsia suggests its possible use as a biomarker. There are however obvious limitations to this. An effective biomarker for pre-eclampsia would allow early stratification of the risk of the disease developing, perhaps at the beginning of the second trimester. This would then enable women to be deemed low or high risk and their level of care adjusted accordingly. However the data show that sST2 levels remain similar to non-pregnant levels in all patient groups until the third trimester which reduces its value as a predictive marker, although it may help improve diagnosis. Of note, one patient who developed severe early onset pre-eclampsia at 22 weeks did have an increased sST2 (43ng/ml) at 20 weeks suggesting that the rise in sST2 is likely to occur at least a few weeks before the onset of clinical symptoms. A larger

study to confirm the time interval between the increase in circulating sST2 and the onset of clinical symptoms would be of benefit in assessing the potential of sST2 to serve as a useful biomarker

5.6 Summary

The findings presented in this chapter show for the first time that sST2 levels are significantly increased in pre-eclampsia compared to normal pregnancy. Of interest, the levels of sST2 are significantly higher in the third trimester in women destined to develop the condition compared to age, parity and gestation matched women who go on to complete a normal pregnancy. This increase in the third trimester is seen even prior to the onset of the disease. This raises the possibility of its use as a novel biomarker for the disease, perhaps independent from other markers such as sFlt-1, endoglin and placental growth factors. The origin of at least some sST2 in pregnancy and pre-eclampsia is likely to be the placenta however its functional significance is as yet unknown.

6. Summary and Conclusions

This thesis covers two areas of reproductive immunology. Firstly it addresses the contentious clinical issue of peripheral blood immune cells and reproductive failure and secondly the role of IL-33 and ST2 in normal and pathological human pregnancy. The major factor limiting the data presented in this thesis is the relatively small numbers of patients used in each of the studies. Although multiple time points were investigated to look for differences over the menstrual cycle or an IVF cycle, or in the trimesters of pregnancy, these studies would have benefitted from larger numbers. The numbers used were a reflection of the time constraints, the difficulty in recruiting and completing studies with multiple cycle dependent samples and the labour intensive nature of the work. Despite the relatively small numbers recruited in each study, a number of interesting findings and significant differences between study groups were identified. The results of each of the studies have given rise to further questions for future investigation which are summarised below.

6.1 Circulating NK and T cells in the normal menstrual cycle and recurrent miscarriage

In chapter 2 of this thesis, a type 1 shift was identified in the mid-luteal phase of the menstrual cycle in both NK^{dim} and CD8+ T cells. Although it must be acknowledged that recent evidence suggests ST2L is not purely a type 2 marker, the type 1 shift in the ratio of IL-18R+ and ST2L+ cells was remarkably consistent. The most obvious difference in the mid-luteal phase of the menstrual cycle compared to baseline is the high plasma progesterone level.

Progesterone receptors have been identified in peripheral NK^{dim} (but not NK^{bright}) cells (Arruvito *et al.*, 2008). Although the physiological relevance of non-classical progesterone receptors is debated, up regulation of the recently identified progesterone membrane receptor mPRα has been shown to occur in CD8+ T cells but not CD4+ T cells (Dosiou *et al.*, 2008) in the mid luteal phase of the menstrual cycle. It is possible that changes in the cell phenotype (towards type 1) may be mediated via progesterone activation of as yet unknown pathways.

Experiments to investigate whether progesterone regulates expression of ST2L or IL-18R are warranted. In addition, lymphocytes obtained from women at the beginning of the menstrual cycle and treated with allo-serum taken during the mid-luteal phase of the previous menstrual cycle in the presence or absence of a progesterone antagonist would identify if other soluble factors present in the mid-luteal phase of the cycle alter IL-18R or ST2L expression. In order to correlate the cell surface changes seen between baseline and the mid-luteal phase, unstimulated and stimulated NK and T cell populations (and their expression of IL-18R and ST2L) could be identified by flow cytometry and the culture supernatant analysed by ELISA for cytokines released.

The recurrent miscarriage study presented in chapter 2 adds to the controversial and often contradictory body of evidence regarding peripheral blood NK cells and miscarriage. Contrary to the hypothesis of the study, women with recurrent unexplained miscarriage were found to have a significantly increased number of NK^{dim} cells in the mid-luteal phase of the

menstrual cycle compared to baseline. In addition, evidence was presented to suggest that the menstrual cycle affected the number of NK^{dim} cells differently in women with a history of recurrent miscarriage compared to controls. One novel finding in this thesis was the significant increase in circulating NK^{bright} cells in women with recurrent unexplained miscarriage. The large number of events collected (compared to most published studies) may be the reason why significant differences were found in contrast to other studies. Of note, IL-18R is very highly expressed by NK^{bright} cells. NK^{bright} cells produce high levels of cytokines and the cells were are strongly deviated to a type 1 phenotype. It is possible that the increased numbers of NK^{bright} cells in the recurrent miscarriage group leads to a relative type 1 cytokine environment. This would be consistent with the findings of previous studies suggesting a pre-pregnancy type 1 phenotype in women with unexplained recurrent miscarriage.

The potential value of measuring circulating NK cells is the possibility that they are a surrogate marker for events in the peri-implantation endometrium. Two studies to date have attempted to correlate uterine and peripheral NK cells. One study found a significant correlation between the mean number of uterine CD56+NK cells and the percentage of CD3-CD56+ and CD3-CD56+CD16+ cells in women with two or more miscarriages (Park *et al.*, 2010). However given that the live birth rate of women with two previous miscarriages is around 85% in subsequent pregnancies, that group cannot be considered to have a distinct pathology (Liddell *et al.*, 1991; Ogasawara *et al.*, 2000). A more recent study found no correlation between uterine NK cells and the percentage of circulating CD56+, CD56+CD16- or CD56+CD16+ cells (Laird *et al.*, 2011)

however the data presented in this thesis showed only absolute counts rather than a percentage of cells to be associated with recurrent miscarriage. Therefore a study comparing uterine NK cells and the absolute count of peripheral cells would be of interest, although technically challenging.

The recurrent miscarriage study would benefit from increased recruitment which could be limited to the mid-luteal phase of the cycle. In addition, experiments to establish whether the numbers of circulating NK^{dim} and NK^{bright} cells are of functional significance in the process of implantation, the cytokine environment or the maintenance of normal first trimester pregnancy are required. Even if a larger study were to confirm the presence of pre-pregnancy differences in peripheral NK cells in women with a history of recurrent miscarriage, it is possible that these are a reflection of an underlying immune dysfunction (that may also affect implantation and the maintenance of pregnancy) rather than a cause of miscarriage itself. Currently the measurement of peripheral blood natural killer cells in clinical practice is carried out by clinicians who believe that there is a sub-group of recurrent miscarriage patients who benefit from therapies such as steroids. This question can only be answered by a large randomised controlled trial of interventions such as steroid treatment, where the primary outcome is the live birth rate.

6.2 Circulating NK and T cells in early pregnancy

The most interesting finding in chapter 3 was the very early up-regulation in the expression of ST2L, identified consistently in the peripheral NK^{dim} cells as well as CD4+ and CD8+ T cells. Although a number of groups have previously identified a shift towards a type 2 phenotype or cytokine production in the late

first trimester, by recruiting women undergoing IVF this study was novel in its approach, enabling access to samples so early after implantation. The findings suggest that the post-implantation conceptus influences the systemic maternal immune system earlier than had previously been shown. These findings give rise to the question as to what is the very early stimulus for an increase in peripheral NK and T cell up-regulation of ST2L. Although HCG is a candidate, as discussed in chapter 3 it is used as a trigger of oocyte maturation 35 hours prior to egg collection and is still detectable in urine at the time of egg collection. No significant differences were seen at that time point compared to baseline or down-regulation. However recombinant HCG, used as an oocyte maturation trigger, may not have the same biological properties as endogenous HCG. It is also possible that the embryo may produce factors, or stimulate the decidual production of factors, that are secreted into the peripheral circulation and in turn up-regulate ST2L.

6.3 IL-33 and ST2 in normal pregnancy

The finding of ST2L up-regulation on NK^{dim} and T cells gave rise to questions that changed the overall direction of this thesis. This led me to investigate ST2 and its ligand IL-33 in normal and pathological human pregnancy and has led to a successful grant application to continue this work. In chapter 4 the possibility that the developing embryo and later the first trimester trophoblast could be a source of IL-33 was investigated. It was shown for the first time that the day 5 pre-implantation human embryo expresses IL-33. ST2 expression was also identified. However not all of the embryos labelled were shown to express IL-33 or ST2 and further experiments with increased embryo numbers and the use of confocal microscopy are planned. It is possible that the genes

for these proteins are switched on at the blastocyst stage explaining why not all the embryos labelled positive for IL-33 and ST2. Experiments utilising real time PCR are planned in order to identify the time point that these proteins are expressed. The function of this cytokine in the process of implantation has never been investigated. Human embryo co-culture experiments with IL-33 (Carver *et al.*, 2003) will be carried out to investigate whether IL-33 has an effect on trophoblast outgrowth and invasion onto human endometrial stromal cells.

Initial data has suggested that IL-33 and ST2 are expressed by endometrial stromal tissue and that there is variable expression of the proteins (increasing in the later first trimester) in the decidua. Difficulties in arranging a material transfer agreement between universities meant that only pilot data has been obtained thus far. Western blotting will be repeated to assess expression of the proteins in first trimester placental lysates. Once further decidual and placental sections are obtained, serial samples will be double labelled using immunofluorescence in order to establish which cells in the decidua are expressing IL-33 and ST2.

No significant differences in either circulating IL-33 or sST2 were identified in early pregnancy however the previous finding by our group that ST2L was up-regulated on NK cells throughout pregnancy (though not in pre-eclampsia) led to the question as to whether circulating IL-33 or sST2 changed in later pregnancy. Circulating sST2 was shown to increase in the 3rd trimester of pregnancy although no changes in IL-33 were identified. Variable placental

expression of both proteins was shown in 3rd trimester placenta and perfusion experiments showed that sST2 can be released by the placenta.

