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Roles of regulatory T cells in endometriosis-associated infertility

by

Azmat Riaz

SID: 420021533

A thesis submitted to the Sydney Medical School in fulfilment of the requirement for the degree of

Master of Philosophy in Medicine

September 2014

Queen Elizabeth II Research Institute for Mothers and Infants
Department of Obstetrics, Gynaecology and Neonatology
Sydney Medical School
The University of Sydney, NSW, 2006
Australia
Declaration

I hereby declare that the contents of this thesis consist of original work carried out by the author unless otherwise stated and duly acknowledged. To the best of my knowledge no part of this thesis has been submitted in whole or part for the award of any other degree of the university or other institution.

Azmat Riaz

September 2014
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First of all, I would like to express my deepest appreciation for my primary supervisor, Dr Alison Hey Cunningham. Without her guidance and help, this thesis would not have been possible within the limited time frame. I would also like to acknowledge, with much appreciation, the crucial role of Dr Marina Berbic for her guidance.

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Finally, I would like to say a big thanks to my family and husband for their encouragement and prayers.
Abstract

Background
Endometriosis is an oestrogen-dependent benign condition which is characterised by the presence of endometrial-like glands and stroma outside the uterus. Women with endometriosis usually present with pelvic pain and/or infertility. Endometriosis is a leading cause of infertility. Endometriosis is commonly thought to result from implantation of viable shed endometrial fragments in the peritoneal cavity after retrograde menstruation through the fallopian tubes; and genetic and endometrial factors, coelomic metaplasia and dissemination of menstrual debris through blood and lymphatic vessels may also contribute to its pathogenesis. In endometriosis, immune cells may be unable to effectively target the shed menstrual debris and allow their implantation.

Regulatory T cells (Tregs) are a group of immuno-suppressive lymphocytes that control other immune cells. In early pregnancy, Tregs suppress maternal immune responses to fetal antigens and prevent fetal rejection. Previous studies have demonstrated that Tregs are disturbed in women with endometriosis. Numbers of Tregs may be inadequate in unexplained infertility and sub-clinical pregnancy loss, yet their precise roles in endometriosis and associated infertility are unclear.

Aims
This project aimed to investigate the proportions and numbers of blood and endometrial Tregs in women with endometriosis and associated infertility.
Methods

Fertile and infertile women with and without endometriosis were recruited. Flow cytometry (n = 50) and immunohistochemistry (n = 88) were performed to analyse circulating and endometrial immune cell proportions and numbers, particularly Tregs, throughout the menstrual cycle.

Results

The overall results showed that numbers of Tregs and other lymphocytes differed in women with endometriosis, infertility and endometriosis-associated infertility in the menstrual cycle compared to controls. Endometrial Treg proportions were significantly increased during the proliferative phase in fertile compared to infertile women. Proportions of blood and endometrial live lymphocyte populations were increased during the proliferative phase in endometriosis, infertility and endometriosis-associated infertility.

Conclusions

This project provides new evidence for alterations in blood and endometrial Tregs and other lymphocyte populations in women with endometriosis, infertility and endometriosis-associated infertility. The immune system is tightly regulated during the normal menstrual cycle and variations in these cyclical patterns at systemic and endometrial levels may contribute to reproductive pathologies such as endometriosis and infertility. There is an indication of increased survival of lymphocytes in blood and the endometrium in endometriosis and infertility before the window of implantation. Increased survival of lymphocytes around the peri-implantation period, possibly secondary to altered Treg count in infertile women, may contribute to an inflammatory endometrial environment and to endometriosis and infertility.
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<th>Full Form</th>
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<tr>
<td>Ang-2</td>
<td>Angiopoietin-2</td>
</tr>
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<td>APCs</td>
<td>Antigen presenting cells</td>
</tr>
<tr>
<td>ART</td>
<td>Assisted reproductive techniques</td>
</tr>
<tr>
<td>Bax</td>
<td>Bcl-2 associated X protein</td>
</tr>
<tr>
<td>Bcl 2</td>
<td>B cell lymphoma 2 gene</td>
</tr>
<tr>
<td>17 β HSD-1</td>
<td>17 beta hydroxysteroid dehydrogenase-1</td>
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<tr>
<td>C3</td>
<td>Complement component C3</td>
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<td>CA 125</td>
<td>Cancer antigen/Carbohydrate antigen 125</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CL</td>
<td>Corpus luteum</td>
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<td>Cytotoxic T lymphocyte associated protein-4</td>
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<td>Chemokine receptor-3</td>
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<td>DCs</td>
<td>Dendritic cells</td>
</tr>
<tr>
<td>DN</td>
<td>Double negative</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxy-ribonucleic acid</td>
</tr>
<tr>
<td>DP</td>
<td>Double positive</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>ER</td>
<td>Oestrogen receptor</td>
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<tr>
<td>FF</td>
<td>Follicular fluid</td>
</tr>
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<td>Foxp3</td>
<td>Forkhead box protein3</td>
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<tr>
<td>FSH</td>
<td>Follicle stimulating hormone</td>
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<td>GAP-43</td>
<td>Growth associated protein-43</td>
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<td>GITR</td>
<td>Glucocorticoid induced tumour necrosis factor receptor</td>
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<td>GnRH</td>
<td>Gonadotropin releasing hormone</td>
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<td>HO-1</td>
<td>Heme-oxygenase-1</td>
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<td>ICSI</td>
<td>Intra-cytoplasmic sperm injection</td>
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<td>IVF</td>
<td>In-vitro fertilisation</td>
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<td>LAG-3</td>
<td>Lymphocyte activation gene-3</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>LH</td>
<td>Luteinizing hormone</td>
</tr>
<tr>
<td>LIF</td>
<td>Leuko-cyte migration Inhibitory Factor</td>
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<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte chemo-attractant protein 1</td>
</tr>
<tr>
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<td>Monthly fecundity rate</td>
</tr>
<tr>
<td>MIF</td>
<td>Macrophage migration inhibitory factor</td>
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<td>MMPs</td>
<td>Matrix metalloproteinases</td>
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<td>Messenger ribo-nucleic acid</td>
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<td>NGF</td>
<td>Nerve growth factor</td>
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<td>Natural Treg</td>
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<td>Peritoneal fluid</td>
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<tr>
<td>PGI₂</td>
<td>Prostaglandin I₂</td>
</tr>
<tr>
<td>POF</td>
<td>Premature ovarian failure</td>
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<tr>
<td>PR</td>
<td>Progesterone receptor</td>
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<tr>
<td>RANTES</td>
<td>Regulated on activation, normal T cell expressed and secreted</td>
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<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
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<tr>
<td>RSA</td>
<td>Recurrent spontaneous abortion</td>
</tr>
<tr>
<td>sICAM</td>
<td>Soluble intercellular adhesion molecule</td>
</tr>
<tr>
<td>suPAR</td>
<td>Soluble urokinase-type plasminogen activator receptor</td>
</tr>
<tr>
<td>sVEGFR</td>
<td>Soluble VEGF receptors</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>Th</td>
<td>Helper T cells</td>
</tr>
<tr>
<td>TIMPs</td>
<td>Tissue inhibitors of matrix metalloproteinases</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor-alpha</td>
</tr>
<tr>
<td>Treg</td>
<td>Regulatory T cell</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
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<td>----------------------------------</td>
</tr>
<tr>
<td>TVS</td>
<td>Trans-vaginal ultrasound</td>
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<tr>
<td>VEGF-A</td>
<td>Vascular endothelial growth factor-A</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organisation</td>
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CHAPTER 1: ENDOMETRIOSIS

1.1 INTRODUCTION

Endometriosis is a benign oestrogen-dependent gynaecological condition. It is characterised by the presence of functional endometrial-like glands and stroma outside the uterus (Sampson, 1927). Endometriosis is a chronic, progressive and debilitating disease and it adversely affects the quality of life of women. It is manifested commonly as pelvic pain and/or infertility (Hwang et al., 2012, Nezhat et al., 2012).

The reported overall incidence of endometriosis is 5–15 % in reproductive age women (Bulun, 2009). Since surgical visualisation is the gold standard for diagnosis, this may just be the ‘tip of the endometriosis iceberg’ and the true incidence of endometriosis be under-reported in asymptomatic population. Between 1-7 % of women having tubal sterilisation are diagnosed with ‘incidental’ endometriosis with visualisation of pelvis (ASRM, 2012).

1.2 UTERUS AND MENSTRUAL CYCLE

The body of the uterus is histologically composed of an inner mucosal endometrium with glands and stroma, an outer fibro-muscular myometrium and the outermost layer of connective tissue covered by peritoneum (the perimetrium). Based on histology and function, the endometrium can be divided into three layers; the superficial stratum compactum and stratum spongiosum (together forming stratum functionalis) and the deeper stratum basalis. The stratum functionalis is sloughed off during menstruation and is the site of cyclic changes in the endometrium. The stratum basalis does not take part in menstruation and is the regenerative zone for zona functionalis (Lebrun, 1993, Salamonsen, 1998, Evans et al., 2012).
Female reproduction depends on the cyclical development of the endometrium (menstrual cycle) and release of eggs (ovulation) from the ovary under influence of certain hormones. Disturbances in the menstrual cycle, ovulation or the balance of hormones can result in reproductive pathology (Mansfield and Emans, 1984). An overview of the female reproductive physiology is given below.

The menstrual cycle and ovulation are dependent on an intricate balance of hormones released by the hypothalamus, pituitary and ovaries - the hypothalamic-pituitary-ovarian (HPO) axis. Gonadotropin releasing hormone (GnRH) is released from the hypothalamus. Pulsatile GnRH secretion stimulates release of follicle stimulating hormone and luteinising hormone from the pituitary, which are transported to the ovary through the blood (Salamonsen, 1998). FSH facilitates the development of ovarian follicles and selection of a dominant follicle for ovulation. LH promotes production of androgenic hormones, which are converted into oestrogens under the influence of FSH (Mansfield and Emans, 1984).

The menstrual cycle is divided into three phases (menstrual, proliferative and secretory), which are based on hormone-dependent histological changes in the functional layer of endometrium (Evans et al., 2012) (Figure 1.1). The average cycle is 28 days but may vary from individual to individual. The normal range is 21 to 35 days (Lebrun, 1993).
Menstruation (days 1-3): In the absence of fertilisation, the corpus luteum (formed in the secretory phase) ceases to produce progesterone and removes negative feedback on the pituitary. Decreased levels of progesterone and LH result in constriction of the spiral arterioles and ischaemia of the endometrium. The arterioles rupture and shedding of the necrotic functional layer takes place (Salamonsen, 1998, Evans et al., 2012) (Figure 1.1).

Proliferative Phase (days 4-14): Under the influence of oestradiol secreted by ovarian follicles, the endometrium thickens, glandular structures in the stratum functionalis open out onto the surface and arteries and connective tissue renews. In the late-proliferative phase, oestradiol exerts a positive feedback on the pituitary and induces a pre-ovulatory surge of LH to facilitate release of egg from the dominant ovarian follicle (ovulation) (Figure 1.1) (Evans et al., 2012).

Secretory Phase (days 15-28): After ovulation, the ovarian follicle becomes a corpus luteum (CL) which is a temporary progesterone-synthesising endocrine structure (Figure 1.1) (Mansfield and Emans, 1984). Progesterone modifies the endometrial lining to allow for embryo implantation (Greydanus and McAnarney, 1982, Salamonsen, 1998). The endometrial glands become cork-screw shaped and secrete a glycogen rich secretion. The
endometrial stromal changes include increased mitosis, oedema and infiltration of white blood cells (leukocytes) (Salamonsen, 1998, Evans et al., 2012).

1.3 ENDOMETRIOTIC LESIONS

Endometriosis commonly involves parts of the female pelvis like the ovaries, anterior and posterior cul-de-sac, fallopian tubes and utero-sacral ligaments as illustrated in Figure 1.2. The bowel can be affected, most often in the advanced stages of the disease (Nezhat et al., 2011); bladder involvement does occur but is uncommon (Mettler et al., 2008); while other organs (like skin and lungs) are rarely affected (Wong et al., 1995, Chatzikokkinou et al., 2009, Huang et al., 2013). In the pelvis, endometriosis may be present in three forms: 1) Peritoneal endometriosis, 2) Ovarian endometriosis and 3) Deep infiltrating endometriosis (DIE).

Figure 1.2. Common locations for endometriotic lesions.
1.3.1 PERITONEAL ENDOMETRIOSIS

Peritoneal lesions are further classified into red, black and white types according to their appearance during laparoscopy (Jansen RP, 1986, Nisolle et al., 1993). Red lesions are considered as the initial form of endometriosis (Figure 1.3a). They have an extensive blood supply, giving their characteristic appearance (Nisolle and Donnez, 1997). Cyclical growth and menstrual ‘shedding’ of the tissue induces an inflammatory reaction and a ‘scarification’ process (Nisolle et al., 1993). As a result, the lesion appears as ‘black’ due to intra-luminal debris (Nisolle and Donnez, 1997). In some cases, the inflammation causes fibrosis and de-vascularisation of the implants, giving a ‘white’ or yellow-brown appearance (Figure 1.3 b).

![Figure 1.3](image.png)

Figure 1.3. Laparoscopic appearances of (a) red lesion and (b) new blood vessels around a scarred white lesion of peritoneal endometriosis (Fraser, 2008).

