



UNIVERSITY OF
LIVERPOOL

The Origin and Role of Uterine Natural Killer Cells in Patients with Recurrent Implantation Failure

By Leena Khan

August 2008-2009

CONTENTS

Acknowledgements	iv
Abstract	v
List of Abbreviations	vii
List of Figures	ix
List of Tables	xi

Chapter 1: Introduction and Literature Review	1
1.1 Infertility	1
1.1.1 Definition	1
1.1.2 Aetiology	3
1.1.2.1 Male Factor Infertility	3
1.1.2.2 Female Factor Infertility	5
1.1.2.3 Unexplained Infertility	6
1.1.3 Treating Infertility	7
1.2 Menstrual Cycle	10
1.3 Recurrent Implantation Failure	13
1.3.1 Overview of fertilisation and implantation	15
1.4 Immune System Overview	19
1.4.1 Uterine Leukocytes	21
1.4.2 Implantation and the Immune System	22
1.5 Natural Killer Cells	28
1.5.1 Uterine Natural Killer Cells	28
1.5.1.1 History and Terminology	28
1.5.1.2 Anatomy of Placentation	28
1.5.1.3 Location	29
1.5.2 Peripheral Natural Killer Cells	30
1.5.3 Recurrent Miscarriage	34
1.5.4 Uterine Natural Killer Cell Receptors	37
1.5.5 Theories of origin of uNK cells	42

1.5.5.1 Trafficking	42
1.5.5.2 Proliferation	44
1.5.6 uNK Cell Function	46
1.5.6.1 Vascular Function	46
1.5.6.2 Trophoblast Development and Implantation	47
1.6 Methodological Limitations of Previous Studies	48
1.7 Conclusion	49
1.8 Hypothesis and Aims	50
1.8.1 Hypothesis	50
1.8.2 Aim	50
Chapter 2: Methods	51
2.1 Patient Selection	52
2.2 Endometrial Biopsy	53
2.3 Endometrial Tissue Reception	54
2.4 Tissue Processing	56
2.5 Embedding	57
2.6 Cutting Sections	58
2.7 Immunohistochemistry	59
2.8 Photographing	64
2.9 Image Analysis	66
2.10 Statistical Methods Used for Analysis	69
Chapter 3: Results	70
3.1 Patient Demographics	70
3.2 Image Analysis Validation	72
3.3 Micrographs	74
3.4 Graphs and statistical results	80
Chapter 4: Discussion	85
4.1 NK Cells distribution	85
4.2 Trafficking	86
4.3 Proliferation	90
4.4 Differentiation and Activation of NK Cells	91

Chapter 5: Conclusion	94
5.1: Limitations in Methodology	95
5.2 Clinical Implications	96
5.3 Future Directions	97
References	98
Appendix	115

ACKNOWLEDGEMENTS

I would like to thank the following people, without whom I would not have been able to complete this research project:

My supervisors, Dr. Siobhan Quenby and Dr. Mark Turner, for their support, encouragement and continued guidance.

Dr. Siobhan Quenby and Dr Ai-Wei Tang for collecting the endometrial samples from patients used in this research.

The laboratory technicians, Lisa Heathecote and Jo Drury for their patience in teaching me the laboratory methods carried out in this research and for cutting of the embedded endometrial sections.

I would finally like to thank my colleague, Asiyah Karam for her support and encouragement throughout the year.

ABSTRACT

Recurrent implantation failure (RIF) is the failure of the embryo to implant. Embryonic causes of RIF have been studied intensely; recently, investigations of endometrial causes of RIF have emerged.

‘Natural Killer’ cells appear in large numbers in the endometrium towards the mid-secretory phase, during the ‘window of impanation’. Their role in recurrent implantation failure needs investigating.

There are two competing hypotheses regarding the origin of uNK cells. They are either the result of proliferation of resident uNK cells or are from the trafficking of peripheral blood cells into the uterus and their subsequent differentiation into their uNK form.

Endometrial biopsies from 19 patients, 10 ‘low’ density uNK and 9 ‘high’ density uNK were stained using immunohistochemistry to compare antibodies associated with trafficking: L-Selectin, with a marker for peripheral blood NK cells, CD16, against makers for proliferation Ki67 and differentiation NKp30, on endometrial tissue, using CD56 as the universal marker for natural killer cells. The endometrial tissue will be obtained 7 days after the LH surge in the luteal phase as this is the window of implantation. Variation in location of these cells within samples was also studied in the following areas: sub-epithelial, areas with low stromal density and perivascular stroma in both groups.

Differences in percentage cell density was observed between both groups, as expected. With both groups observing more positively stained cells of each antibody in perivascular areas.

Few numbers of CD62-L and CD16+, markers for trafficking cells were detected. This suggests that uNK cells are not migrating in from peripheral blood, but arising from the endometrium, possibly from heamatopoietic cells.

CD56+ cells were seen in higher in areas around vessels than in other locations (p=0.003). Their close proximity to vessels enables them to promote vasculature

development. The markers for proliferation and differentiation of uNK cells, Ki67 and NKp30 was also significantly higher in areas around vessels compared to areas near epithelium ($p=0.006$ and $p=0.023$ respectively).

Perhaps this early development of arteries leads to a highly oxygenated state, which is detrimental to the developing embryo.

The paucity of trafficking markers indicates that uNK cells are not trafficking from the peripheral blood, so peripheral blood testing is of no clinical value in patients with recurrent implantation failure.

The consistent perivascular location of these cells indicates that these proliferating and differentiating cells have a role in vascular remodelling, which, may lead to a highly oxygenated state, decreasing the receptivity of the endometrium and causing implantation failure.

List of Abbreviations

ANG-2	Angiopoietin 2
APES	3-Aminopropyltriethoxysilane
β-hCG	Beta Human Chorionic Gonadotrophin
BSA	Bovine Serum Albumin
DAB	3,3-Diaminobenzidine
DNA	Deoxyribonucleic Acid
DPX	Distyrene Plasticiser Xylene
ECM	Extracellular Matrix Proteins
FSH	Follicle-Stimulating Hormone
GIFT	Gamete Intrafalopian Transfer
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
GnRH	Gonadotropin Releasing Hormone
hCG	Human Chorionic Gonadotrophin
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigens
HRP	Horseradish Peroxidase
ICSI	Intracytoplasmic Sperm Injection
IGFBP-1	Insulin-Like Growth Factor Binding Protein-1
IGg	Immunoglobulin G
IFN γ	Inteferon Gamma
IL	Interleukin
IUGR	Intra Uterine Growth Restriction
IVF	In-Vitro Fertilisation
KAR	Killer Activating Receptor
KIR	Killer Inhibitory Receptor
LBR	Live Birth Rate
LGL	Large Granular Lymphocytes
LH	Luteinising Hormone
LIF	Leukaemia Inhibitory Factor
MCI	Microbiological Safety Class
MHC	Major Histocompatibility Complex
NBF	Neutral Buffered Formalin
NK	Natural Killer
pbNK	Peripheral Blood Natural Killer
PCOS	Polycystic Ovarian Syndrome
RIF	Recurrent Implantation Failure
RGD	Arginine-Glycine-Aspartic Acid
TBS	Tris Buffered Saline
Th0	T – helper type 0

Th1	T – helper type 1
Th2	T – helper type 2
TNF α	Tissue Necrosis Factor Alpha
TNF β	Tissue Necrosis Factor Beta
uNK	Uterine Natural Killer
VEGF	Vascular Endothelial Growth Factor
ZIFT	Zygote Intra Fallopian Transfer

List of Figures

Figure 1: Hormonal regulation in sperm production.	3
Figure 2: Late-secretory phase endometrium, showing signs of enlargement of endometrial glands and oedematous areas	11
Figure 3: The change in structure and development of the blastocyst in the first few days from fertilisation	16
Figure 4: Leukocytes cell lineage	17
Figure 5: Relationship between uNK cell numbers detected by phloxine tartrazine staining in decidua basalis and gestational age	29
Figure 6: Diagrammatic representation of the process of lymphocyte migration	43
Figure 7: Schematic of process from tissue sampling to image analysis	51
Figure 8: Graph illustrating the relationship between age and uNK % in recurrent implantation failure patients	71
Figure 9: Bland Altman plot for JD/LK uNK counts	72
Figure 10: Bland Altman plot for AT/LK uNK counts	72
Figure 11: Bland Altman plot for AK/LK uNK counts	73
Figure 12: Micrographs showing subepithelial location of uNK Cells in a 'Low' patient	74
Figure 13: Micrographs showing perivascular location of uNK Cells in a 'High' patient	75
Figure 14: Micrographs showing areas of low stromal density of uNK Cells in a 'Low' patient	76
Figure 15: Micrographs showing subepithelial location of uNK Cells in a 'High' patient	77
Figure 16: Micrographs showing perivascular location of uNK Cells in a 'High' patient	78
Figure 17: Micrographs showing areas of low stromal density in a 'High' patient	79
Figure 18: Graph illustrating difference in antibody staining in 'High' and 'Low' uNK cell density population in peri-epithelial areas	80

Figure 19: Graph illustrating the difference between CD56 counts in ‘High’ and ‘Low’ uNK cell density populations in all observed locations	81
Figure 20: Graph illustrating the difference in antibody stained per location in the ‘Low’ uNK cell density group.	82
Figure 21: Graph illustrating the difference in antibody stained per location in the ‘High’ uNK cell density group.	83
Figure 22: Micrographs showing the staining of an area of lymphocyte infiltration	88
Figure 23: Micrographs of frozen sections stained with NKp30 and Negative control	92
Figure 24: Micrographs of decidua taken at different gestations in the first trimester stained with CD56 or NKp30	93
Figure 25: Micrographs showing cytoplasmic and nuclear double staining	96

List of Tables

Table 1: Cytokines produced by various cell types and their function	22
Table 2: Interleukins and their role in implantation	25
Table 3: Markers to be used in determining distribution and function of uNK cells	41
Table 4: Schedule of automated processor to process NBF fixed endometrial samples	56
Table 5: Dewaxing and Rehydrating schedule	59
Table 6: Citrate Buffer components	60
Table 7: Antibodies used and their respective concentrations used in validation	61
Table 8: Antibodies used and their respective concentrations to investigate hypothesis	61
Table 9: Solutions used in detection kit	61
Table 10: Tris Buffered Saline components	62
Table 11: Dehydration Procedure	63
Table 12: Settings used in image capture of stained endometrial sections	64
Table 13: Demographic distribution of the selected patients	70
Table 14: Results of analysis of data collected in image analysis software in each stain used in both 'high' and 'low' uNK populations.	84

CHAPTER 1 – INTRODUCTION AND LITERATURE REVIEW

1.1 Infertility

1.1.1 Definition

Infertility refers to a couple's inability to conceive after a year of regular, unprotected intercourse in the fertile phase of the menstrual cycle (1). Impaired fertility affects 10-15% of couples (2). It is estimated that on average 7 couples per year per average sized general practice in the UK seek medical advice for fertility problems (3). A large population based study of UK women (n=60,000) found a life time prevalence of infertility to be as high as 2.4%, with no difference among birth cohorts, despite advances in medical treatment (2).

Couples who are diagnosed as being infertile experience many difficulties, putting a strain on their relationship. Psychological stresses stem not only from the social and personal burden of not being able to have children, but also from the many investigations and numerous visits to the hospital. Treatment schedules become the main focus of time spent, and patients also may experience behavioural changes due to treatment drugs (4). When these treatment attempts fail, it can be devastating for couples and strength emotionally and financially to attempt treatment again may not be available.

Infertility can be regarded as either primary or secondary, with primary infertility referring to a woman never having conceived before and secondary when she has previously been pregnant, regardless of the outcome of the pregnancy.

In order for conception to take place the following conditions must be present(5):

- The Ovum: ovulation must occur in the female, anovulation is responsible for approximately 30% of cases of infertility.
- Sperm: Adequate quantities of 'good quality' sperm must be produced.

- Sperm must reach the egg: Sperm must be able to travel from their source of production, the male testis, to the site of the ovum, usually in the fallopian tube of the female, in order for fertilisation to take place.
- Implantation: the fertilised egg must be able to implant in the endometrium for a viable pregnancy to occur. Defective implantation may be as high as 30%.

Investigating inability to conceive is usual after one year of unprotected intercourse, and just six months in couples where the woman is over the age of 35. Initial investigations may reveal a source for the infertility, divided into male and female factors (6).

1.1.2 Aetiology

1.1.2.1 Male factor infertility

Male factor infertility accounts for 32.5% of infertility of all infertility(7).

Disorders of spermatogenesis:

Spermatogenesis is controlled by the hypothalamic-pituitary-axis (see Figure 1 below), producing follicle-stimulating-hormone (FSH) and leutinising hormone (LH). LH stimulates the release of androgens by leydig cells in the testis, FSH stimulates sertoli cells to secrete inhibin which acts as negative feedback on the production of FSH. Spermatogenesis, governed by genes located on the Y chromosome, is the division of spermatogonia into spermatocytes, whereas spermiogenesis is the transformation of spermatogonia into spermatozoa (8).

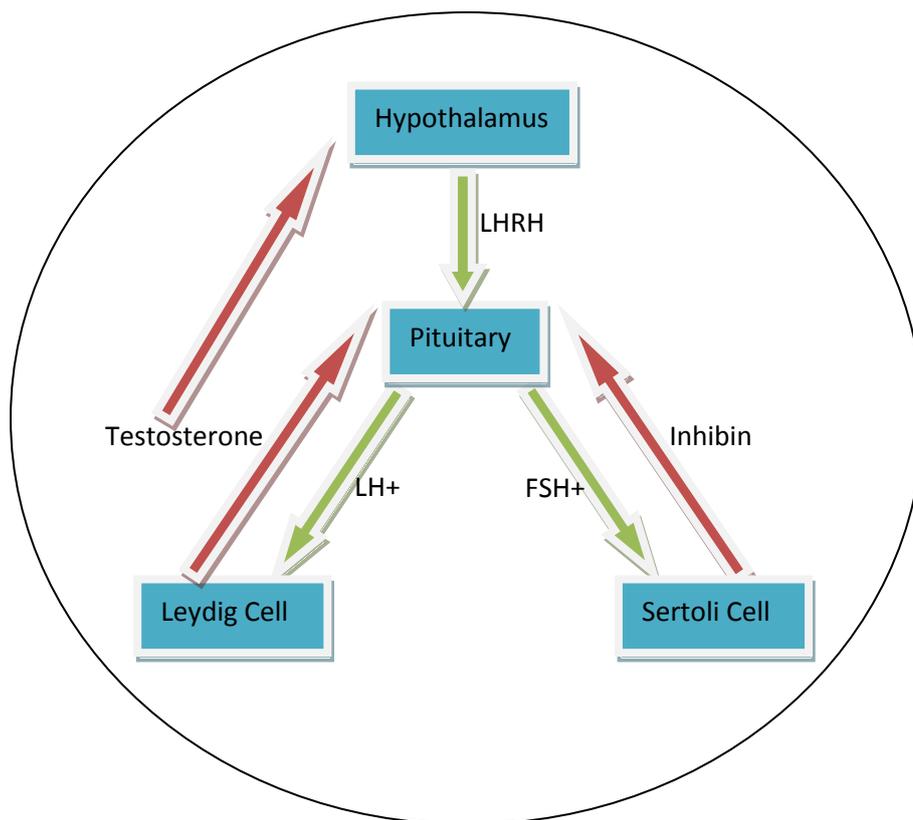


Figure 1: Hormonal regulation in sperm production. Adapted from Human Embryology(8).

Disorders of spermatogenesis may arise when scrotal temperatures rise, due to undescended testes, varicocele, hot baths or tight underwear (9). Microdeletions of

the Y chromosome may also lead to impaired sperm production (10). Also the following medications may have a negative effect: psychotropic drugs, antiepileptics, antihypertensive and antibiotics (11).

Impaired sperm motility and transport:

Spermatozoa, released in the lumen of the seminiferous tubules do not gain their motility until they reach the ampulla of the vas deferens. During ejaculation the semen is released comprising of these spermatozoa and secretions from the prostatic seminal vesicles (5).

Altered motility may be the result of congenital malformations of the epididymis or obstruction due to infection and an inflammation or vasectomy.

Ejaculatory dysfunction:

This can be caused by medications, recreational drug use, idiopathic or caused by metabolic and systemic diseases.

Immunological and Infective factors:

Mumps, caused by the mumps virus (a rubulavirus (12)). Can affect male fertility if it causes orchitis.

Sexually transmitted disease

Although uncommon in western countries, sexually transmitted diseases can affect male fertility, as well as female. Chronic infection with *Neisseria Gonorrhoea* and *Chlamydia Trachomatis* can cause damage to urethral structures and lead to epididymo-orchitis. Also *Ureaplasma Urealyticum* may impair spermatozoa (motility and DNA condensation), and HIV affects the quality of semen (13).

Male infection with *Neisseria Gonorrhoea* and *Chlamydia Trachomatis* can easily spread to the female partner and damage fallopian tubes through pelvic inflammatory disease (14).

It is necessary to test and treat for any of these infections, which if caught early, can be treated successfully with no long-term injury to reproductive structures.

1.1.2.2 Female Factor Infertility

Disorders of ovulation:

An intact hypothalamic-pituitary-ovarian axis is necessary for ovulation. Pulsatile GnRH release from hypothalamus stimulates the pituitary to release the hormones FSH and LH, which in turn stimulate the maturation of follicles within the ovary, and following a surge in LH on day 14 of the menstrual cycle, ovulation occurs.

Impairment in ovulation can occur at any point from the hypothalamus to the ovary.

Several conditions may affect the pulsatile release of GnRH, from psychological stress to structural lesions, including prolactinomas, and systemic conditions such as thyroid disease, which may consequently lead to anovulation. Polycystic Ovarian Syndrome (PCOS) is the most common cause of anovulation.

Impaired oocyte production:

Turner's syndrome: Patients with Turner's syndrome have gonadal dysgenesis which leads to amenorrhoea and sterility (15).

Premature ovarian failure: these patients undergo an early menopause, before the age of 40, either genetic in nature, idiopathic or the result of an oophorectomy, medically indicated for disease, or similarly radiation, chemotherapy to the pelvic region (16).

Tubal Dysfunction:

This has a higher prevalence in populations where sexually transmitted infections are higher, as secondary subfertility. *Chlamydia Trichomatis* is the main organism that leads to cilia lining the lumen of the fallopian tubes to become damaged and thus impair oocyte transportation to the ampulla.

Disorders of implantation:

These factors relate to the endometrium, the site of blastocyst implantation, defects in its development of growth and adhesion molecules may produce a barrier to implantation. It is also thought that structural disorders within the uterus such as fibroids prevent or hinder the implantation of the embryo into the uterine wall(17).

1.1.2.3 Unexplained Infertility

Unexplained infertility accounts for 23.1% of infertility (7). Where causes cannot be found in the rest of the male or female reproductive fact, the problem in conceiving may lie within the endometrium itself (18). Endometrial receptivity has been looked at in depth (19), with various hypotheses to potential pathology. It is currently not known what major endometrial factor contributes to unexplained implantation failure.

1.1.3 Treating Infertility

Addressing causative features:

Initial management of these patients should involve taking a comprehensive history and conduction of a full investigation, identifying whether it may be a male or female factor or mixed, and isolating it and tailoring treatment accordingly.

Assisted Conception:

Assisted conception techniques have been around since the 1970s (7), and have enabled over a million babies to be conceived. The technique centres in allowing the ovum and the sperm to be in close proximity to enable fertilisation to take place.

The most commonly used methods in current practice are intrauterine insemination, in vitro fertilisation and intracytoplasmic sperm injection (ICSI).

Steps in assisted reproduction:

Pituitary down-regulation:

This is necessary to prevent the natural LH surge mid-cycle from causing follicular rupture prior to egg retrieval; this is achieved with the use of GnRH analogue.

Ovarian Stimulation

This is achieved by daily injections with gonadotrophins for 11-14days until the follicles are 18mm in diameter on transvaginal ultrasound. To trigger ovulation an injection of hCG is used in replace of LH to trigger ovulation, approximately 34-36 hours later, these eggs are retrieved in their follicular fluid via a controlled vacuum pump.

Insemination:

Isolated sperm, having undergone a washing procedure to remove unwanted leukocytes, bacteria and seminal plasma, are incubated with the egg 4-6 hours after

collection, for 16-18 hours. ICSI requires an additional step to remove the surrounding cumulus cells surrounding the oocytes.

48 hours after culture the embryos are examined for cleavage and are graded, those with minimal fragmentation are scored more highly than those with any fragments.

Embryo transfer:

Embryos are transferred using a transcervical catheter on the second or third day of culture. Only two embryos are allowed to be transferred in each cycle in the UK, to minimise the complication of multiple births. However, in exceptional circumstances, for instance increasing maternal age, a limit of three embryos per transfer is allowed (7).

Any spare embryos of good quality can be stored via cryopreservation and used at a later date in frozen cycles. Two-thirds of embryos survive the thawing process.

Luteal support can be given in the form of progesterone supplements or low-dose hCG.

Pregnancy is screened for by a urinary pregnancy test or serum β -hCG analysis 14 days after embryo transfer.

Complications:

IVF is not without complications, the risk of multiple pregnancy is higher than with natural conception, this is minimised by limiting the number of embryos transferred to two and careful monitoring throughout ovarian stimulation. Multiple pregnancy rates from IVF have remained steady over the past 15 years, around 24% (7) multiple births increase the morbidity and mortality rate for the mother and her offspring resulting in great healthcare costs. Ovarian hyper-stimulation syndrome arises as a result of an exaggerated response to exogenous gonadotrophins, and is more common in those women who produce 20 or more follicles or those who have PCOS. Patients who develop this may need inpatient care.

Four per cent of pregnancies arising from IVF treatment result in ectopic pregnancies, with an increased risk in patients who have a history of tubal disease or damage.

Despite advances in technology unfortunately 4 per cent of couples will remain infertile.

Most causes of infertility can be isolated and subsequently treated for, with many patients finding the solution in using in vitro fertilisation (IVF), however for those where there is a problem in implantation, this is not a solution. As methods of assisted reproduction can only go so far, allowing the ovum and sperm to meet and for fertilisation to take place, it cannot enhance the receptivity of the uterus. This limiting factor must be investigated.

1.2. Menstrual Cycle(5)

Hormonal regulation of the menstrual cycle prepares the endometrium for implantation if fertilisation occurs.

The implantation window, days 7-10 after LH surge, signifies the period in which implantation is most likely to occur (20). By this point the endometrium has undergone significant changes to enhance its receptivity to the embryo. A surge in LH secreted from the anterior pituitary causes egg release from the mature follicle, ovulation, seven to nine days later is the ideal time for implantation to occur (20). Under the governance of the gonadotrophins, LH and FSH, the endometrium lining proliferates with vascular changes.

Structural Changes to Enable Implantation:

Menstruation: Days 1-4

The lack of implantation stimulates the corpus luteum to lower production of hormones, withdrawing support for the endometrium. The tunica media of the spiral arteries contract, ceasing blood supply to the tissue, necrosis follows and the endometrium is shed, along with blood which has not coagulated due to a local fibrinolytic.

The proliferative phase: days 5-13

FSH release from the anterior pituitary causes follicular growth, which in turn leads to secretion of oestradiol, which causes the endometrium to develop, proliferate, with intense mitosis in the stroma and glandular epithelium, and re-form following menstruation (start of which is day 0). Early during this phase the surface epithelium re-forms and covers the endometrium. Elongation of the spiral arteries and glands occur, with the glands increasing significantly in number.

The luteal/secretory phase: days 14-28

LH surge at day 14 causes ovulation, the remaining follicle from which the egg was released becomes the corpus luteum and continues producing oestradiol and

relatively more progesterone. This stimulates the endometrium to become 'secretory', and for the glands and stromal cells to enlarge (see Figure 2 below). The glands secrete a glycogen rich material. The endometrial stromal tissue can be observed becoming more oedematous (21). If fertilisation has not occurred by the end of this phase the corpus luteum fails thus leading to decreases in the steroid hormones, and menstruation ensues.

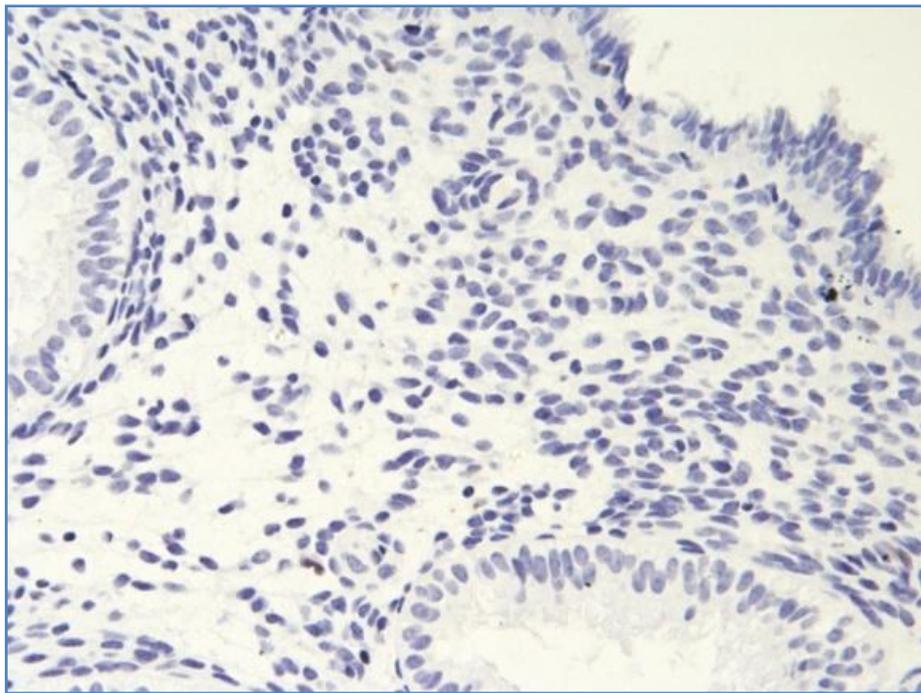


Figure 2: Late-secretory phase endometrium, showing signs of enlargement of endometrial glands and oedematous areas.

Decidualisation

Decidualisation is essential for implantation to take place. The endometrium undergoes a series of changes in order to maximise the receptivity of the uterus to the conceptus.

Stromal Cells:

These cells increase significantly in size (5 fold) and change from spindle-shaped to fleshy decidual cells (22). These cells lay down a matrix of peri-cellular rim of extracellular matrix proteins (ECM), (laminin and fibronectin) (22). They also increase production of various proteins, including prolactin, renin and insulin-like

growth factor binding protein-1 (IGFBP-1) (22). Although the function of the prolactin and renin are poorly understood, it is thought that IGFBP-1 with the matrix serves as a scaffold for trophoblastic cells to invade (22). IGFBP-1 and fibronectin contain RGD (arginine-glycine-aspartic acid) sequences that have the ability to bind to $\alpha_5\beta_1$ integrins expressed by extravillous trophoblastic cells (23). The secretion of these cells is maintained by progesterone (24).

Spiral Arteries:

These arteries modify in a way unique to the endometrium, it does not involve capillary sprouts degrading the basement membrane and migrating into surrounding connective tissue (25). The integral structure of the artery is maintained while it grows and increases in length (26). During placentation walls of the arteries are destroyed and the lumen dilates (22).

Endometrial Glands:

After proliferating in the follicular phase, the glandular epithelium differentiates, allowing it to start secreting. Their secretory ability is maximal through days LH+5 to LH+7 (22).

1.3. Recurrent Implantation Failure

IVF has improved over the past two decades, however its success rates have remained fairly constant, and remain relatively low, the latest figures show of all IVF attempts only 23.1% are successful and result in a live birth (7). Many couples are left childless even after several cycles of IVF. The burden on the couples involved, psychologically and financially is great, with state funding available on a limited basis.

This clinical dilemma leads patients to seek alternative treatment, where evidence base is lacking to prove their efficacy or safety (27). These can range from pregenetic screening, assisted hatching, blastocyst culture, allogenic culture, GIFT or ZIFT or immunosuppression.

Recurrent implantation failure (RIF) is the failure of implantation of the embryo into the uterine wall following embryo transfer in an IVF cycle. The 'recurrent' element is usually defined as three failed IVF cycles, however this varies amongst literature and studies undertaken have often limited this definition to at least two IVF failed IVF (28). It is important to distinguish the difference between these two definitions. Studies following the success of subsequent IVF cycles have found that the likely success of conceiving from IVF remains constant in the first three cycles, however, chances decline thereafter (29-34).

Another popular definition of RIF states that it is the failure of conception following the transfer of 10 'good quality' embryos (35-38). What defines a 'good quality' embryo is subjective, and for those under 40 who would have had only two embryos transferred at each cycle, this would result in a total of 5 failed cycles before falling into this category, equating to much time and even more expense for the couple.

One must also consider the nature of the cycle. Fresh versus frozen embryo transfer. Live birth rate (LBR) has increased steadily over the past fifteen years and is currently around 20% for frozen embryo transfer (7) (5% higher with donated

embryos) and it is higher with fresh cycles 27.5% in 2006 (7) higher with donated sperm.

The proportion of patients using cryothawed embryos has increased (7,28), this is probably in part due to the limitation on the number of embryos allowed for transfer in one cycle and the enhances in ovarian stimulation and embryo cryopreservation protocols.

Another point that needs considering is at what point is the best time to transfer embryos back into the uterus, at days 3, 5, or blastocyst stage. Various studies have been conducted to investigate the ideal day to transfer. Cruz *et al.*(39) conducted a retrospective trial comparing 15 IVF cycles with blastocyst transfer (days 5/6) and 22 cycles transferred at day 3, with patients having completed 4 cycles of IVF previously. They reported increases in implantation and pregnancy rates with blastocyst transfer, however, this is a very small number of cycles, in this retrospective study. In a larger, prospective study by Simon C *et al.* (40), looking at 2 day transfer versus blastocyst transfer, 168 patients underwent blastocyst transfer. Implantation and pregnancy rates (11.9% and 20.2%) compared to just 20 patients undergoing 2 day transfer implantation and pregnancy rates 10.7% and 35% respectively. The authors state these results were comparable to each other, reinforcing that stage of embryo transfer is not the main factor in patients with recurrent implantation failure.

Also what constitutes implantation failure, at what point after embryo transfer is it considered a failed implantation? Is it the lack of a positive pregnancy test at two week post transfer, or the lack of a visible gestational sac at five weeks or the absence of a foetal heartbeat? With different definitions, how can one be sure it is true implantation failure or 'biochemical loss', which may indeed have a completely different aetiology.

Hence infertility, in particular, recurrent implantation failure is a multifactorial disease in which an inhospitable uterine environment might significantly exacerbate the problem.