Currently it is not known whether the commercially available assays for IL-33 measure both free and bound IL-33 and sST2. In order to fully interpret the findings presented in this thesis this question must be further investigated.

6.4 IL-33 and ST2 in pre-eclampsia

In chapter 5 it was shown that although no change was seen in circulating IL-33 there was an increase in sST2 in women with pre-eclampsia. The increase was identified even before the onset of the clinical syndrome giving rise to the possibility that sST2 may be a potential biomarker for pre-eclampsia. This entirely novel finding has led to a patent application. If sST2 is placental rather than endothelial in origin, it may be possible to distinguish women with pregnancy induced hypertension from those who are going to develop pre-eclampsia and this will be investigated.

The increased cell surface expression of ST2L on peripheral blood NK cells may be a reflection of the type 2 bias of normal human pregnancy. It is possible that in normal pregnancy IL-33 stimulates the production of type 2 cytokines such as IL-5 and IL-13, whereas in the strong pro-inflammatory environment of pre-eclampsia it stimulates type 1 cytokines, notably IFN-γ. This theory will be investigated by stimulating lymphocytes obtained from matched non pregnant, normally pregnant and pre-eclamptic women with IL-33 and measuring the cytokines produced. As previous studies from our group have shown that inflammatory cytokine production may be primarily from NK

cells and is exacerbated in pre-eclampsia, isolation of NK cells and stimulation with IL-33 in samples from matched pre-eclamptic and normal pregnant women will also be carried out.

The potential sources of sST2 include the placenta or maternal endothelium. Using a system of placental perfusions, evidence was presented in chapter 5 to suggest that the placenta can be the source of sST2. The data presented in this thesis suggests a significant role for IL-33 and ST2 in human pregnancy however the functional significance remains to be determined. Experiments using placental explants are underway to investigate the effect of inflammatory cytokines and oxidative stress (to model conditions of pre-eclampsia) in order to see if placental secretion of sST2 is increased. Together these experiments will shed more light on the maternal immune system in pregnancy.

References

Allakhverdi Z, Smith DE, Comeau MR & Delespesse G. (2007). Cutting edge: The ST2 ligand IL-33 potently activates and drives maturation of human mast cells. *Journal of Immunology* **179,** 2051-2054.

Alliende ME. (2002). Mean versus individual hormonal profiles in the menstrual cycle. *Fertility and Sterility* **78,** 90-95.

Alves-Filho JC, Sonego F, Souto FO, Freitas A, Verri WA, Auxiliadora-Martins M, Basile-Filho A, McKenzie AN, Xu D, Cunha FQ & Liew FY. (2010). Interleukin-33 attenuates sepsis by enhancing neutrophil influx to the site of infection. *Nature Medicine* **16,** 708-712.

Aoki K, Kajiura S, Matsumoto Y, Ogasawara M, Okada S, Yagami Y & Gleicher N. (1995). Preconceptional natural-killer-cell activity as a predictor of miscarriage. *Lancet* **345,** 1340-1342.

Aoki S, Hayakawa M, Ozaki H, Takezako N, Obata H, Ibaraki N, Tsuru T, Tominaga S & Yanagisawa K. (2010). ST2 gene expression is proliferation-dependent and its ligand, IL-33, induces inflammatory reaction in endothelial cells. *Mollecular and Cellular Biochemistry* **335,** 75-81.

Apps R, Gardner L, Hiby SE, Sharkey AM & Moffett A. (2008). Conformation of human leucocyte antigen-C molecules at the surface of human trophoblast cells. *Immunology* **124,** 322-328.

Aramilena P-D, José T, Pedro S & María Isabel C. (2008). Immunophenotype of Peripheral T Lymphocytes, NK Cells and Expression of CD69 Activation Marker in Patients with Recurrent Spontaneous Abortions, During the Mid-Luteal Phase. *American Journal of Reproductive Immunology* **60,** 66-74.

Arend WP, Palmer G & Gabay C. (2008). IL-1, IL-18, and IL-33 families of cytokines. *Immunology Reviews* **223,** 20-38.

Arruvito L, Giulianelli S, Flores AC, Paladino N, Barboza M, Lanari C & Fainboim L. (2008). NK Cells Expressing a Progesterone Receptor Are Susceptible to Progesterone-Induced Apoptosis. *Journal of Immunoogyl* **180,** 5746-5753.

Ata B, Tan SL, Shehata F, Holzer H & Buckett W. (2011). A systematic review of intravenous immunoglobulin for treatment of unexplained recurrent miscarriage. *Fertility and Sterility* **95**.

Baekkevold ES, Roussigne M, Yamanaka T, Johansen FE, Jahnsen FL, Amalric F, Brandtzaeg P, Erard M, Haraldsen G & Girard JP. (2003). Molecular characterization of NF-HEV, a nuclear factor preferentially expressed in human high endothelial venules. *American Journal of Pathology* **163,** 69-79.

Bartunek J, Delrue L, Van Durme F, Muller O, Casselman F, De Wiest B, Croes R, Verstreken S, Goethals M, de Raedt H, Sarma J, Joseph L, Vanderheyden M & Weinberg EO. (2008). Nonmyocardial Production of ST2 Protein in Human Hypertrophy and Failure Is Related to Diastolic Load. *Journal of the American College of Cardiology* **52,** 2166-2174.

Bdolah Y, Sukhatme VP & Karumanchi SA. (2004). Angiogenic imbalance in the pathophysiology of preeclampsia: newer insights. *Seminars in Nephrology* **24,** 548-556.

Beer AE, Kwak JY & Ruiz JE. (1996). Immunophenotypic profiles of peripheral blood lymphocytes in women with recurrent pregnancy losses and in infertile women with multiple failed in vitro fertilization cycles. *American Journal of Reproductive Immunology* **35,** 376-382.

Beltran CJ, Nunez LE, Diaz-Jimenez D, Farfan N, Candia E, Heine C, Lopez F, Gonzalez MJ, Quera R & Hermoso MA. (2009). Characterization of the novel ST2/IL-33 system in patients with inflammatory bowel disease. *Inflammatory Bowel Disease*.

Benschop RJ, Rodriguez-Feuerhahn M & Schedlowski M. (1996). Catecholamine-induced leukocytosis: early observations, current research, and future directions. *Brain Behavior and Immunology* **10,** 77-91.

Bohn E, Sing A, Zumbihl R, Bielfeldt C, Okamura H, Kurimoto M, Heesemann J & Autenrieth IB. (1998). IL-18 (IFN-gamma-inducing factor) regulates early cytokine production in, and promotes resolution of, bacterial infection in mice. *Journal of Immunology* **160,** 299.

Borzychowski A. (2005). PhD Thesis. The systemic inflammatory response in human pregnancy. University of Guelph, Guelph.

Borzychowski AM, Croy BA, Chan WL, Redman CW & Sargent IL. (2005). Changes in systemic type 1 and type 2 immunity in normal pregnancy and pre-eclampsia may be mediated by natural killer cells. *European Journal of Immunology* **35,** 3054-3063.

Bouman A, Moes H, Heineman MJ, de Leij LF & Faas MM. (2001). The immune response during the luteal phase of the ovarian cycle: increasing sensitivity of human monocytes to endotoxin. *Fertiity and Sterility* **76,** 555-559.

Bourgeois E, Van LP, Samson M, Diem S, Barra A, Roga S, Gombert JM, Schneider E, Dy M, Gourdy P, Girard JP & Herbelin A. (2009). The pro-Th2 cytokine IL-33 directly interacts with invariant NKT and NK cells to induce IFN-gamma production. *European Journal of Immunology* **39,** 1046-1055.

Boyson JE, Nagarkatti N, Nizam L, Exley MA & Strominger JL. (2006). Gestation stage-dependent mechanisms of invariant natural killer T cell-mediated pregnancy loss. *Procedings of the National Acadamy of Science U S A* **103,** 4580-4585.

Boyson JE, Rybalov B, Koopman LA, Exley M, Balk SP, Racke FK, Schatz F, Masch R, Wilson SB & Strominger JL. (2002). CD1d and invariant NKT cells at the human maternal-fetal interface. *Procedings of the National Acadamy of Science U S A* **99,** 13741-13746.

Brint EK, Xu D, Liu H, Dunne A, McKenzie AN, O'Neill LA & Liew FY. (2004). ST2 is an inhibitor of interleukin 1 receptor and Toll-like receptor 4 signaling and maintains endotoxin tolerance. *Nature Immunology* **5,** 373-379.

Brunner M, Krenn C, Roth G, Moser B, Dworschak M, Jensen-Jarolim E, Spittler A, Sautner T, Bonaros N, Wolner E, Boltz-Nitulescu G & Ankersmit HJ. (2004). Increased levels of soluble ST2 protein and IgG1 production in patients with sepsis and trauma. *Intensive Care Medicine* **30,** 1468-1473.

Bulek K, Swaidani S, Qin J, Lu Y, Gulen MF, Herjan T, Min B, Kastelein RA, Aronica M, Kosz-Vnenchak M & Li X. (2009). The Essential Role of Single Ig IL-1 Receptor-Related Molecule/Toll IL-1R8 in Regulation of Th2 Immune Response. *Journal of Immunology* **182,** 2601-2609.

Burger O, Pick E, Zwickel J, Klayman M, Meiri H, Slotky R, Mandel S, Rabinovitch L, Paltieli Y, Admon A & Gonen R. (2004). Placental protein 13 (PP-13): effects on cultured trophoblasts, and its detection in human body fluids in normal and pathological pregnancies. *Placenta* **25,** 608-622.

Burton GJ & Jauniaux E. (2004). Placental oxidative stress: from miscarriage to preeclampsia. *J of the Society of Gynecoogical Investigation* **11,** 342-352.

Burton GJ, Watson AL, Hempstock J, Skepper JN & Jauniaux E. (2002). Uterine glands provide histiotrophic nutrition for the human fetus during the first trimester of pregnancy. *Journal of Clinical Endocrinology and Metabolism* **87,** 2954-2959.