1.3.2 OVARIAN ENDOMETRIOSIS

In the ovaries, endometriosis typically presents as an endometrioma. Cyclical menstrual bleeding in the endometriotic cyst results in accumulation of characteristic thick-brown fluid and gives it the name ‘chocolate cyst’ (Redwine, 1987, Nisolle and Donnez, 1997) (Figure 1.4).
1.3.3 DEEP INFILTRATING

Endometriotic lesions penetrating the peritoneum by at least 5mm are considered to be deep (Kinkel et al., 1999, Mabrouk et al., 2011). Common sites of deep endometriosis include the infiltrative forms involving bladder, ureters, bowel and recto-vaginal tissue (Kinkel et al., 1999). Deep lesions cause inflammation and fibrosis in the surrounding tissues, retraction of the rectal mucosa and nodule formation in the utero-sacral ligaments (Redwine, 1987, Nisolle and Donnez, 1997) (Figure 1.5).

**Figure 1.5.** Deep infiltrating endometriosis showing focal infiltration and retraction of utero-sacral ligament (Chamié et al., 2011).
1.4 PATHOPHYSIOLOGY

1.4.1 GENETIC FACTORS

Endometriosis is a polygenic disorder with multiple allelic variations (Dun et al., 2010, Ballester et al., 2012, Layman, 2013). Women have a seven times greater risk of developing endometriosis if they have one first degree relative with the disease (Moen and Magnus, 1993) and more severe forms are associated with a family history of the disease (Moen and Magnus, 1993, Nouri et al., 2010). Twin studies also support a genetic basis of this disease (Treloar et al., 1999).

An association of endometriosis with genes such as HLA-B7, GALT, ABO, pseudo-cholineesterase, transferrin, C3 and phospho-gluco-mutase has been described (Simpson et al., 1980, Cramer et al., 1996, Treloar et al., 1999). More recently, it is shown that mutations at 7p15.2, 1p36, 2p25, 12q22, 2p14, 6p22.3 and 9p21.3 are associated with the increased risk of developing endometriosis (Painter et al., 2011, Nyholt et al., 2012).

1.4.2 THEORIES OF PATHOGENESIS

While the exact cause and pathogenesis of endometriosis remains unknown, there are a range of theories which have been proposed to explain its development.

1.4.2.1 COELOMIC METAPLASIA

The theory of coelomic metaplasia proposes that endometriosis develops from cells of the peritoneal cavity, such as the germinal epithelium of the ovary, which change by metaplasia into endometrial-like tissue (Iwanoff, 1898). This theory could explain the presence of endometriosis on the ovaries and peritoneum and in the absence of menstruation (Doty et al., 1980, Vinatier et al., 2001, Mok-Lin et al., 2010). However, several observations argue
against this theory: 1) endometriosis does not affect males, 2) metaplasia is not manifested in every tissue derived from coelomic epithelium (for example, pericardium) and 3) the incidence of common metaplasia increases with age. However, the incidence of endometriosis does not (Vinatier et al., 2001).

1.4.2.2 IMPLANTATION

According to the theory of implantation, endometrial tissue shed at menstruation is refluxed through the fallopian tubes and can implant in the abdominal cavity as endometriotic lesions (Sampson, 1927). This theory is widely accepted as the observed location of lesions correspond to tubal reflux and shed endometrial cells are viable and capable of implantation (Tabibzadeh, 1992, Vinatier et al., 2001). In addition, women with increased exposure to menstruation, for example, shorter menstrual cycles of less than or equal to 27 days and longer than one week of flow, have more than double the risk of developing endometriosis (Cramer et al., 1986, Viganò et al., 2004, Larosa et al., 2010). However, this theory fails to explain the presence of disease at distant sites and why only some women develop endometriosis, although retrograde menstruation occurs in almost all women with patent fallopian tubes (Halme et al., 1984).

1.4.2.3 AN ENDOMETRIAL DISEASE

As discussed above, retrograde menstruation is a quasi-universal phenomenon but endometriosis occurs in only 5-15% of women (Bulun, 2009). Evidence shows that in endometriosis, the eutopic endometrium is altered compared to women without the disease in a number of ways. This may facilitate establishment of endometriotic lesions from menstrual tissue following retrograde flow, which suggests that the primary defect in endometriosis may be in the uterine endometrium (Vinatier et al., 2000, Sharpe-Timms, 2001, Ulukus and
Endometrial anomalies in the eutopic endometrium of women with endometriosis are outlined in Table 1.1.

Table 1.1. Endometrial anomalies in eutopic endometrium from women with endometriosis compared to women without endometriosis.

<table>
<thead>
<tr>
<th>Endometrial anomaly</th>
<th>Description and pathogenic implications</th>
<th>Specific molecules</th>
<th>Expression</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apoptosis</td>
<td>Percentage of endometrial cells undergoing apoptosis is significantly ↓ which allows more viable cells to reach the peritoneal cavity</td>
<td>B cell lymphoma 2 gene and Bcl-2 associated X protein mRNA</td>
<td>↓</td>
<td>(Gebel et al., 1998, Meresman et al., 2000, Braun et al., 2002, Johnson et al., 2005, Burlev et al., 2006, Braun et al., 2007)</td>
</tr>
<tr>
<td>Cell adhesion molecules (CAMs)</td>
<td>Facilitate ectopic attachment of endometrial fragments</td>
<td>Soluble intercellular adhesion molecule, integrins, cadherin and selectins</td>
<td>↑ (some conflicting data)</td>
<td>(Lessey et al., 1994, Somigliana et al., 1996, Ota et al., 1996, Viganò et al., 2004, Kyama et al., 2008, Sundqvist et al., 2012)</td>
</tr>
<tr>
<td>Proteases and their inhibitors</td>
<td>↑ proteolytic activity and facilitation of peritoneal invasion</td>
<td>Matrix metalloproteinases, cathepsin B, D and G, plasminogen activator inhibitor-1, soluble urokinase-type plasminogen activator receptor</td>
<td>↑ (some conflicting data)</td>
<td>(Bruner et al., 1997, Chung et al., 2001, Guan et al., 2002, Chung et al., 2002, Collette et al., 2004, Zhou et al., 2004, Collette et al., 2006, Kyama et al., 2006, Gaetje et al., 2007, Di Carlo et al., 2009, Laudanski et al., 2013)</td>
</tr>
</tbody>
</table>

Tissue inhibitor of metalloproteinases ↓ (Chung et al., 2001, Chung et al., 2002, Zhao et al., 2007)
Proliferation  ↑ proliferation of shed endometrial fragments  Ki67 and epidermal growth factor, telomerase and telomerase length and transforming growth factor- beta  ↑ (Wingfield et al., 1995, Jones et al., 1995, Osteen et al., 1996, Jürgensen et al., 1996, Scotti et al., 2000, Burlev et al., 2006, Kim et al., 2007, Park et al., 2009, Aghajanova et al., 2010)

Angiogenesis  Establishment of blood supply to implanted fragments  Vascular endothelial growth factor-A, soluble Vascular endothelial growth factor receptors-1 and 2, angiogenin, and angiopoietin-2 and blood vessel density  ↑ (Donnez et al., 1998, Kressin et al., 2001, Burlev et al., 2005, Burlev et al., 2006, Cosín et al., 2009, Di Carlo et al., 2009, Bourlev et al., 2010, Hey-Cunningham et al., 2010)

Neurogenesis  In growth of nerve fibres and roles in endometriosis associated pain  Nerve growth factor, nerve growth factor receptors, transforming growth factor- α, growth associated protein-43, prostaglandin I₂ and nerve fibre density  ↑ (Ylikorkala and Viinikka, 1983, Okragly et al., 1999, Bergqvist et al., 2001, Tokushige et al., 2006, Bokor et al., 2009)


Steroid receptor expression  State of progesterone resistance inhibits matrix metalloproteinases and contributes to oestrogen dominance  -Progesterone receptors A and B and oestrogen receptor alpha  -Oestrogen receptor beta  ↓ ↑ (Fujishita et al., 1997, Attia et al., 2000, Matsuzaki et al., 2001, Igarashi et al., 2005, Bulun et al., 2006, Trukhacheva et al., 2009)

Eutopic endometrium in women with endometriosis differs from the normal endometrium during all menstrual cycle phases in a variety of ways (Sharpe-Timms, 2001, Akoum et al., 2007, Al-Jefout et al., 2009). As detailed in Table 1.1, several characteristic features including reduced apoptosis, dysregulated immune response and increased adhesiveness, proteolysis, proliferation, angiogenesis, neurogenesis and local oestrogen production are evident in the endometrium from women with endometriosis. These eutopic endometrial anomalies in endometriosis are hypothesised to enable the establishment and persistence of
endometriotic lesions from the refluxed endometrium and indicate that an innate endometrial aberration is central to the pathogenesis of endometriosis.

1.4.2.4 AN IMMUNE DISEASE

As touched upon in section 1.4.2.3 above, it is suggested that immune dysfunction contributes to the implantation of endometrial fragments and progression of endometriosis. The endometrial, peritoneal and blood immune cell populations in women with endometriosis differ from those of women without the disease, leading to the theory of endometriosis as an immune disease (Vinatier et al., 1996, Ho et al., 1997, Ulukus and Arici, 2005). The theory hypothesises that central to endometriosis pathogenesis is failure of the immune system to clear shed and refluxed endometrial tissue and to protect the peritoneal cavity from its adhesion and invasion. Specific immunological changes in endometriosis are discussed in detail in section 1.5. Adding support to this theory, endometriosis also fulfils the criteria for auto-immune diseases such as abnormalities of B and T cell function, production of auto-antibodies, tissue damage and multi-organ involvement (Weed et al., 1980, Mathur, 2000, Eisenberg et al., 2012).

1.5 IMMUNOLOGICAL CHANGES

Endometriosis is an enigmatic disease. The theories of pathogenesis discussed above (in section 1.4) can be linked by a defective immune system. In addition to the hypothesised permissive role of immune dysfunction in the implantation theory, in the metaplasia theory, growth and other factors resulting from immune activation may induce metaplasia of the inflamed mesothelium (Vinatier et al., 1996). Indeed, immunological changes are found to be associated with endometriosis and its consequences (Vinatier et al., 1996, Ulukus and Arici,
2005). It is, however, as yet unknown what triggers the immune system in endometriosis and whether these changes are primary or secondary to the disease (Berbic and Fraser, 2011).

Broadly speaking, the human immune system is divided into innate and adaptive immunity. The innate system is the first line of defence against foreign substances. It is comprised of immune cells such as the phagocytic cells (which ingest dead bacteria or harmful foreign bodies), important cytokines released by the immune cells, antibodies (proteins which identify and neutralise foreign bodies) and the complement system (made up of protein digesting enzymes which “complement” the antibodies, phagocytic cells and released cytokines once activated). In contrast to innate immunity, adaptive immunity is specific to antigens and has a memory of pathogens to which an individual has been previously exposed. An antigen can be a toxin or pathogen, which stimulates antibody production. Adaptive immunity involves the antigen-presenting cells (APCs) such as dendritic cells (DCs), lymphocytes and their released cytokines (lymphokines) and antibodies (Colacurci et al., 1991, Rier and Yeaman, 1997).

1.5.1 IMMUNE CELLS

Immune cells consist of the killer cells (such as the natural killer cells), the phagocytes (such as macrophages), APCs (such as dendritic cells [DCs]) and the lymphocytes (T and B). The Natural killer cells (NKs) are granular lymphocytes of the innate immune system (Manaster and Mandelboim, 2008) which have spontaneous cytotoxicity against tumour cells, virus infected cells and other cells (Gill, 2010). Macrophages engulf cellular debris and pathogens by phagocytosis (Oral et al., 1996). Dendritic cells recognise and capture antigens, process antigen material, present it on their cell surface to T cells and induce an immune response from T and B cells (Banchereau and Steinman, 1998, McKenna et al., 2005). B cells
function to make antibodies against antigens, perform suppressive functions by production of
granzymes (cell death inducing proteases) and can act as antigen-presenting cells (Mauri and

T cells are a subset of lymphocytes which defend the body against tumour cells and
pathogenic organisms, initiate antigen-specific T cell responses to fetal antigens and produce
cytokines that can have a role in maintenance or failure of pregnancy (Piccinni, 2005). They
are further divided into cytotoxic (CD8+), which destroy specific targets and the helper
(CD4+) T cells which transmit signals from the antigen-presenting cells (Osuga et al., 2011).
Helper T cells include the Th1 cells, Th2 cells (Osuga et al., 2011), Th17 cells (Park et al.,
2009) and the regulatory T cells (Robertson et al., 2009). Subpopulations of T cells, including
the regulatory T cells, will be discussed in detail in section 2.5.2.

1.5.2 IMMUNE CELL DYSREGULATION IN ENDOMETRIOSIS

Immune cells differ in numbers and function in various body compartments in different
phases of the menstrual cycle. These differences appear to facilitate the endometrium for
embryo implantation and prevent certain reproductive pathologies (Johnson et al., 1999, El-
Hamarneh et al., 2013). Changes in the numbers and function of different immune cells
during the normal menstrual cycle are summarised in Table 1.2.
Table 1.2. Immune cell population changes during the normal menstrual cycle.