1.3.1 Overview of fertilisation and implantation.

Dissolution of the zona pellucida indicates the blastocyst is ready to implant (see Figure 3 below). When the blastocyst attaches to the endometrium in the secretory phase, it orientates itself so that the inner cell mass is facing the endometrium. The endometrial glands in this area then enlarge and the tissue becomes more vascularised, subsequently the blastocyst firmly implants into and is surrounded by the endometrium (5).

The zona pellucida acts as a barrier to immunological attack, between the mother and embryo, as it lacks histocompatibility (human leukocyte antigens – HLA). It also aims to prevent premature implantation of blastocyst into the fallopian tubes, as implantation cannot occur until the blastocyst ‘hatches’ from this ‘shell’ (5). This process initiates pre-implantation signalling, changing the structure of the uterus and promote orientation of the blastocyst to implantation site (41).

Stages in implantation (42)

Apposition:

Apposition can only occur during the ‘implantation window’, because it is associated with maturation of the endometrium. It is the very first (*loose*) contact of the blastocyst with the endometrium (43).

Adhesion:

The apical surfaces of endometrial epithelial cells express adhesion molecules (such as integrins) that allow implantation to take place. Similarly, trophoblastic cells of the pre-implantation blastocyst also express adhesion molecules at their surfaces. Protrusion of the trophoblast, microvilli, interact through cell surface glycoproteins, with the epithelial lining of the uterus. This stage produces a much stronger connection than that in apposition (8,41). The blastocyst attaches to the endometrial epithelium through the mediation of bridging ligands. This is termed adhesion. Studies (*in vivo* and *in vitro*) have shown that the attachment of the blastocyst to the uterine wall occurs after the rotation of the blastocyst, so that

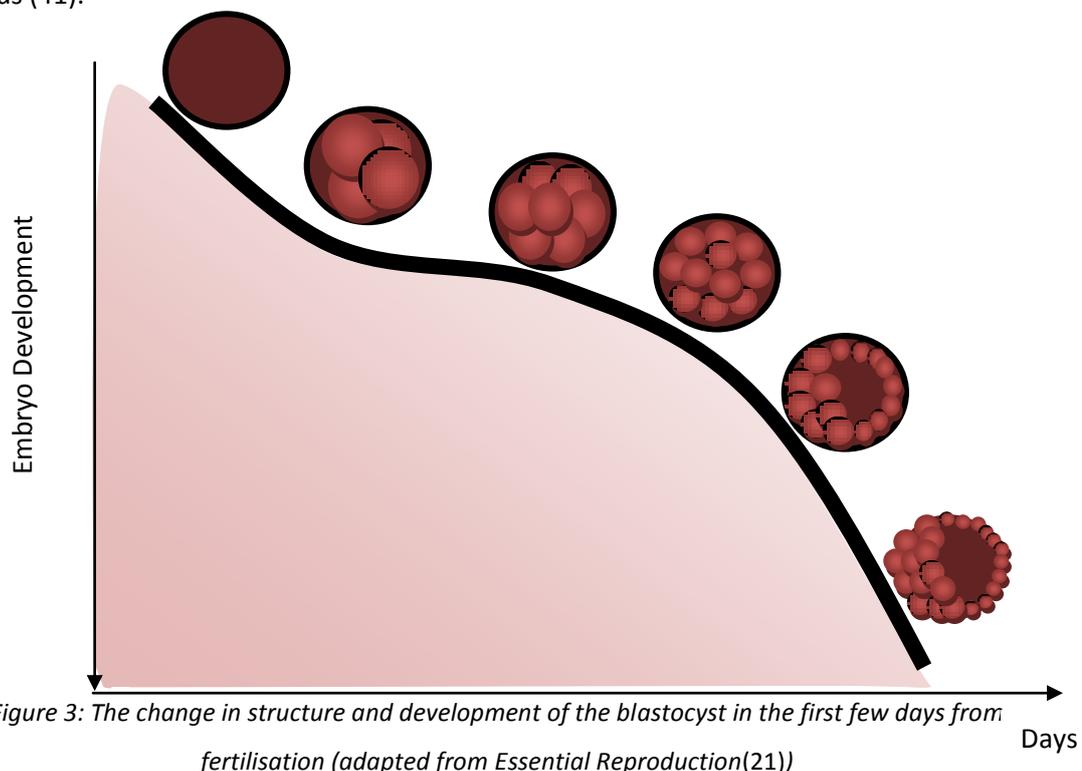
attachment occurs at the embryonic pole. If the attachment site is so specific, it may be that the surface of the blastocyst differs in expression of surface adhesion molecules.

Penetration:

Next stage is termed 'penetration' and involves the penetration of the blastocyst into the uterine epithelium. The trophoblast differentiates into two distinct cell types; syncytiotrophoblast and cytotrophoblast. Small projections of syncytiotrophoblastic cell mass invades between uterine epithelial cells, through the basal lamina, and into the endometrial stroma. This invades into the uterine mucosa to induce a decidual reaction. This stimulates the stromal cells to enlarge and become filled with glycogen thus providing nourishment to developing embryo. (44)

Trophoblastic Invasion:

This is the accumulation of the above stages, with resultant embedding of the embryo completely into the uterine wall, it becomes surrounded by syncytiotrophoblast cells. Lytic activity of the syncytiotrophoblast leads to development of lacunae and subsequent placental development with maternal blood flowing through vessels and lacunae to the foetus (41).



Pinopodes and Implantation

Pinopodes are small, finger like projections from the endometrial surface, thought to have a key role in the adhesion of the blastocyst to the endometrial surface. They appear between days 19 and 21 of the menstrual cycle, some suggest (45) that they are a marker of endometrial receptivity (45). They have a short-life span and appear for approximately 2-3 days. Their development is enhanced by progesterone and inhibited by oestrogens (45). They endocytose uterine fluid and consequently bring the uterine walls closer to the embryo by decreasing volume of the uterine cavity.

If it was shown that pinopodes appeared earlier in stimulated cycles it would indicate a shorter 'window of implantation' in these women than controls and may possibly explain the problems with implantation. Nikas *et al.*(46) found that pinopodes do not appear to be affected by ovarian stimulated cycles in patients undergoing IVF treatment, with their numbers, morphology and life span appearing similar between these patients and controls (47). However they did find that pinopodes appear at slightly different stages in the cycle, appearing earlier in women in stimulated cycles by a day or two. Whereas I. Oborna *et al.*(48) found that pinopodes did not vary in timing under hormonal preparation of the endometrium. The differences in results could be explained by different timings of biopsies and different hormonal concentrations. However Quinn *et al.*(45) have shown that they are not useful in the mouse or human models as consistent markers of endometrial receptivity for implantation. In the human, pinopodes have a prolonged (>5 days) presence in the luteal phase and fail to delineate the brief (24-48 h) window of receptivity

The failure of the embryo to implant is thought to be responsible for 75% of conceptuses lost (49). Mechanisms that can lead to this are either within the conceptus or the uterus. Chromosomal abnormality is estimated at occurring in 60% of conceptuses, if three fertilised oocytes are transferred into the uterus, it gives the chance of 78% of having a normal conceptus (50), when you compare this to the 27.9% success rate for IVF, it is more suggestive that the problem lies within the

uterus. Factors affecting implantation in the uterus are related to structural and hormonal irregularity. Immune factors within the endometrium has gained significant attention for many years (51).

1.4: Immune System Overview

The innate immune system acts as the body's first line of defence against hostile agents, acting in a non-specific nature against foreign bodies. There are four major functions of the innate immune system, they are:

- Recruiting immune cells to sites of infection and inflammation via cytokines
- Initiation of the complement cascade
- Phagocytosis of foreign body material through recognition of self from non-self
- Antigen presentation leading to activation of the adaptive immune system

The innate immune system consists of leukocytes (see Figure 4 below), derived from pluripotent haematopoietic stem cells in the bone marrow. The leukocyte population consists of the following cells: Mast Cells, Eosinophils, Natural Killer Cells, Phagocytic Cells (macrophages, neutrophils and dendritic cells) (52). The main type of cells active in the innate immune system are neutrophils, consisting of 50 – 60% of circulating leukocytes, they are phagocytic in nature, engulfing foreign pathogens, they are classified as granulocytes due to cytoplasmic granules, which contain toxic substances to pathogens.

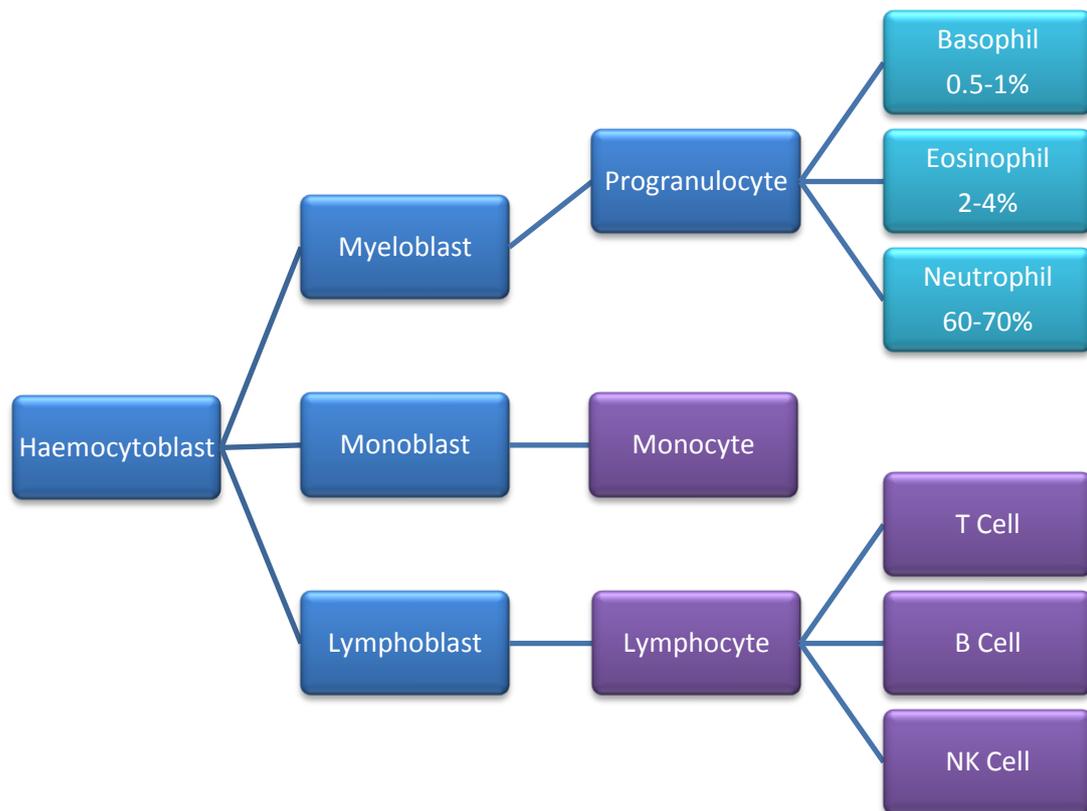


Figure 4: Leukocytes cell lineage. Adapted from CHXA: Leucocytes. (53)

Natural killer cells recognise, attack and kill cells that have been infected with intracellular pathogens. They do not have antigen receptors, unlike B and T cells, but instead recognise phenotypical changes in target cells (54). The decision to kill is based on the balance of signals from inhibiting and activating receptors (53), such as Killer Inhibitory Receptors (KIRs) and NKG2D, an activating receptor(54). KIRs recognise MHC (major histocompatibility complexes), tumour cells, for example, lose MHC class 1 and thus KIRs are not activated and so NK cells are *not* inhibited and thus attack these cells. DNA damage and infection triggers NKG2D and activates NK cells(55).

1.4.1 Uterine Leukocytes

The stratum basalis contains lymphoid aggregate, consisting of T cells. B cells are very minimal and almost absent. In the proliferative phase, the stratum functionalis contains few uNK cells, T cells and macrophages, with macrophages increasing in number prior to menstruation(22). There is also a significant number of macrophages found at the implantation site (22)

Decidualisation, induced by progesterone, is associated with a rise in the number of leukocytes to the endometrium. 70% of the CD45+ leukocytes are CD56bright NK cells, together with some macrophages and a small number of T cells. B Cells are very minimal and are almost completely absent.

Recently IL-15 and prolactin have been implicated in the proliferation and differentiation of these cells. Both of these hormones are produced by the stromal cells of the endometrium and their production is upregulated in the presence of progesterone (56).

1.4.2 Implantation and the immune system

Cytokines and the endometrium

During the 'window of implantation' there must be an appropriate balance of several factors that influence blastocysts adhesion in the endometrium. These factors involve the communication between hormones; paracrine and endocrine, adhesion molecules and cytokines (18). The interaction between these factors must be at its maximal during this period. Subsequent to this period the endometrial stroma undergoes 'decidualisation'; fibroblastic stromal cells differentiate into secretory decidual cells, this is associated with increased production of proteins. Some of which are insulin-like growth factor binding protein and prolactin.

Cytokines are produced by the immune cells. There are three types of these cells, all producing differing cytokines with varied function. See table below (18,57,58):

Table 1: Cytokines produced by various cell types and their function

Cell Type	Cytokines Produced	Effect
Th1	Inteferon Gamma (IFN γ) Tumour necrosis factor β (TNF β), Interleukin 2 (IL-2)	Cell mediated Immune Responses
Th2	Interleukin – 4 (IL-4) Interleukin – 5 (IL-5) Interleukin – 6 (IL-6) Interleukin – 10 (IL-10)	Antibody mediated humoural responses
Th0	Can be converted into either Th1 or Th2 and can produce each of the above respective to each cell as well as: Tumour necrosis factor α (TNF α) Granulocyte-macrophage colony- stimulating factor (GM-CSF)	
Macrophage	Interleukin 1 (IL-1) Interleukin 6 (IL-6)	Pro-Inflammatory cytokines

	TNF α Leukaemia inhibitory factor (LIF)	
Uterine Natural Killer Cells	Colony-Stimulating Factor 1 (CSF-1) TNF α , IFN γ , IL-1 α , Transforming Growth factor β (TGF β), LIF and GM-CSF, angiogenic cytokines-VEGF C	

As well as the above mentioned cells, it has been discovered that non-immune cells within the endometrium, can secrete these cytokines. These cells are active in the pregnant and non-pregnant uterus. It has been suggested, through immunocytochemical studies that epithelial cells are responsible for a large portion of the cytokines produced (59,60). After decidualisation uNK cells secrete a vast array of cytokines (see table above), suggesting that these cytokines are produced for the establishment of the blastocyst-endometrial connection.

Several studies have investigated the interplay of Th1 and Th2 cytokines, looking at the balance between production and how this profile leads to a favourable pregnancy outcome.

Rodent Population:

Studies in rodents nearly fifteen years ago indicated that for a successful pregnancy the balance of production would be toward Th2 cytokines, with Th1 cytokines being harmful to a successful outcome (61). Similar studies in rodents focused on matings between abortion-prone and abortion non-prone mice (62), showed increases in TNF α , IFN γ , IL-2 when challenged in abortion prone mice, compared to abortion non-prone mice (63). Further to this, administration of these specific cytokines made the non-abortion prone mice more susceptible to abortion, and conversely administration of the Th2 cytokine, IL-10, decreased abortion tendencies in the abortion-prone mice (64,65).

Human Population:

During pregnancy diseases with Th1 response decrease in severity, such as rheumatoid arthritis and conversely, diseases with a Th2 response increase, such as systemic lupus erythematosus. Studies investigating concentration of these cytokines in peripheral blood of recurrent miscarriage patients have yielded mixed results, one suggestion for these discrepancies is the timing of the blood sampling and whether they were taken in the absence or presence of miscarriage. Bates *et al.*(66) showed increased Th2 cytokines, IL-4 and IL-10, and decreased TNF α and Th1 cytokines IFN γ from women with recurrent miscarriage, when compared with controls, samples were taken in early pregnancy. This is the opposite from what we would have expected, when using previous rodent data.

When looking at the implantation failure population, Ginsburg *et al.*(67), increased IL-10 was observed in controls when compared to those with unexplained infertility.

Kwak-Kim *et al.*(68) showed increases in the ratios of TNF α /IL-4 and TNF α /IL-10 in patients with RIF. However, excluding this, other studies have shown conflicting results with regard to IFN γ and IVF failures and TNF α . (67,69). Problems with defining the population and timing of blood sampling could explain difference in results.

No studies investigating the profile of Th1 or Th2 cytokines within the endometrium of patients with implantation failure have been conducted.

Lim *et al.*(70) and Shimada *et al.*(71) showed conflicting results in recurrent miscarriage patients, this can be explained by the difference in tissue analysis.

Interleukins:Table 2: Interleukins and their role in implantation.

Interleukin	Role in Implantation	Study
<u>IL-1:</u>	Increased production of metalloproteases and integrins, possible role in implantation.	Meisser <i>et al.</i> (72) Simon <i>et al.</i> 1997 (73)
IL-Ra	Prevents blastocyst implantation	Simon <i>et al.</i> 1994 (74)
Follicular Fluid: Increased IL-1 α and IL-1 β	Correlated with successful IVF pregnancy	Karagouni <i>et al.</i> (75)
Low plasma IL-Ra and High plasma IL-1 β	Association between good quality embryos and clinical pregnancies	Spandorfer <i>et al.</i> (76)
IL-18 (pro-inflammatory, Th1 response via IFNγ)	IL-18 decreased in mice placenta from abortion prone mice Abnormal expression in implantation failure patients, varying degree of expression in epithelial and stromal areas	Chaouat <i>et al.</i> (77) Ledee-Bataille <i>et al.</i> (78)
IL-12 (pro-inflammatory, Th1 activation)	Increased expression in 10/35 IVF implantation failure patients	Ledee-Bataille <i>et al.</i> (78)

These studies are heterogeneous in nature and consequently a direct comparison of results is difficult. Ideally studies investigating this area should clearly define study

population with confounding variables accounted for, in detailed clinical history, for example, cause of infertility, number of cycles previously attempted and number of embryos transferred, whether 'good quality' embryos were replaced. Another factor to consider is which cells were investigated, as mentioned earlier cytokines are not only produced by immune cells, and a comprehensive look at production profile in a well-defined population is needed.

It has been shown that uNK cells are capable of producing cytokines (18,57,58), it has also been shown that these cells have receptors for some cytokines, IL-1, IL-2, TNF α (57,79). IL-2 expression, in vitro on uNK cells has been shown to increase the expression of CD16 on uNK cells (80). It also has been shown to induce cytotoxicity towards the trophoblast (81).

Receptors for cytokines are present on decidual cells as well as trophoblastic cells (18). The interplay between these cells and uNK cells may well directly impact on the outcome of implantation through the direct and indirect action of cytokines.

LIF and implantation

Leukaemia Inhibitory factor (LIF) has been shown to be influential in the implantation of blastocysts in animal studies (82). Its expression on glandular epithelium is induced by oestrogen before implantation occurs. It is said to peak during the mid-late secretory phase of the menstrual cycle (83) and is secreted mainly by the glandular epithelium (84,85), and is not dependent on the presence of an embryo.

Animals lacking LIF gene fail to implant their blastocysts into the endometrium wall, and when injected with recombinant LIF, implantation is restored (86).

LIF is thought to work in a paracrine manner via the luminal endometrial epithelial cells, to prime the endometrium for reception of the blastocyst (87,88).

However in human studies the evidence is conflicting. There have been reports of a decrease of LIF detected in uterine flushings (83,89). Whereas others report no difference in infertile patients when compared to controls (90,91).

There is supporting evidence for a role of LIF in implantation in animals, however more research needs to be conducted to establish a conclusive role for LIF in human endometrial implantation.

1.5. Natural Killer cells

1.5.1 Uterine Natural Killer Cells

1.5.1.1 History and terminology

Presence of granulated cells in the endometrium is not a new discovery. Since as early as 1921 (92) the presence of these cells has been recognised. The presence of these cells was not restricted to human endometrium. Endometrium of mouse and rat also showed these cells (93).

A stromal origin for these cells was generally the accepted even though there were suggestions by some scientists that the origin of these cells were in fact lymphocytic (human (92), mouse (94), monkey (95)). In 1980s it was found that these cells stained positively for the CD45, the leukocyte common antigen (96) and later CD56, which confirmed the NK cell phenotype (97).

Natural killer cells are a type of cytotoxic lymphocyte, approximately 15µm, with a reniform nucleus (22), they can be distinguished from CD8+ T cells by their lack of rearrangement of T-Cell receptor genes. They are large lymphocytes that have an abundant granule-containing cytoplasm (large granular lymphocytes (LGL), they function by cell killing or cytokine production which is enhanced by cytokines mentioned earlier.

1.5.1.2 Anatomy of placentation.

Our innate immune system works to achieve the goal of defending itself from invading foreign bodies. What happens when a semi-allogenic foetus attempts to invade into the endometrium, having initiated decidualisation and is rapidly filling with lymphocytes? A symbiosis must exist between the maternal endometrium and the invading foetal trophoblast. A balance between these two must form to prevent extensive invasion (parasitism), limited invasion (pre-eclampsia) or outright rejection of this semi-allogenic being from the mother (implantation failure or miscarriage).

1.5.1.3. Location

NK cells tempero-spatial relationship within the endometrium was also noted around the 1960s (98-100). It was noted that these cells occurred mainly in the late secretory phase and were visible in early pregnancy, as well as towards the end of pregnancy. These cells are thought to continue to a peak in pregnancy and then decrease in numbers by 20 weeks gestation and are absent in term decidua (see Figure 5 below) (101-104). They still feature in the proliferative phase and early secretory phase but in relatively small numbers (105,106).

The relationship between gestational age and uNK numbers have been investigated with results from one study shown below (93), however a limitation of only 35 patients means it is hard to accurately suggest differences between trimesters.

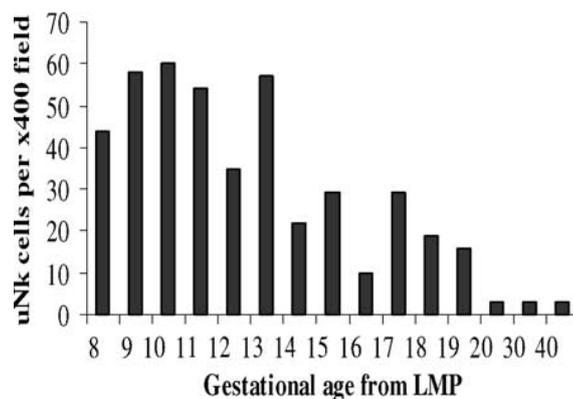


Figure 5. Relationship between uNK cell numbers detected by phloxine tartrazine staining in decidua basalis and gestational age. Cells were counted in 5×400 fields of the central part of the placental bed in pregnancy hysterectomy samples ($n = 35$). (Copied with permission from Bulmer and Lash (93))

It is difficult to quantify the presence of NK cells in placental tissue in varying gestational age as there are confounding variables and the heterogeneity of samples. A factor is the way in which the biopsy was obtained, miscarriage and terminations will inevitably lead to a local inflammatory response, how soon after the event were the samples taken and then processed? Also placental bed biopsies will differ from complete hysterectomy samples as there may be less inflammation in a hysterectomy sample. Additionally we need to look at causality of miscarriage,

whether idiopathic and otherwise 'normal pregnancies' or pathology such as chromosomal abnormality.

1.5.2 Peripheral Blood Natural Killer Cells

Peripheral blood natural killer cells are distinctly phenotypically and functionally different from uterine natural killer cells. uNK cells for example exhibit low levels of cytotoxicity and cytolytic behaviour, most uNK cells are CD56+ and CD16-, whereas only 10% of pbNK cells stain this way with 90% of pbNK cells staining positive for CD16.

It is necessary to determine the origins of these immune cells, how do they come to exist in such high numbers within endometrial tissue, fluctuating at different times in the cycle? The big question is testing the theory between peripheral blood origins and proliferation of locally derived uNK cells. The outcome is one which will affect clinical practice. Currently there are many clinicians who believe that 'high' numbers of uNK cells are a prognostic indicator and negative influential factor to implantation outcome. Subsequently various methods of reducing NK cell levels in the blood have been implemented in some of these patients. The scientific data behind the link between peripheral blood natural killer cells and implantation failure is differing, with opposing thoughts on the impact of these blood cells on the endometrium and implantation.

It is important to look at the distinct differences in the phenotype and function of peripheral blood NK cells and uNK cells.

The majority of peripheral blood NK cells are CD56dim and CD16+. CD16 is a low-affinity receptor for IgG complex and is the receptor responsible for NK-mediated, antibody-dependant cellular cytotoxicity.

Unlike uNK cells peripheral blood NK cells do not vary in number and activity in the menstrual cycle. However in pregnancy their numbers and functional ability have been found to be suppressed during pregnancy (107,108).

Koopman *et al.*(109) investigated the phenotype of these two populations, peripheral blood CD56^{bright}CD16+ and CD56^{bright}CD16- uNK cells, and have concluded that they are phenotypically different. Using microarray analysis, flow-cytometric and RT-PCR studies to obtain results. This suggests that peripheral blood NK cells are not uNK cells and must either differentiate to become these cells or are from a small, distinct population of peripheral blood NK cells. Consequently studies investigating solely peripheral blood NK cells are not reflecting the true picture of what is occurring at the feto-maternal interface.

CD69 is one of the earliest cell surface activation markers expressed by NK cells. uNK cells also express a variety of KIR, killer inhibitory receptors, killing activating receptors (KARs) which recognise HLA-G expressed on extravillous trophoblast (80).

NK cells do 'kill' trophoblastic tissue in vitro when first stimulated by the cytokine IL-2, it is important to note however, that IL-2 is not present at the moment of implantation in the endometrial tissue (110).

Differing results have emerged regarding reproductive outcome in mice studies within the same research group. Croy *et al.* has shown that in Terhost mutants NK cell deficient mice there is an increased rate of miscarriage. However this rate is not increased in IL-15^{-/-} NK cell deficient mice (IL-15 is the activating cytokine for NK cells), similarly with IL-2 receptor knock-out mice, which also lack uNK cells (111). These differing results can be explained partly by the subset of NK cells that is affected by the knock-out mice.

The relationship between peripheral blood NK cells and reproductive outcome after IVF or spontaneous pregnancy has been investigated in several studies. These studies however have been small-scale observational studies (50,112-117), The results from these studies show a connection between NK cell activity and subsequent implantation/conception failure or miscarriage. However, the reasons for reproductive failure in the patient populations were different (118). Methodological flaws within the studies centre around the sample and how it is obtained, whether whole blood samples or fractionated mononuclear cells used, the time of day in which the biopsy was taken, whether the patient had partaken in

physical exercise, stress, age of patient, sex and ethnicity (119) and whether samples were thawed from prior freezing (120-122).

The level for 'high' peripheral blood NK levels, Beer *et al.*(113) was previously set a level of 12% of all lymphocytes, however other studies have a normal range as high as 29%. Eidukaite *et al.*(123) found that amongst normal healthy individuals the percentage of CD56+ NK cells in the blood can range from as little as 5% to as much as 29% (124). Therefore there can be a substantial crossover in of normal patients labelled as 'abnormal'.

The difference in measuring NK cell activity is also an issue. Most commonly the in vitro assay is cytotoxicity, however this may have little resemblance to what is taking place in vivo.

Thum *et al.*(125) 2004, have shown an association with increases in absolute count of CD56DimCD16+CD69+ with implantation failure following IVF. It is important to highlight that that mean number of IVF failures in the sample population was 1.3, not enough to be considered 'implantation failure', there may not be pathology in patients who have only had one failed embryo transfer cycle. These cells were counted using flow cytometry, which is influenced by the variables mentioned earlier and the arbitrary nature in which the lymphocyte gate is set on the flow cytometer. The absolute count of CD56dimCD16+CD69+ is 1.66×10^6 this is out of an overall count for CD56dimCD16+ of 212×10^6 as a percentage, this proportion is merely 0.78%. This very small figure could contribute to a large margin of error. Looking at a ROC (receiver operating characteristic), the authors calculated an area under the graph as 0.63, with a perfect test having an area of 1 and a worthless test having an area of less than 0.5, a value of 0.63 does not infer that testing peripheral blood NK cells is of prognostic value in patients with implantation failure following IVF.

Methods of clinical intervention in this patient population involve attempting to suppress an 'excessive' immune response and lower NK cell numbers and activation. Treatments include LIT, (lymphocyte immunisation therapy); IV immunoglobulins, anti-TNF-alpha and glucocorticoids. These interventions are

associated with significant side effects (126-129), and when rationale behind their use is incomplete, it is unsafe and unethical to subject patients to these unproven treatments.

Fukui *et al.*(114) previously showed an increase in CD16+ NK peripheral blood cells was associated with a failed implantation following IVF treatment.

Fukui *et al.*(130) reported a significant difference in the cytokine profile in peripheral blood cells in a sample of patients with recurrent miscarriage and those with implantation failure, but no difference in the percentage of CD56+, CD56bright and CD56dim between these two groups and a set of controls (n=15). The only differences were in CD56bright/Interferon γ +/TNK-alpha+ and TNF alpha/GSM-CSF expressing CD56bright cell ratio in all three groups, with significant increases in the RM and implantation failure samples. However, sample numbers were small, n=20 in each comparison group and implantation failure of only 4 embryo transfers was set as the inclusion criteria for implantation failure, this, as discussed above does not cover the general consensus on what defines implantation failure. Methods included were flow cytometry, analysing a fraction of the sample of isolated mononuclear cells from peripheral blood sample, with 3×10^4 cells analysed in each sample.

1.5.3 Recurrent Miscarriage (RM)

Much focus and investigation into the subject of uNK cells, their interaction and role within the fetomaternal unit is centred in the pathology of recurrent miscarriage. Similar to implantation failure, RM patients have negative reproductive outcome, coupled with intense emotional turmoil for patients. These patients have managed to overcome the hurdle of conceiving and implantation of embryo firmly into the uterine wall. However, it is no less traumatic for the patients and families involved.

Several studies have highlighted the importance of timing in endometrial sampling of these patients, and necessitate the accuracy of measuring the LH surge, to be sure to obtain samples in this narrow window (131).

Several studies have investigated the prognostic value of NK cells in recurrent miscarriage.

Quenby *et al.* (132) 1999 and Clifford *et al.* (133) showed an increase in number of CD56+ uNK cells in patients with RM in the non-pregnant endometrium, however Michimata *et al.* (134), were unable to show any difference between RM patient and controls. Quenby *et al.* (132) 1999, had a sample population of 12 patients, 4 of which went on to miscarry and 8 of which went on to have a live birth. It was shown that patients who went on to miscarry had significantly higher levels of uNK cells than the live birth patients. However Michimata *et al.* (134), in a study of 17 women, 6 of whom miscarried, failed to show any difference. It is important to highlight that in the later study, the inclusion criteria included women with only two previous miscarriage, this does not conform to the widely accepted definition for this disease of three or more consecutive miscarriages. It could be that the differences in the finding can be explained by the more severe phenotype of patients in the previous study and the lack of appropriate patient selection in the later study.