Burton GJ, Yung HW, Cindrova-Davies T & Charnock-Jones DS. (2009). Placental endoplasmic reticulum stress and oxidative stress in the pathophysiology of unexplained intrauterine growth restriction and early onset preeclampsia. *Placenta* **30 Suppl A,** S43-48.

Carbone J, Gallego A, Lanio N, Chean C, Navarro J & Sarmiento E. (2010). Peripheral blood CD8+DR+ T-cell count: a potential new immunologic marker of unexplained recurrent abortion. *Fertility and Sterility* **94,** 360-361.

Carlino C, Stabile H, Morrone S, Bulla R, Soriani A, Agostinis C, Bossi F, Mocci C, Sarazani F, Tedesco F, Santoni A & Gismondi A. (2008). Recruitment of circulating NK cells through decidual tissues: a possible mechanism controlling NK cell accumulation in the uterus during early pregnancy. *Blood* **111,** 3108-3115.

Carnaud C, Lee D, Donnars O, Park SH, Beavis A, Koezuka Y & Bendelac A. (1999). Cutting edge: Cross-talk between cells of the innate immune system: NKT cells rapidly activate NK cells. *Journal of Immunology* **163,** 4647-4650.

Carriere V, Roussel L, Ortega N, Lacorre DA, Americh L, Aguilar L, Bouche G & Girard JP. (2007). IL-33, the IL-1-like cytokine ligand for ST2 receptor, is a chromatin-associated nuclear factor in vivo. *Procedings of the National Acadamy of Science U S A* **104,** 282-287.

Carver J, Martin K, Spyropoulou I, Barlow D, Sargent I & Mardon H. (2003). An in-vitro model for stromal invasion during implantation of the human blastocyst. *Human Reproduction* **18,** 283-290.

Cayrol C & Girard JP. (2009). The IL-1-like cytokine IL-33 is inactivated after maturation by caspase-1. *Procedings of the National Acadamy of Science U S A* **106,** 9021-9026.

Chackerian AA, Oldham ER, Murphy EE, Schmitz J, Pflanz S & Kastelein RA. (2007). IL-1 receptor accessory protein and ST2 comprise the IL-33 receptor complex. *Journal of Immunology* **179,** 2551-2555.

Chan WL, Pejnovic N, Lee CA & Al-Ali NA. (2001). Human IL-18 Receptor and ST2L Are Stable and Selective Markers for the Respective Type 1 and Type 2 Circulating Lymphocytes. *Journal of Immunology* **167,** 1238-1244.

Chantakru S. (2002). Contributions from self-renewal and trafficking to the uterine NK-cell population of early pregnancy. *Journal of Immunology* **168,** 22-28.

Chaouat G. (2007). The Th1/Th2 paradigm: still important in pregnancy? *Seminars in Immunopathology* **29,** 95-113.

Chaouat G, Dubanchet S & Ledée N. (2007). Cytokines: Important for implantation? *Journal of Assisted Reproduction and Genetics* **24,** 491-505.

Chaouat G, Petitbarat M, Dubanchet S, Rahmati M & Ledée N. (2010). REVIEW ARTICLE: Tolerance to the Foetal Allograft? *American Journal of Reproductive Immunology* **63,** 624-636.

Chappell LC, Seed PT, Briley A, Kelly FJ, Hunt BJ, Charnock-Jones DS, Mallet AI & Poston L. (2002). A longitudinal study of biochemical variables in women at risk of preeclampsia. *American Journal of Obstetrics and Gynecology* **187,** 127-136.

Cherry WB, Yoon J, Bartemes KR, Iijima K & Kita H. (2008). A novel IL-1 family cytokine, IL-33, potently activates human eosinophils. *Journal of Allergy and Clinical Immunology* **121,** 1484-1490.

Choi Y-S, Choi H-J, Min J-K, Pyun B-J, Maeng Y-S, Park H, Kim J, Kim Y-M & Kwon Y-G. (2009). Interleukin-33 induces angiogenesis and vascular permeability through ST2/TRAF6-mediated endothelial nitric oxide production. *Blood* **114,** 3117-3126.

Christiansen OB, Nielsen HS & Kolte AM. (2006). Future directions of failed implantation and recurrent miscarriage research. *Reproductive BioMedicine Online* **13,** 71-83.

Clark D, Coulam C & Stricker R. (2006). Is intravenous immunoglobulins (IVIG) efficacious in early pregnancy failure? A critical review and meta-analysis for patients who fail in vitro fertilization and embryo transfer (IVF). *Journal of Assisted Reproduction and Genetics* **23,** 1-13.

Clark DA. (2008). Immunological Factors in Pregnancy Wastage: Fact or Fiction. *American Journal of Reproductive Immunology* **59,** 277-300.

Clark DA. (2009). COMMENTARY: Should Anti-TNF-α Therapy be Offered to Patients with Infertility and Recurrent Spontaneous Abortion?*. *American Journal of Reproductive Immunology* **61,** 107-112.

Clifford K, Flanagan AM & Regan L. (1999). Endometrial CD56+ natural killer cells in women with recurrent miscarriage: a histomorphometric study. *Human Reproduction* **14,** 2727-2730.

Cole LA. (2010). Biological functions of hCG and hCG-related molecules. *Reproductive Biology and Endocrinology* **8,** 102.

Coulam CB, Goodman C, Roussev RG, Thomason EJ & Beaman KD. (1995). Systemic CD56+ cells can predict pregnancy outcome. *American Journal of Reproductive Immunology* **33,** 40-46.

Croxatto HB, Ortiz ME, Diaz S, Hess R, Balmaceda J & Croxatto HD. (1978). Studies on the duration of egg transport by the human oviduct. II. Ovum location at various intervals following luteinizing hormone peak. *American Journal of Obstetrics and Gynecology* **132,** 629-634.

Croy BA, Chantakru S, Esadeg S, Ashkar AA & Wei Q. (2002). Decidual natural killer cells: key regulators of placental development (a review). *Journal of Reproductive Immunology* **57,** 151-168.

Diedrich K, Fauser BCJM, Devroey P & Griesinger G. (2007). The role of the endometrium and embryo in human implantation. *Human Reproduction Update* **13,** 365-377.

Dieplinger B, Januzzi JL, Jr., Steinmair M, Gabriel C, Poelz W, Haltmayer M & Mueller T. (2009). Analytical and clinical evaluation of a novel high-sensitivity assay for measurement of soluble ST2 in human plasma--the Presage ST2 assay. *Clinica Chimica Acta* **409,** 33-40.

Dosiou C, Hamilton AE, Pang Y, Overgaard MT, Tulac S, Dong J, Thomas P & Giudice LC. (2008). Expression of membrane progesterone receptors on human T lymphocytes and Jurkat cells and activation of G-proteins by progesterone. *Journalof Endocrinology* **196,** 67-77.

Duckitt K & Harrington D. (2005). Risk factors for pre-eclampsia at antenatal booking: systematic review of controlled studies. *British Medical Journal* **330,** 565.

Eaton BM & Oakey MP. (1994). Sequential preparation of highly purified microvillous and basal syncytiotrophoblast membranes in substantial yield from a single term human placenta: inhibition of microvillous alkaline phosphatase activity by EDTA. *Biochimica et Biophysica Acta* **1193,** 85-92.

Eberl G & MacDonald HR. (2000). Selective induction of NK cell proliferation and cytotoxicity by activated NKT cells. *European Journal of Immunology* **30,** 985-992.

Eggers KM, Armstrong PW, Califf RM, Simoons ML, Venge P, Wallentin L & James SK. (2010). ST2 and mortality in non-ST-segment elevation acute coronary syndrome. *American Heart Journal* **159,** 788-794.

Emmer PM, Nelen WLDM, Steegers EAP, Hendriks JCM, Veerhoek M & Joosten I. (2000). Peripheral natural killer cytotoxicity and CD56posCD16pos cells increase during early pregnancy in women with a history of recurrent spontaneous abortion. *Human Reproduction* **15,** 1163-1169.

Faas M, Bouman A, Moesa H, Heineman MJ, de Leij L & Schuiling G. (2000). The immune response during the luteal phase of the ovarian cycle: a Th2-type response? *Fertility and Sterility* **74,** 1008-1013.

Ferguson MM & McDonald FG. (1985). Oestrogen as an inhibitor of human NK cell cytolysis. *FEBS Letters* **191,** 145-148.

Fukui A, Fujii S, Yamaguchi E, Kimura H, Sato S & Saito Y. (1999). Natural killer cell subpopulations and cytotoxicity for infertile patients undergoing in vitro fertilization. *American Journal of Reproductive Immunology* **41,** 413-422.

Funakoshi-Tago M, Tago K, Hayakawa M, Tominaga S, Ohshio T, Sonoda Y & Kasahara T. (2008). TRAF6 is a critical signal transducer in IL-33 signaling pathway. *Cell Signal* **20,** 1679-1686.

Gellersen B, Brosens IA & Brosens JJ. (2007). Decidualization of the human endometrium: mechanisms, functions, and clinical perspectives. *Seminars in Reproductive Medicine* **25,** 445-453.

Germain SJ, Sacks GP, Soorana SR, Sargent IL & Redman CW. (2007a). Systemic Inflammatory Priming in Normal Pregnancy and Preeclampsia: The Role of Circulating Syncytiotrophoblast Microparticles. *Journal of Immunology* **178,** 5949-5956.

Germain SJ, Sacks GP, Soorana SR, Sargent IL & Redman CW. (2007b). Systemic Inflammatory Priming in Normal Pregnancy and Preeclampsia: The Role of Circulating Syncytiotrophoblast Microparticles. *Journal of Immunology* **178,** 5949-5956.

Germain SJ, Sacks GP, Sooranna SR, Sargent IL & Redman CW. (2007c). Systemic inflammatory priming in normal pregnancy and preeclampsia: the role of circulating syncytiotrophoblast microparticles. *Journal of Immunology* **178,** 5949-5956.