<table>
<thead>
<tr>
<th>CELL TYPE</th>
<th>MENSTRUAL CYCLE</th>
<th>REFERENCES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Natural Killer Cells (NKs)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NUMBERS</td>
<td>↑ from proliferative to late secretory phase and ↓ after menstruation</td>
<td>(Starkey et al., 1991, Yovel et al., 2001, Lobo et al., 2004, Lee et al., 2011)</td>
</tr>
<tr>
<td>FUNCTION</td>
<td>↓ in cytotoxicity in secretory phase</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-Remodel spiral arteries, secrete angiogenic and growth factors</td>
<td></td>
</tr>
<tr>
<td></td>
<td>No change or ↑ in secretory phase compared to proliferative</td>
<td>(Sulke et al., 1985, Ho et al., 1996, Yovel et al., 2001, Souza et al., 2001, Lobo et al., 2004, Dosiou and Giudice, 2005, De Carolis et al., 2010, Lee et al., 2011)</td>
</tr>
<tr>
<td>Macrophages</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NUMBERS</td>
<td>-Stable in endometrium in proliferative and secretory phases</td>
<td>Unknown</td>
</tr>
<tr>
<td></td>
<td>- ↑ from mid-menstrual phase with peak in post-menstrual period</td>
<td>(Braun et al., 2002, Berbic et al., 2009)</td>
</tr>
<tr>
<td>FUNCTION</td>
<td>↑ Release of proteases help in degrading endometrium</td>
<td>Unknown</td>
</tr>
<tr>
<td></td>
<td>- Express adhesion and activation markers in proliferative phase, roles in endometrial regeneration</td>
<td>(Arcuri et al., 2001, Lin et al., 2010, Thiruchelvam et al., 2013)</td>
</tr>
<tr>
<td>Dendritic Cells (DCs)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NUMBERS</td>
<td>-Immature DCs ↑ throughout the menstrual cycle in basal layer</td>
<td>Unknown</td>
</tr>
<tr>
<td></td>
<td>- Mature DC numbers remain constant</td>
<td>(King, 2000, Rieger et al., 2004, Schulke et al., 2008, Berbic and Fraser, 2013)</td>
</tr>
<tr>
<td>FUNCTION</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
<tr>
<td>T Lymphocytes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NUMBERS</td>
<td>↓ from late proliferative to the late secretory phases</td>
<td>(Salamonsen and Lathbury, 2000, Flynn et al., 2000, Holtmeier and Kabelitz, 2005)</td>
</tr>
<tr>
<td>FUNCTION</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

In women with endometriosis, immune cells change in numbers and function during the menstrual cycle compared to women without the disease (Gazvani and Templeton, 2002, Larosa et al., 2010). These differences and how they may contribute to endometriosis pathogenesis are summarised in Table 1.3.
Table 1.3. Alterations in immune cell populations in women with endometriosis compared to women without the disease.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Endometrium</th>
<th>Lesions</th>
<th>Peritoneal fluid</th>
<th>Peripheral blood</th>
<th>Implications for pathogenesis</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>NUMBERS</td>
<td>↓ in the mid-secretory phase</td>
<td>↑ in the core lesions</td>
<td>No change</td>
<td>No change</td>
<td>- ↓ cytotoxicity allows fragments to implant -NKs may be recruited to the lesions in attempt to inhibit growth</td>
<td></td>
</tr>
<tr>
<td>FUNCTION</td>
<td>↓ cytotoxicity</td>
<td>Unknown</td>
<td>↓ cytotoxicity</td>
<td>↓ cytotoxicity</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Macrophages</td>
</tr>
<tr>
<td>FUNCTION</td>
<td>↓ phagocytosis</td>
<td>↓ phagocytosis, produce haptoglobin</td>
<td>↑ release of inflammatory cytokines</td>
<td>Unknown</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Dendritic Cells (DCs)</td>
</tr>
<tr>
<td>NUMBERS</td>
<td>-↑ immature DCs in the proliferative phase -↓ mature DCs in the proliferative phase</td>
<td>-↑ immature and mature DCs</td>
<td>↓ mature DCs</td>
<td>Unknown</td>
<td>- Disturbed antigen presentation -↑ DC recruitment in the lesions may be secondary to signalling by peritoneal micro-environment to get rid of shed debris</td>
<td>(Schulke et al., 2009, Tariverdian et al., 2009)</td>
</tr>
<tr>
<td>FUNCTION</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Unknown</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>T Lymphocytes</td>
</tr>
<tr>
<td>NUMBERS</td>
<td>-↓ CD8+ cells and γδ-T cells and ↑Tregs in secretory phase</td>
<td>↑ CD8 cells</td>
<td>↑ CD4 and CD8 cells</td>
<td>↑ in CD4:CD8 ratio</td>
<td>-↓ cytotoxicity allows survival of shed fragments -Altered cytokine secretion by helper T cells may affect macrophage function</td>
<td>(Steele et al., 1984, Gilmore et al., 1992, Ota et al., 1996, Bulmer et al., 1998, Szyllo et al., 2003, Gallinelli et al., 2004, Tariverdian et al., 2009, Berbic et al., 2010, Ganewatta et al., 2010, Osuga et al., 2011)</td>
</tr>
<tr>
<td>FUNCTION</td>
<td>↓ anti-inflammatory action</td>
<td>↓ anti-inflammatory cytokine production</td>
<td>↓ anti-inflammatory cytokine production</td>
<td>↓ anti-inflammatory cytokine production</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Although data is lacking and the exact mechanisms are not well understood, defective immune surveillance in the endometrium, altered recruitment to lesions from systemic circulation (and vice versa) and inflammatory environment in the peritoneum may contribute to the pathogenesis of endometriosis.

1.5.3 CYTOKINES AND GROWTH FACTORS

Cytokines are cell-signalling protein molecules, which play a major role in the initiation and regulation of immune and inflammatory responses. Immune cell activation results in release of a cascade of cytokines from various cells (Iwabe et al., 2002). In endometriosis, cytokines are mainly produced by macrophages but lymphocytes, endometrial lesions and mesothelial cells of the peritoneum also release a variety of cytokines. Cytokines can be pro- or anti-inflammatory, which recruit different cell types to the site of inflammation (Iwabe et al., 2002).

Cytokine secretion is altered in women with endometriosis in the endometrium, peritoneal lesions, peritoneal fluid and peripheral blood compared to women without the disease. Table 1.4 summarises the expression and roles of key cytokines in endometriosis.
Table 1.4. Important cytokines and their possible roles in endometriosis.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Produced by</th>
<th>Function/s</th>
<th>Levels in endometriosis</th>
<th>Possible role/s in endometriosis</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interleukin-1</td>
<td>Macrophages</td>
<td>-Induces angiogenesis and expression of adhesion molecules -Pro-inflammatory</td>
<td>Endometrium-unchanged Lesions-↑ Peritoneal fluid-↑/unchanged Blood -unchanged</td>
<td>-Promotes blood supply to lesions -Promotes endometrial cell peritoneal adhesion</td>
<td>(Fakih et al., 1987, Mori et al., 1991, Keenan et al., 1995, Wang et al., 1995, Bedaiwy et al., 2002, Voronov et al., 2003, Hudelist et al., 2005, Sokolov et al., 2005, Kalu et al., 2007, Guay et al., 2011)</td>
</tr>
<tr>
<td>Interleukin-2</td>
<td>Helper T cells</td>
<td>-Induce Fas-mediated apoptosis</td>
<td>Endometrium-↑ Lesions-unknown Peritoneal fluid-unchanged Blood-unchanged</td>
<td>↑ apoptosis of lymphocytes in the endometrium that normally target shed fragments</td>
<td>(Melioli et al., 1993, Keenan et al., 1995, Hsu et al., 1997, Jaleco et al., 2003, Sokolov et al., 2005)</td>
</tr>
<tr>
<td>Interleukin-4</td>
<td>Th2, natural killer, γδ and mast cells</td>
<td>Induces growth factor independent-1</td>
<td>Endometrium-Unknown Lesions-↑ Peritoneal fluid-↑ Blood-↑</td>
<td>Stimulates proliferation of endometriotic stromal cells</td>
<td>(Ho et al., 1996, Hsu et al., 1997, Zhu et al., 2002, Antsiferova et al., 2005, Ouyang et al., 2010)</td>
</tr>
<tr>
<td>Interleukin-8</td>
<td>Macrophages</td>
<td>Induces angiogenesis</td>
<td>Endometrium-unknown Lesions-↑ Peritoneal fluid-↑ Blood-↑</td>
<td>Improves blood supply and proliferation within endometriotic lesions</td>
<td>(Keenan et al., 1995, Kyama et al., 2006, Kalu et al., 2007, Carmona et al., 2012)</td>
</tr>
<tr>
<td>Interleukin -10</td>
<td>Monocytes, Th2 CD4+ cells, regulatory T cells and activated B cells</td>
<td>Potent growth and differentiation factor for activated B cells</td>
<td>Endometrium-unknown Lesions-unknown Peritoneal fluid-unchanged Blood-unchanged</td>
<td>B cell function ↑ in endometriosis but exact role in disease pathogenesis unknown</td>
<td>(Rousset et al., 1992, Ho et al., 1997)</td>
</tr>
<tr>
<td>----------------</td>
<td>-----------------------------------------------------------------</td>
<td>---------------------------------------------------------------</td>
<td>---------------------------------------------------------------------------------</td>
<td>--------------------------------------------------------------------------------</td>
<td>--------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Interleukin -11</td>
<td>Endometrial stromal and epithelial cells</td>
<td>↑ lympho- &amp; haemato-poiesis, placentation and decidualisation and facilitates implantation</td>
<td>Endometrium- ↓ Lesions-unknown Peritoneal fluid-unknown Blood-unknown</td>
<td>Interferes with uterine receptivity and embryo implantation</td>
<td>(Dimitriadis et al., 2006)</td>
</tr>
<tr>
<td>Monocyte Chemotactic Protein 1</td>
<td>Macrophages and endothelial cells</td>
<td>Chemo-attractant, pro-inflammatory and activating factor for monocytes</td>
<td>Endometrium-unknown Lesions- ↑ Peritoneal fluid- ↑ Blood- ↑</td>
<td>Recruitment and activation of peritoneal macrophages</td>
<td>(Jolicoeur et al., 1998, Kalu et al., 2007)</td>
</tr>
<tr>
<td>Macrophage migration Inhibitory Factor</td>
<td>Macrophages and dendritic cells</td>
<td>Induces the expression of aromatase (enzyme for oestrogen synthesis)</td>
<td>Endometrium- ↑ Lesions, peritoneal fluid and blood-unknown</td>
<td>Oestrogen promotes growth of endometriotic lesions</td>
<td>(Collette et al., 2006, Veillat et al., 2012)</td>
</tr>
<tr>
<td>Tumor Necrosis Factor-alpha</td>
<td>Macrophages and lymphocytes</td>
<td>-Regulate immune cells -Endometrial proliferation and shedding, -Pro-inflammatory</td>
<td>Endometrium, lesions and blood-unknown Peritoneal fluid- ↑</td>
<td>Promotes adhesion of endometrial fragments</td>
<td>(Lebovic et al., 2000, Birt et al., 2013)</td>
</tr>
</tbody>
</table>
The development of endometriosis is a complex process, which includes disturbances in the numbers and function of immune cells and their released cytokines. Cytokine changes in endometriosis appear to increase angiogenesis, growth of lesions, inflammatory cell recruitment, adhesions and oestrogen production and interfere with embryo implantation and production of auto-antibodies against the endometrial cells. These changes may result in a failure of the immune system to clear shed and refluixed endometrial tissue and enhance development of the endometriotic lesions.

1.6 CLINICAL ASPECTS

1.6.1 SYMPTOMS

Endometriosis commonly presents with pelvic pain and/or infertility.

1.6.1.1 PELVIC PAIN

Pain is an unpleasant sensory or emotional experience due to actual or potential tissue damage. In women evaluated for pelvic pain, 40-60 % are diagnosed with endometriosis (Murphy, 2002). Pelvic pain in endometriosis may be chronic or acute, in the form of dysmenorrhea (pain during menstruation), dyspareunia (painful sexual activity) and dyschezia (pain during defecation). Pelvic pain can be cyclic or acyclic. In endometriosis, 90 % of women suffer from dysmenorrhoea, 42 % from deep dyspareunia and 39 % from non-menstrual pelvic pain (Jamieson and Steege, 1996). Although endometriosis is associated with pelvic pain, the mechanisms of pain in this disease are not well understood but likely include the nociceptive, inflammatory or neuropathic pathways (Jamieson and Steege, 1996, Howard, 2003, Howard, 2009).
1.6.1.2 INFERTILITY
Infertility can be the only presenting symptom of endometriosis (Murphy, 2002).
Endometriosis is present in 25-40 % of infertile women (Ozkan et al., 2008) and 30-50 % of women diagnosed with endometriosis present with infertility (Ajossa et al., 1994).
Endometriosis-associated infertility will be discussed in detail in the second chapter of this literature review (section 2.3).

1.6.2 DIAGNOSIS
The gold standard for diagnosing endometriosis is visualisation of the abdominal and pelvic viscera under general anaesthesia by diagnostic laparoscopy (Kennedy et al., 2005).
Laparoscopy involves keyhole surgery through small abdominal incisions and use of a camera to visualise the pelvic cavity. Inspection of the endometriotic implants and endometriomas at laparoscopy is used in conjunction with confirmation by histological examination (Murphy, 2002).

Development of an accurate non-invasive diagnostic method for endometriosis is highly desirable. Imaging techniques like trans-vaginal ultrasound (TVS) and magnetic resonance imaging (MRI) have roles in diagnosing ovarian and deep lesions (Kennedy et al., 2005).
Diagnostic tests using serum bio-markers such as IL-6, IL-8, CA-125, anti-transferrin and anti-2HS glycoprotein antibodies (Pillai et al., 1996, Mathur et al., 1998, Velasco et al., 2010, May et al., 2010, Somigliana et al., 2010) or endometrial biopsy (for example, detection of nerve fibres) have been explored (Al-Jefout et al., 2004), however, no method suitable for routine clinical practice has been identified yet (Somigliana et al., 2010).
1.6.3 TREATMENT

Treatment options for endometriosis depend on the severity of the disease and include medical and surgical therapy, either alone or in combination. Management of endometriosis should be individualised depending on the desired treatment outcome like relief from pain, preventing recurrence or treatment of infertility (Ozkan and Arici, 2009).