Both these studies had a small sample population, which hinders the ability to draw pertinent conclusions.

As with studies in RIF, the ability to make comparisons between studies is made difficult by the heterogeneity of quantifying uNK cells. Clifford *et al.* 1999 (133)

opted to use the mean number of uNK cells, whereas Quenby *et al.*(132) 1999 and Tuckerman *et al.*(60) chose to view these cells as a percentage of stromal cells, Michimata *et al.*(134), elected to quantify the proportion of uNK cells to CD45+leukocytes.

Tuckerman *et al.* (135) 2007, investigated the prognostic value of uNK cells in patients with RM, in 87 women with LH timed endometrial samples, paraffin embedded and counted numbers of CD56+, 51 of the women went on to become pregnant, of the 51 women, 32 proceeded to a live birth and the remainder, 19 miscarried, with a control group of 10 patients, 7 with proven fertility. However, no description on how the cells were counted, or how stromal cells were counted to obtain a percentage.

They found the means of CD56+ in the control group was 6.2% and significantly lower ($p=0.013$) than in the 87 women, 11.2% (CD56+ count), which is higher than observed by Quenby *et al.*(136) 2005, who stated a level of 5% based on the upper interquartile level of their control population ($n=18$).

Tuckerman *et al.*(135) 2007 did not find any difference between the live birth group and the miscarriage group in terms of uNK levels. It is important to note that the levels in the live birth group varied from 1.1 – 41.4%, which is very large. Although this is a better powered study, one must question the accuracy of analysing the count of CD56+ compared to stromal cells in the high-powered fields.

Quenby *et al.*(136) 2005, trialled the administration of prednisolone in women with RM, with aim to lower uNK cells and lead to a subsequent live birth in the next pregnancy in 85 women, with 18 sterile women as controls. Of the 85 women, 29 with uNK levels (from endometrial biopsy at day 21 of menstrual cycle) $>5\%$ consented to take 20mg prednisolone daily from day 1 to 21 of their menstrual cycles. On this cycle, a second biopsy was taken. Women with RM were found to have significantly higher levels of uNK % than controls ($p=0.008$), with prednisolone reducing levels of uNK from a median of 14% (before) to 9% (after) ($p=0.0004$). This

study is the basis for a current randomised trial into the effects of prednisolone vs placebo on RM.

1.5.4. uNK cell Receptors

By the late secretory phase NK cells proliferate to encompass 30% of endometrial stromal cells (119). They continue to increase in number and are found in high concentrations surrounding implantation sites (119). Hence they are in close proximity to the invading trophoblast and must therefore be in contact with them. The suggested function in vascular remodelling occurs here to modify spiral arteries to increase flow of blood across the feto-maternal interface via the placenta (119). The method of interaction between uNK cells and trophoblast tissues is unknown but receptors on uNK cells have been found corresponding to those on trophoblastic cells (80). Major histocompatibility complexes (MHC) 1 molecules have been found to bind to receptors on NK cells.

Activation/Inhibitory Markers

uNK cells express KIRs, killer-inhibitory receptors for HLA-G, which is uniquely expressed by invading trophoblastic cells (137) suggesting, strongly the association between these cells and facilitating the implantation of the trophoblast.

uNK cells also express KIR for HLA-C, which is the most dominant and it is thought that this combined with specific polymorphisms for foetal HLA-C may lead to problems with trophoblast invasion (138,139).

Negative control of cytolytic potential may exist in normal pregnancy as the HLA-E ligand of CD94/NKG2A is expressed by extravillous cytotrophoblast.

CD69 is an early activation marker (140,141,141), present in the proliferative phase, decreasing through menstrual cycle.

Natural cytotoxicity receptors (NCRs) include NKp30, NKp44, and NKp46, responsible for NK cell activation resulting in cytokine production and/or target cell lysis (142)(143)(144). NCRs are part of the immunoglobulin superfamily and are pivotal to NK cell function (143). Vacca *et al.*(143) have reported the presence of NKp46 and NKp30 on resting and activated circulating NK cells.

NKp30 has been found on decidual NK cells (145,146) but their presence on uNK cells has been reported with conflicting results.

El Costa *et al.* (147) showed that activating receptor engagement of NKp30, induces the production of several key pro-inflammatory cytokines IFN- γ , TNF α , MIP-1 α and GM-CSF, *in vitro* on decidual natural killer cells, with dose effect. These effects decreased by addition of NKG2A ($p < 0.0031$). They also showed cytolytic activity induced by engagement of NKp46, effects of which were blocked by NKG2A.

Manaster *et al.* (148), investigated the occurrence of NKp30 receptor on uNK cells in the non-pregnant uterus. They found a lack of expression of NKp30 prior to activation of IL-15 and conclude that these NK cells are 'immature cells' that await pregnancy. Unfortunately there are many flaws with this data. Firstly the patients that were sampled, 25 women, had cycle status confirmed on ultrasound and blood progesterone levels, neither of which are as reliable as the narrow peak of the LH surge. Secondly flow cytometric analysis was used, which one cannot assume was free from any stromal or peripheral blood NK cells in the isolation of uNK cells. They showed presence of NKp46 across each phase, little NKp44, and flow graphs produced show a very slight increase in NKp30.

This study did however, show a substantial presence of NKp30 ligands on endometrial stromal cells.

Ponnampalam *et al.* (149), also studied NKp30, using immunohistochemistry, and found staining of endometrial epithelium with NKp30, however this was performed on frozen sections. NKp30 mRNA was increased in fresh cultured tissue from the late secretory phase

IL-15 has been shown to be necessary for the differentiation of uNK cells in mice (150). uNK cells have also been found absent from the implantation sites of mice deficient in IL-15 and subsequently had no evidence of spiral artery modification (151). IL-15 also activates uNK cells to lyse classical NK cell targets *in vitro* (93). IL-15 levels are higher in the secretory phase, than the proliferative phase endometrium (152) and endometrial stromal fibroblasts have been stimulated to produce it by

progesterone, in vitro (153). Ponnampalam *et al.* (149), also studied NKp30, using immunohistochemistry, and found staining of endometrial glandular epithelium with NKp30.

Proliferation Markers

A previous study investigating proliferation of uNK cells, using immunohistochemical staining in frozen sections found that 40% of CD56 positive uNK cells, in the late-secretory phase endometrium express the antigen Ki67 (154).

Trafficking Markers

L-Selectin

The rise in LH and ovarian steroid stimulate the adhesive properties of natural killer cells to mouse uterine mucosa through the effect of L Selectin and α_4 -dependant mechanisms, it is still unclear whether these effects are direct or indirect (150,155,156). This support the hypothesis that NK cells are recruited from peripheral circulation, however this has been found in mouse, not human tissue.

Human blastocysts utilise L – Selectin to help them adhere to the uterine wall by binding to uterine ligands, to initiate implantation. It is hypothesised that the absence of these ligands on the uterine wall could lead to recurrent implantation failure (157).

A study (158) found that L-Selectin expression varied throughout the menstrual cycle. This expression was highest during ovulation and remained high through to the endometrial mid-secretory phase. This occurs at the same time as implantation therefore any defects in this adhesion mechanism of L-Selectin may explain why embryos fail to implant.

Steroid Receptors

uNK cells have oestrogen receptor β , but not oestrogen receptor α (80,159). uNK cells vary in number and structure through the menstrual cycle. This is thought to be tightly linked to the levels of progesterone (93), as they increase rapidly in number after ovulation (137), which synchronises with the rise in progesterone, and the tailing off of oestrogen levels. As progesterone decreases, uNK cells apoptose (80) this is thought to indicate the first sign of menstruation rather than decidualisation and implantation. However uNK cells do not have progesterone receptors (159). It has been suggested that progesterone exerts its influence on uNK cells via the action of IL-15, which L-Selectin and α increases as progesterone increases (160). Progesterone receptors have been found on stromal cells (24) and stromal cells have been shown to produce IL-15 in the decidualising uterus in both humans and mice, A study (153,161,162) looked at in vitro addition of sex hormones to isolated and highly purified CD56^{bright}CD16⁻ on proliferation, cytolytic behaviour and cytokine production in 7 terminations of pregnancy specimens and 13 histologically normal hysterectomy specimens. They found that activity of NK cells were not directly influenced by progesterone or 17 β -oestradiol. However this study was carried out in *vitro* and may not be a true reflection of in *vivo* circumstances.

To evaluate the function and origin of these cells it is necessary to look at the following markers (80,93,148,154):

Table 3: Markers to be used in determining distribution and function of uNK cells.

Marker	Reasons for Investigation
CD56	This is the universal marker for NK cell and will identify which cells are uNK cells so we can observe the variance in location and staining patterns.
CD16	Marker for the majority of peripheral blood NK cells, will identify which cell are likely to be coming from peripheral blood into the uterus
CD62-L / L-Selectin	A migration marker for leukocytes. If present in the endometrium signals that NK cells have arisen through trafficking of peripheral blood NK cells into the endometrium.
Ki67	A nuclear marker for proliferating cells. This, with location variance might indicate the potential role of NK cells.
NKp30	A novel marker for activation of NK cells. This, with location variance might indicate the potential role of NK cells.

1.5.5 Theories of Origin of uNK Cells

There has been much speculation surrounding the origin of these cells. Two main competing theories have arisen, these are:

- uNK cells arise from the trafficking of peripheral blood NK cells into the human endometrium.
- uNK cells arise from the proliferation and differentiation of resident NK cells already within the uterus.

1.5.5.1 Trafficking:

Substantial work carried out by Croy and colleagues have focused on trying to find evidence to support this theory, with the majority of work done in rodent and porcine models (151,155,156). They have found that endometrium becomes 'sticky' to NK cells from the blood when the homing marker 'L-Selectin' is present (163). The concept is this, leukocyte express adhesion molecules, such as L-Selectin, and once activated by the engagement of a chemokine receptor, they are subsequently able to attach to their ligands, expressed on the endothelium(164). This was investigated using an adapted Stamper-Woodruff adhesion assay (165) using mouse uterus, attaching various other tissues to it, including human endometrium. They were successful in finding an adhesion between these two tissues but this depended heavily on the presence of L-Selectin and alpha-4 integrin, so much so that if the receptors for these molecules were blocked the adhesion was eliminated (163)(163). Subsequent study in IVF patients showed that in sampling peripheral blood lymphocytes their adhesion was irrespective and unaltered by administration of exogenous hormones or establishment of pregnancy comparative to menstrual cycle (166). However an increase in adhesion by LH +40 was shown to be apparent in those with sustained pregnancy compared to those without, indicating that there may be a link between uNK cells secreting molecules aiding implantation.

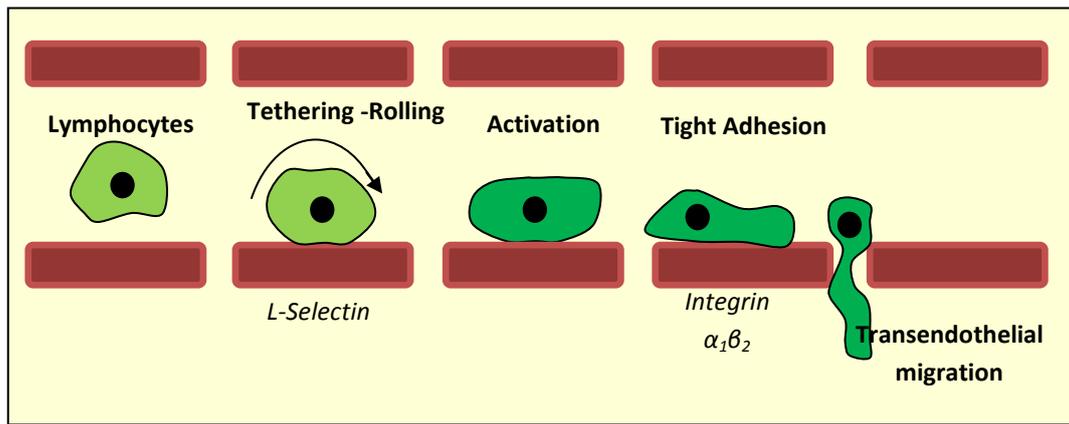


Figure 6: Diagrammatic representation of the process of lymphocyte migration.
Adapted from Kitaya et al.(152)

Studies (167-169) have shown that selectin adhesion systems mediate the initial interactions between leukocytes and vascular endothelial cells (see Figure 6 above). These adhesion systems allow leukocytes to adhere strongly to endothelial wall, against the flow of blood.

Van Den Heuvel (170) also found that in fertile women an increase in L-Selectin (CD62-L) occurs between CD56+ cells and the endothelium around LH surge. They further conclude that this recruitment of peripheral blood NK cells to the endometrium occurs with increases in oestrogen and LH and is limited by increases in progesterone. In the murine model pro-uNK cells (self-renewing progenitors to uNK cells) are developed from pre-uNK cells from peripheral lymphoid sites (155). These cells are thought to get to the decidua via the vessels. Once in the endometrium it is thought that these uNK cells then undergo proliferation (as seen by mitotic figures) and differentiation. NK cells are not solely recruited by the conceptus, as they have been found in conceptus-free decidua (171,172) However it is thought that these cells need the conceptus for sustainability, as without this they show signs of senescence towards end of cycle (173).

Work in porcine endometrium is also supports the concept of trafficking of NK cells from the blood into the decidua (174), however this model uses a completely different form of placentation, involving development of subepithelial capillary plexus, completely non-invasive, contrastingly different to the human placenta.

Differentiation of these uNK cells, in mice is stimulated by IL-15, and mice lacking this interleukin have found to be unable to differentiate their NK cells and as such their arteries resemble those that cannot be modified (175).

There is much evidence regarding the role and function the uNK cells in spiral artery modification and their concentration near vascular structures supports this concept presence near vascular structures.

Conclusively, the work above has been undertaken primarily in murine models and the adhesion factor driving the theory of trafficking depends heavily on the presence of specific adhesion molecules.

1.5.5.2 Proliferation

The presence of NK cells in the human endometrium peaks in the late-secretory phase (176). Though there are only a few CD56Bright cells in the proliferative phase they are still present, it could be that these cells are the remnants from the previous cycle, as the stratum basalis is not shed during menstruation (176).

Mitotic figures are seen and are a recognised feature of premenstrual endometrium. There is an increase in the number of Ki67 (40% based on immunohistochemistry on frozen sections (177)) present in the secretory phase endometrium and deciduas of early pregnancy which supports this theory. Maximum proliferation was also observed in secretory phase endometrium with decreasing numbers with increased gestational age (178).

There are a limited number of CD16+ cells observed in endometrium, at any point in the menstrual cycle. If these CD16+ cells were indeed trafficking from the peripheral blood would there not be increased number of CD16+ (90% of peripheral blood NK cells) cells in the endometrium? The fact that these are lacking would push towards a theory of proliferation of the abundant CD56brightCD16- NK cells in the endometrium. Trafficking supporters may postulate that the peripheral blood NK

cells undergo modification under the uterine microenvironment, in which they subsequently resemble the majority of uNK cells.

Alternatively the rise of CD56BrightCD16- uNK cells could be solely the end result of recruitment of this minute population of peripheral blood NK cells and there subsequent proliferation and differentiation of the more prevalent CD16- into the common uNK cell.

The concentration of IL-15 which stimulates proliferation of uNK cells peaks during the secretory phase (179) at the time when uNK cells density is at its highest.

If indeed these cells are arising from the proliferation of resident uNK cells they could possibly be coming from bone marrow, Taylor (180), found that in 4 women with HLA mismatched bone marrow transplantation, donor HLA-phenotypes were discovered in these women's endometrium, as glandular and stromal cells. Indication that these stem cells can migrate to the uterus and form cells there.

There could also be resident haematopoietic 'stem cells' within the endometrium, from which uNK form and proliferate (152).

1.5.6 uNK cell function

1.5.6.1 Vascular Function

uNK cells tend to localise near blood vessels(181), whether this is because they are recruited from peripheral blood via these vessels or they congregate around the vessels having developed from proliferating resident uNK cells. The link between uNK cells and vessels is further highlighted with the decrease of uNK cells around 20 weeks gestation where vascular remodelling is complete.

Studies in mice(151) have highlighted the importance of uNK and their functional role in the establishment of spiral arteries. Modification of these arteries is essential in the formation of the human placenta. It allows the necessary transfer of nutrients between mother and foetus. Failing to establish good arterial flow from mother to foetus can result in complications for the mother e.g. pre-eclampsia, and complications for the foetus, IUGR and miscarriage. uNK cells have been found to produce a number of key angiogenic factors such as VEGF-C, placental growth factor, angiopoietin 1 and 2 and transforming growth factor β (131,181,182) in the non-pregnant uterus as well as in early pregnancy decidua.

Angiopoietin is only expressed by those tissues with vessels undergoing remodelling, therefore it may be an important mediator for spiral artery transformation.

Hiby *et al.* (183) compared the genotypes of maternal uNK cell in patients with and without pre-eclampsia, they found that patients with the genotypes that were inhibitory on uNK cells had higher rates of pre-eclampsia. This would suggest that uNK cells that are maximally inhibited allow trophoblast to prematurely cease remodelling of spiral arteries pre-disposing to pre-eclampsia.

Interferon γ , produced by uNK cells is also thought to play a role in artery modification as shown in mice studies, where those mice deficient in either this cytokine or uNK cells had abnormalities in implantation site and lack of decidual artery transformation.

A recent study conducted by Qeunby *et al.* 2009 (181), showed an association between uNK cell density and the formation of blood vessels, lymphatics, spiral artery smooth muscle differentiation and endometrial oedema. Additionally increased density of uNK cells associated with a clinically reduced uterine artery resistance to blood flow. A theory proposed by this research group into how this directly leads to a negative reproductive outcome is one of excess blood flow leading to oxidative stress on the feto-placental unit.

1.5.6.2 Trophoblast Development and implantation

Early trophoblastic invasion is necessary for implantation of the embryo into the uterine wall. Lack of receptivity of the uterus to the conceptus or lack of adhesion from embryo to deciduas can result in failed implantation.

With uNK cells being the predominant leukocyte population in the uterus in the first trimester, they have been considered to play a substantial role in implantation and early placentation (184).

It has been suggested that uNK cells are not directly involved in the process of implantation (184). uNK cells have been shown, in mice, unnecessary for implantation, as uNK-deficient mice became pregnant. However uNK and IL-15 deficient mice were unsuccessful in pregnancy (175).

Also, in humans, at site of implantation in ectopic pregnancies, uNK cells are not present in the large numbers they are in uterine pregnancies. They are also present in large number in the uterus, despite a non-uterine (ectopic) implantation (185,186).

1.6 Methodological Limitations of Previous Studies

Flow cytometry is often used to analyse uNK cells, but this method has some disadvantages compared to immunohistochemistry including, the possibility of incorporation of other cell types cannot be excluded as the cell sorting is rarely 100% pure, and mechanical and enzymatic processing of the tissue will lead to loss of information regarding certain antigens and location of cells. Hence there are discrepancies between studies using immunohistochemistry and flow cytometry.

CD69 was found on uNK cells by flow cytometry (187,188) but found predominantly on T cells within the endometrium, when using immunohistochemistry (189).

The advantages of using immunohistochemistry over flow cytometry, is that you are able to look at the tissue as a whole and the interaction between cellular activity and structure.

1.7: Conclusion

The role and value of uterine natural killer cells in reproductive failure and especially recurrent implantation failure is a contentious issue. Since their discovery in the endometrium nearly 100 years ago, scientists and clinicians alike have been trying to locate their function and influence on the fetomaternal interface.

The origins of these cells further sits in controversy, with current speculation surrounding two main theories. These cells are coming from the peripheral blood and trafficking their way into the endometrium to exert their effect or they are proliferating and differentiating from cells already resident in the uterus, possibly from the stratum basalis which is not shed during menstruation.

The clinical implication on discovering the origins of these cells is important. There are currently many centres offering peripheral blood testing for NK cells (119) and attaching prognostic value to these tests, which is yet to be adequately proven. Patients with recurrent implantation failure not only have the cost of their emotions but the additional costs of these tests to contend with, if the NK cells in the blood bear little, if no resemblance bar a shared name to uNK cells then these tests must stop and focus needs to be drawn back to the endometrium.

It is therefore necessary to investigate the character (expression of key receptors Ki67, CD16, CD62-L, NKp30) and location of these cells to determine primarily the origin of these cells and their spatial relationship within endometrial tissue.

1.8: Hypothesis and Aim

1.8.1 Hypothesis

We have two competing hypotheses:

1. Uterine natural killer cells in the endometrial tissue are the result of proliferation of resident uNK cells
2. Uterine natural killer cells in the endometrial tissue are from the trafficking of peripheral blood cells into the uterus and their subsequent differentiation into their uNK form.

1.8.2 Aim

We aim to test these hypotheses by using immunohistochemistry to compare antibodies associated with trafficking: L-Selectin, with a marker for peripheral blood NK cells, CD16, against markers for proliferation Ki67 and differentiation NKp30, on endometrial tissue, using CD56 as the universal marker for natural killer cells. The endometrial tissue will be obtained 7 days after the LH surge in the luteal phase as this is the window of implantation.

Furthermore we aim to compare and contrast the occurrence of these markers in the tissue of patients already identified as having 'high' and 'low' density uNK cells.

Additionally we aim to investigate the spatial relationship of these cells to three main structures within endometrial tissue: sub-epithelial, areas with low stromal density and perivascular stroma in both groups.

If hypothesis 1 is correct then the majority of uNK cells will be positive for ki67 and NKp30 and very few positive for L- selectin or CD16. Further more in cases with high density of uNK cells there will be more KI67 positive cells.

If hypothesis 2 is correct then the NK cells surrounding the blood vessel will be highly positive for L- Selectin and CD16 and those away from the blood vessels in areas of stromal oedema and subepitheelial areas will show increased markers of differentiation (NKp30) and loss of CD16.

CHAPTER 2: METHODS

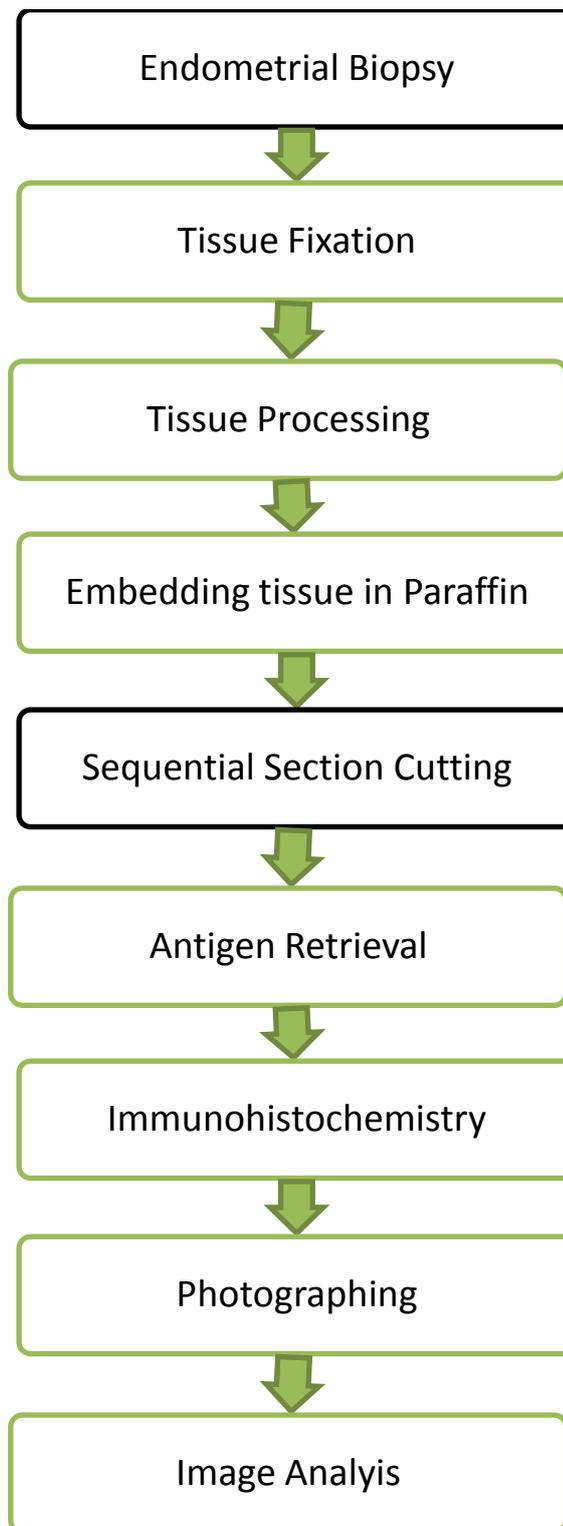


Figure7: Schematic of process from tissue sampling to image analysis, all steps in green carried out by myself with resources from the tissue bank also, all steps in black carried out by a member of the department.

2.1 Patient Selection

From a database and tissue bank of 500 patients with endometrial samples taken at LH+7, 19 patients were selected who fit the criteria detailed below. The population comprised of a group of 9 high uNK cell-density patients and a group of 10 low uNK cell-density patients.

Inclusion Criteria: Patients had to have recurrent implantation failure, defined as having a history of infertility and at least 3 failed attempts at IVF including at least 5 failed embryo transfers of apparently good quality embryos. uNK result of >5% (136) for 'high' patients and <3% for 'low' uNK patients. Patients were biopsied at days LH+ 7 +/- 2 days.

Exclusion Criteria: Mixed diagnosis of both IVF failure and miscarriage. Patients who did not have enough paraffin embedded tissue in the tissue bank.

2.2 Endometrial Biopsy

Consent was taken from each patient prior to performing the biopsy.

At sample collection all women had a transvaginal scan to ensure normal endometrial thickness, as assessed by the attending clinician.

The samples were obtained using a Wallach endometrial sampler to take a biopsy of the endometrium.

Prior to biopsy all women were asked to do a home LH urine test and then the biopsy was taken 5 to 9 days after this. These cycles were 'out-of-treatment' cycles and so not influenced by hormone therapy. The mid-luteal phase was chosen because it is important to investigate the pathology in this 'window of implantation'.

After obtaining the biopsy, the sampler was immediately transferred to the laboratory for freezing and fixation according to the procedure described below:

2.3 Endometrial Tissue Reception

Correct and rapid fixation of tissues is necessary to preserve the structure of the tissue and cell morphology. If tissue fixation is delayed it may lead to a decrease in the antibody binding capacity(190). This is why all specimens were fixed in 10% NBF within 20 minutes of the endometrial sample being taken.

Equipment

- Virkon and laboratory plastics (e.g. universals etc) are purchased from Liverpool Women's Hospital (NHS) purchasing department.
- Forceps and scissors are supplied by Raymond A Lamb Ltd. Manufacturer's address: Units 4 & 5, Parkview industrial estate, Eastbourne, East Sussex, BN23 6QE England.
- TriGene Advance is obtained from Medichem International. Supplier address: PO Box 237, Seven Oaks, Kent, TN15 0ZJ. Telephone: 01732 763555.
- Neutral Buffered Formalin solution is purchased from Sigma Aldrich. Supplier address: Fancy road, Poole, Dorset, BH12 4QH. Telephone 01202 712300.
- Biomat² Recirculating microbiological safety cabinet was purchased from Bioquell. Supplier information: Walworth road, Andover, Hampshire, SP10 5AA.

Laboratory Preparation:

The surface was cleaned Clean the surface of the microbiological safety class II (MCII) cabinet with 1% TriGene Advance solution and dried with absorbent towels.

The fume hood was set up to contain the following:

- Universal tube containing 4% neutral buffered formalin (NBF). The Universal should be labelled with the date and the RM number.
- Small weighing boats

- Forceps and scissors
- Discard pot containing 1% Virkon solution.
- Equipment decontamination container (small plastic container filled to approximately 50% capacity with 2% TriGene Advance Solution).
- Small bottle of sterile PBS
- A labelled Nunc cryotube and pair of clean forceps are placed in a box of dry ice.
- Liquid nitrogen.

Biopsy Processing Procedure:

The biopsy was removed from the Wallach endometrial sampler and washed with sterile PBS if a large amount of blood was present. Mucous and blood clots were removed from the tissue using forceps. Tissue was then placed in a labelled tube of 4% NBF for 24 hours. If there was excess tissue available then it was frozen. The tissue to be frozen was then placed in a clean weighing boat and frozen using liquid nitrogen, to be retrieved with forceps and placed in a Nunc cryotube to be stored in a -80°C freezer. All information was recorded in a database and consent forms filed away in a locked cabinet.

2.4 Tissue Processing

Formalin fixed tissue was dehydrated, cleared then impregnated with paraffin wax using the automated Shandon Citadel 1000 processing machine according to the schedule detailed below. The total processing time was 18 ¾ hours.

Processing Schedule:

Table 4: Schedule of automated processor to process NBF fixed endometrial samples:

Step	Solution	Time
1	4% formalin in neutral buffer	45 minutes
2	60% Ethanol	1 hour
3	70% Ethanol	1 hour
4	90% Ethanol	1 hour
5	100% Ethanol	1 hour
6	100% Ethanol	1 ½ hours
7	100% Ethanol	2 hours
8	Xylene 1	1 hour
9	Xylene 2	1 ½ hours
10	Xylene 3	2 hours
11	Wax 1	2 ½ hours
12	Wax 2	3 ½ hours

Equipment and solvent information:

- Citadel processor manufacturer: Thermo electron Corporation.
Manufacturer's address: Chadwick Road, Astmoor, Runcorn, Cheshire, WA7 1PR England
- Ethanol and xylene are purchased from Chemistry solvent stores (University of Liverpool).
- The wax (Paramat Paraplast Gurr) is purchased from Histoplast PE which comes from Thermo.

Preparation of Solvent Containers on Processing Machine:

To access the processor solvent containers and check solution levels, the **raise** button was pressed. The buttons: **rotate** or **check/fill** were used to check each solution in turn and they were topped up/changed if required.

Sample Processing Procedure

Plastic cassettes were labelled with unique sample ID using pencil. Tissue was then retrieved from the formalin and placed into small wire inserts and subsequently placed into the plastic cassettes, which were then placed in the processing baskets and lowered into slot 1. The processor was started. When the processing cycles were completed the cassettes were taken out and placed on the heating element in the Shandon Histocentre 3 embedding machine.

2.5 Embedding

Paraffin embedding allows adequate demonstration of most tissue antigens with the use of antigen retrieval. Some cell antigens do not survive fixation and paraffin embedding, and require samples to be frozen with liquid nitrogen. However cutting these frozen samples into sections is very difficult, with sequential sectioning almost impossible, also the cell morphology and tissue structure is very poor(191), this is why paraffin sections are better when needing to analyse tissue structure.