Gumperz JE, Miyake S, Yamamura T & Brenner MB. (2002). Functionally distinct subsets of CD1d-restricted natural killer T cells revealed by CD1d tetramer staining. *Journal of Experimental Medicine* **195,** 625-636.

Hanna J, Goldman-Wohl D, Hamani Y, Avraham I, Greenfield C, Natanson-Yaron S, Prus D, Cohen-Daniel L, Arnon TI, Manaster I, Gazit R, Yutkin V, Benharroch D, Porgador A, Keshet E, Yagel S & Mandelboim O. (2006). Decidual NK cells regulate key developmental processes at the human fetal-maternal interface. *Nature Medicine* **12,** 1065-1074.

Hanson LA, Korotkova M, Lundin S, Haversen L, Silfverdal SA, Mattsby-Baltzer I, Strandvik B & Telemo E. (2003). The transfer of immunity from mother to child. *Annals of the New York Acadamy of Science* **987,** 199-206.

Haraldsen G, Balogh J, Pollheimer J, Sponheim J & Kuchler AM. (2009). Interleukin-33 - cytokine of dual function or novel alarmin? *Trends in Immunology* **30,** 227-233.

Harrington LE, Hatton RD, Mangan PR, Turner H, Murphy TL, Murphy KM & Weaver CT. (2005). Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nature Immunology* **6,** 1123-1132.

Heilmann L, Schorsch M & Hahn T. (2010). SHORT COMMUNICATION: CD3− CD56+ CD16+ Natural Killer Cells and Improvement of Pregnancy Outcome in IVF/ICSI Failure After Additional IVIG-Treatment. *American Journal of Reproductive Immunology* **63,** 263-265.

Herzenberg LA, Tung J, Moore WA, Herzenberg LA & Parks DR. (2006). Interpreting flow cytometry data: a guide for the perplexed. *Nature Immunoogyl* **7,** 681-685.

Hiby SE, Apps R, Sharkey AM, Farrell LE, Gardner L, Mulder A, Claas FH, Walker JJ, Redman CW, Morgan L, Tower C, Regan L, Moore GE, Carrington M & Moffett A. (2010). Maternal activating KIRs protect against human reproductive failure mediated by fetal HLA-C2. *Journal of Clinical Investigation* **120,** 4102-4110.

Hiby SE, Regan L, Lo W, Farrell L, Carrington M & Moffett A. (2008). Association of maternal killer-cell immunoglobulin-like receptors and parental HLA-C genotypes with recurrent miscarriage. *Human Reproduction* **23,** 972-976.

Hiby SE, Walker JJ, O'Shaughnessy K M, Redman CW, Carrington M, Trowsdale J & Moffett A. (2004). Combinations of maternal KIR and fetal HLA-C genes influence the risk of preeclampsia and reproductive success. *Journal of Exerimental Medicine* **200,** 957-965.

Higuma-Myojo S, Sasaki Y, Miyazaki S, Sakai M, Siozaki A, Miwa N & Saito S. (2005). Cytokine profile of natural killer cells in early human pregnancy. *American Journal of Immunology* **54,** 21-29.

Hoogerwerf JJ, Tanck MW, van Zoelen MA, Wittebole X, Laterre PF & van der Poll T. (2010). Soluble ST2 plasma concentrations predict mortality in severe sepsis. *Intensive Care Med* **36,** 630-637.

Hori S, Nomura T & Sakaguchi S. (2003). Control of Regulatory T Cell Development by the Transcription Factor Foxp3. *Science* **299,** 1057-1061.

Hoshino K, Kashiwamura S, Kuribayashi K, Kodama T, Tsujimura T, Nakanishi K, Matsuyama T, Takeda K & Akira S. (1999). The absence of interleukin 1 receptor-related T1/ST2 does not affect T helper cell type 2 development and its effector function. *Journal of Exerimental Medicine* **190,** 1541.

Houghton-Trivino N, Salgado DM, Rodriguez JA, Bosch I & Castellanos JE. (2010). Levels of soluble ST2 in serum associated with severity of dengue due to tumour necrosis factor alpha stimulation. *Journal of General Virology* **91,** 697-706.

Hughes DA, Haslam PL, Townsend PJ & Turner-Warwick M. (1985). Numerical and functional alterations in circulatory lymphocytes in cigarette smokers. *Clinical and Experimenta Immunology* **61,** 459-466.

Hung TH & Burton GJ. (2006). Hypoxia and reoxygenation: a possible mechanism for placental oxidative stress in preeclampsia. *Taiwan Journal of Obstetrics and Gynecol*ogy **45,** 189-200.

Iwahana H, Hayakawa M, Kuroiwa K, Tago K, Yanagisawa K, Noji S & Tominaga S. (2004). Molecular cloning of the chicken ST2 gene and a novel variant form of the ST2 gene product, ST2LV. *Biochimica and Biophysica Acta* **1681,** 1-14.

Jacobs R. (2001). CD56bright cells differ in their KIR repertoire and cytotoxic features from CD56dim NK cells. *European Journal of Immunoogyl* **31,** 3121-3126.

Jauniaux E, Watson AL, Hempstock J, Bao YP, Skepper JN & Burton GJ. (2000). Onset of maternal arterial blood flow and placental oxidative stress. A possible factor in human early pregnancy failure. *American Journal of Pathoogyl* **157,** 2111-2122.

Kakkar R & Lee RT. (2008). The IL-33/ST2 pathway: therapeutic target and novel biomarker. *Nature Reviews of Drug Discovories* **7,** 827-840.

Karumanchi SA & Bdolah Y. (2004). Hypoxia and sFlt-1 in preeclampsia: the "chicken-and-egg" question. *Endocrinology* **145,** 4835-4837.

King A. (2000). Uterine leukocytes and decidualization. *Human Reproduction Update* **6,** 28-36.

King A, Balendran N, Wooding P, Carter NP & Loke YW. (1991). CD3- leukocytes present in the human uterus during early placentation: phenotypic and morphologic characterization of the CD56++ population. *Developmental Immunology* **1,** 169-190.

King A, Wellings V, Gardner L & Loke YW. (1989). Immunocytochemical characterization of the unusual large granular lymphocytes in human endometrium throughout the menstrual cycle. *Human Immunology* **24,** 195-205.

King A, Wooding P, Gardner L & Loke YW. (1993). Expression of perforin, granzyme A and TIA-1 by human uterine CD56+ NK cells implies they are activated and capable of effector functions. *Human Reproduction* **8,** 2061-2067.

King K, Smith S, Chapman M & Sacks G. (2010). Detailed analysis of peripheral blood natural killer (NK) cells in women with recurrent miscarriage. *Human Reproduction d* **25,** 52-58.

Knight M, Redman CW, Linton EA & Sargent IL. (1998). Shedding of syncytiotrophoblast microvilli into the maternal circulation in pre-eclamptic pregnancies. *British Journal of Obstetrics and Gynaecology* **105,** 632-640.

Kroeger KM, Sullivan BM & Locksley RM. (2009). IL-18 and IL-33 elicit Th2 cytokines from basophils via a MyD88- and p38alpha-dependent pathway. *Journal of Leukocyte Biology* **86,** 769-778.

Kruse N, Greif M, Moriabadi NF, Marx L, Toyka KV & Rieckmann P. (2000). Variations in cytokine mRNA expression during normal human pregnancy. *Clinical and Experimental Immunology* **119,** 317-322.

Kumar S, Tzimas MN, Griswold DE & Young PR. (1997). Expression of ST2, an Interleukin-1 Receptor Homologue, Is Induced by Proinflammatory Stimuli. *Biochemical and Biophysical Research Communications* **235,** 474-478.

Kuroiwa K, Arai T, Okazaki H, Minota S & Tominaga S-i. (2001). Identification of Human ST2 Protein in the Sera of Patients with Autoimmune Diseases. *Biochemical and Biophysical Research Communications* **284,** 1104-1108.

Kurowska-Stolarska M, Kewin P, Murphy G, Russo RC, Stolarski B, Garcia CC, Komai-Koma M, Pitman N, Li Y, Niedbala W, McKenzie AN, Teixeira MM, Liew FY & Xu D. (2008). IL-33 induces antigen-specific IL-5+ T cells and promotes allergic-induced airway inflammation independent of IL-4. *Journal of Immunology* **181,** 4780-4790.

Kwak-Kim JYH, Chung-Bang HS, Ng SC, Ntrivalas EI, Mangubat CP, Beaman KD, Beer AE & Gilman-Sachs A. (2003). Increased T helper 1 cytokine responses by circulating T cells are present in women with recurrent pregnancy losses and in infertile women with multiple implantation failures after IVF. *Human Reproduction* **18,** 767-773.

Lachapelle MH, Miron P, Hemmings R & Roy DC. (1996). Endometrial T, B, and NK cells in patients with recurrent spontaneous abortion. Altered profile and pregnancy outcome. *Journal of Immunology* **156,** 4027-4034.

Laird SM, Mariee N, Wei L & Li TC. (2011). Measurements of CD56+ cells in peripheral blood and endometrium by flow cytometry and immunohistochemical staining in situ. *Human Reproduction*.

Lamkanfi M & Dixit VM. (2009). IL-33 raises alarm. *Immunity* **31,** 5-7.

Landmann R. (1992). Beta-adrenergic receptors in human leukocyte subpopulations. *European Journal of Clinical Investigation* **22 Suppl 1,** 30-36.

Lash GE, Schiessl B, Kirkley M, Innes BA, Cooper A, Searle RF, Robson SC & Bulmer JN. (2006). Expression of angiogenic growth factors by uterine natural killer cells during early pregnancy. *Journal of Leukocyte Biology* **80,** 572-580.

Lecart S, Lecointe N, Subramaniam A, Alkan S, Ni D, Chen R, Boulay V, Pene J, Kuroiwa K, Tominaga S & Yssel H. (2002). Activated, but not resting human Th2 cells, in contrast to Th1 and T regulatory cells, produce soluble ST2 and express low levels of ST2L at the cell surface. *European Journal of Immunology* **32,** 2979-2987.