1.6.3.1 MEDICAL

Medical options include analgesia, such as over the counter pain killers (for example, non-steroidal anti-inflammatory drugs), and the hormonal therapies, such as oral contraceptive pills (OCPs), progestogens (oral, injectable, sub-dermal implants or intra-uterine device) and gonadotropin-releasing hormone (GnRH) analogues (Olive and Pritts, 2001, Olive et al., 2003, Ozawa et al., 2006). All hormonal options in general aim to reduce oestrogen dominance of the disease and by inducing an amenorrhoeic state, arrest the growth of endometriotic lesions (Ozkan and Arici, 2009). All hormonal agents have almost the same efficacy, although they differ in their side effect profile (Ozkan and Arici, 2009). Medical treatment generally improves pain scores for endometriosis-related pain (Ailawadi et al., 2004, Batzer, 2006, Abushahin et al., 2011). Medical therapy is not suitable if fertility is desired, as most of the options are contraceptive (Olive and Pritts, 2001, Ozkan and Arici, 2009).

1.6.3.2 SURGICAL

Endometriosis surgery aims to remove all the apparent lesions in the pelvis and abdomen and restore normal anatomy, either through the laparoscope or via laparotomy with access to the abdomino-pelvic cavity through a wide incision (Hershlag and Markovitz, 2005). Surgical approaches include conservative options such as excision of lesions, destruction by electro-
cautery or lasers through to radical surgery involving removal of uterus (hysterectomy) and/or bilateral fallopian tubes and ovaries (salpingo-oophorectomy) and deep resection of bowel and bladder lesions (Kim and Adamson, 1999, Osuga et al., 2002). Cautery can be used to ablate the peritoneal lesions (Valle and Sciarra, 2003) but it is ineffective in clearing DIE and can cause injury to the underlying structures such as vessels and the ureters (Milingos et al., 2003, Valle and Sciarra, 2003, Abbott et al., 2003). Therefore, surgical excision is the preferred method for removal of endometriosis, particularly DIE (Milingos et al., 2003). Laparoscopy and laparotomy are equally effective in treating endometriosis but laparoscopy has the added advantage of better visualisation, less tissue trauma and faster recovery (Vercellini et al., 2010).

Although both surgical and medical treatments can effectively relieve pain associated with endometriosis, recurrence rates remain high (Olive and Pritts, 2001, Olive et al., 2003, Ozawa et al., 2006). Following conservative surgery, endometriosis has a recurrence rate of 20-40% within five years. Whether endometriosis will recur post-operatively depends on how severe the disease was at the time of surgery and how completely the lesions were removed (Vercellini et al., 2010).
CHAPTER 2: ENDOMETRIOSIS-ASSOCIATED INFERTILITY AND REGULATORY T CELLS

2.1 INTRODUCTION

As touched upon in chapter 1, women with endometriosis may present with infertility. Immunological disturbances in endometriosis may adversely affect the fertility of these women. Regulatory T cells are a sub-population of T cells with immuno-suppressive properties, which are crucial for female fertility and appear to be dysregulated in endometriosis. In this chapter, female infertility and its associations with immune alterations, particularly those of Tregs, will be discussed.

2.1.1 NORMAL CONCEPTION AND IMPLANTATION

In normal conception, fertilisation occurs in the fallopian tube, the embryo travels to the uterus and implants in the endometrium in the mid-secretory phase of the menstrual cycle (the ‘implantation window’) (Lee et al., 2011). Since half of the fetal antigens are of paternal origin, successful implantation depends on maternal immune tolerance of the fetus (feto-maternal tolerance) (Aagaard-Tillery et al., 2006). The immunological relationship between mother and fetus is complex. Endometrial function and embryo-endometrial interaction involve multiple immune and molecular signalling cascades (Simon et al., 1995). The endometrial immune cells and cytokines are altered in the peri-implantation period to establish and maintain pregnancy through various mechanisms, which are not yet fully understood.
2.2 INFERTILITY

The World Health Organisation (WHO) defines infertility as the inability of a sexually active couple, having coitus at least three times per month, to conceive in one year while not using any contraception (Hamada et al., 2012). Infertility occurs in around 15 % of couples (Kokcu et al., 2012). Infertility may be due to male or female factors or a combination of both. The main causes for female infertility, which may be associated with ovarian, tubal or endometrial immune disturbances, are absence of ovulation (anovulation), anatomical and/or functional abnormalities in the fallopian tubes (tubal factors) and endometriosis (Muse and Wilson, 1982, Mardh, 2004, Kokcu et al., 2012, Samsami Dehaghani et al., 2013). In male infertility, sperm numbers and/or function are altered that may be secondary to genetic or inherited diseases, specific abnormalities in the Y chromosome, anatomical abnormalities, hormonal disturbances, infections, chemicals, drugs and tumours (Nwabuisi and Onile, 2001, Costabile and Spevak, 2001, Poongothai et al., 2009, Ahmed et al., 2010).

2.2.1 IMMUNE ASPECTS OF FEMALE INFERTILITY

As mentioned in section 2.1.1, the endometrium ‘tolerates’ the fetus by immune mechanisms (Aagaard-Tillery et al., 2006). Disturbances in the numbers and/or function of the endometrial immune cells and their produced cytokines during the ‘implantation window’ may result in difficulty conceiving and pregnancy failure (Lee et al., 2011). Immune cell disturbances implicated in female infertility are summarised in Table 2.1.
Table 2.1. Immune disturbances in female infertility.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Roles in normal embryo implantation and development</th>
<th>Levels in infertility</th>
<th>Possible roles in infertility</th>
<th>References</th>
</tr>
</thead>
</table>
| **Dendritic Cells** | - ↑ in decidua  
- Pro-apoptotic, regulate angiogenesis and tissue remodelling and enhance development of Tregs            | ↑ in peritoneal fluid | Stimulate autoimmune response against oocyte                                                   | (Błaszkowska et al., 2001, Plaks et al., 2008, Pollard, 2008)               |
| **Macrophages**   | - Release pro-inflammatory cytokines  
- Enhance tissue and vascular remodelling and immune tolerance                                                              | ↑ in peritoneal fluid and fallopian tubes | Phagocytosis of sperm                                                                         | (Haney et al., 1983)                                                      |
| **Natural Killer cells** | - Secrete cytokines, help in migration of trophoblasts into spiral arteries and increase blood flow to fetus  
- Promote fetal-tolerance by regulating pro-inflammatory cells                                                      | Either no change or ↓ numbers in endometrium | Targeting of trophoblast cells can result in fetal rejection                                  | (Moffett et al., 2004, Vujisić et al., 2004, Hanna et al., 2006, Manaster and Mandelboim, 2008, Kumar and Medhi, 2008, Manaster and Mandelboim, 2010, Tang et al., 2011, Fu et al., 2013) |
| **T Cells**       | **Th1** – Numbers ↓ but cytokine production ↑, (Tumor Necrosis Factor-α and interferon-γ in 1st trimester)  
- Pro-inflammatory  
- Facilitate embryo implantation by ↑ endometrial invasion  
**Th2** - Numbers ↓ but cytokine production ↑, (interleukin-4 and 10 in 2nd trimester)  
- Anti-inflammatory  
- Facilitate fetal growth and development | No change in peritoneal fluid and blood | Unknown                                                                                        | (Veenstra et al., 2003, Vujisić et al., 2004, Mor et al., 2011)            |
| **B Cells**       | - Roles in folliculogenesis uncertain  
- Facilitate implantation, placentation, and early embryonic development                                                   | Immunoglobulin A & M ↓ in peritoneal fluid and blood  
In summary, immune alterations can contribute to unexplained female infertility by mechanisms such as disturbed ovulation, folliculogenesis and implantation and phagocytosis of sperm.

### 2.3 ENDOMETRIOSIS-ASSOCIATED INFERTILITY

The prevalence of infertility is up to 50% in women with endometriosis, much higher than in women without the disease in whom it is 5-10% (D'Hooghe et al., 2003, Koch et al., 2012, Stilley et al., 2012). In addition, about 25–50% of infertile women have endometriosis compared to 0.5-5% of fertile women (Ozkan et al., 2008, Bulletti et al., 2010, Falcone and Lebovic, 2011).

#### 2.3.1 MECHANISMS

It is well established that extensive endometriosis can distort the pelvic anatomy that may result in infertility. However, so called milder forms of the disease may also result in infertility (Falcone and Lebovic, 2011). Therefore, anatomical abnormalities alone are not responsible for endometriosis-associated infertility, suggesting other mechanisms like hormonal, cytokine or immunological abnormalities may have roles (Gupta et al., 2008, Holoch and Lessey, 2010). A range of processes implicated in endometriosis-associated infertility are reviewed below.

#### 2.3.1.1 FOLLICULOGENESIS AND OVULATION

In endometriosis, there are pro-inflammatory changes in the follicular fluid which may adversely affect the follicular growth and function (Garrido et al., 2000). Increased interleukin-6 (IL-6) and decreased vascular endothelial growth factor levels in follicular fluid in endometriosis compared to women with unexplained infertility result in slower growth rate
of ovarian follicles and reduced size and vascularisation of the dominant follicle, respectively (Pellicer et al., 1998, Cahill et al., 2000, Garrido et al., 2000, Trinder and Cahill, 2002, Yoshida et al., 2004). In endometriosis, elevated numbers of macrophages and leukocytes in follicular and peritoneal fluid produce reactive oxygen species, which interfere with ovarian steroidogenesis and ovulation (Das et al., 2006). Reactive oxygen species are chemically reactive molecules concerned with cell signalling and if increased in concentration, they cause cellular damage (Van et al., 2002). Altered folliculogenesis may contribute to ovulatory disturbances such as reduced circulating concentrations of oestradiol in the pre-ovulatory phase, delayed LH surge, anovulation or delayed release of oocyte from ovary (Trinder and Cahill, 2002).

2.3.1.2 OOCYTE QUALITY AND FUNCTION

Alterations within follicles may result in oocytes and embryos of lower quality in women with endometriosis (Pellicer et al., 2000). In endometriosis, the number of oocytes retrieved for in-vitro fertilisation (IVF), embryo cleavage rates and pregnancy rates are markedly decreased compared to women without the disease (Du et al., 2013). In addition, evidence suggests lower pregnancy rates with oocytes derived from women with endometriosis when donated to controls compared to oocytes derived from women without the disease (Simon et al., 1994). Reduced oestrogen levels in the follicular fluid (Wunder et al., 2005) may be the underlying cause of low quality and number of oocytes in endometriosis as oocyte quality and number is dependent on the follicular fluid oestrogen levels (Díaz et al., 2008). In addition, elevated levels of prostaglandin E2, a fatty acid derivative and regulator of oestrogen producing enzyme aromatase, in the follicular fluid and IL-10 in the peritoneal fluid in endometriosis may inhibit oocyte maturation by interfering with granulosa cell development (Punnonen et al., 1996, Noble et al., 1997, Díaz et al., 2008, Lin et al., 2012, Du
et al., 2013). Brizek and co-workers reported micro-tubular and chromosomal abnormalities in oocytes from women with endometriosis (Brizek et al., 1995). Although these findings are confirmed in mouse and bovine oocytes grown in-vitro in follicular fluid from infertile women with endometriosis (Mansour et al., 2009, Da Broi et al., 2014), more recent studies on human oocytes fail to support such abnormalities (Barcelos et al., 2008, Dib et al., 2013).

2.3.1.3 SPERM FUNCTION

In endometriosis, sperms available for fertilisation decrease in numbers due to their increased binding to the epithelium in the ampulla of fallopian tubes (Reeve et al., 2005). In addition, in women with endometriosis the environment within the reproductive tract increases sperm DNA fragmentation and membrane permeability, loss of membrane integrity, spermatid enzyme inactivation (Agarwal et al., 2006) and decreased sperm-oocyte fusion (Baker and Aitken, 2004). Elevated levels of reactive oxygen species in the peritoneal fluid in endometriosis may cause DNA fragmentation and other damage to the sperm cells (Van et al., 2002). In addition, increased peritoneal fluid levels of cytokines such as IL-6, Regulated on Activation, T cell Expressed and Secreted (RANTES) and Tumor Necrosis Factor-alpha reduce sperm motility, fertilising capacity, sperm counts and sperm-oocyte binding by enhanced reactive oxygen species production, respectively (Punnonen et al., 1996, Said et al., 2005, Bersinger et al., 2006, Barbonetti et al., 2008).

2.3.1.4 FERTILISATION AND IMPLANTATION

Lower pregnancy rates in women with endometriosis may largely be accounted for by abnormalities of fertilisation and/or implantation in the endometrium, resulting in failure of pregnancy establishment (Kao et al., 2003, Dechaud et al., 2009). Peritoneal fluid in endometriosis inhibits oocyte-sperm binding (Faber et al., 2001) due to altered cytokine and
growth factor balance (Vassiliadis et al., 2005). Possible mechanisms affecting implantation in endometriosis may include endometrial anomalies in endometriosis as reviewed in section 1.4.2.3 such as progesterone resistance with luteal phase deficiency, altered immune environment, altered expression of genes involved in embryonic attachment and apoptotic responses (Kao et al., 2003) and reduced or absent expression of the adhesion molecule integrin which facilitates implantation (Lessey et al., 1994). Collectively, these mechanisms create a hostile endometrial environment for the embryo.