Procedure

The endometrial tissue samples were embedded using the Shandon Histocentre 3 embedding machine. The samples were placed into the heated reservoir of the machine to stop the wax from setting. The sample was removed from the cassette using forceps and transferred to a small metal mould on the cooling unit. Hot wax was then applied over the sample, then the same cassette that initially housed the sample was placed above it, using forceps to compress it over the wax. Tapping of the cassette was done to remove any air bubbles. The cassette and wax block was left to cool on the cooling compartment. Once fully cooled the cassette and wax block separated from the metal mould. They were then stored in the fridge prior to having sections cut.

2.6 Cutting Sections

- Micron HM335 rotary microtome is supplied by MICROM company. Manufacture's address: Microm UK Ltd, 8 Thame Park Business Centre, Leinman Road, Thame, OX9 3XA. <http://www.micron-online.com>
- Microscope slides (super premium twin frost, catalogue number 631-0111) are purchased from VWR International. UK based distributor is located at: Hunter Boulevard, Magna Park, Lutterworth, Leicestershire, LE17 4XN.
- Microtome blades (feather, stainless steel S35) are purchased from Liverpool Women's Hospital (NHS) purchasing department.
- Forceps, paintbrush, section dryer and water bath are supplied by Raymond A Lamb Ltd. Manufacture's address; Units 4 & 5, Parkview industrial estate, Eastbourne, East Sussex, BN23 6QE England.

The water bath set was set at 40°C prior to commencing cutting.

The cassettes were placed into the cutting machine and trimmed back until the tissue was seen and then placed in the fridge for cooling. Sections were cut at 4µm thick, sequentially and placed on to the surface of the water bath. They were then picked up with slide pre-coated with APES (3-aminopropyltriethoxysilane) solution, in the same sequence in which they were cut, they were then labelled and left to dry.

2.7 Immunohistochemistry(192)

Immunohistochemistry is the detection of antigens using specific antibodies and reagents.

The demonstration of antigens can be greatly improved by the process of antigen retrieval. This involves the pretreatment of the tissue sections with an antigen retrieval agent, in this case Citrate Buffer in heat (Heat Induced Epitope Retrieval). A pressure cooker allows adequate temperature to be reached in order to break cross-links formed between proteins in formalin fixation, uncovering antigenic sites.

Background staining can be common in samples that were inadequately fixed. This can result in false positive staining, interfering with results. This can be minimised with the use of the blocking agent Hydrogen Peroxide prior to the application of the primary antibody. This eliminates the endogenous peroxidase activity, which is found in many tissues.

Controls were used in every staining run. Positive controls were used to ensure staining intensity was consistent and if staining was absent on tissues it was a tissue-specific result and not the result of the failure of a specific antibody to stain to specific antigen, i.e. to make sure it was a true negative. The positive control was most commonly a high uNK density sample which was known to stain well with the antibodies used. In all validation staining tonsil was used as the positive control, as this is a known positive control for natural killer cells antigens. Negative controls were used in the form of IgG antibody, which would highlight any staining due to other factors than the primary antibody being studied.

There are two main methods of staining in immunohistochemistry. Direct method involves a simple one-step procedure with a labelled antibody reacting directly with a specific antigen. This procedure is quick but not sensitive due to poor amplification. The Indirect method involves using an unlabelled primary antibody (first layer), this reacts with the specific tissue antigen being studied, then a second layer is added, the labelled secondary antibody (either goat or mouse polymer HRP

– respective species to the antibody being used) which reacts with the first layer. This is much more sensitive due to the amplification signal thorough several secondary antibody reactions

Procedure

Prior to immunohistochemical staining, slides were baked for 1 hour at 60°C or overnight at 37°C to improve adhesion. They were then dewaxed and rehydrated (see below) and pressure cooker treated in citrate buffer (pH=6.0 see below), brought to boil and immersed for 1 min.

Table 5: Dewaxing and Rehydrating schedule:

Solution	Time
Xylene	10mins
Xylene	10mins
100% Ethanol	3mins
100% Ethanol	3mins
90% Ethanol	1mins
70% Ethanol	1mins
Water	

Table 6: Citrate Buffer components:

Component	Manufacturer	Quantity
Citric Acid	VWR, Magna Park, Lutterworth, Leicestershire, LE17 4XN	12.0g
dH₂O		1.5L
pH 6	VWR, , Magna Park, Lutterworth, Leicestershire, LE17 4XN	

Equipment:

Staining dishes, Humidified chamber, Cover slips

Antibody information:

Validation for each antibody was performed to find the optimum concentration to produce the best results. The varying concentrations are detailed below.

For each antibody validation staining procedure, tonsil was used a positive control.

Table 7: Antibodies used and their respective concentrations used in validation:

Antigen	Antibody / Catalogue Number	Company	Dilution For validation	Polymer	Clone
Ki67	NCL-Ki67- MM1	Novocastra	1:100, 1:150, 1:200, 1:400	Mouse	MM1
CD56	NCL-L- CD56-1B6	Novocastra	1:50	Mouse	1B6
CD16	DJ13OC	Dako	1:50, 1:100, 1:200	Mouse	VIFcRIII
CD62-L	AB49508- 500	Abcam	1:25, 1:50, 1:100	Mouse	9H6
NKp30	SC-20477	Santa Cruz	1:100, 1:200, 1:400	Goat	Sc-24077
Mouse IgG	MCA928	Serotech	1:100	Mouse	IgG1
Goat IgG	Sc-2028	Santa Cruz	1:200	Goat	Sc-2028

Table 8: Antibodies used and their respective concentrations to investigate hypothesis:

Antigen	Antibody / Catalogue Number	Company	Dilution	Polymer	Clone
Ki67	NCL-Ki67-MM1	Novocastra	1:150	Mouse	MM1
CD56	NCL-L-CD56-1B6	Novocastra	1:50	Mouse	1B6
CD16	DJ13OC	Dako	1:100	Mouse	VIFcRIII
CD62-L	AB49508-500	Abcam	1:25	Mouse	9H6
NKp30	SC-20477	Santa Cruz	1:100	Goat	Sc-24077
Mouse IgG	MCA928	Serotech	1:100	Mouse	IgG1
Goat IgG	Sc-2028	Santa Cruz	1:200	Goat	Sc-2028

For each antibody staining procedure, a known 'high' uNK density patient was used as a positive control.

Table 9: Solutions used in detection kit

Detection Kit	Kit /cat#	Company	Dilution	Duration
DAB EnVision (Substrate & Chromagen)	K4006	Dako	1:50	10 mins
Dako anti-mouse	K4003	Dako	1 Drop	10 mins
Vector IMMPress Anti-Goat Ig (Peoxidase)	MP-7405	Vector Labs	1 Drop	30 mins

Table 10: Tris Buffered Saline components:

Component	Manufacturer	Quantity
Tris	VWR, Magna Park, Lutterworth, Leicestershire, LE17 4XN	12.0g
NaCl	VWR, Magna Park, Lutterworth, Leicestershire, LE17 4XN	17.4g
dH₂O		1.5L
pH 7.6 → add 500mL dH₂O to make 2L		

Procedure:

Slides were placed into a staining dish containing TBS and incubated for 5 minutes at room temperature. The slides were then incubated in a 0.3% H₂O₂/TBS for 10 minutes at room temperature. The slides were then reincubated in TBS for a further 5 minutes, and then each specimen was outlined with a hydrophobic marker pen (DAKO). The slides were incubated in TBS for 5 minutes. They were then placed in the humidifying chamber, and antibody (see table of antibodies above) (made with antibody diluent TBS/0.5 % BSA and respective antibody in respective concentration) was applied, 50 µl per section. These slides were then incubated with the antibody for 60 minutes at room temperature. Antibody was tapped off and the slides were then placed in TBS for 5 mins, decanted and refilled with TBS.

The slides were then returned to the humidifying chamber and 1 drop of labelled polymer-HRP (see table above) was applied. These slides were then incubated for 30 minutes at room temperature. The polymer was then tapped off and the staining dish was filled with TBS, incubated for 5 minutes, decanted and refilled with fresh TBS and incubated for a further 5 minutes. The slides were then returned to the humidifying chamber and substrate/chromagen solution (20 μ l/1ml substrate) 50 μ l per section was applied. After 10 minutes the slides were immersed immediately in water to stop the reaction. The slides were then incubated for 1 minute, 30 seconds in Gill 2 filtered haematoxylin. The slides were then rinsed with water until the water runs clear, then dipped in acid alcohol briefly, and immersed immediately back in tap water for several minutes to allow the haematoxylin to “blue”. The slides were then put through the dehydration procedure at the following:

Table 11: Dehydration Procedure:

Solution	Time
70% Ethanol	1 mins
90% Ethanol	1 mins
100% Ethanol	3 mins
100% Ethanol	3 mins
Xylene	5 mins
Xylene	10 mins

Slides were then wet mounted by placing a cover slip upon each section with the aid of DPX, and air bubbles removed.

2.8 Photographing

Images were photographed using Nikon technology (Nikon eclipse 50i, lens is Nikon DSFi1) and NIS elements F2.30 software with the following settings:

Table 12: Settings used in image capture of stained endometrial sections:

Feature	Level
Mode	Normal
Fast(focus)	640x480 pixels
Quality Capture	1280x960 pixels
Mode	Autoexposure
AE Compensation	+1.6v
Exposure	3ms
AE Lock	Yes
Gain	1.70x
Colour Contrast	High
Scale	1:1

Autowhite using 'AE', focus to full level and capture, all images are saved as .tiff files.

Location of Areas:

To enable accurate photographing, a log was produced enabling a record to be kept for each patient photographed. The purpose of this log was to prevent the same area being photographed twice and labelled as two separate areas. Initially a rough diagram of the section of tissue was drawn and areas on sequential tissue where tissue had not adhered to the slide, or other artefact, was highlighted on the diagram, to prevent the photographing of this area. The log also allowed comments to be made regarding any unusual staining or interesting differences in staining or general tissue appearance.

Subepithelial: Tissue was orientated to view an area with epithelial edge, corresponding sequential sections to CD56 were also checked to observe adequate tissue to photograph.

Low stromal cell density: As above tissue was orientated to view areas of low stromal cell density and the same area located on corresponding CD56 stained sections before photographing.

Perivascular: As above, with areas of high-density vascular structures.

In all the locations above the confounding influence of these structures overlapping in fields were minimised by excluding areas where more than one structure was visible in field, where possible.

2.9 Image Analysis

Image analysis software was chosen to analyse micrographs because this offers the benefit of reducing inter-observer variability. This method has previously been validated by our department. There are two parts to image analysis, firstly to produce a number for the count of stromal cells and secondly to count positively stained cells, with the aim to produce a percentage stromal staining for each micrograph, each stain for each area to be looked at.

The advantage of producing three micrographs per stain per location, would enhance accuracy by enabling to use this average in the analysis of results.

Equipment Information

Setup 1

- Nikon Biophot Microscope, Nikon Corporation, Tokyo 100-8331, Japan
- Nikon DS-5M camera head 5M pixel, Nikon Corporation, Tokyo 100-8331, Japan
- Nikon Digital Sight DS-U1 digital control unit, Nikon Corporation, Tokyo 100-8331, Japan
- Nikon C-Mount TV adaptor 0.6x, Nikon Corporation, Tokyo 100-8331, Japan
- Eclipsenet software, developed by Laboratory Imaging s.r.o. for Nikon Instruments Europe B.V., 1170AE Badhoevedorp, The Netherlands
- Adobe Photoshop CS2 software, Education Version. Adobe Systems Incorporated. 345 Park Avenue, San Jose, CA 95110-2704 USA www.adobe.com

Setup 2 (supplied by Jencons-PLS)

- Nikon Eclipse 50i Microscope, Nikon Corporation, Tokyo 100-8331, Japan
- Nikon DS-Fi1 digital camera Head 5M pixel, Nikon Corporation, Tokyo 100-8331, Japan
- Nikon Digital control unit DS-U2 USB, Nikon Corporation, Tokyo 100-8331, Japan
- Nikon C-Mount TV adaptor, 0.63x, Nikon Corporation, Tokyo 100-8331, Japan
- NIS-Elements-F software, developed for Nikon Instruments
- Adobe Photoshop CS3 software, Education Version. Adobe Systems Incorporated. 345 Park Avenue, San Jose, CA 95110-2704 USA www.adobe.com

Both systems

- Personal computer (minimum specification 1GB RAM, 2.8GHz processor)
- WCIF Image J <http://www.uhnresearch.ca/facilities/wcif/fdownload.html>

Procedure: Stromal Count

Adobe Photoshop was used to prepare the micrographs for image analysis. Each file to be analysed was opened in Adobe Photoshop and had each gland and vessel manually removed by using the Magic Lasso tool, creating an outline of each structure, to be deleted. Following this, it was important to remove the DAB staining to just leave the stromal cells. This was achieved by using the Magic wand tool, to select and delete areas of 'brown' positive staining. At each step images were saved in TIFF format, to prevent detail being lost in other compression formats.

To obtain a count for the stromal cells, ImageJ software was used. Each file with DAB removed (stroma images) were opened in ImageJ and subsequently saved as 8-bit grey scale images (Image|Type|8 bit). This allows a threshold to be applied to the image (Image|Adjust|Threshold) set at level which accurately represents the majority of cells visible in 'red' without background being selected also. The threshold number was noted to enable adjustment at a later stage if necessary. If needed 'smooth' function was applied to smooth edges and have more 'cellular' outlines. The threshold was then applied. If a significant proportion of the cells are patchy they can be filled using Process|Binary|Fill. To enable distinct cells to be counted, instead on one cluster, cells were separated using the watershed function (Process|Binary|Watershed). To count the number of nuclei, Analyze Particles (Analyze|Analyze Particles) was used with the following settings (Setup 1 Size: 0.0015-0.1, Setup 2 Size: 36-2250, with both setups using the following: Circularity: 0.3-1, Show outline, Clear results, Summarise). An outline of the nuclei was then displayed, allowing, after inversion, colour merging of this image onto the original, to check the majority of cells were correctly identified. A summary box showing

total count was then available, providing the total number of stromal cells identified. This number was noted down.

Procedure: positively stained cell count

ImageJ software was also used for the identification of positively stained cells. Each original file was opened up and positive cells counted using the 'point picker' tool (Plugins|Particle Analysis|Pointpicker), after applying a grid (Plugins|Particle Analysis|Grid Average per point = 15170, pixel width = 2). For cytoplasmic markers, NKp30 and Ki67, positive stained cells are those in which there is a brown stained nucleus instead of the haematoxylin stained blue stromal nuclei. Cytoplasmic markers CD56, CD16 and CD62-L/L-Selectin are cytoplasmic markers. Positively identified cells were marked with a cross by left-mouse clicking on the cell. Markers can be removed and moved by selecting corresponding buttons on the menu bar. When finished selecting positively stained cells, a results box was obtained by selecting 'show' on the menu bar. The total number of cells identified was noted.

To obtain a percentage of stromal cells stained divide the stained cell count by the total stromal count and multiplied by 100.

2.10 Statistical Methods Used For Analysis

The statistical analysis was carried out using SPSS version 16.

More than one measurement was performed per sample patient. This varied from 2 to 9 replicates per condition. The mean value was calculated for each condition for each individual and this value was used in the analysis.

To detect a relationship between the age of the sample population and uNK cell density, a correlation analysis, using Spearman rank was performed.

To validate my measuring of the density of uNK cells per field, comparisons were made against experienced cell counters within the department. Bland-Altman plots were produced to highlight any systematic differences in cell counts resulting from methodical differences.

Graphical representation of the expression of each antibody in each location was then prepared categorising by 'high' or 'low' uNK cell density group, with errors bars at 95% confidence intervals.

Analysis of Variance was used with a contrast. There was a degree of repeated measurement in the analysis because each woman contributed data for each antibody in three conditions. Therefore study patient number was used as a covariate, a practical measure to overcome any effects of repeated measurements. Post hoc tests were not possible with a covariate, therefore a simple contrast was used to compare the location sites, with epithelium set as a reference group.

An interaction term was also included to determine whether the difference in location were attributable to uNK cell density groups. This interaction term was not significant in any of the analyses and so is not presented in the results.

CHAPTER 3: RESULTS

3.1 Patient Demographics

Table 13: Demographic distribution of the selected patients:

Patient Demographics	High Group	Low Group
Age	37 (n=23-45)	37.5 (32-41)
uNK count	7.7 (6.1-11)	2.2 (0.3-3.1)
Number (n)	9	10
IVF Cycles	3 (3-6)	4.5 (3-12)
Embryo Transfers	8 (6-12)	8.5 (6-33)

The median values for embryo transfer and age were the same amongst both ‘high’ and ‘low’ density uNK groups. Additionally the median number of IVF cycles undertaken and previous embryo transfer attempts by these patients were similar in both the ‘low’ and ‘high’ uNK groups.

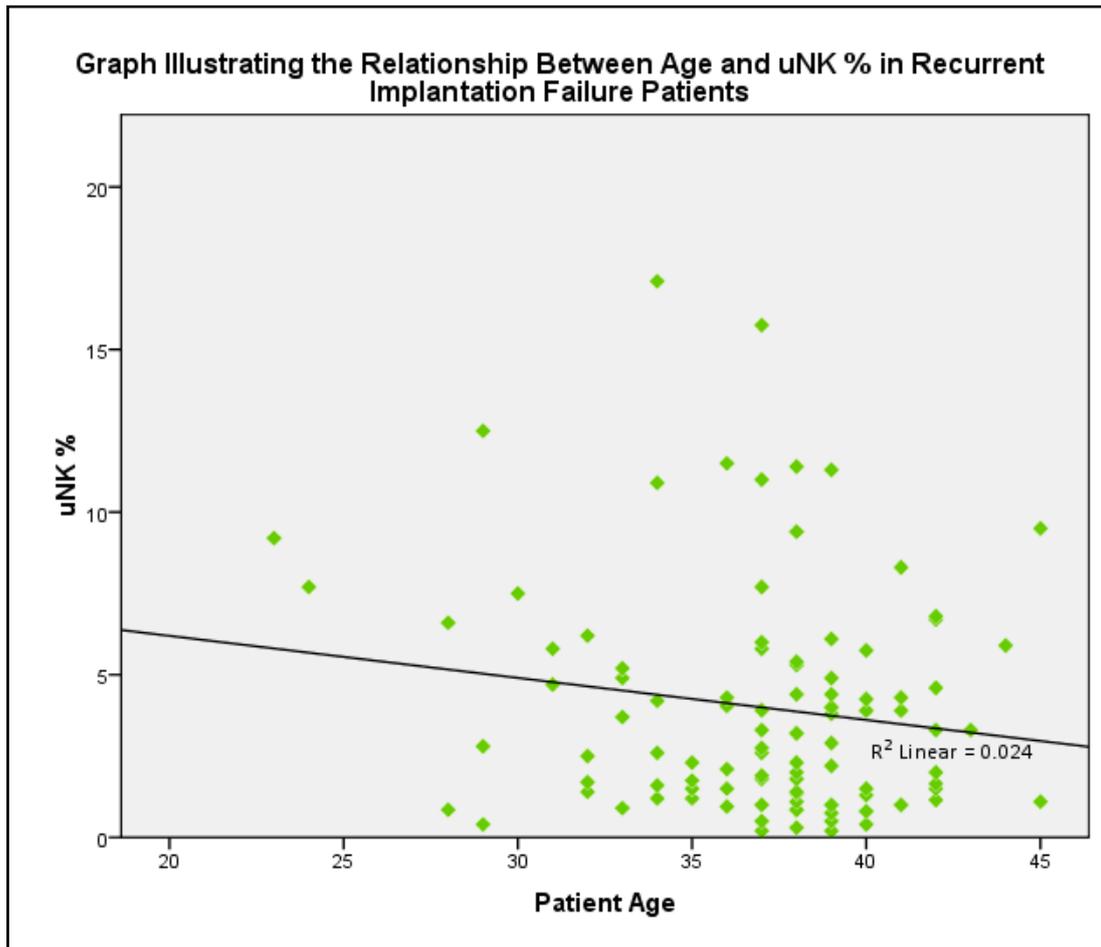


Figure 8: Graph illustrating the relationship between age and uNK % in recurrent implantation failure patients.

Spearman rank correlation was performed comparing age and IVF failures in patients to determine whether 'age' would be a confounding variable (see Figure 8 above). Many believe that the quality of eggs produced by females of a younger age are better than women of an older age (193). On a database of 101 recurrent implantation failure patients, statistical tests were performed to detect the level of correlation. A correlation coefficient of -0.087 was detected which had no statistical significance ($p=0.387$). This shows, that in our sample population, uNK cell values were not attributed to age.

3.2 Image Analysis Validation

Bland Altman Plots

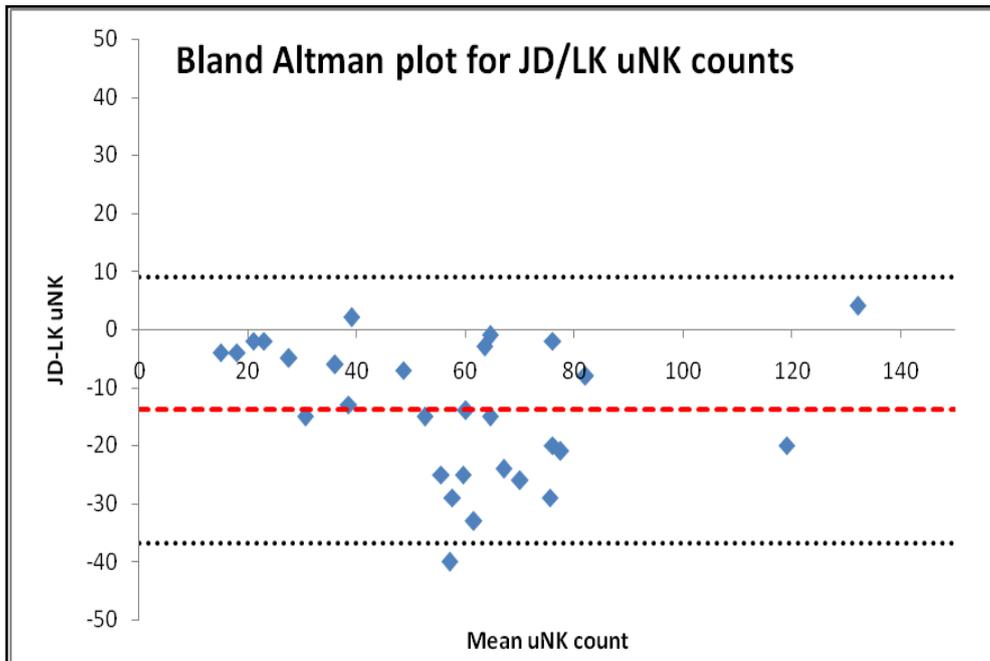


Figure 9: Bland Altman plot for JD/LK uNK counts

The stromal count (counting stromal cells) was based on a selection of 78 images, and compared to JD (who was already validated using the same procedure and same selection of images) to ensure direct comparison (see Figure 9 above). As can be observed on the plot above most values centre closely the mean, outliers are not common.

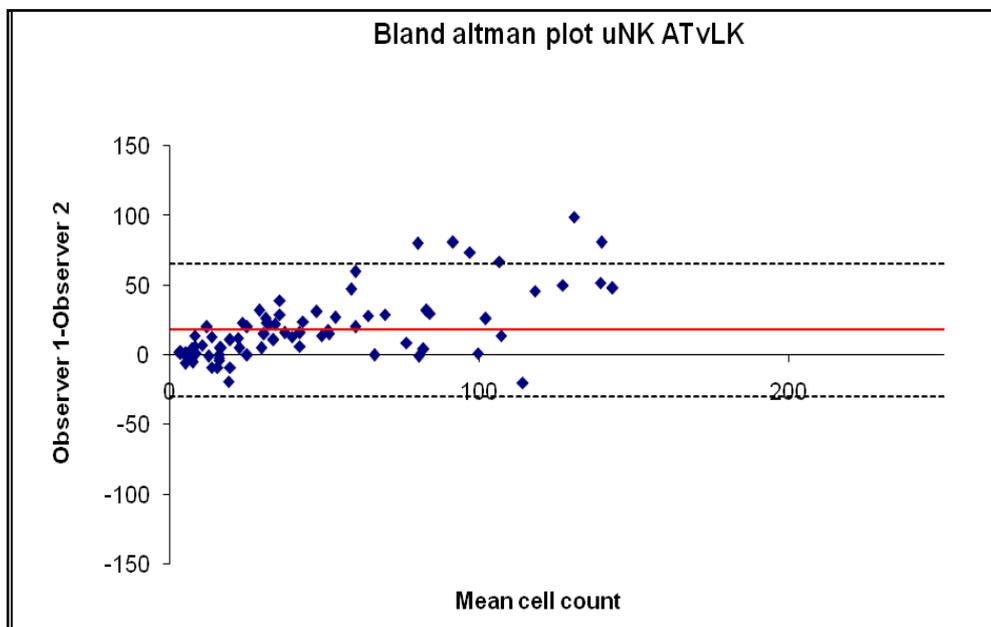


Figure 10: Bland Altman plot for AT/LK uNK counts

The uNK count (counting positive cells) was based on a selection of 61 images, and compared to AT (who was already validated using the same procedure and same selection of images) to ensure direct comparison (see Figure 10 above). As can be observed by the plot above there is a close association between both interpreters at lower levels of uNK cell counting, with observer variance increasing at higher levels of cell density, as can be expected.

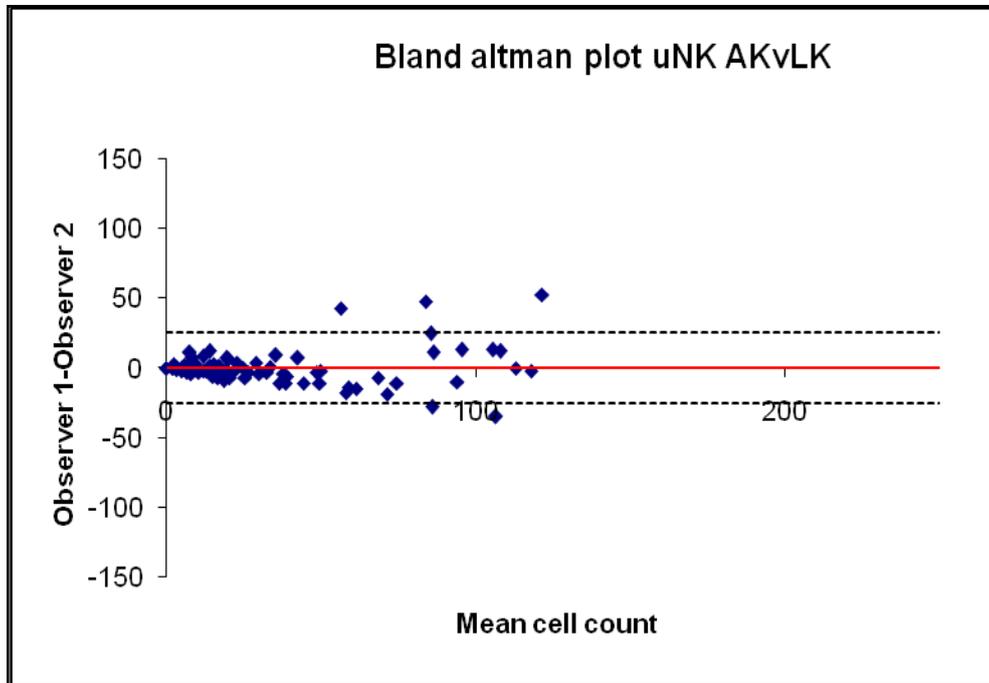


Figure 11: Bland Altman plot for AK/LK uNK counts

The uNK count (counting positive cells) was based on a selection of 61 images, and compared to AK (undergoing validation at the same time point) (see Figure 11 above) using the same procedure and same selection of images to ensure direct comparison. As can be observed on the plot above there is a close association between both interpreters, ensuring consistency of results.