Lee PT, Benlagha K, Teyton L & Bendelac A. (2002). Distinct functional lineages of human V(alpha)24 natural killer T cells. *Journal of Experimental Medicine* **195,** 637-641.

Lee S, Kim J, Jang B, Hur S, Jung U, Kil K, Na B, Lee M, Choi Y, Fukui A, Gilman-Sachs A & Kwak-Kim JY. (2010). Fluctuation of Peripheral Blood T, B, and NK Cells during a Menstrual Cycle of Normal Healthy Women. *The Journal of Immunology* **185,** 756-762.

Levine RJ, Lam C, Qian C, Yu KF, Maynard SE, Sachs BP, Sibai BM, Epstein FH, Romero R, Thadhani R & Karumanchi SA. (2006). Soluble Endoglin and Other Circulating Antiangiogenic Factors in Preeclampsia. *New England Journal of Medicine* **355,** 992-1005.

Levine RJ, Maynard SE, Qian C, Lim KH, England LJ, Yu KF, Schisterman EF, Thadhani R, Sachs BP, Epstein FH, Sibai BM, Sukhatme VP & Karumanchi SA. (2004). Circulating angiogenic factors and the risk of preeclampsia. *New England Journal of Medicine* **350,** 672-683.

Lewis Ge. (2007). The Confidential Enquirey into maternal and Child Health (CEMACH). Saving Mothers' Lives: reviewing maternal deaths to make motherhood safer-2003-2005. The Seventh Report on Confidential Enquiries int Maternal deaths in the united Kingdom. London.

Li XF, Charnock-Jones DS, Zhang E, Hiby S, Malik S, Day K, Licence D, Bowen JM, Gardner L, King A, Loke YW & Smith SK. (2001). Angiogenic growth factor messenger ribonucleic acids in uterine natural killer cells. *Journal of Clinical Endocrinology and Metabolism* **86,** 1823-1834.

Liddell HS, Pattison NS & Zanderigo A. (1991). Recurrent miscarriage--outcome after supportive care in early pregnancy. *Australia and New Zealand Journal of Obstetrics and Gynaecology* **31,** 320-322.

Liew FY, Pitman NI & McInnes IB. (2010). Disease-associated functions of IL-33: the new kid in the IL-1 family. *Nature Reviews in Immunology*.

Lohning M, Stroehmann A, Coyle AJ, Grogan JL, Lin S, Gutierrez-Ramos JC, Levinson D, Radbruch A & Kamradt T. (1998). T1/ST2 is preferentially expressed on murine Th2 cells, independent of interleukin 4, interleukin 5, and interleukin 10, and important for Th2 effector function. *Procedings of the National Acadamy of Science U S A* **95,** 6930-6935.

Luthi AU, Cullen SP, McNeela EA, Duriez PJ, Afonina IS, Sheridan C, Brumatti G, Taylor RC, Kersse K, Vandenabeele P, Lavelle EC & Martin SJ. (2009). Suppression of interleukin-33 bioactivity through proteolysis by apoptotic caspases. *Immunity* **31,** 84-98.

Mahnke YD & Roederer M. (2007). Optimizing a multicolor immunophenotyping assay. *Clinical and Laboratory Medicine* **27,** 469-485, v.

Makhseed M, Raghupathy R, Azizieh F, Farhat R, Hassan N & Bandar A. (2000). Circulating cytokines and CD30 in normal human pregnancy and recurrent spontaneous abortions. *Human Reproduction* **15,** 2011-2017.

Makhseed M, Raghupathy R, Azizieh F, Omu A, Al-Shamali E & Ashkanani L. (2001b). Th1 and Th2 cytokine profiles in recurrent aborters with successful pregnancy and with subsequent abortions. *Human Reproduction* **16,** 2219-2226.

Male V, Hughes T, McClory S, Colucci F, Caligiuri MA & Moffett A. (2010). Immature NK Cells, Capable of Producing IL-22, Are Present in Human Uterine Mucosa. *Journal of Immunology* **185,** 3913-3918.

Manaster I, Mizrahi S, Goldman-Wohl D, Sela HY, Stern-Ginossar N, Lankry D, Gruda R, Hurwitz A, Bdolah Y, Haimov-Kochman R, Yagel S & Mandelboim O. (2008). Endometrial NK Cells Are Special Immature Cells That Await Pregnancy. *Journal of Immunology* **181,** 1869-1876.

Marianne JvdH, Crystal GP, Kota H, Victor KH & David AC. (2007). Decline in number of CD3CD56 positive NKT Cells in Response to Intravenous Immunoglobulin Treatment Correlates with Successful Pregnancy. *American Journal of Reproductive Immunology* **58,** 447-459.

Marzi M, Vigano A, Trabattoni D, Villa ML, Salvaggio A, Clerici E & Clerici M. (1996). Characterization of type 1 and type 2 cytokine production profile in physiologic and pathologic human pregnancy. *Clinical and Experimental Immunol*ogy **106,** 127-133.

McMaster MT, Newton RC, Dey SK & Andrews GK. (1992). Activation and distribution of inflammatory cells in the mouse uterus during the preimplantation period. *Journal of Immunology* **148,** 1699-1705.

Medawar PB. (1953). Some immunological and endocrinological problems raised by evolution of viviparity in vertebrates. *Symposium of the Society of Experimental Biology* **7,** 320-328.

Meisel C, Bonhagen K, Lohning M, Coyle AJ, Gutierrez-Ramos JC, Radbruch A & Kamradt T. (2001). Regulation and function of T1/ST2 expression on CD4+ T cells: induction of type 2 cytokine production by T1/ST2 cross-linking. *Journal of Immunology* **166,** 3143-3150.

Melchiorre K, Sutherland GR, Baltabaeva A, Liberati M & Thilaganathan B. (2011). Maternal cardiac dysfunction and remodeling in women with preeclampsia at term. *Hypertension* **57,** 85-93.

Merviel P, Carbillon L, Challier JC, Rabreau M, Beaufils M & Uzan S. (2004). Pathophysiology of preeclampsia: links with implantation disorders. *Eur J Obstetrics, Gynecology and Reproductive Biology* **115,** 134-147.

Michou VI, Kanavaros P, Athanassiou V, Chronis GB, Stabamas S & Tsilivakos V. (2003). Fraction of the peripheral blood concentration of CD56+/CD16âˆ'/CD3âˆ' cells in total natural killer cells as an indication of fertility and infertility. *Fertility and Sterility* **80,** 691-697.

Mildner M, Storka A, Lichtenauer M, Mlitz V, Ghannadan M, Hoetzenecker K, Nickl S, Dome B, Tschachler E & Ankersmit HJ. (2010). Primary sources and immunological prerequisites for sST2 secretion in humans. *Cardiovascular Research* **87,** 769-777.

Moffett-King A. (2002). Natural killer cells and pregnancy. *Nature Rev iews in Immunology* **2,** 656-663.

Moffett A, Regan L & Braude P. (2004). Natural killer cells, miscarriage, and infertility. *British Medical Journal* **329,** 1283-1285.

Morikawa M, Yamada H, Kato EH, Shimada S, Ebina Y, Yamada T, Sagawa T, Kobashi G & Fujimoto S. (2001). NK cell activity and subsets in women with a history of spontaneous abortion. Cause, number of abortions, and subsequent pregnancy outcome. *Gynecological and Obstetric Invesigation* **52,** 163-167.

Mosmann TR, Cherwinski H, Bond MW, Giedlin MA & Coffman RL. (1986). Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *Journal of Immunology* **136,** 2348-2357.

Moulin D, Donze O, Talabot-Ayer D, Mezin F, Palmer G & Gabay C. (2007). Interleukin (IL)-33 induces the release of pro-inflammatory mediators by mast cells. *Cytokine* **40,** 216-225.

Moussion C, Ortega N & Girard JP. (2008). The IL-1-like cytokine IL-33 is constitutively expressed in the nucleus of endothelial cells and epithelial cells in vivo: a novel 'alarmin'? *PLoS One* **3,** e3331.

Muttukrishna S, North RA, Morris J, Schellenberg JC, Taylor RS, Asselin J, Ledger W, Groome N & Redman CW. (2000). Serum inhibin A and activin A are elevated prior to the onset of pre-eclampsia. *Human Reprouction* **15,** 1640-1645.

Nelson JL. (2003). Microchimerism in human health and disease. *Autoimmunity* **36,** 5-9.

Ness RB & Roberts JM. (1996). Heterogeneous causes constituting the single syndrome of preeclampsia: a hypothesis and its implications. *American Journal of Obstetrics and Gynecology* **175,** 1365-1370.

Noble A, Macary PA & Kemeny DM. (1995). IFN-gamma and IL-4 regulate the growth and differentiation of CD8+ T cells into subpopulations with distinct cytokine profiles. *Journal of Immunology* **155,** 2928-2937.

Northern AL, Rutter SM & Peterson CM. (1994). Cyclic changes in the concentrations of peripheral blood immune cells during the normal menstrual cycle. *Procedings of the Society of Experimental Biology and Medicine* **207,** 81-88.

Norwitz ER. (2006). Defective implantation and placentation: laying the blueprint for pregnancy complications. *Reproductive Biomed Online* **13,** 591-599.

Ntrivalas EI, Kwak-Kim JYH, Gilman-Sachs A, Chung-Bang H, Ng SC, Beaman KD, Mantouvalos HP & Beer AE. (2001). Status of peripheral blood natural killer cells in women with recurrent spontaneous abortions and infertility of unknown aetiology. *Human Reproduction* **16,** 855-861.

Oboki K, Ohno T, Kajiwara N, Arae K, Morita H, Ishii A, Nambu A, Abe T, Kiyonari H, Matsumoto K, Sudo K, Okumura K, Saito H & Nakae S. (2010). IL-33 is a crucial amplifier of innate rather than acquired immunity. *Procedings og the National Acadamy of Science U S A* **107,** 18581-18586.

Ogasawara M, Aoki K, Okada S & Suzumori K. (2000). Embryonic karyotype of abortuses in relation to the number of previous miscarriages. *Fertility and Sterility* **73,** 300-304.