### 2.3.1.5 PERITONEAL ENVIRONMENT

Peritoneal environment is altered in women with endometriosis, which may contribute to infertility in these women (Iwabe et al., 2002). Fertility may be affected in endometriosis by disruption of the pelvic anatomy and angiogenic and immunological alterations (Iwabe et al., 2002). While pelvic adhesions usually affect fertility in advanced stages of the disease, adhesion formation is mainly dependent on pro-inflammatory and angiogenesis-related cytokines (such as IL-6 and IL-8) in the peritoneal fluid (Barcz et al., 2012, Tagashira et al., 2009). Moreover, immune alterations in the peritoneal cavity may be the main culprit in the milder forms of the disease (Barcz et al., 2012). Immune cell populations (predominantly macrophages) and their released cytokines are altered in the peritoneal cavity of women with endometriosis compared to controls, which may adversely affect a variety of reproductive functions (Hou et al., 2009, Liu et al., 2000). Tubal macrophages increase in numbers secondary to peritoneal fluid macrophages in endometriosis (Haney et al., 1983). It is also suggested that elevated concentrations of apoptosis-inducing Fas-ligand in the peritoneal fluid enhance apoptosis of the granulosa cells of the ovarian follicle and interfere with quality of the oocyte (Garcia-Velasco et al., 2002). Increased embryo-toxicity of oocytes can be another mechanism of reduced fertility in women with endometriosis, which has been
demonstrated in mouse oocytes developed in human peritoneal fluid (Gomez-Torres et al., 2002).

In summary, fertility in endometriosis appears to be negatively affected at almost every stage of ovulation and establishment of pregnancy. Most of these mechanisms are related to immunological disturbances. Processes that are affected include folliculogenesis, ovulation, oocyte and sperm function, fertilisation, implantation and blastocyst development (Gupta et al., 2008).

2.4 ARTIFICIAL REPRODUCTIVE TECHNIQUES IN ENDOMETRIOSIS-ASSOCIATED INFERTILITY

Assisted reproductive technology (ART) is the application of laboratory or clinical techniques to gametes (human egg or sperm) and/or embryos for the purposes of reproduction. The most effective treatment for endometriosis-associated infertility is IVF (Senapati and Barnhart, 2011). As discussed in section 2.3, endometriosis affects fertility by various mechanisms. IVF can overcome several of these mechanisms such as disturbances of ovulation, defects of the fallopian tubes and tubal transport, sperm function and quality of gametes and embryos to some extent. However, disturbances in the eutopic endometrium and implantation may not be overcome by IVF.

Overall, women with endometriosis have lower pregnancy rates after IVF than those with other causes of infertility (Senapati and Barnhart, 2011). In infertile women without endometriosis, pregnancy rates are around 34% after each IVF cycle (Senapati and Barnhart, 2011). A large meta-analysis has suggested that pregnancy rates after IVF are reduced to half
in women with endometriosis compared to those without (Meldrum et al., 1998, Barnhart et al., 2002).

Intra-cytoplasmic sperm injection (ICSI) is an advanced form of ART in which a single sperm is injected directly into an egg (Sherins et al., 1995). ICSI offers the added advantage of higher fertilisation rates. It is, therefore, a better treatment option than conventional IVF in endometriosis-associated infertility complicated with male factor infertility (Mínguez et al., 1997, Bukulmez et al., 2001, Ballester et al., 2012, Komsky-Elbaz et al., 2013). But like IVF, ICSI may not alter the endometrial microenvironment to improve implantation.

In summary, current methods of ART improve chances of pregnancy and live birth rates in women with endometriosis-associated infertility but have limitations. It is highly desirable to develop better treatment options to increase chances of conception in infertile women with endometriosis.

2.5 T CELL LINEAGE

As discussed in section 2.3, a range of factors may contribute to endometriosis-associated infertility. Immune aspects, including immune cells and cytokines, are altered in the disease and may have a role in its pathophysiology and associated infertility (Holoch and Lessey, 2010). T cells are a group of immune cells which perform a wide range of functions. Their developmental pathway, subgroups, functions and how they may contribute to endometriosis-associated infertility will be discussed below.
2.5.1 T CELL DEVELOPMENT AND ANTIGEN RECOGNITION

T cells develop in the thymus from T cell progenitors (Bhandoola et al., 2003). Mature T cells express specific surface-markers such as T cell receptors (TCRs), CD4 and CD8. Progenitor T cells are CD4 and CD8 negative. Once they start to be expressed on T cells in the thymus, these cells are referred to as double positive thymocytes. At this stage, central tolerance mechanisms operate to ensure T cells that are capable of reacting against foreign-antigens are selected for further differentiation (positive selection). Those double positive cells which may initiate an auto-immune response are eliminated before they reach systemic circulation (negative selection) (Xiong and Raulet, 2007). Positive selection of double positive thymocytes allows their survival and interaction with self-major histocompatibility complex (MHC). MHC is a cell-surface molecule which mediates interaction between leukocytes and other cells. Depending on their structure, there are two classes of MHC (I and II). Double positive cells that interact well with MHC class II molecules will eventually become single positive CD4+ cells, while thymocytes that interact well with MHC class I molecules mature into single positive CD8+ cells. Double positive thymocytes that interact with MHC molecules either too strongly or too weakly die by neglect or programmed cell death (apoptosis), respectively (Xing and Hogquist, 2012).

Negative selection removes the thymocytes that interact too strongly with self-antigens and may cause auto-immunity. Negative selection occurs by clonal deletion, receptor editing or clonal diversion, ensuring tolerance of self-antigens (self-tolerance). Clonal deletion occurs during the fetal life when immunocytes with self-antigen reactivity are deleted (Palmer, 2003, Baldwin and Hogquist, 2007). Receptor editing changes TCR specificity by secondary gene rearrangements at the TCR loci to prevent auto-reactivity (Wang et al., 1998, Holman et al., 2003). Clonal diversion occurs when thymocytes get signals through their auto-reactive TCR
but are not able to receive co-stimulatory signals. In contrast to clonal deletion that ‘deletes’ self-reactive cells, clonal diversion imparts suppressive or regulatory function to self-reactive cells (Xing and Hogquist, 2012) but cells derived from clonal diversion are non-responsive (clonal anergy). Anergy can be clonal or adaptive. Clonal anergy is a growth-arrest state, which develops in the thymus and may be reversed by an interleukin-2-rich micro-environment. Adaptive anergy can be central (develop in the thymus) or peripheral and represent generalised inhibition of further proliferation of T cells at varying stages of development in persistent presence of a specific antigen. Adaptive anergy reverses if the particular antigen is removed (Schwartz, 2003, Baldwin and Hogquist, 2007).

The generation of Tregs in the thymus begins as a result of an altered negative selection process. Naïve T cells differentiate to Tregs when their TCR/antigen affinity is just weaker than that needed for negative selection but at the extremity of threshold for positive selection (Jordan et al., 2001) (Figure 2.1). Regulatory cells which receive co-stimulatory signals (by CD28) and CD86 expression acquire Foxp3 in the thymus (Chatenoud and Bach, 2006).

![Figure 2.1. Regulatory T cells are selected in the thymus when the avidity of their T cell receptors for self-major histocompatibility complex is lower than the threshold for negative selection by apoptosis but higher than the threshold for positive selection of T cells (Guerin et al., 2009).](image-url)
After positive and negative selection, remaining thymocytes mature as naïve T cells (Wang et al., 1998, Buch et al., 2002, Holman et al., 2003). Naïve T cells in the thymus are activated after binding with antigen-MHC complexes, facilitated by certain co-receptors (CD4 for helper and CD8 for cytotoxic T cells) (Davis and Bjorkman, 1998). Naïve T cells then leave the thymus (Fessler et al., 2013).

Central tolerance mechanisms in the thymus cannot remove all self-responsive T cells as not all antigens are expressed in the thymus (Xing and Hogquist, 2012). To ensure further self-tolerance, when T cells first encounter antigens after maturing and entering the periphery, they become functionally anergic to TCR signals (adaptive anergy) (Xing and Hogquist, 2012). Once in the periphery, T cells are presented antigens by antigen-presenting cells through MHC I and II (Burster, 2013). T cells in the periphery differentiate into T cell subtypes, which perform different functions, depending mainly on the cytokine micro-environment and antigenic type (Fietta and Delsante, 2009, Fessler et al., 2013).

2.5.2 SUB-POPULATIONS OF T CELLS

Classically, T cell sub-populations include cytotoxic, gamma-delta (γδ), helper (Th), memory and natural killer T (NKT) cells (Zheng et al., 2004, Wang et al., 2006) (Table 2.2).
### Table 2.2. Classification of T cells.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Function</th>
<th>Sub-classes</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytotoxic (CD8+)</td>
<td>- Kill tumour or virus infected cells - Release cytotoxins</td>
<td>-</td>
<td>(Milstein et al., 2011)</td>
</tr>
<tr>
<td>Gamma delta (γδ)</td>
<td>- First line of defence in epithelial cell-rich compartments - Produce cytokines - Potent cytotoxic activity</td>
<td>-</td>
<td>(Kabelitz and Wesch, 2003, Holtmeier and Kabelitz, 2005)</td>
</tr>
<tr>
<td>Helper (CD4+)</td>
<td>- Release cytokines - Help B cells to produce antibodies - Activate CD8+ cells - ↑ bactericidal activity of phagocytes</td>
<td>Helper type I (Th1) - Produce interferon-γ, interleukin-2, and tumor necrosis factor- β - Activate macrophages and phagocytosis</td>
<td>(Romagnani, 1999, Zheng et al., 2004, Wang et al., 2006, Sakaguchi, 2006, Rautajoki et al., 2008, Korn et al., 2009)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Helper type II (Th2) - Release interleukin-4, 5, 9 and 13 - Activate mast cells and eosinophils - Induce B lymphocytes - Production of Immunoglobulin E</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Helper type 17 (Th17) - Produce interleukin-17 and 21 - Pro-inflammatory - Recruit neutrophils to sites of inflammation</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Regulatory T cells (Tregs) - Control and suppress range of immune functions</td>
<td></td>
</tr>
<tr>
<td>Memory T cells</td>
<td>- Provide memory against past exposure - Quickly expand after re-exposure to antigen</td>
<td>-</td>
<td>(Akbar et al., 1988)</td>
</tr>
<tr>
<td>Natural killer T (NKT) cells</td>
<td>- Helper and cytotoxic functions - Produce cytokines and bridge gap between innate and adaptive immunity</td>
<td>-</td>
<td>(Van Kaer and Joyce, 2005, Tupin et al., 2007)</td>
</tr>
</tbody>
</table>

### 2.6 REGULATORY T CELLS

Regulatory T cells (Tregs) were first described in 1972 when T cells with suppressive properties were identified and termed ‘suppressive’ cells (Gershon et al., 1972). However, their existence as a distinct T cell sub-population remained controversial until the mid-1990s...
(Green and Webb, 1993). They are now considered a separate specialised T cell sub-group (Sakaguchi et al., 1995, Burzyn et al., 2013).

2.6.1 LINEAGE OF REGULATORY T CELLS

Based on origin, protein expression, mechanism of action and function, there are two classes of Tregs: natural/naïve/professional (nTregs) and induced/adaptive/memory (iTregs). Recently, other T cells such as γδ cells, natural killer T cells and double negative also thought to have regulatory functions have been proposed to be included in the regulatory T cell group (Fessler et al., 2013). However, their immunosuppressive roles are less well known (Guerin et al., 2009, Raffin et al., 2013) and in this literature review, Tregs will be restricted to nTregs and iTregs only.

Naturally occurring Tregs originate from the thymus as CD4+CD25+Foxp3+ cells (Figure 2.2) (Chatenoud and Bach, 2006). Naturally occurring Tregs are long-lived, foreign-antigen non-specific and directly control other T cells by T cell-T cell interaction (Suciu-Foca et al., 2003, Tanaka and Sakaguchi, 2005). As discussed in section 2.5.1 and illustrated in Figure 2.1, cells destined to become nTregs are different from other T cells as they interact with self-antigens neither weakly nor too strongly to protect themselves from negative selection (Maggi et al., 2005, Xing and Hogquist, 2012). In the thymus, T cells with highly reactive TCRs are rescued from deletion by cytokines or a second TCR and go on to become regulatory T cells (Tregs) with suppressive functions (Xing and Hogquist, 2012).
Figure 2.2. Developmental pathways and markers of Treg sub-groups. Natural Tregs use cytokines to exert their action. Induced Tregs can be generated from T cell precursors. Naïve T cells become Foxp3- Tr1 cells or Foxp3+ Th3 cells by cytokines secreted by antigen-presenting cells. TEC= thymic epithelial cell; T\text{conv}= conventional T cell; APC= antigen presenting cell & DC= dendritic cell Adapted from (Workman et al., 2009).

Naturally occurring Tregs differ from iTregs in many aspects, which are summarised in Table 2.3. However, the features which differentiate the two groups are not universally accepted (Lin et al., 2013).
Table 2.3. Differences between regulatory T cell subsets.

<table>
<thead>
<tr>
<th>Generation</th>
<th>Natural Tregs</th>
<th>Induced Tregs</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Co-stimulation</td>
<td>Thymus</td>
<td>Gut-associated lymphoid tissue, spleen, lymph nodes &amp; inflamed tissue</td>
<td>(Curotto et al., 2004)</td>
</tr>
<tr>
<td>requirement</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytokine</td>
<td>CD28</td>
<td>Cytotoxic T lymphocyte-associated protein-4</td>
<td>(Curotto et al., 2004)</td>
</tr>
<tr>
<td>requirement</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Specificity</td>
<td>Self</td>
<td>Allergens, commensal bacteria, neo-antigens (tumor), allo-antigens (fetus)</td>
<td>(Curotto et al., 2004)</td>
</tr>
<tr>
<td>Life span</td>
<td>Longer than iTregs but unstable in inflammatory conditions</td>
<td>Short-lived but remain stable in inflammatory conditions</td>
<td>(Yadav et al., 2013)</td>
</tr>
<tr>
<td>Foxp3</td>
<td>Positive</td>
<td>May be positive (Th3) or negative (Tr1)</td>
<td>(Curotto et al., 2004)</td>
</tr>
</tbody>
</table>

Adapted from (Curotto and Lafaille, 2009).