3.3 Micrographs

Sample Results:

Low 'uNK' patients:

Epithelial Edge

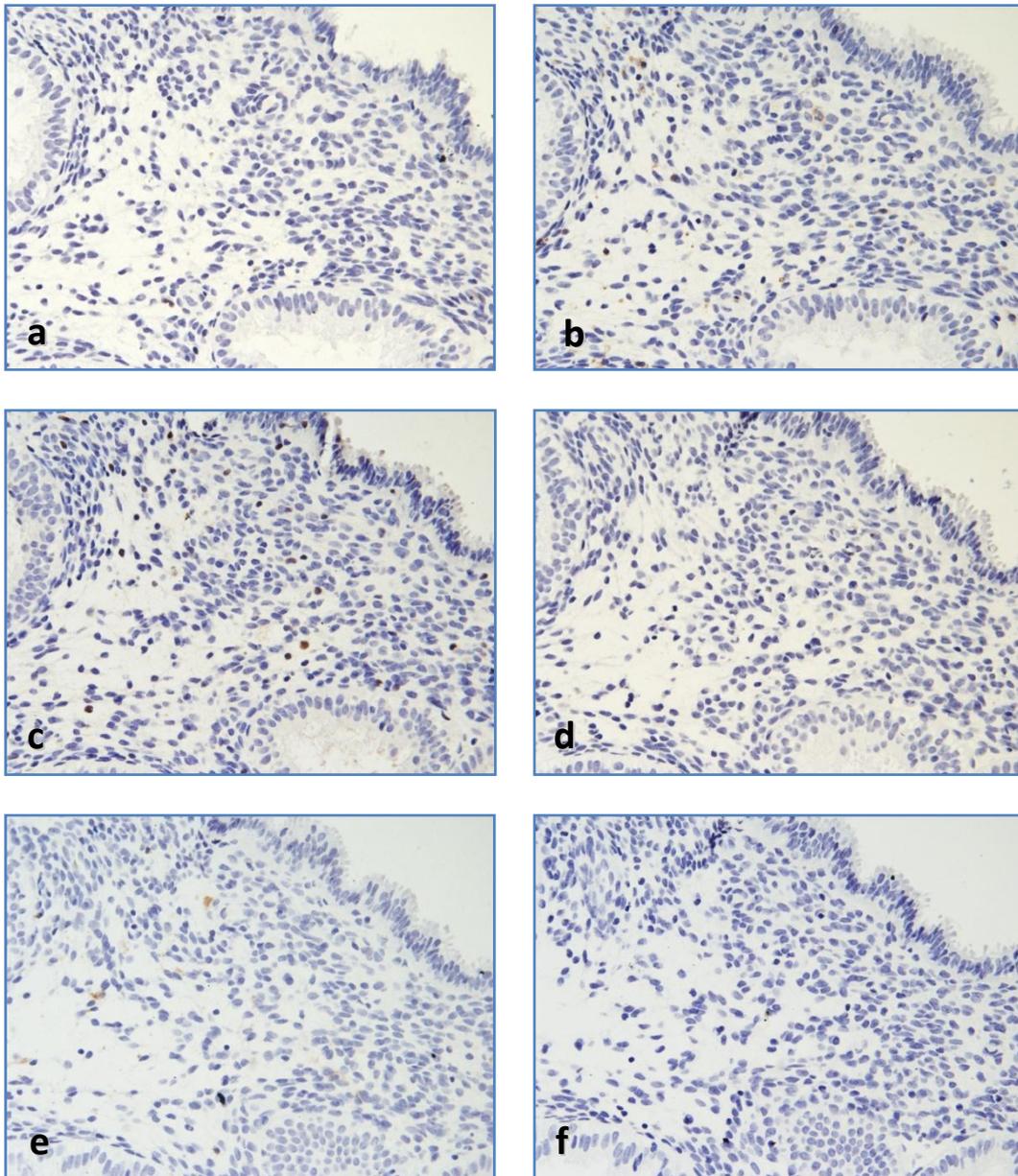


Figure 12: Micrographs showing subepithelial location of uNK Cells in a 'Low' patient, with the following stains: a) Ki67, b) CD56, c) NKp30, d) CD62-L, e) CD16 f) IgG Mouse (negative control)

Perivascular:

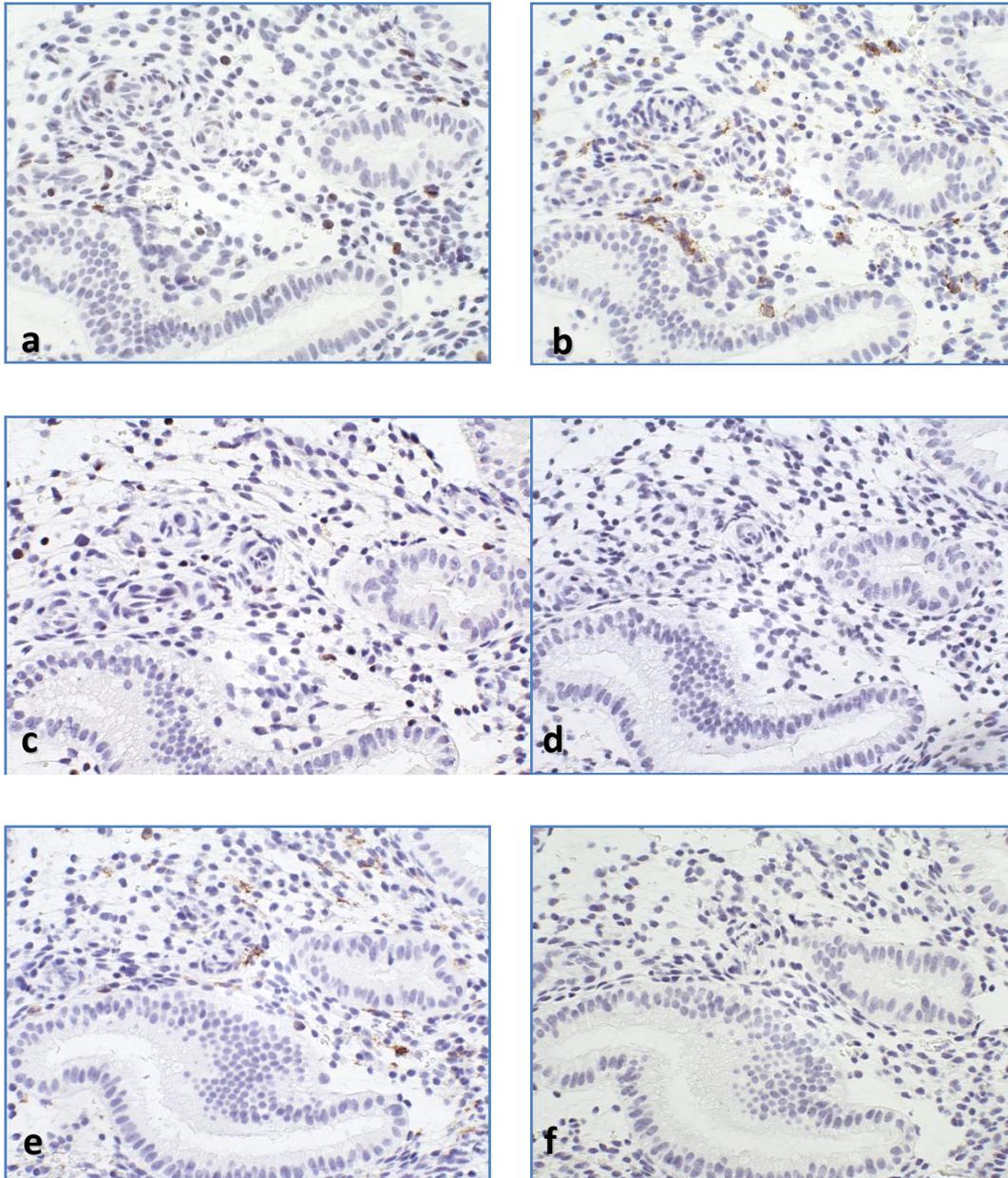


Figure 13: Micrographs showing perivascular location of uNK Cells in a 'High' patient, with the following stains: **a)** Ki67, **b)** CD56, **c)** NKp30, **d)** CD62-L, **e)** CD16 **f)** IgG Mouse (negative control)

Areas of low stromal density (Oedema):

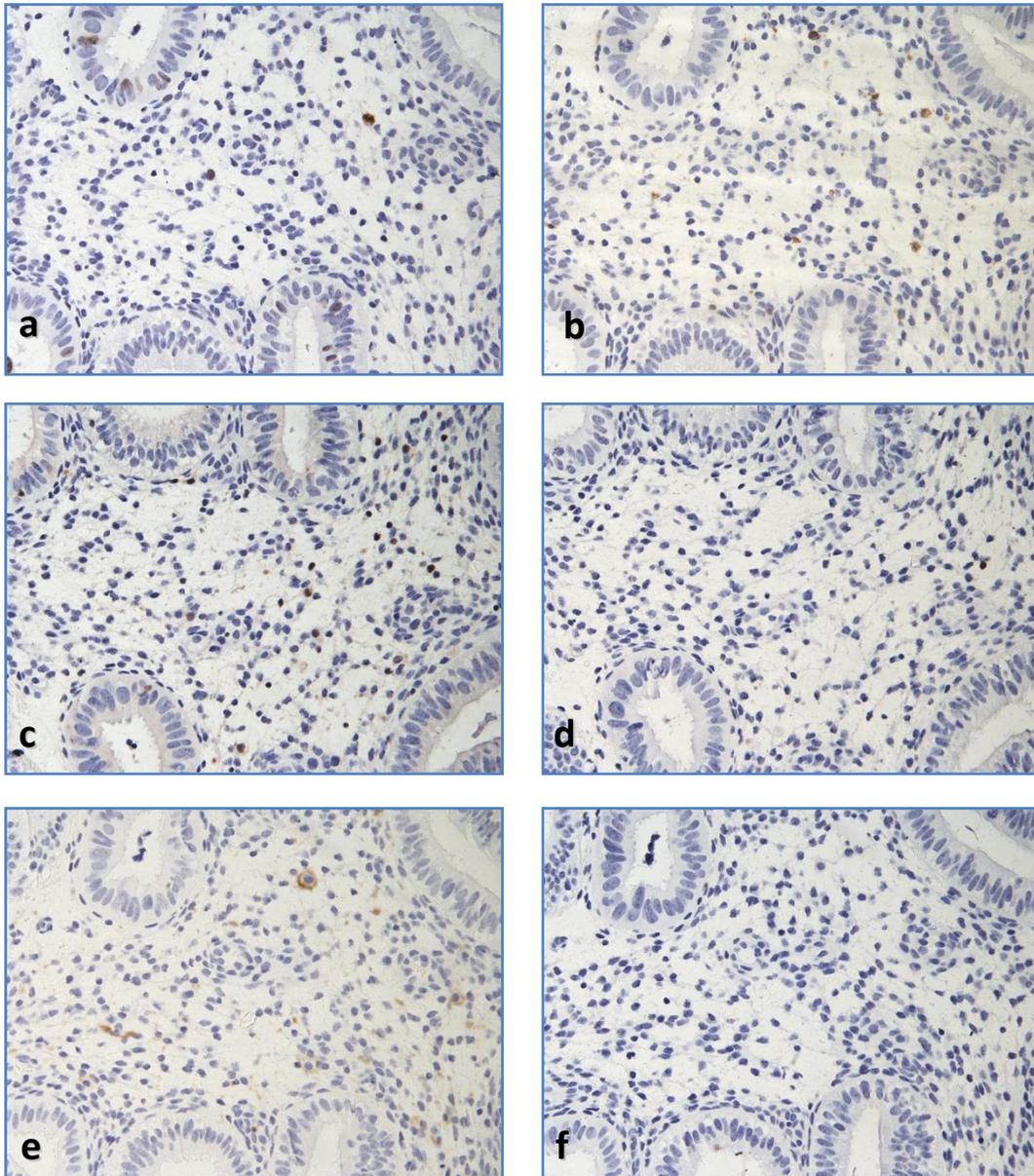


Figure 14: Micrographs showing areas of low stromal density of uNK Cells in a 'Low' patient, with the following stains: **a)** Ki67, **b)** CD56, **c)** NKp30, **d)** CD62-L, **e)** CD16 **f)** IgG Mouse (negative control)

High 'uNK' patients:

Epithelial Edge:

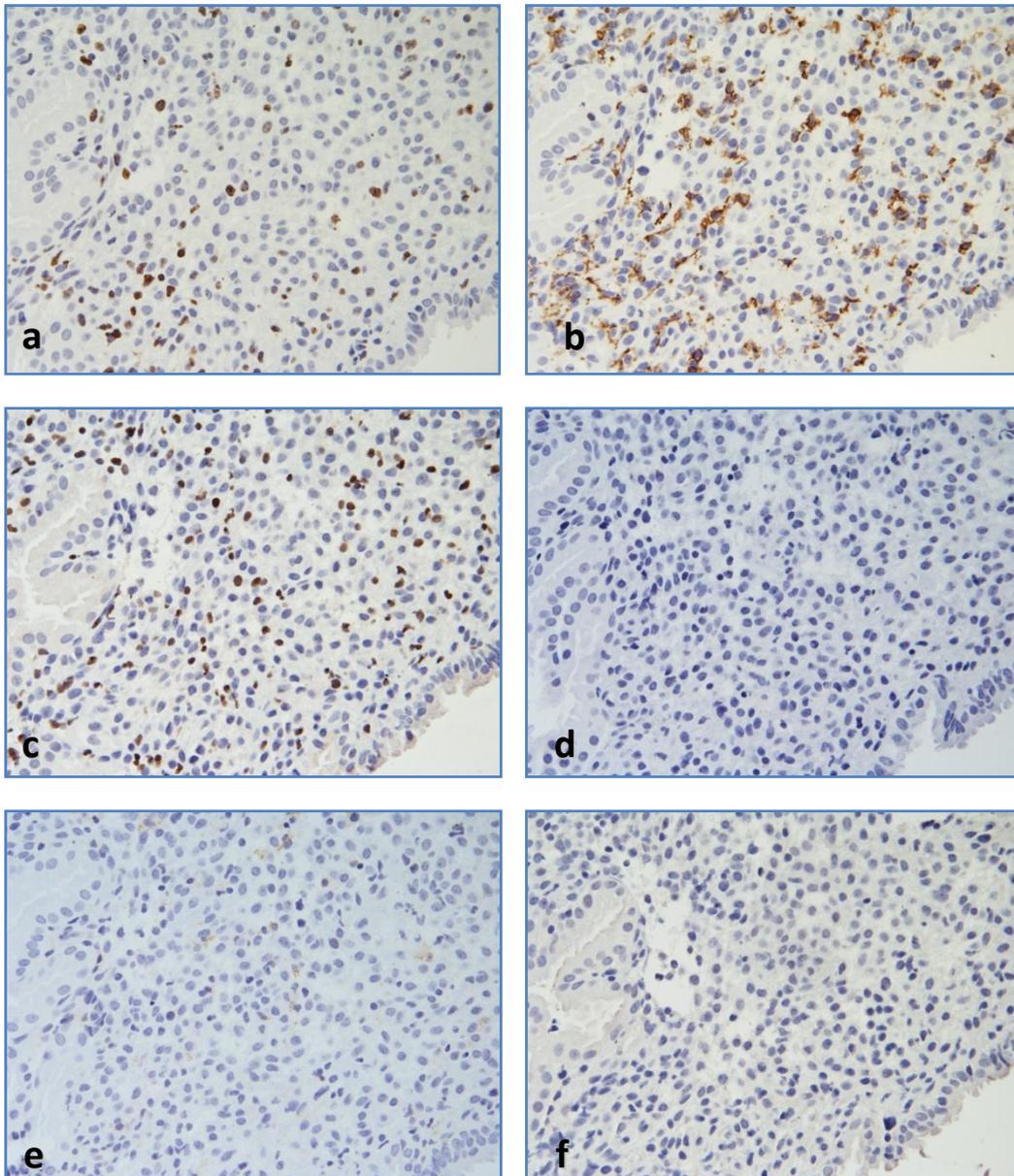


Figure 15: Micrographs showing subepithelial location of uNK Cells in a 'High' uNK patient, with the following stains: **a)** Ki67, **b)** CD56, **c)** NKp30, **d)** CD62-L, **e)** CD16 **f)** IgG Goat (negative control)

Perivascular:

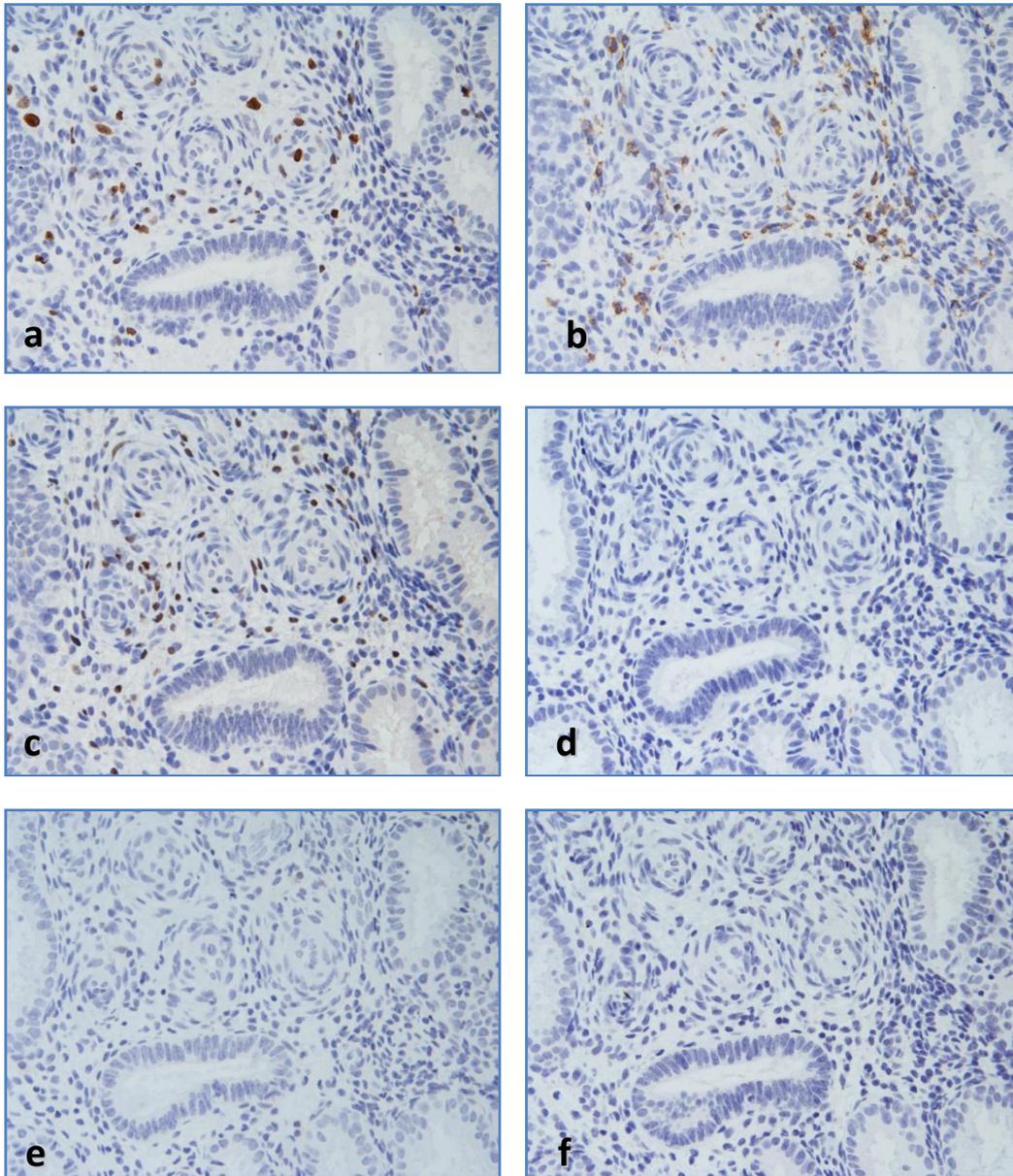


Figure 16: Micrographs showing perivascular location of uNK Cells in a 'High' patient, with the following stains: **a)** Ki67, **b)** CD56, **c)** NKp30, **d)** CD62-L, **e)** CD16 **f)** IgG Mouse (negative control)

Areas of low stromal density (Oedema):

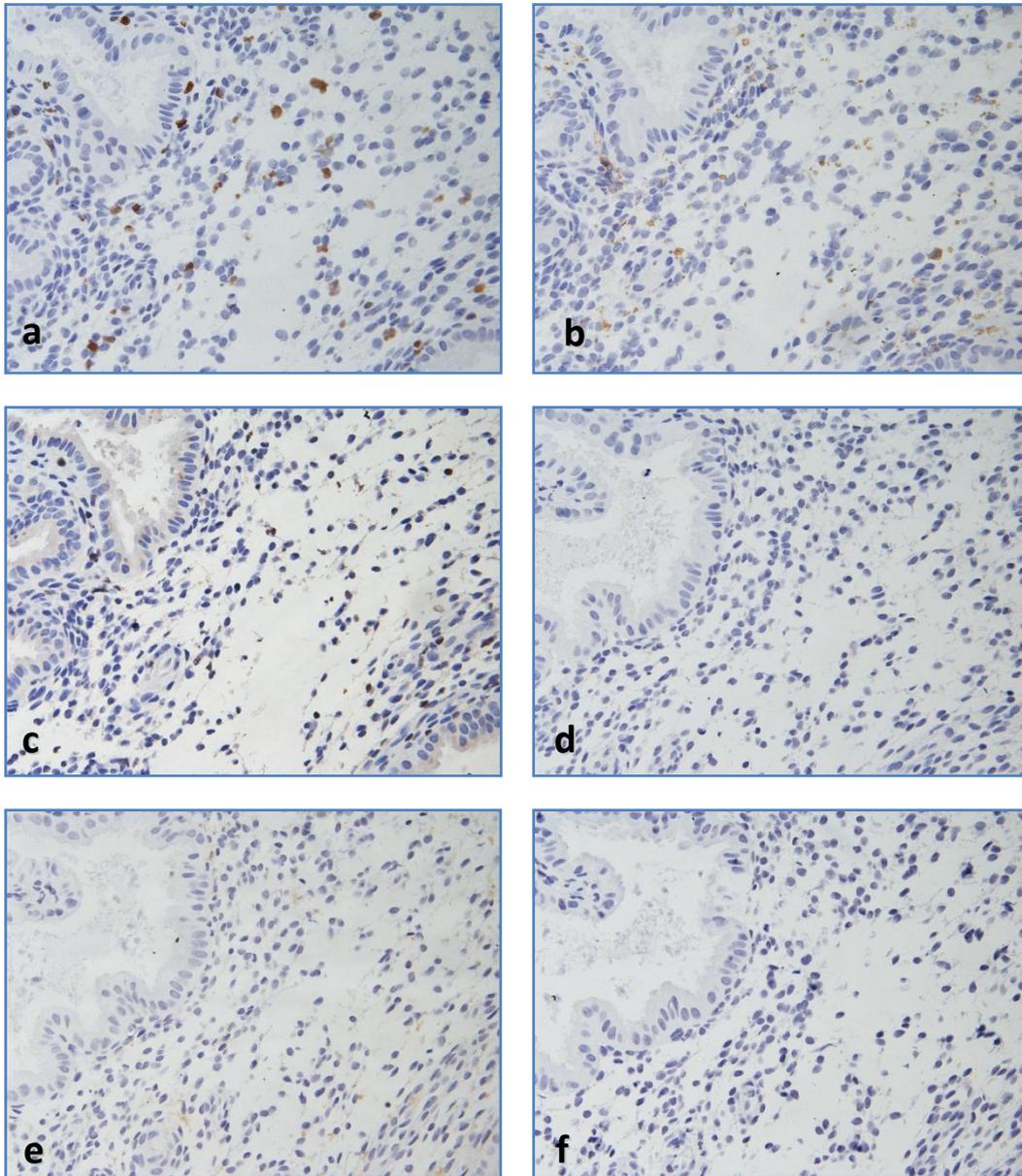


Figure 17: Micrographs showing areas of low stromal density of uNK Cells in a 'High' patient, with the following stains: a) Ki67, b) CD56, c) NKp30, d) CD62-L, e) CD16 f) IgG Goat (negative control)

3.4 Graphs and Statistical Results

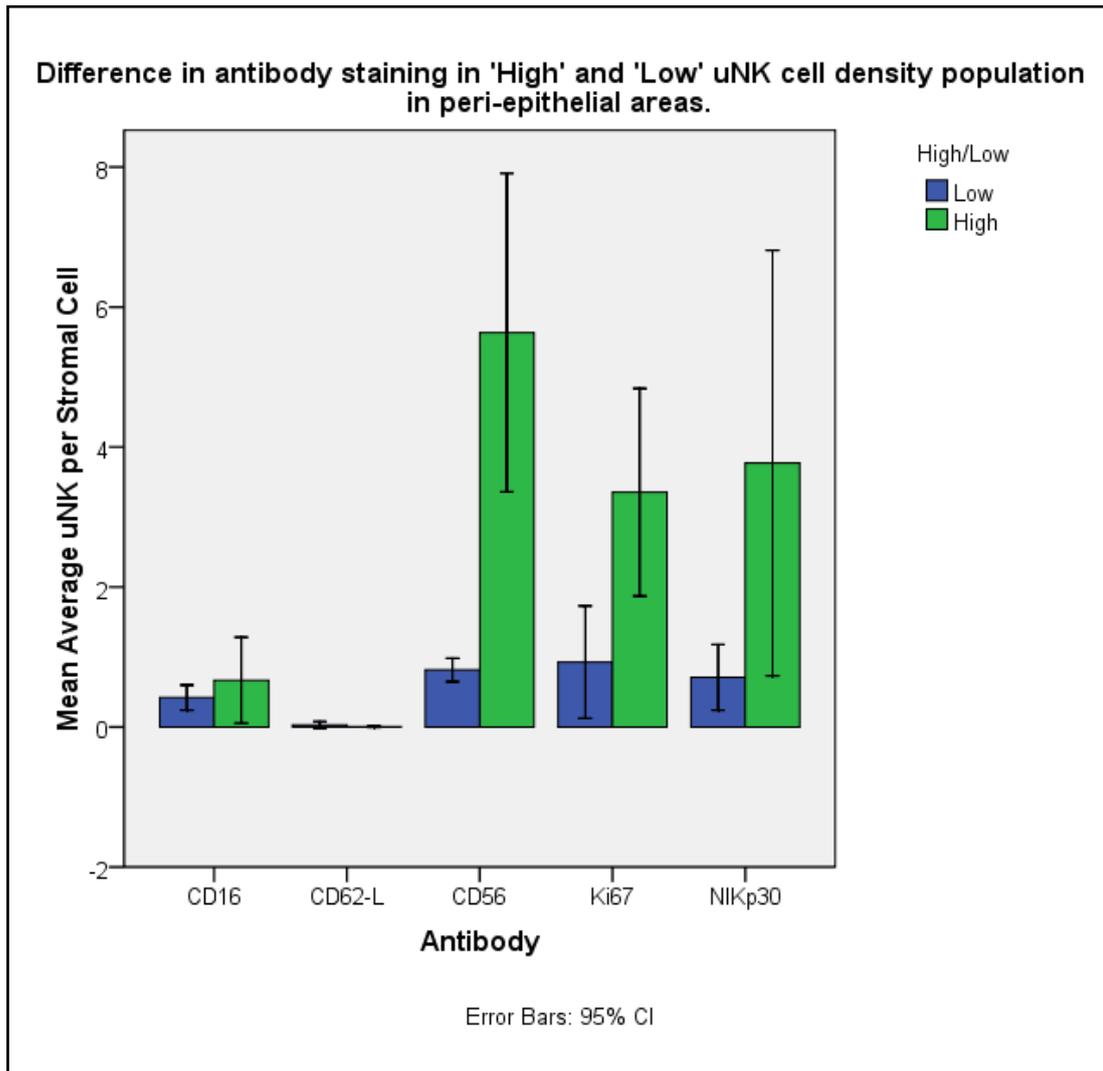


Figure 18: Graph illustrating difference in antibody staining in 'High' and 'Low' uNK cell density population in peri-epithelial areas.

As demonstrated from the graph above (see Figure 18 above), as expected uNK (CD56+) percentage in the 'high' uNK cell density group were significantly higher than uNK (CD56+) percentage in the 'low' uNK cell density group. This difference was also expressed in the Ki67 staining.

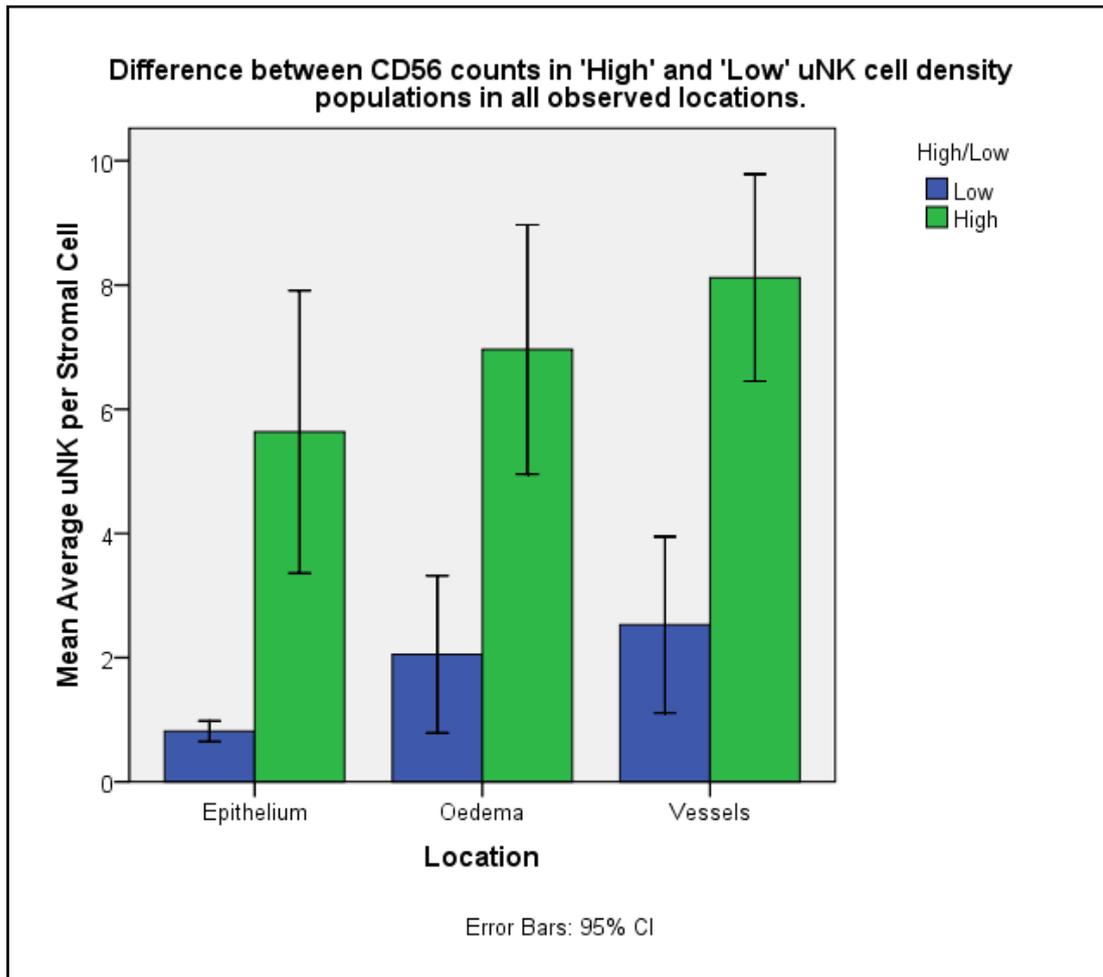


Figure 19: Graph illustrating the difference between CD56 counts in 'High' and 'Low' uNK cell density populations in all observed locations.

There was significant difference in the staining of CD56 in all three locations between the 'high' and 'low' uNK groups (see Figure 19 above). This difference was expected. The error bars at 95% confidence show no overlap. The locations show a similar pattern of staining, with the least percentage positive stained of CD56 observed near epithelium, and the maximal staining observed in the perivascular areas.