Oshikawa K, Kuroiwa K, Tago K, Iwahana H, Yanagisawa K, Ohno S, Tominaga SI & Sugiyama Y. (2001). Elevated soluble ST2 protein levels in sera of patients with asthma with an acute exacerbation. *American Journal of Respiritory and Critical Care Medicine* **164,** 277-281.

Pala P, Hussell T & Openshaw PJM. (2000). Flow cytometric measurement of intracellular cytokines. *Journal of Immunological Methods* **243,** 107-124.

Palmer G, Lipsky BP, Smithgall MD, Meininger D, Siu S, Talabot-Ayer D, Gabay C & Smith DE. (2008). The IL-1 receptor accessory protein (AcP) is required for IL-33 signaling and soluble AcP enhances the ability of soluble ST2 to inhibit IL-33. *Cytokine* **42,** 358-364.

Park DW, Lee HJ, Park CW, Hong SR, Kwak-Kim J & Yang KM. (2010). Peripheral blood NK cells reflect changes in decidual NK cells in women with recurrent miscarriages. *American Journal of Reproductive Immunology* **63,** 173-180.

Pastorelli L, Garg RR, Hoang SB, Spina L, Mattioli B, Scarpa M, Fiocchi C, Vecchi M & Pizarro TT. (2010). Epithelial-derived IL-33 and its receptor ST2 are dysregulated in ulcerative colitis and in experimental Th1/Th2 driven enteritis. *Procedings og the National Acadamy of Science U S A* **107,** 8017-8022.

Pecaric-Petkovic T, Didichenko SA, Kaempfer S, Spiegl N & Dahinden CA. (2009). Human basophils and eosinophils are the direct target leukocytes of the novel IL-1 family member IL-33. *Blood* **113,** 1526-1534.

Peralta CG, Han VK, Horrocks J, Croy BA & van den Heuvel MJ. (2008a). CD56bright cells increase expression of {alpha}4 integrin at ovulation in fertile cycles. *Journal of Leukocyte Biology* **84,** 1065-1074.

Peralta CG, Han VK, Horrocks J, Croy BA & van den Heuvel MJ. (2008b). CD56bright cells increase expression of {alpha}4 integrin at ovulation in fertile cycles. *Journal of Leukocyte Biology* **84,** 1065-1074.

Peritt D, Robertson S, Gri G, Showe L, Aste-Amezaga M & Trinchieri G. (1998). Differentiation of human NK cells into NK1 and NK2 subsets. *Journal of Immunology* **161,** 5821-5824.

Perricone R, Muzio GD, Perricone C, Giacomelli R, Nardo DD, Fontana L & Carolis CD. (2006). High Levels of Peripheral Blood NK Cells in Women Suffering from Recurrent Spontaneous Abortion are Reverted from High-Dose Intravenous Immunoglobulins. *American Journal of Reproductive Immunology* **55,** 232-239.

Piccinni MP, Giudizi MG, Biagiotti R, Beloni L, Giannarini L, Sampognaro S, Parronchi P, Manetti R, Annunziato F, Livi C & et al. (1995). Progesterone favors the development of human T helper cells producing Th2-type cytokines and promotes both IL-4 production and membrane CD30 expression in established Th1 cell clones. *Journal of Immunology* **155,** 128-133.

Pierdominici M, Maselli A, Colasanti T, Giammarioli AM, Delunardo F, Vacirca D, Sanchez M, Giovannetti A, Malorni W & Ortona E. (2010). Estrogen receptor profiles in human peripheral blood lymphocytes. *Immunology Letters* **132,** 79-85.

Pollheimer J., Zeisler H. & M K. (2010). Expression and function of IL-33/ST2 during trophoblast invasion. *Journal of Reproductive Immunology* **86,** 99.

Polliotti BM, Fry AG, Saller DN, Mooney RA, Cox C & Miller RK. (2003). Second-trimester maternal serum placental growth factor and vascular endothelial growth factor for predicting severe, early-onset preeclampsia. *Obstetrics and Gynecology* **101,** 1266-1274.

Poon LC, Akolekar R, Lachmann R, Beta J & Nicolaides KH. (2010). Hypertensive disorders in pregnancy: screening by biophysical and biochemical markers at 11-13 weeks. *Ultrasound in Obstetrics and Gynecology* **35,** 662-670.

Porcelli S, Yockey CE, Brenner MB & Balk SP. (1993). Analysis of T cell antigen receptor (TCR) expression by human peripheral blood CD4-8- alpha/beta T cells demonstrates preferential use of several V beta genes and an invariant TCR alpha chain. *Journal of Experimental Medicine* **178,** 1-16.

Porter TF, LaCoursiere Y & Scott JR. (2006). Immunotherapy for recurrent miscarriage. *Cochrane Database Syst Rev***,** CD000112.

Prado-Drayer A, Teppa J, Sánchez P & Camejo MI. (2008). ORIGINAL ARTICLE: Immunophenotype of Peripheral T Lymphocytes, NK Cells and Expression of CD69 Activation Marker in Patients with Recurrent Spontaneous Abortions, During the Mid-Luteal Phase. *American Journal of Reproductive Immunology* **60,** 66-74.

Prussin C & Foster B. (1997). TCR V alpha 24 and V beta 11 coexpression defines a human NK1 T cell analog containing a unique Th0 subpopulation. *Journal of Immunology* **159,** 5862-5870.

Pushparaj PN, Tay HK, H'Ng S C, Pitman N, Xu D, McKenzie A, Liew FY & Melendez AJ. (2009). The cytokine interleukin-33 mediates anaphylactic shock. *Proceding of the National Acadamy of ScienceU S A* **106,** 9773-9778.

Quenby S, Bates M, Doig T, Brewster J, Lewis-Jones DI, Johnson PM & Vince G. (1999). Pre-implantation endometrial leukocytes in women with recurrent miscarriage. *Human Reproduction* **14,** 2386-2391.

Quenby S, Kalumbi C, Bates M, Farquharson R & Vince G. (2005). Prednisolone reduces preconceptual endometrial natural killer cells in women with recurrent miscarriage. *Fertility and Sterrility* **84,** 980-984.

Quenby SM & Farquharson RG. (1993). Predicting recurring miscarriage: what is important? *Obstetrics and Gynecology* **82,** 132-138.

Quinn CE & Casper RF. (2009). Pinopodes: a questionable role in endometrial receptivity. *Human Reproduction Update* **15,** 229-236.

Raghupathy R, Makhseed M, Azizieh F, Omu A, Gupta M & Farhat R. (2000a). Cytokine production by maternal lymphocytes during normal human pregnancy and in unexplained recurrent spontaneous abortion. *Human Reproduction* **15,** 713-718.

Raghupathy R, Makhseed M, Azizieh F, Omu A, Gupta M & Farhat R. (2000b). Cytokine production by maternal lymphocytes during normal human pregnancy and in unexplained recurrent spontaneous abortion. *Human Reproduction* **15,** 713-718.

Rai R. (2008). Immunological Testing and Interventions for Reproductive Failure. In *Scientific Advisory Committee Opinion Paper*.RCOG. London

Rai R & Regan L. (2006). Recurrent miscarriage. *The Lancet* **368,** 601-611.

Rai R, Sacks G & Trew G. (2005). Natural killer cells and reproductive failure--theory, practice and prejudice. *Human Reproduction* **20,** 1123-1126.

Rai RS, Clifford K, Cohen H & Regan L. (1995). High prospective fetal loss rate in untreated pregnancies of women with recurrent miscarriage and antiphospholipid antibodies. *Human Reproduction* **10,** 3301-3304.

Red-Horse K, Zhou Y, Genbacev O, Prakobphol A, Foulk R, McMaster M & Fisher SJ. (2004). Trophoblast differentiation during embryo implantation and formation of the maternal-fetal interface. *The Journal of Clinical Investigation* **114,** 744-754.

Redman CW. (1991). Current topic: pre-eclampsia and the placenta. *Placenta* **12,** 301-308.

Redman CW & Sargent IL. (2003). Pre-eclampsia, the placenta and the maternal systemic inflammatory response--a review. *Placenta* **24 Suppl A,** S21-27.

Redman CW & Sargent IL. (2009). Placental stress and pre-eclampsia: a revised view. *Placenta* **30 Suppl A,** S38-42.

Redman CWG & Sargent IL. (2008). Circulating Microparticles in Normal Pregnancy and Pre-Eclampsia. *Placenta* **29,** 73-77.

Roberts JM, Taylor RN, Musci TJ, Rodgers GM, Hubel CA & McLaughlin MK. (1989). Preeclampsia: an endothelial cell disorder. *American Journal of Obstetrics and Gynecology* **161,** 1200-1204.

Rößler U, Thomassen E, Hütner L, Baier S, Danescu J & Werenskiold AK. (1995). Secreted and Membrane-Bound Isoforms of T1, an Orphan Receptor Related to IL-1-Binding Proteins, Are Differently Expressed in Vivo. *Developmental Biology* **168,** 86-97.

Sabatine MS, Morrow DA, Higgins LJ, MacGillivray C, Guo W, Bode C, Rifai N, Cannon CP, Gerszten RE & Lee RT. (2008). Complementary roles for biomarkers of biomechanical strain ST2 and N-terminal prohormone B-type natriuretic peptide in patients with ST-elevation myocardial infarction. *Circulation* **117,** 1936-1944.

Sacks GP, Clover LM, Bainbridge DRJ, Redman CWG & Sargent IL. (2001). Flow Cytometric Measurement of Intracellular Th1 and Th2 Cytokine Production by Human Villous and Extravillous Cytotrophoblast. *Placenta* **22,** 550-559.

Sacks GP, Studena K, Sargent K & Redman CW. (1998). Normal pregnancy and preeclampsia both produce inflammatory changes in peripheral blood leukocytes akin to those of sepsis. *American Journal of Obstetrics and Gynecology* **179,** 80-86.