Induced Tregs (CD4+CD25+) develop from mature CD4+ conventional T cells outside of the thymus (Haribhai et al., 2011) after stimulation by cognate antigens (which can stimulate nTreg and iTregs) and immune-regulatory cytokines (such as TGF-β, IL-10, and IL-4) (Seddon and Mason, 1999, Maggi et al., 2005, Chatenoud and Bach, 2006, Akbar et al., 2007, Peterson, 2012). Two main subsets of iTregs, based on the cytokines that cause their induction and are then produced by iTreg subsets, include Foxp3- type 1 regulatory T cells (Tr1) that are induced by IL-10 and Foxp3+ T helper 3 (Th3), which are induced by TGFβ (Figure 2.2) (Sakaguchi et al., 1995, Groux et al., 1997, Jonuleit and Schmitt, 2003, Workman et al., 2009, Kushwah and Hu, 2011, Peterson, 2012, Duhen et al., 2012, Lin et al., 2013). Peripheral generation of iTregs produces suppressive cells with affinity for antigens not expressed in the thymus (Guerin et al., 2009). Tissue-specific antigen recognition by iTregs is important to protect against foreign antigens (Samy et al., 2006) (Figure 2.2).

2.6.2 MARKERS

Tregs are identified on the basis of expression of various cell surface and intracellular markers which are detailed in Table 2.4 (Figure 2.2). Despite a long list of available markers,
it has not been possible to accurately distinguish Tregs from other T cells or iTregs from nTregs due to low specificity and variable expression of markers (Tran et al., 2007).

Table 2.4. Treg markers in humans.

<table>
<thead>
<tr>
<th>Markers</th>
<th>Structure</th>
<th>Expressed by</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aiolos</td>
<td>Intracellular transcription factor</td>
<td>iTregs</td>
<td>(Raffin et al., 2013)</td>
</tr>
<tr>
<td>CCR4 (CD194)</td>
<td>Cell surface protein receptor</td>
<td>nTregs, iTregs</td>
<td>(Kevin et al., 2010)</td>
</tr>
<tr>
<td>CCR6 (CD196)</td>
<td>Cell surface protein receptor</td>
<td>iTregs</td>
<td>(Chen et al., 2003, Kitamura et al., 2010)</td>
</tr>
<tr>
<td>CCR7</td>
<td>Cell surface protein receptor</td>
<td>iTregs</td>
<td>(Raffin et al., 2013)</td>
</tr>
<tr>
<td>CD25</td>
<td>Trans-membrane protein</td>
<td>Anergic &amp; activated nTregs, iTregs</td>
<td>(Scotto et al., 2004)</td>
</tr>
<tr>
<td>CD45RB</td>
<td>Trans-membrane protein</td>
<td>Anergic nTregs</td>
<td>(Scotto et al., 2004)</td>
</tr>
<tr>
<td>CD73</td>
<td>Trans-membrane protein</td>
<td>Higher expression in nTregs than iTregs</td>
<td>(Lin et al., 2013)</td>
</tr>
<tr>
<td>CD95</td>
<td>Trans-membrane protein</td>
<td>nTregs, iTregs</td>
<td>(Scotto et al., 2004)</td>
</tr>
<tr>
<td>CD127</td>
<td>Trans-membrane protein</td>
<td>Absent on Treg (expression inversely correlates with suppressive function of Foxp3+ cells)</td>
<td>(Scotto et al., 2004)</td>
</tr>
<tr>
<td>CTLA-4 (CD152)</td>
<td>Cell surface protein receptor</td>
<td>Anergic and activated nTregs, iTregs</td>
<td>(Scotto et al., 2004)</td>
</tr>
<tr>
<td>CXCR3</td>
<td>G protein-coupled receptor</td>
<td>iTregs</td>
<td>(Hoerning et al., 2011)</td>
</tr>
<tr>
<td>Eos</td>
<td>Intracellular transcription factor</td>
<td>nTregs</td>
<td>(Raffin et al., 2013)</td>
</tr>
<tr>
<td>Foxp3</td>
<td>Intracellular transcription factor (master regulator of Treg development &amp; function)</td>
<td>nTregs, Th3</td>
<td>(Korn et al., 2009)</td>
</tr>
<tr>
<td>GITR</td>
<td>Cell surface protein receptor</td>
<td>Anergic and activated nTregs, iTregs</td>
<td>(Scotto et al., 2004)</td>
</tr>
<tr>
<td>Helios (Izkf2)</td>
<td>Intracellular transcription factor</td>
<td>Higher expression in nTregs than iTregs</td>
<td>(Raffin et al., 2013)</td>
</tr>
<tr>
<td>IL-1R1</td>
<td>Cell surface protein receptor</td>
<td>iTregs</td>
<td>(Raffin et al., 2013)</td>
</tr>
<tr>
<td>LAG-3 (CD223)</td>
<td>Cell surface protein</td>
<td>nTregs, Th3</td>
<td>(Scotto et al., 2004)</td>
</tr>
<tr>
<td>Nrp-1</td>
<td>Cell surface protein receptor</td>
<td>Higher expression in nTregs compared to iTregs</td>
<td>(Hey-Cunningham et al., 2013, Lim et al., 2006)</td>
</tr>
<tr>
<td>Pdcd-1</td>
<td>Cell surface protein</td>
<td>Higher expression in nTregs compared to iTregs</td>
<td>(Lin et al., 2013)</td>
</tr>
</tbody>
</table>

CTLA-4= Cytotoxic T lymphocyte-associated protein-4, CXCR3= Chemokine receptor 3, GITR= Glucocorticoid induced tumour necrosis factor receptor, IL-1R1= Interleukin receptor 1 type 1, LAG-3 = Lymphocyte activation gene-3, Nrp-1= Neuropilin-1, Pdcd-1= Programmed cell death-1.

The combination of staining for CD4, CD25 and CD127 is a common and reliable method for identification of Tregs which are CD4+CD25+CD127$^{-/\text{dim}}$ (Liu et al., 2006, Seddiki et al., 2006). Forkhead box p3 (Foxp3) has been the best known single marker for identification of
Tregs (Giatromanolaki et al., 2008). It is now realised that Foxp3 alone may not be an ideal marker to recognise Tregs as human Foxp3 is also up-regulated in activated T cells without suppressive functions (such as inducible CD8+ cells) (Chen et al., 2003, Raffin et al., 2013). It has recently been suggested that neuropilin-1 and Helios expression in conjunction may provide a superior way to identify Tregs, particularly nTregs, however this has not yet been conclusively established (Kim et al., 2012, Yadav et al., 2012).

2.6.3 ROLES OF DENDRITIC CELLS AND INTERLEUKINS IN DEVELOPMENT AND FUNCTION

The development and differentiation of iTregs is mediated by tissue-resident plasmacytoid dendritic cells (DCs) and cytokines present in the local microenvironment (Steinman et al., 2003, Zou et al., 2010, Kushwah and Hu, 2011). Plasmacytoid DCs represent a distinctive DC population (Gehrie et al., 2011) that secrete IL-27, IL-10 and TGF-β, which induce Foxp3 in naïve T cells and drive their differentiation into iTregs (Kushwah and Hu, 2011) (Figure 2.2). Plasmacytoid DCs express indoleamine-2,3-dioxygenase (IDO), an enzyme which directly activates resting Tregs and maintains Treg suppressive activity (Chen et al., 2009). Blockage of IDO causes conversion of Tregs to a pro-inflammatory phenotype resembling Th-17 cells (Brandacher et al., 2008, Chen et al., 2009). Plasmacytoid DCs can down-regulate the suppressive function of Tregs by certain signals such as CD137 which is a DC cell-surface molecule with co-stimulatory activity and cross-links with Tregs (Choi et al., 2004, Kanamaru et al., 2004).

Various ILs also enhance Treg development and release them from anergic state (Figure 2.2). Roles of important ILs in Treg development are summarised in Table 2.5.
Table 2.5. Roles of important interleukins (ILs) in regulatory T cell (Treg) development.

<table>
<thead>
<tr>
<th>Interleukin</th>
<th>Roles in Treg development</th>
<th>References</th>
</tr>
</thead>
</table>
| IL-1        | - Releases iTregs from anergic state  
 |             | - ↑ iTreg response to proliferation signals | (Kubo et al., 2004, Brinster and Shevach, 2008) |
| IL-2        | - Converts CD25− into CD25+ phenotype  
 |             | - ↑ nTreg proliferation  
 |             | - Releases nTregs from anergic state | (Takahashi et al., 1998, Thornton and Shevach, 1998, Yagi et al., 2004, Malek et al., 2008) |
| IL-4        | - ↑ survival and proliferation | (Thornton et al., 2004, Pace et al., 2005) |
| IL-6        | - Releases iTreg cells from anergic state  
 |             | - ↑ iTreg response to other cytokines  
 |             | - Inhibits transforming growth factor-beta induced iTreg differentiation  
 |             | - ↓ Foxp3 expression | (Kubo et al., 2004, King and Segal, 2005) |
| IL-7        | Survival factor for iTregs | (Harnaha et al., 2006, Li et al., 2011) |
| IL-12       | - ↑ anti-inflammatory IL-10 production  
 |             | - ↓ suppressive ability (but Foxp3 expression not significantly altered) | (Pasare and Medzhitov, 2003, King and Segal, 2005) |
| IL-15       | Enhances iTreg survival and expansion and can replace IL-2 function | (Koenen et al., 2003, Curotto and Lafaille, 2009) |

2.6.4 MECHANISMS OF IMMUNE SUPPRESSION

Broadly, Tregs have four suppressive mechanisms but their function may be the result of one, two or a combination of all of these mechanisms (Vignali et al., 2008, Guerin et al., 2009).

**Suppression by inhibitory cytokines:** Induced Tregs (Tr1 and Th3) suppress immune responses by secreting inhibitory cytokines such as IL-10 and TGF-β (Figure 2.3) (Dieckmann et al., 2001, Green et al., 2003, Annacker et al., 2003, Stassen et al., 2004, Fahlén et al., 2005, Loser et al., 2007, Bergmann et al., 2007, Erhardt et al., 2007, Collison et al., 2007). In contrast, nTregs exert their suppressive functions through cytokine independent and cell-to-cell contact-dependent mechanisms, although paracrine action through TGF-β and IL-10 may enhance contact-dependent manner of suppression (Annacker et al., 2003).
Figure 2.3. Treg have four mechanisms of action which include suppression by inhibitory cytokines, cytolysis, metabolic disruption and targeting dendritic cells. Adapted from (Workman et al., 2009).

**Suppression by cytolysis:** Naturally occurring Tregs produce and release granzymes A and B and perforins to induce death in target cells (Grossman et al., 2004, Cao et al., 2007). In a mouse model, nTregs with deficient expression of granzymes have depressed suppressive action (Gondek et al., 2005) (Figure 2.3).

**Suppression by metabolic disruption:** Induced Tregs act on helper T cells through mechanisms which cause ‘metabolic disruption’ (Vignali et al., 2008). For example, iTregs may use all available IL-2, which regulates the expansion of newly activated T lymphocytes and NKs and deplete other target cells of IL-2 required for their development and survival, leading to cell death (de la Rosa et al., 2004, Sakaguchi, 2004). In addition, iTregs produce
anti-inflammatory adenosine that binds to its receptor on helper T cells and inhibits their function and enhances generation of iTregs by inhibiting IL-6 expression and promoting TGF-β secretion (Zarek et al., 2008) (Figure 2.3).

**Suppression by targeting dendritic cells:** Interactions between CTLA-4, a protein receptor present on iTregs, and CD80 and 86 on DCs condition DCs to express IDO that suppresses helper T cells as described in section 2.6.2 (Figure 2.3).

### 2.6.5 FUNCTIONS

Tregs are potent suppressors of the immune system (Guerin et al., 2009) and perform their functions by suppressing proliferation and cytokine production of immune cells (Sakaguchi et al., 2009). Natural and iTregs perform slightly different functions. While nTregs are concerned with establishment and maintenance of immune-tolerance against self-antigens to prevent auto-immune diseases, iTregs have mainly anti-inflammatory function against foreign antigens (Guerin et al., 2009, Corthay, 2009, Yadav et al., 2013). However, roles of nTregs against non-self-antigens cannot be completely ruled out (Ray et al., 2010). Induced Tregs, on the other hand, appear to have limited roles in establishing self-tolerance in the absence of nTregs. In a mouse model, removal of thymus on day three after birth results in organ-specific autoimmune diseases due to lack of nTreg development, which can be prevented by adoptive transfer of Tregs (Sakaguchi et al., 1995, Asano et al., 1996). Induced Tregs, by controlling the inflammatory responses, protect airways and the intestinal mucosae from non-self-antigens (invading microbes and environmental factors) and commensal bacteria from elimination by the immune system. Their local control of inflammation in tissues may be transient due to short lifespan of iTregs (Yadav et al., 2013). Induced Tregs also prevent inflammation of the testes and ovaries (orchitis and oophoritis, respectively) and
rejection of organs in recipients. Sperms and oocytes express a range of antigens and when administered to recipients can induce autoimmune inflammation (Guerin et al., 2009).