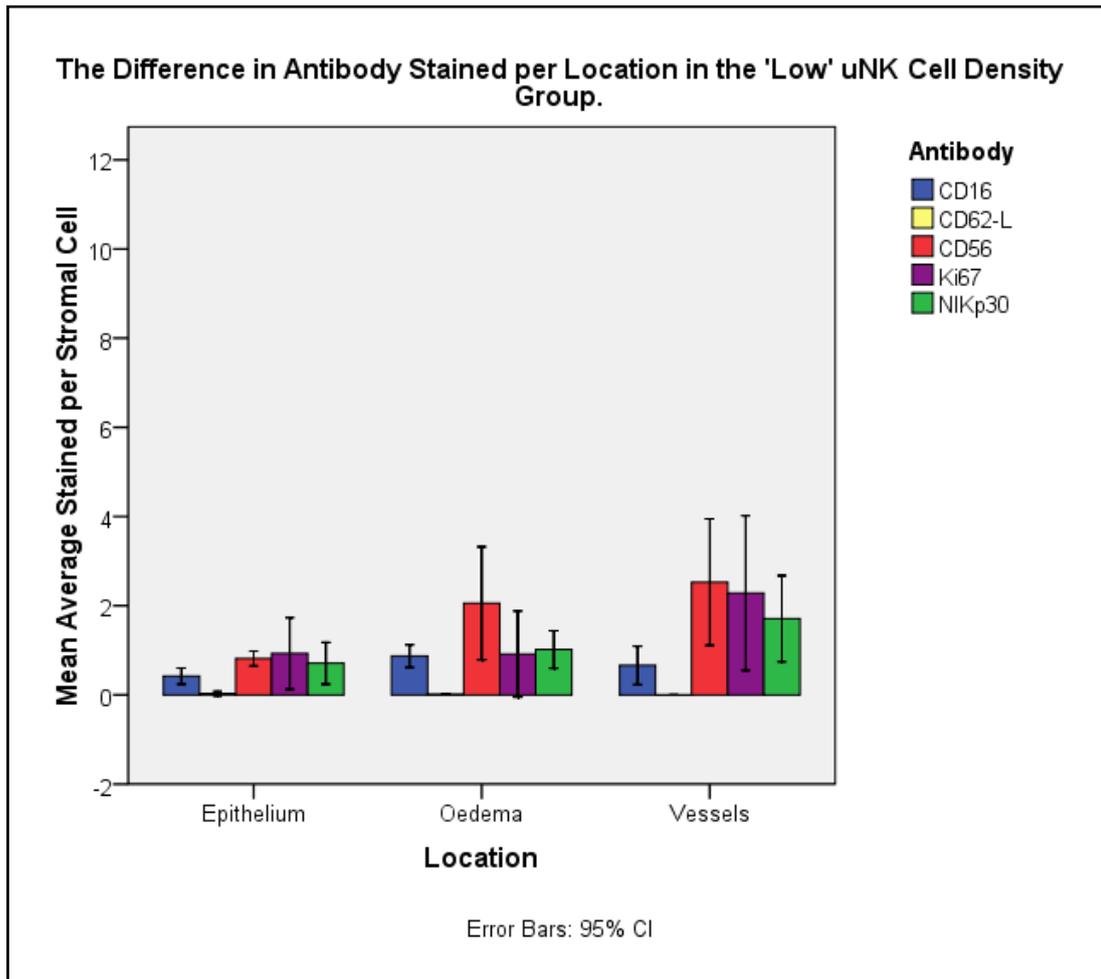


Figure 20: Graph illustrating the difference in antibody stained per location in the 'Low' uNK cell density group.

The expression of CD62-L (L-Selectin) in the 'low' uNK group was minimal. The expression of CD16 in the 'low' uNK group was also relatively low (see Figure 20 above). However the levels of Ki67 and NKp30 were significantly higher than CD62-L in areas surrounding epithelium and vessels.

There were no significant differences in the staining of Ki67, NKp30 and CD56 in all three locations. However there were higher levels of CD56 than CD16 in areas surrounding vessels and epithelium.

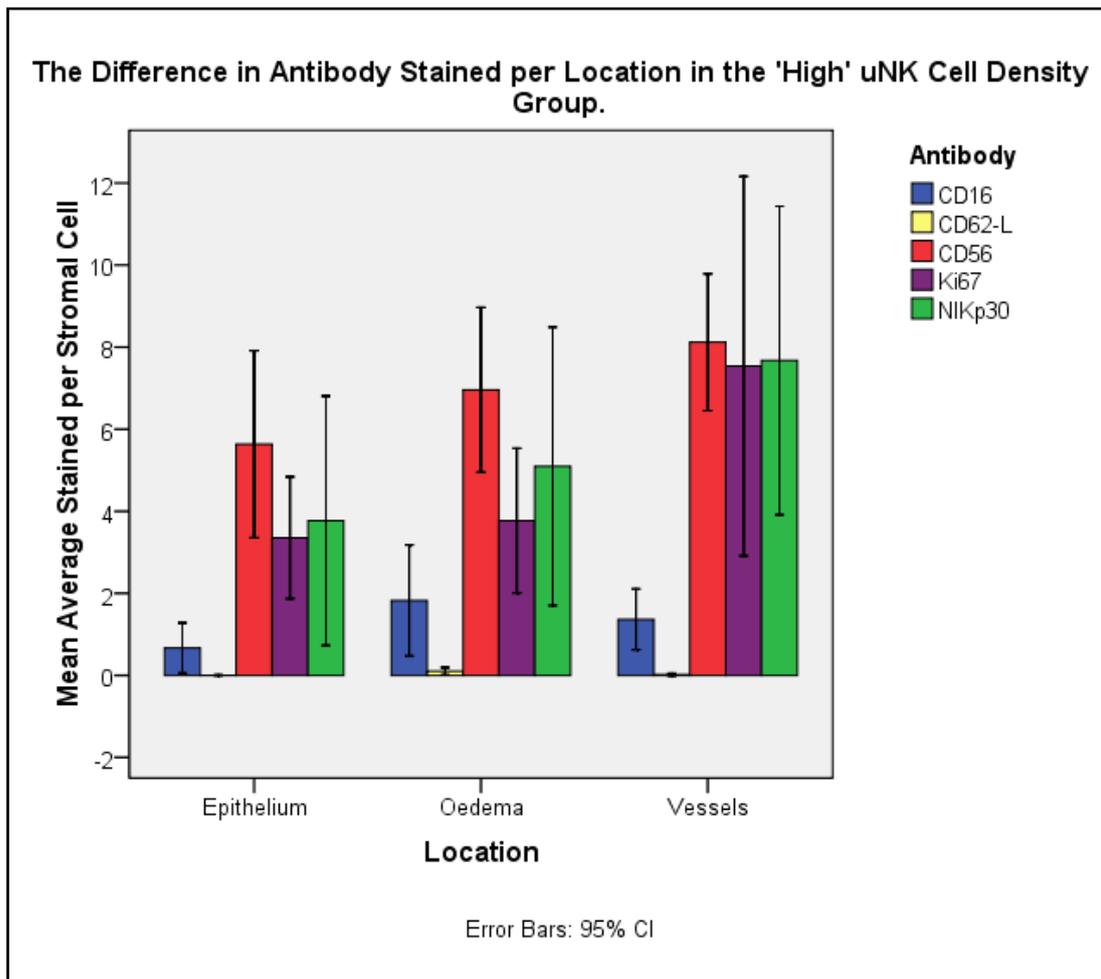


Figure 21: Graph illustrating the difference in antibody stained per location in the 'High' uNK cell density group.

The expression of CD62-L here in the 'high' uNK group is minimal, with the expression of CD16 also relatively low (see Figure 21 above). There were no significant difference between the staining of NKp30, Ki67 and CD56 in all three locations. However, there was a difference between CD56 and CD16 staining in all three areas, most remarkably in areas near vasculature. Also, in perivascular areas there is significant difference between CD16 and CD62-L and the other stains, CD56, Ki67 and NKp30.

Antibody	High/Low Group		Location	Comparison of Location Sites		
	Overall % Mean Difference	% Significant Difference (p-value)		Overall Significant Difference Between Locations (p-value)	Location	Contrast to Explore Difference
CD16	0.652	0.013	0.033	Oedema vs Epithelium	0.804	0.01
				Vessel vs Epithelium	0.468	NS
CD62-L / L-Selectin	0.026	NS	0.040	Oedema vs Epithelium	0.04	0.045
				Vessel vs Epithelium	-0.007	NS
CD56	4.8567	0.000	0.011	Oedema vs Epithelium	1.283	NS
				Vessel vs Epithelium	2.099	0.003
Ki67	3.354	0.000	0.009	Oedema vs Epithelium	0.201	NS
				Vessel vs Epithelium	2.770	0.006
NKp30	4.366	0.000	0.067	Oedema vs Epithelium	0.018	NS
				Vessel vs Epithelium	2.451	0.023

Table 14: Results of analysis of data collected in image analysis software in each stain used in both 'high' and 'low' uNK populations.

Univariate analysis of variance (ANOVA) showed significant differences between 'high' and 'low' uNK cell density groups in antibody staining and locations. Analysis was performed with patient study number as a covariate. A simple contrast was used to compare antibody staining at each location with epithelium as a reference group.

CHAPTER 4: DISCUSSION

This project describes the endometrial expression of markers of cell differentiation and proliferation in cases of RIF with, and without high uNK density.

We have examined the distribution of each cell marker. This allows us to comment on the spatial differentiation of NK cells and the implications for function of that distribution. We can also comment on whether cases of high uNK cell density are associated with markers of uNK cell trafficking and/or proliferation. This allows us to address a controversial area of clinical relevance in endometrial biology.

4.1 NK Cells distribution

CD56+ cells were seen higher in areas around vessels than in other locations ($p=0.003$). This finding is consistent with theories relating to the function of these cells (181). Their close proximity to vessels makes them ideally situated to engage in promoting vasculature development. Perhaps this early development of arteries leads to a highly oxygenated state, which is detrimental to the developing embryo.

In both the 'high' and the 'low' uNK groups the majority of staining for CD56+ cells was observed around vasculature. The relative decrease of uNK cells observed at the epithelial edge could be a reason for the high incidence of implantation failure in these patients. The epithelial edge of the endometrium is part of the foetal-maternal interface, and the first point of call in adhesion of the blastocyst to the epithelial edge. If uNK cells assist in the invasion of the trophoblast it could be possible that in this group of patients they are not situated most optimally.

4.2 Trafficking

The majority of uNK cells have the phenotype CD56brightCD16-, in contrast to the majority of peripheral blood NK cells, CD56dimCD16-. The results show low levels of CD16+ cells in the endometrium. This is consistent with previous reports (93). If peripheral blood NK cells were trafficking to the endometrium they would have to be of the subtype CD56brightCD16-, which form only 10% of the peripheral blood NK cells, this would indicate a selection method to only recruit the CD56brightCD16+ phenotype.

The difference observed in the CD16 distribution across the three locations showed a difference in the trends of the other antibodies studied. With a significant increase in these cells seen in areas of low stromal density (oedema) compared to subepithelial areas ($p=0.01$). This association could be explained by the association of lymph vessels with areas of low stromal cell density which was general observation when carrying out this research. CD16 antigen is not specific to NK cells, it stains other leukocytes such as monocytes, macrophages and neutrophils. Observing staining whilst photographing it became obvious that some CD16+ cells were not NK in morphology, but resembled neutrophils and monocytes also. When looking at the overall numbers of CD16+ cells, we must note this may include other lymphocytes stained, this makes the actual number of CD16+ NK cells even smaller.

Very few stromal cells stained positive for CD62-L (L-Selectin), this is very strongly suggestive that NK cells in the endometrium are not the result of migration, trafficking of peripheral blood NK cells, from the blood into the endometrium. One would expect the adhesion module to be present on all cells migrating from the blood, as it is necessary for successful adherence to endothelial lining of vessels and subsequent migration (152).

Previous work carried out with L-Selectin was performed in mice (156), and *in vitro*, and may not reflect what is actually occurring at the foetal-maternal interface, as murine models exhibit different modes of placentation (80).

With these two results it becomes increasingly more suggestive that trafficking of peripheral blood NK cells into the endometrium does not explain the high numbers

of these cells in the endometrium. Importantly, the group with high density of uNK cells did not have more L-selectin positive cells than the low uNK cell density group. Further more the L-selectin positive cells were not clumped round blood vessel as would have been expected if trafficking had occurred. Hence the increased uNK cell density seen in some women did not appear to be the result of trafficking.

Given the extremely small number of stained cells for L-Selectin, it would be understandable to consider the validity of the antibody used to detect this receptor. However, all antibodies used were validated, using known positive controls. Furthermore in one patient from the 'high' group, on microscopy, a dense area of leukocyte infiltrate was noted, as these cells appeared atypical and stained heavily with CD62-L (see Figure 22 below). These findings were confirmed by H&E (haematoxylin and eosin stain) where the area was structurally abnormal. However this area did not stain in a similar fashion with CD56, so these cells were not NK cells. (Please see micrographs below)

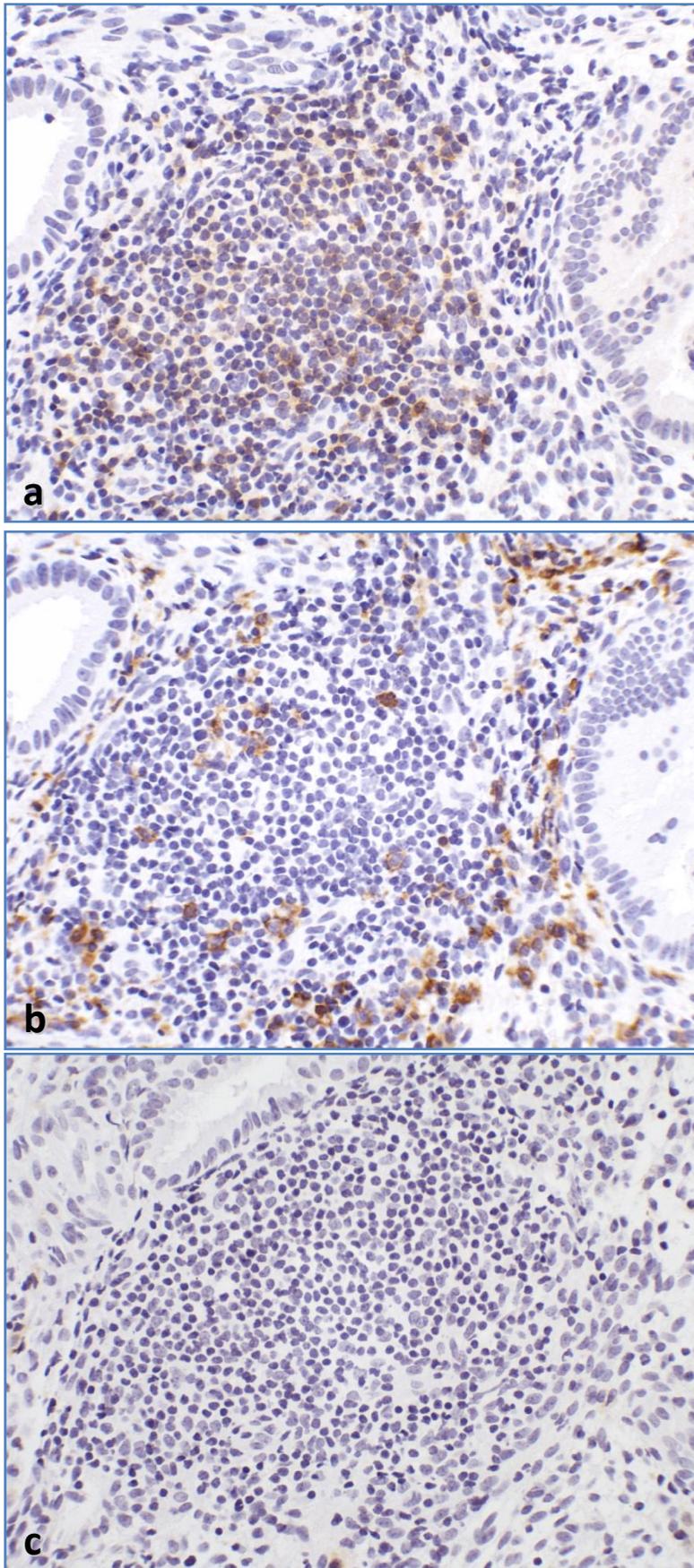


Figure 22: Micrographs showing the staining of an area of lymphocyte infiltration with the following antibodies to **a)** CD62-L, **b)** CD56, **c)** CD16.

Staining in micrograph **a** does not correlate with staining in the other two stains, indicating that the staining is not of NK cells or CD16+ Lymphocytes.

Another explanation for our data, is that if these cells were migrating, that they, upon activation would 'lose' their L-Selectin by binding to endothelial lining of vessels (194). Levels of NKp30+ cells (differentiation and activation marker) were found to be much higher in the stroma than that of CD62-L, so the majority of these cells were activated. However, the lumens of vessels at LH + 5-9 are not developed, and many still exist as endothelial cords only. This means that there was unlikely to have been enough time for the cells to migrate in this fashion to 'lose' their L-Selectin status.

Previous work on finding L-Selectin expressed on trophoblast does not necessarily suggest any direct function of this receptor to uNK cells, as endometrial epithelial cells are found to express a ligand to L-Selectin, which makes embryo adhesion more efficient (195).

Work on leukocyte migration in mice, in other tissues with inflammation, has shown that L-Selectin is necessary for migration of leukocytes (196). Migration of leukocytes is severely inhibited in L-Selectin deficient mice, and those challenged with L-Selected neutralising antibodies and L-Selectin IgG chimera (197).

When mice were injected with thioglycollate (196) into their peritoneal cavity to induce an inflammatory response, inhibited leukocyte migration was observed in L-Selectin deficient mice, with a significant difference compared to controls ($p=0.03$). This shows that L-Selectin is needed in adequate migration of leukocytes to non-primary lymphoid organs. These mouse experiments can be related to the endometrium, albeit a unique tissue within the body, as similar migration pathways would be employed if leukocytes were trafficking here. The paucity of L-Selectin detected in this study suggests that leukocytes, NK cells from peripheral blood, are not migrating from the peripheral blood to the endometrium to any significant degree.

4.3 Proliferation

The marker for proliferation of uNK cells, Ki67 was significantly different between 'high' and 'low' uNK groups ($p < 0.0001$). It was also found to vary statistically between locations ($p = 0.009$) and was significantly higher in areas around vessels compared to areas near epithelium ($p = 0.006$). This suggests that those uNK cells surrounding vessels are proliferating. The locality of these profliering cells could suggest that they have a role in vascular remodelling or they are proliferating near to vessels because this is their mode of transport into the endometrium. The later is unlikely due to the low levels of CD16+ and L-Selectin+ cells that were found in these areas, as discussed previously.

The origin of uNK cells, much debated, can be suggested by the results of our study. The levels of Ki67 found in previous studies (154) as high as 40% of uNK cells, suggesting that these cells proliferate and differentiate from cells already resident in the stratum basalis, which is not shed in menstruation.

Lynch *et al.*(198) have found heamatopoetic stem cells phenotype in the endometrium. These cells have the ability to differentiate and proliferate into uNK cells. Cho *et al.*(199), also showed the presence of stem cell markers in the stroma of the basalis. This evidence, along with our and others recognition of Ki67 stained uNK cells (93), is strongly supportive of the concept that uNK cell arise of proliferation and differentiation of haematopoetic stem cells within the endometrium.

4.4 Differentiation and Activation of NK cells.

Numbers of cells staining positive to NKp30 were significantly higher in the 'high' uNK group, than the low (p<0001).

We found higher number of NKp30+ cells in areas of vessels compared to epithelium (p=0.023), with no significant difference between oedema and epithelial areas.

The NKp30 staining confirms that these cells are activated in the endometrium at LH + 5-9. During this 'window of implantation' it would be hard to ignore that these cells have a large role in the process of implantation. Their high numbers and activated state in patients with recurrent implantation failure, most likely plays a role in the pathology of this unique condition.

Our staining of NKp30 differed to previous results from other studies. Manaster *et al.* (148) found no positive cells for NKp30 without prior activation of the endometrium by IL-15, *in vivo*. Ponnampalam *et al.*(149), only found NKp30 staining on the glandular epithelium and luminal epithelium and the staining was cytoplasmic, rather than our nuclear staining. Manaster *et al.* (148) used flow cytometry and thus may have lost some cells during the digestion needed to separate the cell from solid tissue prior to flow cytometry or during the "gating" or cell size sorting. Also nuclear staining will be far less effective in detecting than cytoplasmic staining using flow cytometry.

Although Ponnampalam *et al.*(149) used the same antibody to detect NKp30 (sc-20477) as we did, they used it on frozen sections. To determine whether the difference in our staining were due to the difference in tissue type used we performed immunohistochemistry with the same antibody on frozen sections on a small selection of our study sample. The results were similar to paraffin staining and are shown below. The staining did not change, it remained nuclear and was not shown in glandular epithelium or luminal epithelium. This persistent difference may be explained by an increase in background staining observed in Ponnampalam *et al.*(149) micrographs, as goat polymer in the detection of NKp30 can produce background staining more than mouse polymer systems.

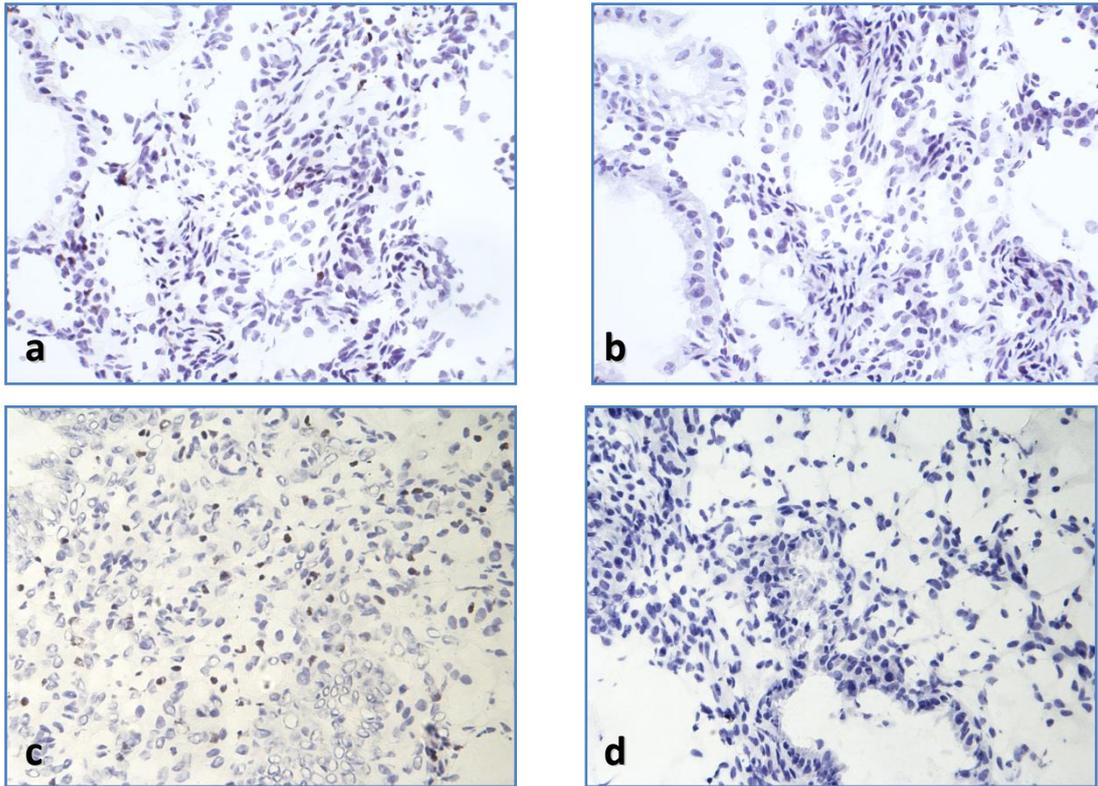


Figure 23: Micrographs of frozen sections stained with NKp30 and Negative control, IgG Goat. **a)** 'Low' patient with NKp30 staining, **b)** 'Low' patient with IgG Goat staining, **c)** 'High' patient with NKp30 staining, **d)** 'High' patient with IgG Goat staining. These micrographs were cut and stained by an experienced member of our department.

To address the differences observed by Manaster *et al.* (148), we looked at the staining pattern of NKp30 in deciduas, of varying weeks gestation in first trimester (see Figure 23 above, Figure 24 below). If their NKp30 positive peaks were only observed once stimulated with IL-15, *in vitro*, it could be that changes in nuclear to cytoplasmic staining could occur at different differentiated levels for these cells. However, no change in staining pattern was seen in any of the decidual staining, (see below). We must conclude from this that our staining of novel NKp30, is the first to be truly observed in frozen and paraffin late-secretory phase endometrium and first trimester decidua.

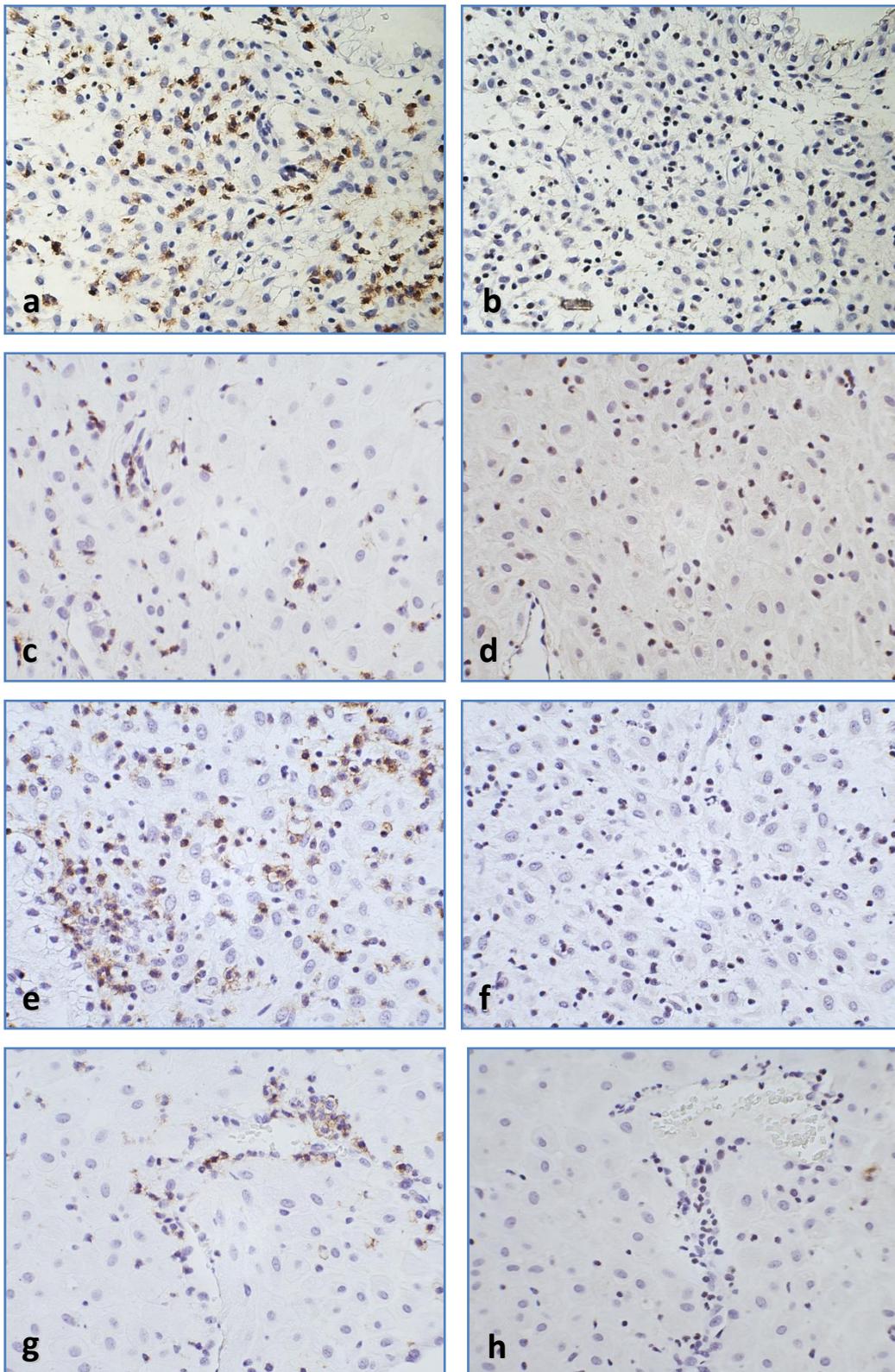


Figure 24: Micrographs of decidua taken at different gestations in the first trimester stained with CD56 or NKp30. **a)** 6 weeks - CD56, **b)** 6 weeks - NKp30, **c)** 8 weeks - CD56, **d)** 8 weeks - NKp30, **e)** 10 weeks - CD56, **f)** 10 weeks - NKp30, **g)** 12 weeks - CD56, **h)** 12 weeks - NKp30. These micrographs were cut and stained by an experienced member of our department.

CHAPTER 5: CONCLUSION

The results of this study strongly suggest the origin of uNK cells population in patients with recurrent implantation failure are within the endometrium and migration of peripheral blood cells into the endometrium is unlikely to be significant. The majority of peripheral blood NK cells differ phenotypically (being CD16+) from uNK cells and are more cytotoxic in their behaviour. However the minority population of peripheral blood NK cells share the same phenotype as the observed uNK cells. A strong competing hypothesis was that these cells migrate to the uterus, in order to do this they use the homing marker, L-Selectin to adhere to endothelial cells. Our results found very little CD16+ and L-Selectin+ cells in the samples selected, with possible explanations discussed previously, the most likely being that these cells are not trafficking to the uterus from peripheral blood.

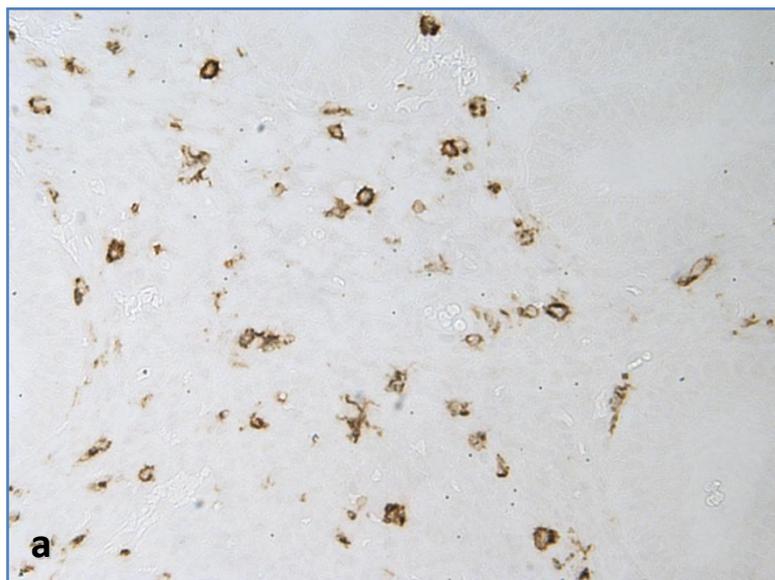
This study also showed that these cells are activated and proliferating in the endometrium, mostly in areas surrounding vessels. Previous work in the same laboratory found a positive correlation between uNK cells density in endometrial vasculature and blood flow (181). However, this correlation could have been found because the uNK cells secrete angiogenic factors that promoted angiogenesis or the uNK cells could have trafficked through the vessel walls and hence be located next to the vessel. Our new data favours the suggestion that uNK cells have a functional role and aid in vascular remodelling.

5.1 Limitations in Methodology.

Immunohistochemistry was chosen as the method to detect these receptors and their expression by uNK cells. We also wanted to gain information about the distribution of these cells in three specific locations. The disadvantage to our method was the inability to label cells with both CD56 and another marker, so one could ensure each labelled cell was a uNK cell when comparing each marker. One way of overcoming this was to use sequential sections, with micrographs of each area being in close proximity to both a uNK cell stain and another antibody stain. However, the sections were only able to be cut at 4µm due to the necessity of sequential sections, cutting at 3µm was not possible. It became clear on photographing stained sections that although the microstructure of the section was similar to sequential sections, the variations between cells made it difficult to locate corresponding cells on sequential sections.