Saito S, Sakai M, Sasaki Y, Tanebe K, Tsuda H & Michimata T. (1999). Quantitative analysis of peripheral blood Th0, Th1, Th2 and the Th1:Th2 cell ratio during normal human pregnancy and preeclampsia. *Clin Exp Immunol* **117,** 550-555.

Saito S, Shiozaki A, Nakashima A, Sakai M & Sasaki Y. (2007). The role of the immune system in preeclampsia. *Molecular Aspects of Medicine* **28,** 192-209.

Sancho D, Gómez M & Sánchez-Madrid F. (2005). CD69 is an immunoregulatory molecule induced following activation. *Trends in Immunology* **26,** 136-140.

Sanford TR, De M & Wood GW. (1992). Expression of colony-stimulating factors and inflammatory cytokines in the uterus of CD1 mice during days 1 to 3 of pregnancy. *Journal of Reproduction and Fertility* **94,** 213-220.

Sargent I, Swales A, Ledee N, Kozma N, Tabiasco J & Le Bouteiller P. (2007). sHLA-G production by human IVF embryos: Can it be measured reliably? *Journal of Reproductive Immunology* **75,** 128-132.

Schmitz J, Owyang A, Oldham E, Song Y, Murphy E, McClanahan TK, Zurawski G, Moshrefi M, Qin J, Li X, Gorman DM, Bazan JF & Kastelein RA. (2005). IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines. *Immunity* **23,** 479-490.

Schneider E, Petit-Bertron AF, Bricard R, Levasseur M, Ramadan A, Girard JP, Herbelin A & Dy M. (2009). IL-33 activates unprimed murine basophils directly in vitro and induces their in vivo expansion indirectly by promoting hematopoietic growth factor production. *Journal of Immunology* **183,** 3591-3597.

Seidelin JB, Bjerrum JT, Coskun M, Widjaya B, Vainer B & Nielsen OH. (2009). IL-33 is upregulated in colonocytes of ulcerative colitis. *Immunology Letters*.

Shakhar K, Rosenne E, Loewenthal R, Shakhar G, Carp H & Shamgar B-E. (2006). High NK cell activity in recurrent miscarriage: what are we really measuring? *Human Reproduction* **21,** 2421-2425.

Shi Y, Ling B, Zhou Y, Gao T, Feng D, Xiao M & Feng L. (2007). Interferon-gamma expression in natural killer cells and natural killer T cells is suppressed in early pregnancy. *Cellular and Molecular Immunology* **4,** 389-394.

Shimpo M, Morrow DA, Weinberg EO, Sabatine MS, Murphy SA, Antman EM & Lee RT. (2004). Serum levels of the interleukin-1 receptor family member ST2 predict mortality and clinical outcome in acute myocardial infarction. *Circulation* **109,** 2186-2190.

Sibai B, Dekker G & Kupferminc M. (2005). Pre-eclampsia. *Lancet* **365,** 785-799.

Singh N, Hong S, Scherer DC, Serizawa I, Burdin N, Kronenberg M, Koezuka Y & Van Kaer L. (1999). Cutting edge: activation of NK T cells by CD1d and alpha-galactosylceramide directs conventional T cells to the acquisition of a Th2 phenotype. *Journal of Immunology* **163,** 2373-2377.

Siu Chui NG, Alice G-S, Poonam T, Kenneth D B, Alan E B & Joanne K-K. (2002). Expression of Intracellular Th1 and Th2 Cytokines in Women with Recurrent Spontaneous Abortion, Implantation Failures after IVF/ET or Normal Pregnancy. *American Journal of Reproductive Immunology* **48,** 77-86.

Smarason AK, Sargent IL, Starkey PM & Redman CW. (1993). The effect of placental syncytiotrophoblast microvillous membranes from normal and pre-eclamptic women on the growth of endothelial cells in vitro. *British Journal of Obstetrics and Gynaecology* **100,** 943-949.

Smithgall MD, Comeau MR, Yoon BR, Kaufman D, Armitage R & Smith DE. (2008). IL-33 amplifies both Th1- and Th2-type responses through its activity on human basophils, allergen-reactive Th2 cells, iNKT and NK cells. *International Immunology* **20,** 1019-1030.

Socrates T, deFilippi C, Reichlin T, Twerenbold R, Breidhardt T, Noveanu M, Potocki M, Reiter M, Arenja N, Heinisch C, Meissner J, Jaeger C, Christenson R & Mueller C. (2010). Interleukin family member ST2 and mortality in acute dyspnoea. *Journal of Internal Medicine* **268,** 493-500.

Soliman S, Daya S, Collins J & Hughes EG. (1994). The role of luteal phase support in infertility treatment: a meta-analysis of randomized trials. *Fertility and Sterility* **61,** 1068-1076.

Southcombe J, Redman C & Sargent I. (2010). Peripheral blood invariant natural killer T cells throughout pregnancy and in preeclamptic women. *Journal of Reproductive Immunology* **87,** 52-59.

Southcombe J, Tannetta D, Redman C & Sargent I. (2011). The immunomodulatory role of syncytiotrophoblast microvesicles. *PLoS One* **6,** e20245.

Souza SS, Castro FA, Mendonça HC, Palma PVB, Morais FR, Ferriani RA & Voltarelli JC. (2001). Influence of menstrual cycle on NK activity. *Journal of Reproductive Immunology* **50,** 151-159.

Souza SS, Ferriani RA, Santos CMP & Voltarelli JC. (2002). Immunological evaluation of patients with recurrent abortion. *Journal of Reproductive Immunology* **56,** 111-121.

Stolarski B, Kurowska-Stolarska M, Kewin P, Xu D & Liew FY. IL-33 exacerbates eosinophil-mediated airway inflammation. *Journal of Immunology* **185,** 3472-3480.

Sulke AN, Jones DB & Wood PJ. (1985a). Hormonal modulation of human natural killer cell activity in vitro. *Journal of Reproductive Immunology* **7,** 105-110.

Sulke AN, Jones DB & Wood PJ. (1985b). Variation in natural killer activity in peripheral blood during the menstrual cycle. *British Medical Journal (Clinical Research Ed)* **290,** 884-886.

Sunderland CA, Naiem M, Mason DY, Redman CW & Stirrat GM. (1981). The expression of major histocompatibility antigens by human chorionic villi. *Journal of Obstetrics and Gynecology* **3,** 323-331.

Suzukawa M, Koketsu R, Iikura M, Nakae S, Matsumoto K, Nagase H, Saito H, Matsushima K, Ohta K, Yamamoto K & Yamaguchi M. (2008). Interleukin-33 enhances adhesion, CD11b expression and survival in human eosinophils. *Laboratory Investigation* **88,** 1245-1253.

Sweet MJ, Leung BP, Kang D, Sogaard M, Schulz K, Trajkovic V, Campbell CC, Xu D & Liew FY. (2001). A novel pathway regulating lipopolysaccharide-induced shock by ST2/T1 via inhibition of Toll-like receptor 4 expression. *Journal of Immunology* **166,** 6633-6639.

Szereday L, Spath P & Szekeres-Bartho J. (1999). Natural killer cell activity and cytokine production after in vitro immunoglobulin treatment of lymphocytes derived from pregnant women with or without risk for spontaneous abortion. *American Journal of Reproductive Immunology* **42,** 282-287.

Tabiasco J, Perrier d'Hauterive S, Thonon F, Parinaud J, Leandri R, Foidart JM, Chaouat G, Munaut C, Lombroso R, Selva J, Bergere M, Hammoud I, Kozma N, Aguerre-Girr M, Swales AK, Sargent IL, Le Bouteiller P & Ledee N. (2009). Soluble HLA-G in IVF/ICSI embryo culture supernatants does not always predict implantation success: a multicentre study. *Reproductive Biomedicine Online* **18,** 374-381.

Takahashi T, Chiba S, Nieda M, Azuma T, Ishihara S, Shibata Y, Juji T & Hirai H. (2002). Cutting edge: analysis of human V alpha 24+CD8+ NK T cells activated by alpha-galactosylceramide-pulsed monocyte-derived dendritic cells. *Journal of Immunology* **168,** 3140-3144.

Takahashi T, Nieda M, Koezuka Y, Nicol A, Porcelli SA, Ishikawa Y, Tadokoro K, Hirai H & Juji T. (2000). Analysis of human V alpha 24+ CD4+ NKT cells activated by alpha-glycosylceramide-pulsed monocyte-derived dendritic cells. *Journal of Immunology* **164,** 4458-4464.

Takezako N, Hayakawa M, Hayakawa H, Aoki S, Yanagisawa K, Endo H & Tominaga S-i. (2006). ST2 suppresses IL-6 production via the inhibition of I[kappa]B degradation induced by the LPS signal in THP-1 cells. *Biochemical and Biophysical Research Communications* **341,** 425-432.

Talabot-Ayer D, Lamacchia C, Gabay C & Palmer G. (2009). Interleukin-33 is biologically active independently of caspase-1 cleavage. *Journal of Biological Chemistry* **284,** 19420-19426.

Than NG, Pick E, Bellyei S, Szigeti A, Burger O, Berente Z, Janaky T, Boronkai A, Kliman H, Meiri H, Bohn H, Than GN & Sumegi B. (2004). Functional analyses of placental protein 13/galectin-13. *Eur J Biochem* **271,** 1065-1078.

Than NG, Romero R, Hillermann R, Cozzi V, Nie G & Huppertz B. (2008). Prediction of preeclampsia - a workshop report. *Placenta* **29 Suppl A,** S83-85.

Thomassen E, Bird TA, Renshaw BR, Kennedy MK & Sims JE. (1998). Binding of interleukin-18 to the interleukin-1 receptor homologous receptor IL-1RrP1 leads to activation of signaling pathways similar to those used by interleukin-1. *Journal of Interferon and Cytokine Research* **18,** 1077.

Thum M-Y, Bhaskaran S, Abdalla HI, Ford B, Sumar N & Bansal A. (2008). ORIGINAL ARTICLE: Prednisolone Suppresses NK Cell Cytotoxicity In Vitro in Women With a History of Infertility and Elevated NK Cell Cytotoxicity. *American Journal of Reproductive Immunology* **59,** 259-265.