Natural Tregs can induce suppressive properties or ‘infectious tolerance’ in other T cell populations, which is due to nTreg mediated conversion of suppressed T cells into iTregs (Curotto and Lafaille, 2009). Co-culture of nTregs with naïve CD4+ T cells causes iTreg formation with IL-10 or TGF-β-dependent suppressive activity (Qiao et al., 2007).

2.6.6 ROLES IN DISEASES

Natural Tregs and iTregs must maintain a fine balance between suppressing potential autoimmune responses while controlling responses to foreign antigens at the same time (Workman et al., 2009). Achieving this balance can be contradictory as Treg sub-populations may over- or under-perform. Occasionally Tregs are unable to mediate peripheral tolerance leading to an exacerbated inflammatory/allergic reaction or autoimmunity. Similarly, imbalances can be detrimental by preventing effective anti-tumour responses and immunity against certain chronic infectious agents (Workman et al., 2009).

In humans, reduced numbers and/or dysfunction of nTregs may result in auto-immune diseases such as rheumatoid arthritis, inflammatory bowel disease, multiple sclerosis, type I diabetes mellitus, systematic lupus erythematosus and X-linked syndromes such as IPEX and XLAAD (De Kleer et al., 2004, Viglietta et al., 2004). IPEX is characterised by dysregulation of immune system, endocrine glands and bowel and XLAAD by diarrhoea, dermatitis, insulin-dependent diabetes, thyroiditis and anemia (Li et al., 2007, Brusko et al., 2008).
Induced Tregs may have roles in providing immunity in chronic viral infections (such as hepatitis C) (Robertson and Hasenkruig, 2006) in addition to allergies, asthma and parasitic and bacterial infections (such as H.pylori) (Robinson, 2009). However, Treg response during infections may be an important barrier for vaccination (Workman et al., 2009). Similarly, Tregs may obstruct tumor response to immune-therapies as tumor tissue promotes conversion of naive T cells into nTregs, which can then induce ‘infectious tolerance’ and local accumulation of Tregs, and impair the development of effector responses (Nishikawa et al., 2003).

2.6.7 EFFECT OF AGING ON Treg NUMBERS AND FUNCTION

After puberty, the thymus progressively degenerates and its capacity to produce new T cells substantially declines (Lin et al., 2013). In adult life, thymic T cell production declines by 3 % per year until reaching a very low level by 45 years of age (Berzins et al., 2002). However, the total number of T cells in the periphery remains unchanged due to peripheral mechanisms of T cell renewal (Steinmann and Muller-Hermelink, 1985, Berzins et al., 2002).

The effect of aging on Treg numbers and function in humans have not been studied in detail but available evidence suggests only minor changes in the circulating total Treg pool (Dejaco et al., 2006, Raynor et al., 2012). Cord blood at birth contains the highest numbers of total Tregs, which decline until the third year of life and remain relatively stable thereafter (Sullivan et al., 2002). Tregs comprise 0.6-15 % of the total CD4+ cell pool in healthy adults (Dejaco et al., 2006). It is suggested that in adult life the total Treg pool is maintained by increased peripheral conversion of non-regulatory T cells into Tregs (Curotto de Lafaillle et al., 2004).
Treg function in terms of anti-inflammatory cytokine production is significantly reduced in the aged (> 60 years) compared to the younger (< 40 years) population (Hwang et al., 2009). Decreased Treg function may contribute to increased risk of certain auto-immune diseases and malignancies in the elderly (Fessler et al., 2013). It is suggested that rather than the total numbers of Tregs, Treg function and relative proportions of nTregs and iTregs in the total pool may be more important in preventing inflammatory changes and immune responses (Fessler et al., 2013, Schlossberger et al., 2013).

2.7 REGULATORY T CELLS IN FEMALE REPRODUCTION

The roles of other T cells in the normal menstrual cycle, endometriosis and unexplained infertility have been previously summarised in tables 1.2, 1.3 and 2.1, respectively. Tregs play important roles in many aspects of female reproduction (Robertson et al., 2009). These roles are reviewed below. It should be noted that, except in the case of normal pregnancy, roles of iTregs in female reproduction are currently less well known and it has been indicated in a mouse model that they may be less crucial than those of nTregs (Guerin et al., 2009, Guerin et al., 2011).

2.7.1 IN THE OVARY

Tregs are present in the ovaries and have roles in self-tolerance of ovarian antigens, corpus luteal function and premature ovarian failure (Facciabene A et al., 2012). There are several ovarian-related antigens (such as zona pellucida antigens) which are not expressed until sexual maturity. Induced Tregs ensure that normally there is no auto-reactivity against ovarian self-antigens (Robertson et al., 2009). Abundance of Tregs in the mid-cycle corpus luteum may support normal progesterone release (and pregnancy) by suppressing pro-inflammatory cytokine synthesis (Robertson et al., 2009). Bovine studies have shown that
there is a sharp decline in Foxp3+ Treg numbers soon after the corpus luteum starts degenerating (Poole and Pate, 2012). In a mouse model, premature ovarian failure has been shown to be associated with disturbances in regulatory mechanisms in the ovary and its draining lymph nodes (Alard et al., 2001, Samy et al., 2006). In humans, altered Treg numbers and/or their dysfunction may adversely affect ovarian function and contribute to premature ovarian failure (Robertson et al., 2009, Facciabene A et al., 2012).

2.7.2 THE MENSTRUAL CYCLE

Although early studies failed to detect changes in the numbers of circulating Tregs during the various phases of the menstrual cycle (Prieto and Rosenstein, 2006), more recently it has been found that Tregs change in numbers in different phases of the menstrual cycle in the endometrium (Berbic et al., 2010) and peripheral blood (Arruvito et al., 2007). Endometrial density of Foxp3+Tregs increases linearly from early to the late proliferative phase but decreases significantly in the secretory phase (Berbic et al., 2010). The pre-ovulatory surge of Tregs in the endometrium may reflect the importance of these cells in inducing immune-tolerance for successful implantation (Guerin et al., 2009). In a mouse model, natural and iTregs are increased in the endometrium and peripheral blood around the peri-implantation period (Aluvihare et al., 2004). Similarly, in fertile non-pregnant women, Tregs in circulation reach the maximum numbers during day 9-13 of the menstrual cycle (late proliferative phase) just before ovulation but there is a dramatic reduction in the numbers of Tregs during the secretory phase. Treg numbers and oestrogen concentration in peripheral blood positively correlate (Arruvito et al., 2007).
2.7.3 NORMAL PREGNANCY

For survival and development of the embryo, it is essential that the peripheral tolerance mechanisms suppress maternal immune responses to paternal-origin fetal antigens (Aluvihare et al., 2004, Guerin et al., 2009, Hsu et al., 2012). Tregs play a part in preventing fetal rejection during pregnancy (Trowsdale and Betz, 2006) by various mechanisms which include suppression of pro-inflammatory Th1 cells and cytotoxic CD8+ cells and enhanced expression of anti-apoptotic molecule heme-oxygenase-1 (HO-1) (Mellor and Munn, 2001, Cosmi et al., 2004, Sollwedel et al., 2005, Brandacher et al., 2008, Chen et al., 2009).

EARLY PREGNANCY: Treg numbers increase in the endometrium or decidua very early in the normal pregnancy that is associated with increased expression of Foxp3 mRNA soon after implantation (Aluvihare et al., 2004, Somerset et al., 2004, Thuere et al., 2007, Hsu et al., 2012). The uterine draining lymph nodes appear to be the main site of iTreg proliferation in early pregnancy (Zhao et al., 2007). Although the mechanisms are not well understood, the decidual antigen-presenting cells help iTregs over-express in the decidua during early pregnancy in relation to nTregs (Hsu et al., 2012). In contrast to nTreg, which raise the threshold for all immune responses, iTregs may specifically maintain a non-inflammatory environment in the uterus in early pregnancy to suppress immune responses to the fetal antigens (Curotto and Lafaille, 2009). Increased oestrogen levels during pregnancy may be the underlying drive for expansion of Treg numbers (Polanczyk et al., 2004). Other pregnancy hormones, such as human chorionic gonadotropin, also have the capacity to attract Tregs to the feto-maternal interface and enhance their Foxp3 expression and suppressive activity (Schumacher et al., 2009).
MID PREGNANCY: During normal pregnancy, there is continued expansion of the peripheral and decidual Treg populations over the first two trimesters. Treg numbers peak in the second trimester and are higher in the decidual tissue than in the peripheral blood (Sasaki et al., 2004, Somerset et al., 2004, Guerin et al., 2009).

LATE PREGNANCY: Initially it was indicated that endometrial Treg numbers start decreasing from mid-pregnancy and reach non-pregnant levels just before delivery at term (Zhao et al., 2007). Lower levels of Tregs at term were thought to have a role in the initiation of labour and fetal expulsion (Guerin et al., 2009). More recent research reports that Treg numbers are not changed before or during labour, but their suppressive activity is decreased in labouring women compared with non-labouring women at term (Schober et al., 2012, Gomez-Lopez and Laresgoiti-Servitje, 2012). At present, changes in Treg function at term and possible mechanisms in initiation of spontaneous term labour are not well understood (Schober et al., 2012).

2.7.4 PREGNANCY RELATED COMPLICATIONS

Tregs have been implicated in pathological processes in a range of pregnancy-related complications, including unexplained infertility, recurrent miscarriage, pre-eclampsia and pre-term birth. The known roles of Tregs in these pregnancy-related complications are reviewed below. Possible roles of Tregs in endometriosis-associated infertility will be discussed separately in section 2.7.6.

INFERTILITY: As discussed in section 2.2.1, infertility is associated with a range of cellular and molecular defects in the endometrium (Lee et al., 2011). Disturbances in the numbers/function of Tregs may be associated with unexplained infertility or sub-clinical
pregnancy loss (Guerin et al., 2009). In women with unexplained infertility, endometrial Treg Foxp3 mRNA expression is significantly reduced in the mid-secretory phase compared to fertile controls (Jasper et al., 2006). It is suggested that unexplained infertility may result from impaired endometrial recruitment of Tregs or differentiation of uterine T cells into iTregs (iTregs) (Jasper et al., 2006, André et al., 2011). In a mouse model, adequate Treg function is necessary to suppress Th1-mediated pro-inflammatory maternal immune responses to the embryo (Aluvihare et al., 2004). Treg dysfunction may, therefore, cause fetal rejection and unexplained infertility (Guerin et al., 2009).

Relative proportions of circulating Treg sub-populations are altered in infertile women with successful ART outcome compared to those who failed to conceive after such treatment despite no differences in the total Treg numbers (Schlossberger et al., 2013). It is hypothesised that Treg sub-populations may have more influence on establishing pregnancy rather than their absolute numbers (Schlossberger et al., 2013).

**MISCARRIAGE:** Recurrent miscarriage is the loss of at least three consecutive pregnancies before 20 weeks (Ford and Schust, 2009). In women with recurrent miscarriage, Treg numbers are markedly reduced in the endometrium and peripheral blood compared to normal pregnancies or isolated cases of spontaneous abortion (Sasaki et al., 2004, Arruvito et al., 2007, Yang et al., 2008, Cao et al., 2014). Furthermore, in women with a history of recurrent miscarriage, the expected increase in endometrial Treg numbers in the late proliferative phase is not observed (Arruvito et al., 2007). Tregs may also be functionally less effective in these women due to an intrinsic defect (Arruvito et al., 2007). If Tregs are depleted in numbers or function, insufficient suppression of pro-inflammatory Th1 mediates fetal loss (Aluvihare et al., 2004). As such, Treg depletion after implantation in a mouse model causes early
pregnancy failure (Zenclussen, 2005). However, if Tregs are transferred from normal pregnant mice to abortion-prone mice, the increased risk of abortion is reversed (Zenclussen, 2005).

**PRE-ECLAMPSIA:** Pre-eclampsia is characterised by the development of new onset raised blood pressure (hypertension) in association with significant protein loss in urine after 20 weeks of gestation (Chanprapaph, 2004, Sibai, 2006). It is part of an abnormally increased maternal inflammatory response to pregnancy where pro-inflammatory Th1 responses dominate and there is exaggerated immune reaction against the fetus (Can et al., 2011). This may be secondary to insufficient Treg numbers and/or function (Saito and Sakai, 2003, Sargent et al., 2006, Guerin et al., 2009). Recent studies favour a significant decrease in the numbers of Tregs in the decidua and peripheral blood of women with pre-eclampsia (Paeschke et al., 2005, Darmochwal-Kolarz et al., 2007, Quinn et al., 2011, Quinn and Parast, 2013, Zeng et al., 2013). It is thought that in pre-eclampsia, diminished numbers and/or function of Tregs cause decreased IDO levels in the placenta or insufficient suppression of the pro-inflammatory Th17 cells. Decreased IDO levels alter Treg function by converting them to a phenotype resembling the pro-inflammatory Th17 cells (Santoso et al., 2002, Grohmann and Puccett, 2002, Nishizawa et al., 2007, Brandacher et al., 2008, Liu et al., 2011) as discussed in section 2.6.3.

**PRETERM LABOR:** Preterm delivery is associated with significantly reduced placental Treg density and decidual Treg suppressive activity compared to uncomplicated term pregnancies (Steinborn et al., 2012, Schober et al., 2012, Quinn and Parast, 2013). In preterm labour, a pro-inflammatory micro-environment in the absence of adequate Treg numbers and/or function may facilitate the initiation of labour. However, the exact mechanisms
underlying preterm labor are not understood (Gomez-Lopez and Laresgoiti-Servitje, 2012, Quinn and Parast, 2013).