A way to overcome this would have been to do double staining on the sections. Some samples of the sample population were sent to laboratories in Newcastle to be double stained, as they have double staining validated, to see if it would be a viable option (see Figure 25 below).

The results of the duel-labelling are shown below:



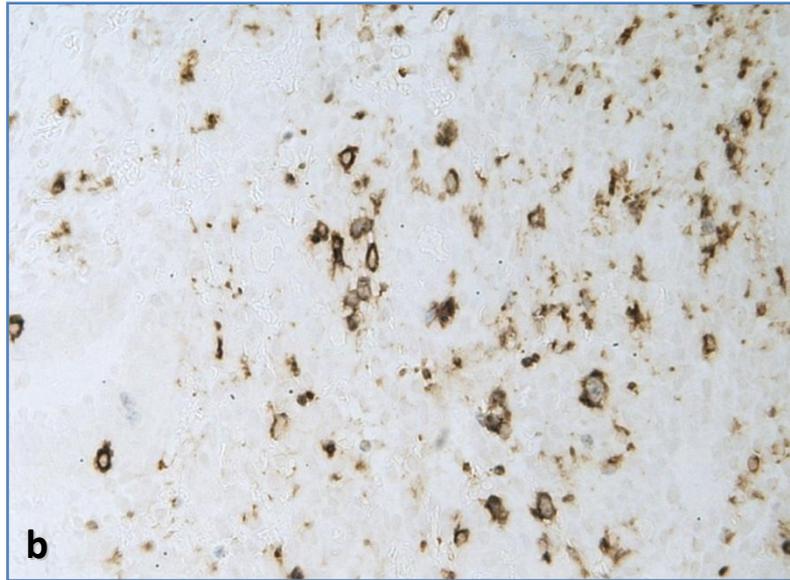


Figure 25: Micrographs showing cytoplasmic and nuclear double staining. Cytoplasmic, brown staining is CD56, nuclear staining, Silver-Grey is Ki67. a) Patient from 'low' uNK group b) patient from 'high' uNK group. These micrographs were cut and stained by an experienced member of a collaborative department.

As you can see the dual labelling has worked as some cells were clearly CD56+ and ki67+, but the lack of counterstain with haematoxylin means that we are unable to see structures such as glands, vessels and epithelial edge. Consequently this method was unable to be used because we would lose information on location if using this method.

5.2 Clinical Implications

With results suggesting that NK cells are not trafficking from peripheral blood, there is no scientific rationale for clinical sampling of peripheral blood for NK cell testing, as it cannot accurately reflect what is happening in the endometrium of these patients. Additionally consequent treatment consisting of NK cell modification in the systemic circulation should cease, as it is not without unwanted and potentially harmful side effects (118).

Advances in diagnostic tests and treatment should move away from peripheral blood NK cell augmentation to uNK cells. With further research in the area of uNK

cells and recurrent implantation failure, hopefully an endometrial factor will be found that treatment options can be tailored to.

5.3 Future Directions

The results of this study need to be replicated on a larger scale to strengthen conclusions made. It would be beneficial to look at endometrium throughout the menstrual cycle, with the receptors used, to identify the pattern of behaviour for these cells. To conclusively reject the competing hypothesis of migration of these cells from peripheral blood, it would be useful to study, not only L-Selectin, but other leukocyte trafficking markers, at various stages in the cycle.

The activation and proliferation of these uNK cells in the unique and dynamic tissue of the endometrium cannot be ignored, and their functional role at the fetomaternal interface must be determined. Prospective observational study of endometrial factors and IVF outcome would help to determine endometrial factors in IVF failure.

Elucidation of the multifactorial causes for recurrent implantation failure will alleviate the emotional distress of this devastating disease for these patients.

References

- (1) Evers JL. Female subfertility. *The Lancet* 2002 7/13;360(9327):151-159.
- (2) Oakley L, Doyle P, Maconochie N. Lifetime prevalence of infertility and infertility treatment in the UK: results from a population-based survey of reproduction. *Human Reproduction* 2008;23(2):447-450.
- (3) Wilkes S, Chinn DJ, Murdoch A, Rubin G. Epidemiology and management of infertility: a population-based study in UK primary care. *Fam.Pract.* 2009.
- (4) Czyba C. Psychosomatic and sexual disorders in infertile couples. 2008; Available at: http://www.gfmer.ch/Books/Reproductive_health/Psychosomatic.html. Accessed 01 Jan, 2009.
- (5) Johnson M, Everitt BJ. *Essential reproduction*. 6th ed. Oxford: Blackwell Publishing; 2007.
- (6) NHS. Infertility: Diagnosing Infertility. 2007; Available at: <http://www.nhs.uk/Conditions/Infertility/Pages/Diagnosis.aspx>. Accessed 01 Jan, 2009.
- (7) HFEA. Facts and figures for researchers and the media. 2009; Available at: <http://www.hfea.gov.uk/99.html>. Accessed 1 Feb, 2009.
- (8) Human Embryology. Gametogenesis: Spermatogenesis. Available at: <http://www.embryology.ch/anglais/cgametogen/spermato03.html#zeitlicher>. Accessed 10 Jan, 2009.
- (9) Jung A, Schill WB, Schuppe HC. Genital heat stress in men of barren couples: a prospective evaluation by means of a questionnaire. *Andrologia* 2002;34(6):349-355.
- (10) Diemer T, Desjardins C. Developmental and genetic disorders in spermatogenesis. *Hum.Reprod.Update* 1999;5(2):120-140.
- (11) Drife J. Drugs and sperm. *Br.Med.J.* 1982;284(6319):844-845.
- (12) Ulane CM, Rodriguez JJ, Parisien JP, Horvath CM. STAT3 ubiquitylation and degradation by mumps virus suppress cytokine and oncogene signaling. *J.Virol.* 2003;77(11):6385-6393.
- (13) Ochsendorf F. Sexually transmitted infections: impact on male fertility. *Andrologia* 2008;40(2):72-75.

- (14) American Academy of Family Physicians. Pelvic Inflammatory Disease. 2008; Available at: <http://familydoctor.org/online/famdocen/home/women/reproductive/sti/213.html>. Accessed 20 Jan, 2009.
- (15) Kaneko N, Kawagoe S, Hiroi M. Turner's syndrome--review of the literature with reference to a successful pregnancy outcome. *Gynecol.Obstet.Invest.* 1990;29(2):81-87.
- (16) Conway GS. Premature ovarian failure. *Br.Med.Bull.* 2000;56(3):643-649.
- (17) Ng EH, Chan CC, Tang OS, Yeung WS, Ho PC. The role of endometrial and subendometrial vascularity measured by three-dimensional power Doppler ultrasound in the prediction of pregnancy during frozen-thawed embryo transfer cycles. *Human Reproduction* 2006 Jun;21(6):1612-1617.
- (18) Laird S, Tuckerman E, LI TC. Cytokine expression in the endometrium of women with implantation failure and recurrent miscarriage. *Reproductive biomedicine online(Print)* 2006;13(1):13-23.
- (19) Sharkey AM, Smith SK. The endometrium as a cause of implantation failure. *Best Practice & Research Clinical Obstetrics & Gynaecology* 2003 4;17(2):289-307.
- (20) Wilcox AJ, Baird DD, Weinberg CR. Time of implantation of the conceptus and loss of pregnancy. *N.Engl.J.Med.* 1999 Jun 10;340(23):1796-1799.
- (21) Johnson MH, Everitt BJ. *Essential reproduction*. 6 rev ed.: Blackwell Pub; 2007.
- (22) King A. Uterine leukocytes and decidualization. *Hum.Reprod.Update* 2000;6(1):28-36.
- (23) Hamilton GS, Lysiak JJ, Han VKM, Lala PK. Autocrine-paracrine regulation of human trophoblast invasiveness by insulin-like growth factor (IGF)-II and IGF-binding protein (IGFBP)-1. *Exp.Cell Res.* 1998;244(1):147-156.
- (24) Loke Y, King A. *Human implantation: cell biology and immunology*. : Cambridge University Press; 1995.
- (25) Kam EPY, Gardner L, Loke YW, King A. The role of trophoblast in the physiological change in decidual spiral arteries. *Human Reproduction* 1999;14(8):2131-2138.
- (26) Rogers PAW, Lederman F, Taylor N. Endometrial microvascular growth in normal and dysfunctional states. *Hum.Reprod.Update* 1998;4(5):503-508.
- (27) Urman B. Recurrent implantation failure in assisted reproduction: how to counsel and manage. B. Treatment options that have not been proven to benefit the couple. *Reproductive biomedicine online(Print)* 2005;11(3):382-391.

(28) El-Toukhy T, Taranissi M. Towards better quality research in recurrent implantation failure: standardizing its definition is the first step. *Reproductive biomedicine online(Print)* 2006;12(3):383-385.

(29) Croucher CA, Lass A, Margara R, Winston RM. Predictive value of the results of a first in-vitro fertilization cycle on the outcome of subsequent cycles. *Human Reproduction* 1998;13(2):403-408.

(30) Templeton A, Morris JK. Reducing the risk of multiple births by transfer of two embryos after in vitro fertilization. *N.Engl.J.Med.* 1998;339(9):573-577.

(31) Osmanagaoglu K, Tournaye H, Camus M, Vandervorst M, Van Steirteghem A, Devroey P. Cumulative delivery rates after intracytoplasmic sperm injection: 5 year follow-up of 498 patients. *Hum.Reprod.* 1999;14(10):2651-2655.

(32) Nargund G, Waterstone J, Bland JM, Philips Z, Parsons J, Campbell S. Cumulative conception and live birth rates in natural (unstimulated) IVF cycles. *Human Reproduction* 2001;16(2):259-262.

(33) Sharma V, Allgar V, Rajkhowa M. Factors influencing the cumulative conception rate and discontinuation of in vitro fertilization treatment for infertility.

(34) Sharma V, Allgar V, Rajkhowa M. Factors influencing the cumulative conception rate and discontinuation of in vitro fertilization treatment for infertility. *Fertil.Steril.* 2002;78(1):40-46.

(35) Stern C, Pertile M, Norris H, Hale L, Baker HWG. Chromosome translocations in couples with in-vitro fertilization implantation failure. *Human Reproduction* 1999;14(8):2097-2101.

(36) Voullaire L, Wilton L, McBain J, Callaghan T, Williamson R. Chromosome abnormalities identified by comparative genomic hybridization in embryos from women with repeated implantation failure. *Mol.Hum.Reprod.* 2002;8(11):1035-1041.

(37) Inagaki N, Stern C, McBain J, Lopata A, Kornman L, Wilkinson D. Analysis of intra-uterine cytokine concentration and matrix-metalloproteinase activity in women with recurrent failed embryo transfer. *Human Reproduction* 2003;18(3):608-615.

(38) Thornhill A, deDie-Smulders C, Geraedts J, Harper J, Harton G, Lavery S, et al. ESHRE PGD Consortium'Best practice guidelines for clinical preimplantation genetic diagnosis (PGD) and preimplantation genetic screening (PGS)'. *Human Reproduction* 2005;20(1):35-48.

(39) Cruz JR, Dubey AK, Patel J, Peak D, Hartog B, Gindoff PR. Is blastocyst transfer useful as an alternative treatment for patients with multiple in vitro fertilization failures? *Fertil.Steril.* 1999 Aug;72(2):218-220.

(40) Simon C, Mercader A, Garcia-Velasco J, Nikas G, Moreno C, Remohi J, et al. Coculture of Human Embryos with Autologous Human Endometrial Epithelial Cells in Patients with Implantation Failure 1. *Journal of Clinical Endocrinology & Metabolism* 1999;84(8):2638-2646.

(41) Human Embryology:. Implantation. Available at: <http://www.embryology.ch/anglais/gnidation/etape02.html>. Accessed 5 Feb, 2009.

(42) Norwitz ER, Schust DJ, Fisher SJ. Implantation and the survival of early pregnancy. *N.Engl.J.Med.* 2001;345(19):1400-1408.

(43) Wikipedia. Implantation. 2009; Available at: <http://en.wikipedia.org/wiki/Implantation>. Accessed 5 Feb, 2009.

(44) Childs GV. Female Reproductive System: Implantation and formation of placenta. 2002; Available at: http://www.cytochemistry.net/microanatomy/medical_lectures/Implantation_and_Placenta_development.htm. Accessed 5 Feb, 2009.

(45) Quinn CE, Casper RF. Pinopodes: a questionable role in endometrial receptivity. *Hum.Reprod.Update* 2009;15(2):229-236.

(46) Nikas G, Drakakis P, Loutradis D, Mara-Skoufari C, Koumantakis E, Michalas S, et al. Uterine pinopodes as markers of the 'nidation window' in cycling women receiving exogenous oestradiol and progesterone. *Human Reproduction* 1995 May;10(5):1208-1213.

(47) Nikas G, Develioglu OH, Toner JP, Jones HW. Endometrial pinopodes indicate a shift in the window of receptivity in IVF cycles. *Human Reproduction* 1999;14(3):787-792.

(48) Oborna I, Novotny R, Brezinova J, Petrova P, Lichnovsky V, Fingerova H. Changes in the development of uterine pinopodes in steroid hormone supplemented cycles. *Physiological Research* 2004;53:423-430.

(49) Wilcox AJ, Weinberg CR, O'Connor JF, Baird DD, Schlatterer JP, Canfield RE, et al. Incidence of early loss of pregnancy. 1988;17(2):469-471.

(50) Yamada H, Morikawa M, Kato EH, Shimada S, Kobashi G, Minakami H. Pre-conceptional natural killer cell activity and percentage as predictors of biochemical pregnancy and spontaneous abortion with normal chromosome karyotype. *Am. J. Reprod. Immunol* 2003;50(4):351-354.

(51) Loke YW, King A. Recent developments in the human maternal-fetal immune interaction. *Curr.Opin.Immunol.* 1991 Oct;3(5):762-766.

(52) Wikipedia. White blood cell. Available at: http://en.wikipedia.org/wiki/White_blood_cell. Accessed 5 March, 2009.

(53) CHXA. Leukocytes. Available at: <http://www.chxa.com/img/leukocytes-white-blood-cells-500x350.gif>. Accessed 15 Feb, 2009.

(54) Scott GB, Meade JL, Cook GP. Profiling killers; unravelling the pathways of human natural killer cell function. *Brief Funct.Genomic Proteomic* 2008 Jan;7(1):8-16.

(55) Lanier LL. Missing self, NK cells, and the white album. *The Journal of Immunology* 2005;174(11):6565-6565.

(56) Gubbay O, Critchley HOD, Bowen JM, King A, Jabbour HN. Prolactin induces ERK phosphorylation in epithelial and CD56 natural killer cells of the human endometrium. *Journal of Clinical Endocrinology & Metabolism* 2002;87(5):2329-2335.

(57) Saito S, Nishikawa K, Morii T, Enomoto M, Narita N, Motoyoshi K, et al. Cytokine production by CD16-CD56bright natural killer cells in the human early pregnancy decidua. *Int.Immunol.* 1993;5(5):559-563.

(58) Jokhi PP, King A, Sharkey AM, Smith SK, Loke YW. Screening for cytokine messenger ribonucleic acids in purified human decidual lymphocyte populations by the reverse-transcriptase polymerase chain reaction. *The Journal of Immunology* 1994;153(10):4427-4435.

(59) Linjawi S, Li T.C., Tuckerman E.M., Blakemore A.I., Laird S.M. Expression of interleukin-11 receptor alpha and interleukin-11 protein in the endometrium of normal fertile women and women with recurrent miscarriage. *J.Reprod.Immunol.* 2004;64(1-2):145-155.

(60) Tuckerman E, Laird SM, Stewart R, Wells M, Li TC. Markers of endometrial function in women with unexplained recurrent pregnancy loss: a comparison between morphologically normal and retarded endometrium. *Human Reproduction* 2004 Jan;19(1):196-205.

(61) Wegmann TG, Lin H, Guilbert L, Mosmann TR. Bidirectional cytokine interactions in the maternal-fetal relationship: is successful pregnancy a TH2 phenomenon? *Immunol Today* 1993;14(7):353-356.

(62) Tangri S, Raghupathy R. Expression of cytokines in placentas of mice undergoing immunologically mediated spontaneous fetal resorptions. *Biol.Reprod.* 1993;49(4):850-857.

(63) Tangri S, Wegmann TG, Lin H, Raghupathy R. Maternal anti-placental reactivity in natural, immunologically-mediated fetal resorptions. *The Journal of Immunology* 1994;152(10):4903-4911.

(64) Chaouat G, Assal Meliani A, Martal J, Raghupathy R, Elliot J, Mosmann T, et al. IL-10 prevents naturally occurring fetal loss in the CBA x DBA/2 mating combination, and local defect in IL-10 production in this abortion-prone combination is corrected by in vivo injection of IFN-tau. *The Journal of Immunology* 1995;154(9):4261-4268.

(65) Chaouat G, Menu E, Clark DA, Dy M, Minkowski M, Wegmann TG. Control of fetal survival in CBA x DBA/2 mice by lymphokine therapy. *Reproduction* 1990;89(2):447-458.

(66) Bates M, Quenby S, Takakuwa K, Johnson P, Vince G. Aberrant cytokine production by peripheral blood mononuclear cells in recurrent pregnancy loss? *Human Reproduction* 2002;17(9):2439-2444.

(67) Ginsburg ES, Xiao L, Gargiulo AR, Kung FT, Politch JA, Schust DJ, et al. T-helper 2 and 3 type immunity to trophoblast in successful in vitro fertilization-embryo transfer. *Fertil.Steril.* 2005;83(6):1659-1664.

(68) Kwak-Kim JYH, Chung-Bang HS, Ng SC, Ntrivalas EI, Mangubat CP, Beaman KD, et al. 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* 2003;18(4):767-773.

(69) Fasouliotis S, Spandorfer S, Witkin S, Schattman G, Liu H, Roberts J, et al. Maternal serum levels of interferon- γ and interleukin-2 soluble receptor- α predict the outcome of early IVF pregnancies. *Human Reproduction* 2004;19(6):1357-1363.

(70) Lim KJ, Odukoya OA, Ajjan RA, Li TC, Weetman AP, Cooke ID. The role of T-helper cytokines in human reproduction. *Fertil.Steril.* 2000 Jan;73(1):136-142.

(71) Shimada S, Kato EH, Morikawa M, Iwabuchi K, Nishida R, Kishi R, et al. No difference in natural killer or natural killer T-cell population, but aberrant T-helper cell population in the endometrium of women with repeated miscarriage. *Human Reproduction* 2004;19(4):1018-1024.

(72) Meisser A, Chardonens D, Campana A, Bischof P. Effects of TNF α , IL1 α , M-CSF and TGF β on trophoblastic matrix metalloproteinases. *Mol.Hum.Reprod.* 1999;5:252-260.

(73) Simon C, Gimeno MJ, Mercader A, O'Connor JE, Remohi J, Polan ML, et al. Embryonic regulation of integrins beta 3, alpha 4, and alpha 1 in human endometrial epithelial cells in vitro. *J.Clin.Endocrinol.Metab.* 1997 Aug;82(8):2607-2616.

(74) Simon C, Frances A, Piquette GN, el Danasouri I, Zurawski G, Dang W, et al. Embryonic implantation in mice is blocked by interleukin-1 receptor antagonist. *Endocrinology* 1994 Feb;134(2):521-528.

(75) Karagouni EE, Chryssikopoulos A, Mantzavinos T, Kanakas N, Dotsika EN. Interleukin-1beta and interleukin-1alpha may affect the implantation rate of patients undergoing in vitro fertilization-embryo transfer. *Fertility & Sterility* 1998 Sep;70(3):553-559.

(76) Spandorfer SD, Neuer A, Liu HC, Rosenwaks Z, Witkin SS. Involvement of Interleukin-1 and the Interleukin-1 Receptor Antagonist in In Vitro Embryo Development Among Women Undergoing In Vitro Fertilization–Embryo Transfer. *J Assist Reprod Genet* 2004;20:502-505.

(77) Chaouat G, Ledee-Bataille N, Dubanchet S, Zourbas S, Sandra O, Martal J. Reproductive immunology 2003: reassessing the Th1/Th2 paradigm? *Immunol.Lett.* 2004 Apr 15;92(3):207-214.

(78) Ledee-Bataille N, Dubanchet S, Coulomb-L'hermine A, Durand-Gasselien I, Frydman R, Chaouat G. A new role for natural killer cells, interleukin (IL)-12, and IL-18 in repeated implantation failure after in vitro fertilization. *Fertility & Sterility* 2004 Jan;81(1):59-65.

(79) Jokhi PP, King A, Loke YW. Cytokine production and cytokine receptor expression by cells of the human first trimester placental–uterine interface. *Cytokine* 1997;9(2):126-137.

(80) Moffett-King A. Natural killer cells and pregnancy. *Nature Reviews Immunology* 2002;2(9):656-663.

(81) Ferry BL, Sargent IL, Starkey PM, Redman CWG. Cytotoxic activity against trophoblast and choriocarcinoma cells of large granular lymphocytes from human early pregnancy decidua. *Cellular immunology(Print)* 1991;132(1):140-149.

(82) Stewart CL, Kaspar P, Brunet LJ, Bhatt H, Gadi I, Köntgen F, et al. Blastocyst implantation depends on maternal expression of leukaemia inhibitory factor. 1992.

(83) Laird S, Tuckerman E, Dalton C, Dunphy B, Li T, Zhang X. The production of leukaemia inhibitory factor by human endometrium: presence in uterine flushings and production by cells in culture. *Human Reproduction* 1997;12(3):569-574.

(84) Charnock-Jones DS, Sharkey AM, Fenwick P, Smith SK. Leukaemia inhibitory factor mRNA concentration peaks in human endometrium at the time of implantation and the blastocyst contains mRNA for the receptor at this time. *J.Reprod.Fertil.* 1994 Jul;101(2):421-426.

(85) Vogiagis D, Marsh MM, Fry RC, Salamonsen LA. Leukaemia inhibitory factor in human endometrium throughout the menstrual cycle. *J.Endocrinol.* 1996 Jan;148(1):95-102.

(86) Chen JR, Cheng JG, Shatzer T, Sewell L, Hernandez L, Stewart CL. Leukemia inhibitory factor can substitute for nidatory estrogen and is essential to inducing a receptive uterus for implantation but is not essential for subsequent embryogenesis. *Endocrinology* 2000 Dec;141(12):4365-4372.

(87) Cheng Jr G, Chen JR, Hernandez L, Alvord WG, Stewart CL. Dual control of LIF expression and LIF receptor function regulate Stat3 activation at the onset of uterine receptivity and embryo implantation. *Proceedings of the National Academy of Sciences* 2001;98(15):8680.

(88) Nichols J, Davidson D, Taga T, Yoshida K, Chambers I, Smith A. Complementary tissue-specific expression of LIF and LIF-receptor mRNAs in early mouse embryogenesis. *Mech.Dev.* 1996 Jul;57(2):123-131.

(89) Delage G, Moreau JF, Taupin JL, Freitas S, Hambartsoumian E, Olivennes F, et al. In-vitro endometrial secretion of human interleukin for DA cells/leukaemia inhibitory factor by explant cultures from fertile and infertile women. *Hum.Reprod.* 1995 Sep;10(9):2483-2488.

(90) Cullinan EB, Abbondanzo SJ, Anderson PS, Pollard JW, Lessey BA, Stewart CL. Leukemia inhibitory factor (LIF) and LIF receptor expression in human endometrium suggests a potential autocrine/paracrine function in regulating embryo implantation. *Proc.Natl.Acad.Sci.U.S.A.* 1996 Apr 2;93(7):3115-3120.

(91) Sherwin JR, Smith SK, Wilson A, Sharkey AM. Soluble gp130 is up-regulated in the implantation window and shows altered secretion in patients with primary unexplained infertility. *J.Clin.Endocrinol.Metab.* 2002 Aug;87(8):3953-3960.

(92) Weill P. Les cellules granuleuses des muqueuses intestinale et uterine. *Arch.Anat.Microsc.* 1921;17:77-82.

(93) Bulmer JN, Lash GE. Human uterine natural killer cells: a reappraisal. *Mol.Immunol.* 2005;42(4):511-521.

(94) Smith LJ. Metrial gland and other glycogen containing cells in the mouse uterus following mating and through implantation of the embryo. *Am.J.Anat.* 1966;119(1):15-23.

(95) Bartelmez GW, Corner GW, Hartman CG. Cyclic changes in the endometrium of the rhesus monkey (*Macaca mulatta*). *Contrib Embryol* 1951;34:99-144.

(96) Bulmer JN, Sunderland CA. Immunohistological characterization of lymphoid cell populations in the early human placental bed. *Immunology* 1984;52(2):349-357.

- (97) Ritson A, Bulmer JN. Extraction of leucocytes from human decidua. A comparison of dispersal techniques. *J.Immunol.Methods* 1987;104(1-2):231-236.
- (98) Dallenbach-Hellweg G. The normal histology of the endometrium. *Histopathology of the endometrium*.4th ed.Berlin: Springer-Verlag 1987:36-39.
- (99) Hamperl H, Hellweg G. Granular endometrial stroma cells. *Obstet.Gynecol.Surv.* 1958;13(6):891.
- (100) Kazzaz BA. Specific endometrial granular cells. A semiquantitative study. *Specific endometrial granular cells.A semiquantitative study* 1972;2(3):77-84.
- (101) Sunderland CA, Bulmer JN, Luscombe M. Immunohistological and biochemical evidence for a role for hyaluronic acid in growth and development of the placenta. *J.Reprod.Immunol.* 1985;8(2-3):197-212.
- (102) Bulmer JN, Sunderland CA. Immunohistological characterization of lymphoid cell populations in the early human placental bed. *Immunology* 1984;52(2):349-357.
- (103) Bulmer JN, Morrison L, Longfellow M, Ritson A, Pace D. Granulated lymphocytes in human endometrium: Histochemical and immunohistochemical studies. *Human Reproduction* 1991;6(6):791-798.
- (104) Trundley A, Moffett A. Human uterine leukocytes and pregnancy. *Tissue Antigens* 2004;63(1):1.
- (105) Bulmer JN, Morrison L, Longfellow M, Ritson A, Pace D. Granulated lymphocytes in human endometrium: histochemical and immunohistochemical studies. *Hum.Reprod.* 1991;6(6):791-798.
- (106) King A, Wellings V, Gardner L, Loke YW. Immunocytochemical characterization of the unusual large granular lymphocytes in human endometrium throughout the menstrual cycle. *Hum.Immunol.* 1989 Mar;24(3):195-205.
- (107) Sacks G, Sargent I, Redman C. An innate view of human pregnancy. *Immunol.Today* 1999;20(3):114-118.
- (108) Yovel G, Shakhar K, Ben-Eliyahu S. The effects of sex, menstrual cycle, and oral contraceptives on the number and activity of natural killer cells. *Gynecol.Oncol.* 2001;81(2):254-262.
- (109) Koopman LA, Kopcow HD, Rybalov B, Boyson JE, Orange JS, Schatz F, et al. Human decidual natural killer cells are a unique NK cell subset with immunomodulatory potential. *J.Exp.Med.* 2003;198(8):1201.

(110) Abadia-Molina AC, Ruiz C, Montes MJ, King A, Loke YW, Olivares EG. Immune phenotype and cytotoxic activity of lymphocytes from human term decidua against trophoblast. *J.Reprod.Immunol.* 1996;31(1-2):109-123.

(111) Barber EM, Pollard JW. The Uterine NK Cell Population Requires IL-15 but These Cells Are Not Required for Pregnancy nor the Resolution of a *Listeria monocytogenes* Infection 1. *The Journal of Immunology* 2003;171(1):37-46.

(112) Aoki K, Kajiuura S, Matsumoto Y, Ogasawara M, Okada S, Yagami Y, et al. Preconceptional natural-killer-cell activity as a predictor of miscarriage. *Lancet(British edition)* 1995;345(8961):1340-1342.

(113) Beer AE, Kwak JYH, Ruiz JE. 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(1989)* 1996;35(4):376-382.

(114) Fukui A, Fujii S, Yamaguchi E, Kimura H, Sato S, Saito Y. Natural killer cell subpopulations and cytotoxicity for infertile patients undergoing in vitro fertilization. *Am. J. Reprod. Immunol* 1999;41:413-422.

(115) Emmer PM, Nelen WLD, Steegers EAP, Hendriks JCM, Veerhoek M, Joosten I. Peripheral natural killer cytotoxicity and CD56posCD16pos cells increase during early pregnancy in women with a history of recurrent spontaneous abortion. *Human Reproduction* 2000;15(5):1163-1169.

(116) Ntrivalas EI, Kwak-Kim JYH, Gilman-Sachs A, Chung-Bang H, Ng SC, Beaman KD, et al. Status of peripheral blood natural killer cells in women with recurrent spontaneous abortions and infertility of unknown aetiology. *Hum.Reprod.* 2001 May 1;16(5):855-861.

(117) Putowski L, Darmochwal-Kolarz D, Rolinski J, Oleszczuk J, Jakowicki J. The immunological profile of infertile women after repeated IVF failure (Preliminary study). *European Journal of Obstetrics & Gynecology and Reproductive Biology* 2004 2/10;112(2):192-196.

(118) Rai R, Sacks G, Trew G. Natural killer cells and reproductive failure-theory, practice and prejudice. *Human Reproduction* 2005;20(5):1123-1126.

(119) Moffett A, Regan L, Braude P. Natural killer cells, miscarriage, and infertility. *Br.Med.J.* 2004;329(7477):1283-1285.

(120) Strong DM, Ortaldo JR, Pandolfi F, Maluish A, Herberman RB. Cryopreservation of human mononuclear cells for quality control in clinical immunology. I. Correlations in recovery of K-and NK-cell functions, surface markers, and morphology. *J.Clin.Immunol.* 1982;2(3):214-221.