Thum MY, Bhaskaran S, Abdalla HI, Ford B, Sumar N, Shehata H & Bansal AS. (2004). An increase in the absolute count of CD56dimCD16+CD69+ NK cells in the peripheral blood is associated with a poorer IVF treatment and pregnancy outcome. *Human Reproduction* **19,** 2395-2400.

Thum MY, Bhaskaran S, Bansal AS, Shehata H, Ford B, Sumar N & Abdalla HI. (2005). Simple enumerations of peripheral blood natural killer (CD56+ NK) cells, B cells and T cells have no predictive value in IVF treatment outcome. *Human Reproduction* **20,** 1272-1276.

Tidwell SC, Hong-Nerng H, Wen-Hsin C, Ronald JT & Donald ST. (2001). Low maternal serum levels of placenta growth factor as an antecedent of clinical preeclampsia. *American journal of Obstetrics and Gynecology* **184,** 1267-1272.

Tilburgs T, Scherjon SA & Claas FHJ. (2010). Major histocompatibility complex (MHC)-mediated immune regulation of decidual leukocytes at the fetal-maternal interface. *Journal of Reproductive Immunology* **85,** 58-62.

Tilburgs T, Scherjon SA, van der Mast BJ, Haasnoot GW, Versteeg-v.d.Voort-Maarschalk M, Roelen DL, van Rood JJ & Claas FHJ. (2009a). Fetal-maternal HLA-C mismatch is associated with decidual T cell activation and induction of functional T regulatory cells. *Journal of Reproductive Immunology* **82,** 148-157.

Tilburgs T, van der Mast BJ, Nagtzaam NM, Roelen DL, Scherjon SA & Claas FH. (2009b). Expression of NK cell receptors on decidual T cells in human pregnancy. *J Reprod Immunol* **80,** 22-32.

Tollerud DJ, Clark JW, Brown LM, Neuland CY, Mann DL, Pankiw-Trost LK, Blattner WA & Hoover RN. (1989). Association of cigarette smoking with decreased numbers of circulating natural killer cells. *American Review of Respiritory Disease* **139,** 194-198.

Tominaga S, Kuroiwa K, Tago K, Iwahana H, Yanagisawa K & Komatsu N. (1999). Presence and expression of a novel variant form of ST2 gene product in human leukemic cell line UT-7/GM. *Biochemical and Biophysical Research Commun*ications **264,** 14-18.

Trajkovic V, Sweet MJ & Xu D. (2004). T1/ST2--an IL-1 receptor-like modulator of immune responses. *Cytokine Growth Factor Reviews* **15,** 87-95.

Trzonkowski P, Mysliwska J, Tukaszuk K, Szmit E, Bryl E & Mysliwski A. (2001). Luteal phase of the menstrual cycle in young healthy women is associated with decline in interleukin 2 levels. *Hormone and Metabolic Research* **33,** 348-353.

Tuckerman E, Laird SM, Prakash A & Li TC. (2007). Prognostic value of the measurement of uterine natural killer cells in the endometrium of women with recurrent miscarriage. *Human Reproduction* **22,** 2208-2213.

Uksila J. (1985). Human NK cell activity is not inhibited by pregnancy and cord serum factors and female steroid hormones in vitro. *Journal of Reproductive Immunology* **7,** 111-120.

Vacca P, Vitale C, Montaldo E, Conte R, Cantoni C, Fulcheri E, Darretta V, Moretta L & Mingari MC. (2011). CD34+ hematopoietic precursors are present in human decidua and differentiate into natural killer cells upon interaction with stromal cells. *Proceedings of the National Academy of Sciences*.

van 't Veer C, van den Pangaart PS, van Zoelen MA, de Kruif M, Birjmohun RS, Stroes ES, de Vos AF & van der Poll T. (2007). Induction of IRAK-M is associated with lipopolysaccharide tolerance in a human endotoxemia model. *Journal of Immunology* **179,** 7110-7120.

van den Heuvel M, Peralta C, Bashar S, Taylor S, Horrocks J & Croy BA. (2005). Trafficking of peripheral blood CD56bright cells to the decidualizing uterus--new tricks for old dogmas? *Journal of Reproductive Immunology* **67,** 21-34.

Van Den Heuvel MJ, Peralta CG, Hatta K, Han VK & Clark DA. (2007). Decline in Number of Elevated Blood CD3+ CD56+ NKT Cells in Response to Intravenous Immunoglobulin Treatment Correlates with Successful Pregnancy. *American Journal of Reproductive Immunology* **58,** 447-459.

Venkatesha S, Toporsian M, Lam C, Hanai J, Mammoto T, Kim YM, Bdolah Y, Lim KH, Yuan HT, Libermann TA, Stillman IE, Roberts D, D'Amore PA, Epstein FH, Sellke FW, Romero R, Sukhatme VP, Letarte M &

Karumanchi SA. (2006). Soluble endoglin contributes to the pathogenesis of preeclampsia. *Nature Medicine* **12,** 642-649.

Verma S, King A & Loke YW. (1997). Expression of killer cell inhibitory receptors on human uterine natural killer cells. *European Journal of Immunology* **27,** 979-983.

Vukmanovic-Stejic M, Vyas B, Gorak-Stolinska P, Noble A & Kemeny DM. (2000). Human Tc1 and Tc2/Tc0 CD8 T-cell clones display distinct cell surface and functional phenotypes. *Blood* **95,** 231-240.

W.H.O. (2005). The World Health Report: Make every mother and child count. WHO, Geneva.

Wegmann TG, Lin H, Guilbert L & Mosmann TR. (1993). Bidirectional cytokine interactions in the maternal-fetal relationship: is successful pregnancy a TH2 phenomenon? *Immunology Today* **14,** 353-356.

Weinberg EO, Shimpo M, Hurwitz S, Tominaga S, Rouleau JL & Lee RT. (2003). Identification of serum soluble ST2 receptor as a novel heart failure biomarker. *Circulation* **107,** 721-726.

Widmer M, Villar J, Benigni A, Conde-Agudelo A, Karumanchi SA & Lindheimer M. (2007). Mapping the theories of preeclampsia and the role of angiogenic factors: a systematic review. *Obstetrics and Gynecology* **109,** 168-180.

Wilcox AJ, Baird DD & Weinberg CR. (1999). Time of Implantation of the Conceptus and Loss of Pregnancy. *New England Journal of Medicine* **340,** 1796-1799.

Wilson R, Jenkins C, Miller H, McInnes IB, Moore J, McLean MA & Walker JJ. (2004a). Abnormal cytokine levels in non-pregnant women with a history of recurrent miscarriage. *European Journal of Obstetrics Gynecoogy andl Reproductive Biology* **115,** 51-54.

Wilson R, Moor J, Jenkins C, Miller H, Walker JJ, McLean MA, Norman J & McInnes IB. (2004b). Abnormal first trimester serum interleukin 18 levels are associated with a poor outcome in women with a history of recurrent miscarriage. *American Journal of Reproductive Immunology* **51,** 156-159.

Xie X, He H, Colonna M, Seya T, Takai T & Croy BA. (2005). Pathways participating in activation of mouse uterine natural killer cells during pregnancy. *Biology and Reproduction* **73,** 510-518.

Xu D, Chan WL, Leung BP, Huang Fp, Wheeler R, Piedrafita D, Robinson JH & Liew FY. (1998a). Selective expression of a stable cell surface molecule on type 2 but not type 1 helper T cells. *Journal of Experimental Medicine* **187,** 787.

Xu D, Chan WL, Leung BP, Hunter D, Schulz K, Carter RW, McInnes IB, Robinson JH & Liew FY. (1998b). Selective expression and functions of interleukin 18 receptor on T helper (Th) type 1 but not Th2 cells. *J Exp Med* **188,** 1485.

Yagami A, Orihara K, Morita H, Futamura K, Hashimoto N, Matsumoto K, Saito H & Matsuda A. IL-33 mediates inflammatory responses in human lung tissue cells. *Journal of Immunology* **185,** 5743-5750.

Yagami A, Orihara K, Morita H, Futamura K, Hashimoto N, Matsumoto K, Saito H & Matsuda A. (2010). IL-33 mediates inflammatory responses in human lung tissue cells. *Journal of Immunology* **185,** 5743-5750.

Yamada G, Shijubo N, Shigehara K, Okamura K, Kurimoto M & Abe S. (2000). Increased levels of circulating interleukin-18 in patients with advanced tuberculosis. *Am J Respir Crit Care Med* **161,** 1786.

Yamada H, Morikawa M, Kato EH, Shimada S, Kobashi G & Minakami H. (2003). Pre-conceptional Natural Killer Cell Activity and Percentage as Predictors of Biochemical Pregnancy and Spontaneous Abortion with Normal Chromosome Karyotype. *American Journal of Reproductive Immunology* **50,** 351-354.

Yang KM, Ntrivalas E, Cho HJ, Kim NY, Beaman K, Gilman-Sachs A & Kwak-Kim J. (2010). Women with multiple implantation failures and recurrent pregnancy losses have increased peripheral blood T cell activation. *American Journal of Reproductive Immunology* **63,** 370-378.

Yovel G, Shakhar K & Ben-Eliyahu S. (2001). The Effects of Sex, Menstrual Cycle, and Oral Contraceptives on the Number and Activity of Natural Killer Cells. *Gynecologic Oncology* **81,** 254-262.

Zhou A, Carrell RW, Murphy MP, Wei Z, Yan Y, Stanley PL, Stein PE, Broughton Pipkin F & Read RJ. (2010). A redox switch in angiotensinogen modulates angiotensin release. *Nature* **468,** 108-111.

Ziegler SF, Ramsdell F, Hjerrild KA, Armitage RJ, Grabstein KH, Hennen KB, Farrah T, Fanslow WC, Shevach EM & Alderson MR. (1993). Molecular characterization of the early activation antigen CD69: a type II membrane glycoprotein related to a family of natural killer cell activation antigens. *Eur J Immunol* **23,** 1643-1648.