- (121) Porzolt F, Gaus W, Heimpel H. The evaluation of serial measurements of the NK cell activity in man. *Immunobiology* 1983 Dec;165(5):475-484.
- (122) Plackett TP, Boehmer ED, Faunce DE, Kovacs EJ. Aging and innate immune cells. *J.Leukoc.Biol.* 2004 August 1;76(2):291-299.
- (123) Eidukaite A, Siaurys A, Tamosiunas V. Differential expression of KIR/NKAT2 and CD94 molecules on decidual and peripheral blood CD56bright and CD56dim natural killer cell subsets. *Fertil.Steril.* 2004;81:863-868.
- (124) Bisset LR, Lung TL, Kaelin M, Ludwig E, Dubs RW. Reference values for peripheral blood lymphocyte phenotypes applicable to the healthy adult population in Switzerland. *Eur.J.Haematol.* 2004;72(3):203.
- (125) Thum MY, Bhaskaran S, Abdalla HI, Ford B, Sumar N, Shehata H, et al. 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. *Hum.Reprod.* 2004 October 1;19(10):2395-2400.
- (126) Fleischmann RM, Iqbal I, Stern RL. Considerations with the use of biological therapy in the treatment of rheumatoid arthritis. *eds* 2004;3(5):391-403.
- (127) Toder V, Fein A, Carp H, Torchinsky A. TNF-alpha in pregnancy loss and embryo maldevelopment: a mediator of detrimental stimuli or a protector of the fetoplacental unit. *J.Assist.Reprod.Genet.* 2003;20:73-81.
- (128) Laskin CA, Bombardier C, Hannah ME, Mandel FP, Ritchie K, Farewell V, et al. Prednisone and Aspirin in Women with Autoantibodies and Unexplained Recurrent Fetal Loss. *N.Engl.J.Med.* 1997 July 17;337(3):148-154.
- (129) Sherer Y, Levy Y, Langevitz P, Rauova L, Fabrizzi F, Shoenfeld Y. Adverse effects of intravenous immunoglobulin therapy in 56 patients with autoimmune diseases. *Pharmacology* 2001;62:133-137.
- (130) Fukui A, Kwak-Kim J, Ntrivalas E, Gilman-Sachs A, Lee SK, Beaman K. Intracellular cytokine expression of peripheral blood natural killer cell subsets in women with recurrent spontaneous abortions and implantation failures. *Fertil.Steril.* 2008;89(1):157-165.
- (131) Li TC, Tuckerman EM, Laird SM. Endometrial factors in recurrent miscarriage. *Hum.Reprod.Update* 2002;8(1):43-52.
- (132) Quenby S, Bates M, Doig T, Brewster J, Lewis-Jones DI, Johnson PM, et al. Pre-implantation endometrial leukocytes in women with recurrent miscarriage. *Human Reproduction* 1999 Sep;14(9):2386-2391.

(133) Clifford K, Flanagan AM, Regan L. Endometrial CD56 natural killer cells in women with recurrent miscarriage: a histomorphometric study. *Human Reproduction* 1999;14(11):2727-2730.

(134) Michimata T, Ogasawara Ms, Tsuda H, Suzumori K, Aoki K, Sakai M, et al. Distributions of endometrial NK cells, B cells, T cells, and Th2/Tc2 cells fail to predict pregnancy outcome following recurrent abortion. *Am. J. Reprod. Immunol* 2002;47(4):196-202.

(135) Tuckerman E, Laird SM, Prakash A, Li TC. Prognostic value of the measurement of uterine natural killer cells in the endometrium of women with recurrent miscarriage. *Hum.Reprod.* 2007 Aug;22(8):2208-2213.

(136) Quenby S, Kalumbi C, Bates M, Farquharson R, Vince G. Prednisolone reduces preconceptual endometrial natural killer cells in women with recurrent miscarriage. *Fertility & Sterility* 2005 Oct;84(4):980-984.

(137) King A, Burrows T, Verma S, Hiby S, Loke Y. Human uterine lymphocytes. *Hum.Reprod.Update* 1998 September 1;4(5):480-485.

(138) Hiby SE, Walker JJ, O'shaughnessy KM, Redman CW, Carrington M, Trowsdale J, et al. Combinations of maternal KIR and fetal HLA-C genes influence the risk of preeclampsia and reproductive success. *J.Exp.Med.* 2004 Oct 18;200(8):957-965.

(139) Moffett A, Hiby SE. How Does the maternal immune system contribute to the development of pre-eclampsia? *Placenta* 2007 Apr;28 Suppl A:S51-6.

(140) Dosiou C, Hamilton AE, Pang Y, Overgaard MT, Tulac S, Dong J, et al. Expression of membrane progesterone receptors on human T lymphocytes and Jurkat cells and activation of G-proteins by progesterone. *J.Endocrinol.* 2008 Jan;196(1):67-77.

(141) Dosiou C, Giudice LC. Natural killer cells in pregnancy and recurrent pregnancy loss: endocrine and immunologic perspectives. *Endocr.Rev.* 2005;26(1):44-62.

(142) Moretta L, Bottino C, Pende D, Vitale M, Mingari MC, Moretta A. Different checkpoints in human NK-cell activation. *Trends Immunol.* 2004;25(12):670-676.

(143) Vacca P, Cantoni C, Prato C, Fulcheri E, Moretta A, Moretta L, et al. Regulatory role of NKp44, NKp46, DNAM-1 and NKG2D receptors in the interaction between NK cells and trophoblast cells. Evidence for divergent functional profiles of decidual versus peripheral NK cells. *Int.Immunol.* 2008 Nov;20(11):1395-1405.

(144) Moretta A, Bottino C, Vitale M, Pende D, Cantoni C, Mingari MC, et al. Activating receptors and coreceptors involved in human natural killer cell-mediated cytotoxicity. *Annu.Rev.Immunol.* 2001;19:197-223.

- (145) Lewis BJ, Croker S, Newton DJ, Lennon GP, Johnson PM, Christmas SE. Natural killer cell receptor expression by human first trimester decidual granular leukocytes and T-lymphocytes. *Am.J.Reprod.Immunol.* 2002 Aug;48(2):103-109.
- (146) Kopcow HD, Allan DS, Chen X, Rybalov B, Andzelm MM, Ge B, et al. Human decidual NK cells form immature activating synapses and are not cytotoxic. *Proc.Natl.Acad.Sci.U.S.A.* 2005 Oct 25;102(43):15563-15568.
- (147) El Costa H, Casemayou A, Aguerre-Girr M, Rabot M, Berrebi A, Parant O, et al. Critical and differential roles of NKp46- and NKp30-activating receptors expressed by uterine NK cells in early pregnancy. *J.Immunol.* 2008 Sep 1;181(5):3009-3017.
- (148) Manaster I, Mizrahi S, Goldman-Wohl D, Sela HY, Stern-Ginossar N, Lankry D, et al. Endometrial NK cells are special immature cells that await pregnancy. *J.Immunol.* 2008 Aug 1;181(3):1869-1876.
- (149) Ponnampalam AP, Gargett CE, Rogers PAW. Identification and hormonal regulation of a novel form of NKp30 in human endometrial epithelium. *Eur.J.Immunol.* 2008;38(1):216-226.
- (150) Croy A, van den Heuvel MJ, Borzychowski AM, Tayade C. Uterine natural killer cells: a specialized differentiation regulated by ovarian hormones. *Immunol.Rev.* 2006;214(1):161.
- (151) Croy BA, Esadeg S, Chantakru S, van den Heuvel M, Paffaro VA, He H, et al. Update on pathways regulating the activation of uterine Natural Killer cells, their interactions with decidual spiral arteries and homing of their precursors to the uterus. *J.Reprod.Immunol.* 2003 Aug;59(2):175-191.
- (152) Kitaya K, Yamaguchi T, Yasuo T, Okubo T, Honjo H. Post-ovulatory rise of endometrial CD16 (-) natural killer cells: in situ proliferation of residual cells or selective recruitment from circulating peripheral blood? *J.Reprod.Immunol.* 2007;76(1-2):45-53.
- (153) Kitaya K, Yasuda J, Yagi I, Tada Y, Fushiki S, Honjo H. IL-15 expression at human endometrium and decidua. *Biol.Reprod.* 2000;63(3):683-687.
- (154) Jones RK, Searle RF, Stewart JA, Turner S, Bulmer JN. Apoptosis, bcl-2 expression, and proliferative activity in human endometrial stroma and endometrial granulated lymphocytes. *Biol.Reprod.* 1998;58(4):995-1002.
- (155) Chantakru S, Miller C, Roach LE, Kuziel WA, Maeda N, Wang WC, et al. Contributions from Self-Renewal and Trafficking to the Uterine NK Cell Population of Early Pregnancy 1. *The Journal of Immunology* 2002;168(1):22-28.

(156) Heuvel MJ, Chantakru S, Xie X, Evans SS, Tekpetey F, Mote PA, et al. Trafficking of circulating pro-NK cells to the decidualizing uterus: regulatory mechanisms in the mouse and human. *Immunol.Invest.* 2005;34(3):273-293.

(157) Foulk RA, Zdravkovic T, Genbacev O, Prakobphol A. Expression of L-selectin ligand MECA-79 as a predictive marker of human uterine receptivity. *J.Assist.Reprod.Genet.* 2007 Jul;24(7):316-321.

(158) Lai T, Shih IM, Vlahos N, Ho CL, Zhao Y. Differential expression of L-selectin ligands in the endometrial epithelium during menstrual cycle. *Fertil.Steril.* 2004;82:75-76.

(159) Henderson TA, Saunders PT, Moffett-King A, Groome NP, Critchley HO. Steroid receptor expression in uterine natural killer cells. *Journal of Clinical Endocrinology & Metabolism* 2003 Jan;88(1):440-449.

(160) Okada H, Nakajima T, Sanezumi M, Ikuta A, Yasuda K, Kanzaki H. Progesterone enhances interleukin-15 production in human endometrial stromal cells in vitro. *J.Clin.Endocrinol.Metab.* 2000 Dec;85(12):4765-4770.

(161) Dunn CL, Critchley HO, Kelly RW. IL-15 regulation in human endometrial stromal cells. *J.Clin.Endocrinol.Metab.* 2002 Apr;87(4):1898-1901.

(162) Kitaya K, Yasuda J, Nakayama T, Fushiki S, Honjo H. Effect of female sex steroids on human endometrial CD16neg CD56bright natural killer cells. *Fertil.Steril.* 2003;79:730-734.

(163) Heuvel MJ, Chantakru S, Xie X, Evans SS, Tekpetey F, Mote PA, et al. Trafficking of circulating pro-NK cells to the decidualizing uterus: regulatory mechanisms in the mouse and human. *Immunol.Invest.* 2005;34(3):273-293.

(164) Fabbri M, Bianchi E, Fumagalli L, Pardi R. Regulation of lymphocyte traffic by adhesion molecules. *Inflammation Res.* 1999;48(5):239-246.

(165) Stamper H,Jr, Woodruff J. Lymphocyte homing into lymph nodes: in vitro demonstration of the selective affinity of recirculating lymphocytes for high-endothelial venules. *J.Exp.Med.* 1976 September 1;144(3):828-833.

(166) van den Heuvel MJ, Horrocks J, Bashar S, Taylor S, Burke S, Hatta K, et al. Menstrual cycle hormones induce changes in functional interactions between lymphocytes and decidual vascular endothelial cells. *Journal of Clinical Endocrinology & Metabolism* 2005;90(5):2835-2842.

(167) Alon R, Feigelson S. From rolling to arrest on blood vessels: leukocyte tap dancing on endothelial integrin ligands and chemokines at sub-second contacts. *Semin.Immunol.* 2002 Apr;14(2):93-104.

- (168) McEver RP. Selectins: lectins that initiate cell adhesion under flow. *Curr.Opin.Cell Biol.* 2002;14(5):581.
- (169) Rosen SD. Ligands for L-selectin: homing, inflammation, and beyond. *Annu.Rev.Immunol.* 2004;22:129-156.
- (170) van den Heuvel MJ, Horrocks J, Bashar S, Hatta K, Burke S, Evans SS, et al. Periovulatory increases in tissue homing potential of circulating CD56bright cells are associated with fertile menstrual cycles. *J.Clin.Endocrinol.Metab.* 2005;90(6):3606-3613.
- (171) Peel S, Bulmer JN. Lectin histochemistry of pregnant rat uterine tissues. *J.Anat.* 1996;188(1):197-205.
- (172) Peel S, Stewart I. Rat granulated metrial gland cells differentiate in pregnant chimeric mice and may be cytotoxic for mouse trophoblast. *Cell Differ.Dev.* 1989 Oct;28(1):55-64.
- (173) Paffaro VA,Jr, Bizinotto MC, Joazeiro PP, Yamada AT. Subset classification of mouse uterine natural killer cells by DBA lectin reactivity. *Placenta* 2003 May;24(5):479-488.
- (174) Engelhardt H, Croy BA, King GJ. Conceptus influences the distribution of uterine leukocytes during early porcine pregnancy. *Biol.Reprod.* 2002 Jun;66(6):1875-1880.
- (175) Ashkar AA, Black GP, Wei Q, He H, Liang L, Head JR, et al. Assessment of requirements for IL-15 and IFN regulatory factors in uterine NK cell differentiation and function during pregnancy. *J.Immunol.* 2003 Sep 15;171(6):2937-2944.
- (176) Bulmer JN, Lash GE. Human uterine natural killer cells: A reappraisal. *Mol.Immunol.* 2005;42(4 SPEC. ISS.):511-521.
- (177) Jones RK, Bulmer JN, Searle RF. Phenotypic and functional studies of leukocytes in human endometrium and endometriosis. *Hum.Reprod.Update* 1998;4(5):702-709.
- (178) King A, Balendran N, Wooding P, Carter NP, Loke YW. CD3- leukocytes present in the human uterus during early placentation: phenotypic and morphologic characterization of the CD56++ population. *Dev.Immunol.* 1991;1(3):169-190.
- (179) Kitaya K, Yamaguchi T, Honjo H. Central role of interleukin-15 in postovulatory recruitment of peripheral blood CD16 (-) natural killer cells into human endometrium. *Journal of Clinical Endocrinology & Metabolism* 2005;90(5):2932-2940.

(180) Taylor HS. Endometrial cells derived from donor stem cells in bone marrow transplant recipients. *JAMA* 2004;292(1):81-85.

(181) Quenby S, Nik H, Innes B, Lash G, Turner M, Drury J, et al. Uterine natural killer cells and angiogenesis in recurrent reproductive failure. *Hum.Reprod.* 2009;24(1):45-54.

(182) Lash GE, Otun HA, Innes BA, Bulmer JN, Searle RF, Robson SC. Low oxygen concentrations inhibit trophoblast cell invasion from early gestation placental explants via alterations in levels of the urokinase plasminogen activator system. *Biol.Reprod.* 2006;74(2):403-409.

(183) Hiby SE, Walker JJ, O'shaughnessy KM, Redman CW, Carrington M, Trowsdale J, et al. Combinations of maternal KIR and fetal HLA-C genes influence the risk of preeclampsia and reproductive success. *J.Exp.Med.* 2004 Oct 18;200(8):957-965.

(184) Le Bouteiller P, Piccinni MP. Human NK Cells in Pregnant Uterus: Why There? *Am.J.Reprod.Immunol.* 2008;59(5):401-406.

(185) Vassiliadou N, Bulmer JN. Characterization of endometrial T lymphocyte subpopulations in spontaneous early pregnancy loss. *Human Reproduction* 1998;13(1):44-47.

(186) Proll J, Bensussan A, Goffin F, Foidart JM, Berrebi A, Le Bouteiller P. Tubal versus uterine placentation: similar HLA-G expressing extravillous cytotrophoblast invasion but different maternal leukocyte recruitment. *Tissue Antigens* 2000;56(6):479-491.

(187) King A, Balendran N, Wooding P, Carter NP, Loke YW. CD3- leukocytes present in the human uterus during early placentation: phenotypic and morphologic characterization of the CD56++ population. *Dev.Immunol.* 1991;1(3):169-190.

(188) Nishikawa K, Saito S, Morii T, Hamada K, Ako H, Narita N, et al. Accumulation of CD16-CD56 natural killer cells with high affinity interleukin 2 receptors in human early pregnancy decidua. *Int.Immunol.* 1991;3(8):743-750.

(189) Vassiliadou N, Bulmer JN. Expression of CD69 activation marker by endometrial granulated lymphocytes throughout the menstrual cycle and in early pregnancy. *Immunology* 1998;94(3):368-375.

(190) Grizzle W. Special symposium: fixation and tissue processing models. *Biotech.Histochem.* 2009 Jun 22:1-9.

(191) Institute for Cancer and Genetics. *Immunohistochemistry.* , 2009.

(192) IHC World Team. Knowledge Centre, Introduction to Immunohistochemistry. 2007; Available at: <http://www.ihcworld.com/introduction.htm>, 2009.

(193) Janny L, Menezo YJ. Maternal age effect on early human embryonic development and blastocyst formation. *Mol.Reprod.Dev.* 1996 Sep;45(1):31-37.

(194) Munro JM, Briscoe DM, Tedder TF. Differential regulation of leucocyte L-selectin (CD62L) expression in normal lymphoid and inflamed extralymphoid tissues. *J.Clin.Pathol.* 1996 Sep;49(9):721-727.

(195) Genbacev OD, Prakobphol A, Foulk RA, Krtolica AR, Ilic D, Singer MS, et al. Trophoblast L-selectin-mediated adhesion at the maternal-fetal interface. *Science* 2003;299(5605):405-408.

(196) Tedder TF, Steeber DA, Pizcueta P. L-selectin-deficient mice have impaired leukocyte recruitment into inflammatory sites. *J.Exp.Med.* 1995 Jun 1;181(6):2259-2264.

(197) Watson SR, Fennie C, Lasky LA. Neutrophil influx into an inflammatory site inhibited by a soluble homing receptor-IgG chimaera. *Nature* 1991 Jan 10;349(6305):164-167.

(198) Lynch L, Golden-Mason L, Eogan M, O'Herlihy C, O'Farrelly C. Cells with haematopoietic stem cell phenotype in adult human endometrium: relevance to infertility? *Human Reproduction* 2007;22(4):919.

(199) Cho NH, Park YK, Kim YT, Yang H, Kim SK. Lifetime expression of stem cell markers in the uterine endometrium. *Fertil.Steril.* 2004 Feb;81(2):403-407.

APPENDIX

Abstract Submitted and Accepted For A Poster Presentation For British Maternal And Foetal Medicine Society On 18-19/06/09.

HIGH NUMBERS OF UNK CELLS LEAD TO A HOSTILE ENDOMETRIAL ENVIRONMENT FOR IMPLANTATION IN PATIENTS WITH RECURRENT IMPLANTATION FAILURE

L Khan, A Karam, J Drury, A W Tang, M Turner, S Quenby
University of Liverpool, Liverpool, United Kingdom

Recurrent implantation failure (RIF) is thought to be the worst end of a spectrum of disorders of placentation. Natural Killer (NK) cells have been associated with RIF, however, the importance of endometrial compared to peripheral blood NK cells is debated.

Hypothesis: uNK cells are originate in the endometrium and do not occur due to trafficking of peripheral blood NK cells

Methods: 20 patients with recurrent implantation failure (RIF: >5 failed embryo transfers) had endometrial biopsies taken at LH+ 7-9. Serial paraffin sections were stained using immunohistochemistry with the following: CD56 (all NK), CD16 and L-Selectin (peripheral NK), NKp30 (differentiated NK) and Ki67 (proliferation). The same areas in samples were analysed using a semi-quantative, single-blind approach observing areas surrounding vessels, oedema, and epithelium. Cases with high-uNK or low-uNK cell density were compared.

Results: Minimal numbers of CD16+ and L-Selectin+ cells were observed in all cases. In the high-uNK cases, a substaintial increase in NKp30+ cells were observed in areas surrounding vessels, with a slight increase of Ki67+ cells.

Conclusion: Low expression of markers for peripheral NK cells at LH+ 7-9 challenges the theory that uNK cells migrate from the blood. On the other hand, in patients with RIF and high-uNK cell density, differentiated and activated uNK cells surround vessels. The differentiation of resident uNK cells could be involved in angiogenesis and the induction of a hostile endometrial environment.

Abstract published as follows:

L. Khan, A. Karam, J. Drury, A. Tang, M. Turner, S. Quenby. High Numbers of Uterine Natural Killer Cells Lead to a Hostile Endometrial Environment For Implantation in Patients with Recurrent Implantation Failure. *Arch Dis Child Fetal Neonatal Ed* 2009;94:Fa58-Fa71

HIGH NUMBERS OF uNK CELLS LEAD TO A HOSTILE ENDOMETRIAL ENVIRONMENT FOR IMPLANTATION IN PATIENTS WITH RECURRENT IMPLANTATION FAILURE

Leena Khan¹, Asiyah Karam¹, Jo Drury¹, Ai-Wei Tang^{1,2}, Mark Turner^{1,2}, Siobhan Quenby^{1,2}

¹University of Liverpool, Liverpool, UK, ²Liverpool Women's Hospital



Introduction

Recurrent implantation failure (RIF) is thought to be the worst end of a spectrum of disorders of placentation. Natural Killer (NK) cells have been associated with RIF, however, the importance of endometrial compared to peripheral blood NK cells is debated.

Hypothesis

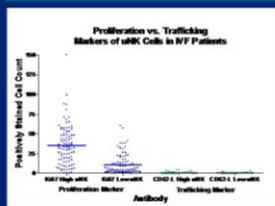
uNK cells originate in the endometrium and do not occur due to trafficking of peripheral blood NK cells.

Methods

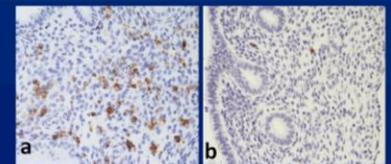
20 patients with recurrent implantation failure (RIF: >5 failed embryo transfers) had endometrial biopsies taken at LH+ 7-9. Serial paraffin sections were stained using immunohistochemistry with the following: CD56 (all NK), CD16 and L-Selectin (peripheral NK), Nkp30 (differentiated NK) and Ki67 (proliferation). The same areas in samples were analysed using a quantitative, single-blind approach observing areas surrounding vessels, oedema, and epithelium. Cases with high-uNK (n=9) or low-uNK (n=10) cell density were compared.

Results

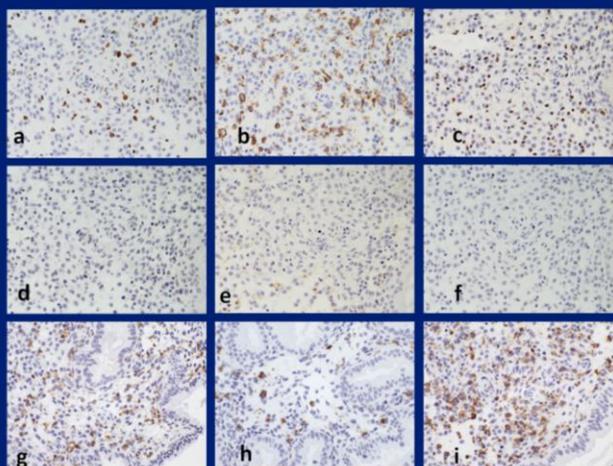
Minimal numbers of CD16+ and L-Selectin+ cells were observed in all cases. In the high-uNK cases there was a significant increase in the percentage staining in Nkp30 and Ki67 in vessels (p values 0.007 and 0.0001 respectively, when compared to areas of oedema and epithelium).



Sample Group	Age Median (range)	uNK count (%) Median (range)	IVF cycles Median (range)	Embryos transferred Median (range)
High uNK	37 (28-45)	7.7 (6.1-11)	3 (2-6)	8 (6-12)
Low uNK	37 (32-41)	2.1 (0.3-3.1)	4 (2-12)	8 (6-33)



Left: Clinical data for patient sample groups; 9 High and 10 Low uNK patients. Above: Micrographs of a) High uNK and b) Low uNK Patient



Micrographs of High uNK Patient:
 a → f: Micrographs of endometrial sections demonstrating area surrounding vessels
 a – Ki67 proliferation marker,
 b – CD56 uNK cell marker,
 c – Nkp30 differentiation marker, d – CD62-L, L-selectin trafficking marker,
 e – CD16 trafficking marker,
 f – IgG Mouse Negative control.
 g → i: Micrographs of a High uNK patient showing variation in distribution of CD56 positive cells
 g – Epithelium edge,
 h – Oedema,
 i – vessels

Antibody	Location	Average Antibody per stromal cell		Mann Whitney U-test Distribution compared to vessels	
		High	Low	High	Low
CD16	Epithelium	0.67	0.423	p=0.055	NS
	Oedema	1.83	0.9	NS	p=0.022
CD62-L	Epithelium	0.13	0.03	NS	NS
	Oedema	0.09	0.01	NS	NS
Nkp30	Epithelium	3.71	1.02	p=0.007	NS
	Oedema	4.89	1.08	NS	NS
Ki67	Epithelium	3.36	1.224	p=0.0001	NS
	Oedema	3.77	0.641	p=0.005	p=0.003
CD56	Epithelium	5.56	0.798	p=0.003	p=0.0001
	Oedema	7.003	1.456	p=0.006	p=0.003
	Vessels	8.12	1.784	-	-

Conclusion: Low expression of markers for peripheral NK cells at LH+ 7-9 challenges the theory that uNK cells migrate from the blood. On the other hand, in patients with RIF and high-uNK cell density, differentiated and activated uNK cells surround vessels. The differentiation of resident uNK cells could be involved in angiogenesis and the induction of a hostile endometrial environment.



University of Liverpool
 School of Reproductive and Developmental Medicine

Abstract Submitted and Accepted For A Poster Presentation For Blair Bell Meeting On 05/11/10.

High numbers of uNK cells lead to a hostile endometrial environment for implantation in patients with recurrent implantation failure.

L. Khan, A. Karam, A. Tang, J. Drury, M. Turner, S. Quenby

Department of Reproductive and Developmental Medicine, University of Liverpool, Liverpool Women's Hospital, Liverpool, L8 7SS

Background: Natural Killer (NK) cells have been associated with Recurrent implantation failure (RIF), however, the importance of endometrial compared to peripheral blood NK cells is debated.

Methods: Endometrial biopsies (LH 7-9) from 19 patients with RIF were stained with the following: CD56 (all NK), CD16 and L-Selectin (peripheral NK), NKp30 (differentiated NK) and Ki67 (proliferation). Areas surrounding vessels, oedema, and epithelium were observed in patients with high-uNK or low-uNK cell density groups.

Results: Minimal numbers of CD16+ and L-Selectin+ cells were observed universally, with substantial increases in NKp30+ ($p=0.023$) and Ki67+ ($p=0.006$) observed in areas surrounding vessels in both groups.

Conclusion: Low expression of peripheral NK markers challenges the theory that uNK cells migrate from the blood. In both high and low-uNK cell density patients, differentiated and activated uNK cells surround vessels. The differentiation of resident uNK cells could be involved in angiogenesis and the induction of a hostile endometrial environment.

HIGH NUMBERS OF uNK CELLS LEAD TO A HOSTILE ENDOMETRIAL ENVIRONMENT FOR IMPLANTATION IN PATIENTS WITH RECURRENT IMPLANTATION FAILURE

Leena Khan¹, Asiyah Karam¹, Jo Drury¹, Ai-Wei Tang^{1,2}, Mark Turner^{1,2}, Siobhan Quenby^{1,2}

¹University of Liverpool, Liverpool, UK, ²Liverpool Women's Hospital



Introduction

Recurrent implantation failure (RIF) is thought to be the worst end of a spectrum of disorders of placentation. Natural Killer (NK) cells have been associated with RIF, however, the importance of endometrial compared to peripheral blood NK cells is debated.

Hypothesis

uNK cells are originate in the endometrium and do not occur due to trafficking of peripheral blood NK cells.

Methods

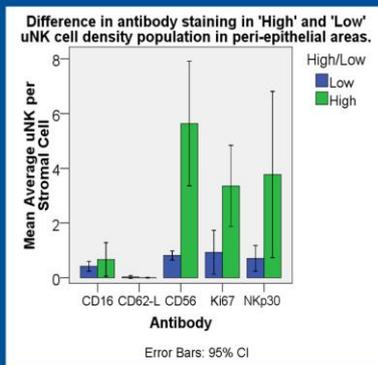
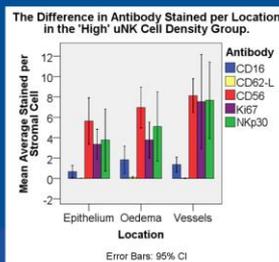
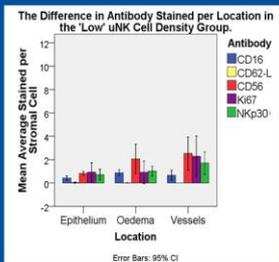
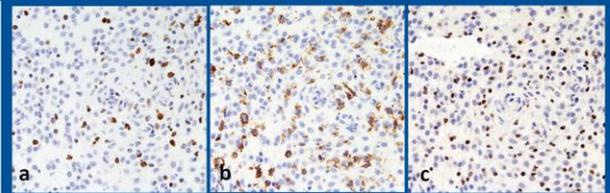
19 patients with recurrent implantation failure (RIF: >5 failed embryo transfers) had endometrial biopsies taken at LH+ 7-9. Serial paraffin sections were stained using immunohistochemistry with the following: CD56 (all NK), CD16 and L-Selectin (peripheral NK), Nkp30 (differentiated NK) and Ki67 (proliferation). The same areas in samples were analysed using a quantitative, single-blind approach observing areas surrounding vessels, oedema, and epithelium. Cases with high-uNK (n=9) or low-uNK (n=10) cell density were compared. **Analysis:** ANOVA with contrast.

Results

Differences in cell density were observed between both groups as expected. Few numbers of CD62-L+ and CD16+ were observed. The density of CD56+ (p=0.003), Ki67+ (p=0.006) and Nkp30+ (p=0.023) was also significantly higher in perivascular areas compared to peri-epithelial areas.

Sample Group	Age Median (range)	uNK count (%) Median (range)	IVF cycles Median (range)	Embryos transferred Median (range)
High uNK	37 (23-45)	7.7 (6.1-11)	3 (2-6)	8 (6-12)
Low uNK	37 (32-41)	2.1 (0.3-3.1)	4 (2-12)	8 (6-33)

Left: Clinical data for sampled groups: 9 High and 10 Low uNK patients
 Right: Micrographs of High uNK Patient: a - Ki67 proliferation marker, b - CD56 uNK cell marker, c - Nkp30 differentiation marker



Antibody	High/Low Group		Location	Comparison of Location Sites		
	Overall % Mean Difference	% Significant Difference (p-value)		Overall Significant Difference Between Locations (p-value)	Location (O = Oedema, E = Epithelium, V = Vessels)	Contrast to Explore Difference
CD16	0.652	0.013	0.033	O vs E	0.804	0.01
				V vs E	0.468	NS
CD62-L / L-Selectin	0.026	NS	0.040	O vs E	0.04	0.045
				V vs E	-0.007	NS
CD56	4.8567	0.000	0.011	O vs E	1.283	NS
				V vs E	2.099	0.003
Ki67	3.354	0.000	0.009	O vs E	0.201	NS
				V vs E	2.770	0.006
Nkp30	4.366	0.000	0.067	O vs E	0.018	NS
				V vs E	2.451	0.023

Conclusion: Low expression of markers for peripheral NK cells at LH+ 7-9 challenges the theory that uNK cells migrate from the blood, limiting effectiveness of peripheral blood testing as a screening tool in patients with RIF. In this population, differentiated and activated uNK cells surround vessels. The consistent perivascular location of these cells indicates that these cells have a role in vascular remodelling. The differentiation of resident uNK cells could be involved the induction of a hostile endometrial environment in patients with recurrent implantation failure.



University of Liverpool
 School of Reproductive and Developmental Medicine