2.7.5 ENDOMETRIOSIS

Tregs are dysregulated in women with endometriosis (Berbic et al., 2010). As described earlier in section 2.7.2, in the normal menstrual cycle, endometrial Treg numbers decrease during the secretory phase (Arruvito et al., 2007). In the eutopic endometrium of women with endometriosis, the numbers of Foxp3+ Tregs are significantly increased in the secretory phase of the menstrual cycle compared to women without the disease (Berbic et al., 2010) (Figure 2.4).

Figure 2.4. Comparison of endometrial Treg numbers in women with and without endometriosis. * Treg numbers are significantly increased in the secretory phase compared to controls (Berbic et al., 2010).

It is suggested that increased presence of Tregs in the secretory phase in endometriosis may suppress the recruited endometrial leucocytes and hence allow survival of the shed endometrial fragments, facilitating their implantation in the peritoneal cavity (Berbic et al.,
2010. The exact mechanism of Treg immuno-suppression of other cells in this scenario is not well understood but may include interference with phagocytic function of macrophages and maturation of dendritic cells (Berbic et al., 2010). Local oestrogen production in the endometrium of women with endometriosis may provide positive feedback for increased Treg proliferation (Zeitoun et al., 1998, Berbic et al., 2010).

Tregs appear to mobilise from blood to the peritoneal cavity in the early proliferative phase in women with endometriosis as circulating Treg numbers are markedly decreased in blood compared to the peritoneal lesions (Olkowska-Truchanowicz et al., 2013). In women with endometriomas, the density of Tregs is increased in the peritoneal fluid compared to women without the disease (Olkowska-Truchanowicz et al., 2013). Foxp3+ Tregs are also present in some peritoneal endometriotic lesions and in lesions in a baboon model of endometriosis (Berbic et al., 2010, Braundmeier et al., 2012). It is thought that presence of Tregs in the lesions may be dependent on the progression and stage of endometriotic lesions. Active trafficking of Treg cells to the endometrium, peritoneal lesions and/or peritoneal fluid may be a compensatory anti-inflammatory mechanism in women with endometriosis, a known inflammatory condition (Olkowska-Truchanowicz et al., 2013).

In addition to changes in the Treg populations in the uterus, circulation and the peritoneal cavity, certain genetic polymorphisms of Foxp3 mRNA in the endometrium are positively and significantly correlated with the presence of endometriosis (André et al., 2011). Foxp3 gene polymorphisms can cause altered numbers or function of Tregs, which can contribute to the pathogenesis of endometriosis (Barbosa et al., 2012). It is hypothesised that in endometriosis, Tregs over-suppress immune cell responses and allow the menstrual
fragments to implant at ectopic sites (Berbic et al., 2010), however, the exact mechanisms of Treg action in endometriosis are yet unknown.

**2.7.6 POSSIBLE ROLES OF Tregs IN ENDOMETRIOSIS-ASSOCIATED INFERTILITY**

The possible roles of Tregs in endometriosis-associated infertility are currently unknown and may be different to those in other types of infertility. However, given the implications of Tregs in both infertility (Jasper et al., 2006, André et al., 2011) and endometriosis pathophysiology (Berbic et al., 2010, Olkowska-Truchanowicz et al., 2013), important roles of Tregs in endometriosis-associated infertility are indicated. The fertility status of women was not reported or unknown in studies that suggested roles of endometrial or circulating Tregs in endometriosis (Berbic et al., 2010, Olkowska-Truchanowicz et al., 2013). Similarly, presence or absence of endometriosis was laparoscopically confirmed in only one (André et al., 2011) of the two studies that suggested reduced endometrial expression of Foxp3 in unexplained infertility (Jasper et al., 2006). Therefore, it is now of considerable interest to better understand roles of Tregs in the pathophysiology of infertility in endometriosis.
CHAPTER 3: HYPOTHESES, AIMS AND OBJECTIVES

3.1 INTRODUCTION

Endometriosis is an enigmatic disease with a number of postulated theories of pathogenesis. It is widely thought that during menstruation, shed endometrial fragments reach the peritoneal cavity via retrograde flow and can implant to form endometriotic lesions. Evidence suggests that endometriosis is an endometrial disease where the eutopic endometrium is altered compared to the endometrium of women without the disease in a number of ways, which may facilitate establishment of endometriotic lesions. This indicates that the primary defect in endometriosis may be in the uterine endometrium. Importantly, immunological responses, which recognise and target the shed endometrial cells, are disturbed in endometriosis. Endometriosis is a leading cause of infertility but the exact aetiology of this infertility remains unknown. It has been shown that specific immunological mechanisms play crucial roles in the establishment and maintenance of pregnancy. Immunological alterations may contribute to the pathogenesis of endometriosis-associated infertility.

Regulatory T cells control and suppress a range of immune responses at local and systemic levels. In the endometrium of women with endometriosis, Treg numbers are high in the secretory phase of the menstrual cycle. In the endometrium of women without endometriosis, their numbers greatly decrease. It is suggested that in endometriosis, increased presence of endometrial Tregs may suppress the ability of other immune cells in the endometrium to effectively target shed menstrual fragments, although this is not well understood. Preliminary indications are that numbers and/or function of Tregs may be inadequate in infertility. However, it is currently unknown whether Tregs are altered in endometriosis-associated infertility.
3.2 HYPOTHESES

3.2.1 PRIMARY HYPOTHESIS
The primary hypothesis of this work is that the numbers of Tregs are altered across the menstrual cycle in circulation and the endometrium between fertile and infertile women with and without endometriosis.

3.2.2 SECONDARY HYPOTHESES
The secondary hypotheses of this project are as follows:

- Circulating and endometrial Treg numbers are increased in the secretory phase in women with endometriosis compared to women without the disease.
- The numbers of Tregs are increased in circulation and the endometrium of fertile women compared to infertile women.
- Tregs are decreased in numbers in circulation and the endometrium of infertile women with endometriosis compared to fertile women with and without endometriosis.

3.3 AIMS
To test the hypotheses stated above, this project aimed:

- To examine Tregs in circulation and the endometrium during the menstrual cycle in women with endometriosis and associated infertility compared women without endometriosis (both fertile and infertile).
3.4 OBJECTIVES

The main objective of this study was to investigate the roles that Tregs may play in the pathogenesis of endometriosis and particularly in the mechanisms of endometriosis-associated infertility. Specifically, the objectives included:

- Study of circulating and endometrial Tregs in fertile and infertile women with and without endometriosis during the menstrual cycle.
- Determination of Tregs change during the menstrual cycle in circulation and the endometrium of fertile and infertile women with and without endometriosis.
- Determination of differences in Tregs in circulation and the endometrium between fertile and infertile women with and without endometriosis.
CHAPTER 4: METHODOLOGY

Treg analyses were performed by two methods: flow cytometry and immunohistochemistry.

4.1 ETHICS APPROVALS

Ethics approvals for the study were granted by the Human Ethics Committees of Sydney Local Health District (Royal Prince Alfred Hospital RPAH zone; Protocol No X12-0344, HREC/12/RPAH/525 & SSSA/13/RPAH/36) and The University of Sydney (Project No 2013/112).

4.2 SAMPLE SIZE CALCULATION

Sample size was calculated to determine statistical power of the study. Sample size calculation was performed by using WHO sample size calculator with the fixed level of significance at 5% (to exclude type I statistical errors) and fixed power of the test at 90% (to rule out type II errors). The statistically calculated required sample size was 108 (54 in each group).

4.3 FLOW CYTOMETRY

Blood and endometrial Treg populations were studied with multi-colour flow cytometry.

4.3.1 PATIENT RECRUITMENT

Patients were recruited for one full year. Infertile and fertile women of reproductive age (18-45 yrs) with an intact uterus and with or without laparoscopically-confirmed endometriosis (peritoneal, ovarian and/or DIE lesions observed and histologically confirmed) were included in the study (n = 50). For the purpose of this experiment, infertility was defined as inability to
conceive for at least one year while having regular sexual intercourse and not using any method of contraception (Hamada et al., 2012). Women on hormonal or immuno-suppressive therapy, with a history of autoimmune disease or recent infection (within the past two weeks) were excluded. Due to strict inclusion criteria and difficulty in finding controls, the ideal sample size according to power calculations could not be achieved.

All participants gave written, informed consent. Detailed clinical history was taken at the time of recruitment in a standard format. Age, date of last menstrual period, fertility history and parity, symptoms such as dysmenorrhea, pelvic pain and abdominal bloating, previous gynaecological diagnoses and surgeries, and current medications were noted. Menstrual cycle phase was determined by dating of the histological appearance of the endometrium by an experienced gynaecological histopathologist (Noyes et al., 1950).

4.3.2 COLLECTION OF SAMPLES

4.3.2.1 BLOOD

Blood samples were collected from infertile and fertile women with (n = 37) and without (n = 13) endometriosis by routine phlebotomy, immediately before or after receiving general anaesthesia for diagnostic/operative laparoscopy. One sample was collected from each patient. Blood was collected into cell preparation tubes (Vacutainer CPT, BD Biosciences, USA). The vacutainer cell preparation tube is an 8 mL draw capacity blood collection tube with anticoagulant (sodium citrate) and a density gradient fluid layer (polysaccharide/sodium diatrizoate). A polyester gel barrier acts as a blood separation medium and with centrifugation; low density white blood cells and plasma are separated from the denser blood components. At least 7 mL blood was collected from each participant. Tubes were gently
inverted to mix the contents then kept upright at room temperature. Samples were processed
within 1 hour of blood collection.

4.3.2.2 ENDOMETRIUM
A hysteroscopically-directed endometrial sample was taken from infertile and fertile women
with (n = 20) and without (n = 13) endometriosis, immediately before or after laparoscopy,
and collected into gentleMACS dissociator C tube (Miltenyi Biotec, Germany) containing 5
mL of buffer (See appendix A). The tubes were weighed before and after tissue collection.
Time of collection and processing of samples were noted and all samples processed within
one hour of collection.

4.3.3 PROCESSING OF BLOOD AND ENDOMETRIAL SAMPLES
4.3.3.1 BLOOD PROCESSING
Blood was mixed by gently inverting the tube before centrifugation at 1600 relative
centrifugal force (RCF), 21 °C and the brake off (Deceleration 0) for 15 minutes.
Centrifugation separated the cells of interest (white blood cells) from red blood cells and
platelets. After centrifugation, white blood cells accumulated in the buffy coat layer, which
separated the platelet-rich serum (above) from the packed red cells (below) (Figure
4.1). Supernatant (serum) was aspirated and discarded and the white cell layer collected in a
15 mL falcon tube. Cells were washed twice, tube was filled to the top with phosphate
buffered saline (PBS) (See appendix B) and centrifuged at 300 RCF, 5 °C, 7 minutes,
deceleration 9 (DEC 9), supernatant was discarded and cells resuspended in PBS.
Figure 4.1. Components of a blood sample in a BD Vacutainer Cell Preparation Tube after centrifugation.

4.3.3.2 DISSOCIATION OF TISSUE

Tissue samples were dissociated using gentleMACS, a bench top instrument for automated tissue dissociation to obtain single-cell suspensions with a high viability rate (Miltenyi Biotec, Germany). It ensures safety and reproducibility. The plastic rotor uniformly dissociates samples in the C tube by spinning in clock- and counter-clock directions to generate single-cell suspensions. It is equipped with 23 specific and 5 (A-E) general programmes. Programme A is suitable for dissociation of soft tissues, whereas, programme E is used for harder tissues. In this experiment, the endometrial tissue was processed with gentleMACS using pre-set general programme C followed by B (31 seconds, setting B_01).

After gentleMACS, tissue was further dissociated using 5 % type IV collagenase (0.0043 g Worthington, USA), which was added to the sample in the tube. The sample was incubated on a rocker for 20 minutes at 37 °C. The sample was then filtered through a 70 µm Filcon filter (BD Biosciences, USA) into a 15 mL falcon tube to remove large fragments. The tube was filled to the top with buffer. After centrifugation (300 RCF, 5 °C, 7 minutes, DEC 9),
cells were resuspended in 4 mL pre-diluted 1 in 10 red blood cell lysis (RCL) solution (Miltenyi Biotec, Germany). RCL solution lyses erythrocytes but has minimal effects on leukocytes. Lysing samples were incubated in the dark for 10 minutes at room temperature. After 10 minutes, samples were centrifuged (300 RCF, 5 °C, 7 minutes, DEC 9), supernatant was discarded and cells were washed with PBS.

After these initial steps, blood and tissue samples were processed in a similar fashion. Samples were transferred to a 5 mL falcon tube as clean, single cell suspensions that were stained as described in the following section.

4.3.4 CELL STAINING

4.3.4.1 LIVE/DEAD CELL STAINING

Endometrial cells and peripheral blood leukocytes were initially labelled with a fluorescent dye to differentiate between live and dead cell populations. Live/dead fixable dead cell stain (near-infrared [Near-IR] fluorescent reactive dye; Life Technologies, Australia) consists of a reactive dye, which easily penetrates necrotic/dead cell membranes. Dead cells generate a strong signal while viable/live cells produce a relatively dim signal. Near-IR dye requires excitation at approximately 633-635 nm and emits light at 665 nm.

The Live/Dead Fixable Dead Cell Stain Kit was stored at -20 °C, away from light. Prior to use fluorescent reactive dye (Component A) and anhydrous dimethylsulfoxide (DMSO) (Component B) vials were brought to room temperature and 50 µL of Component B was added to a vial containing a pre-determined mass of Component A. Blood and tissue single cell suspensions in 5 mL falcon tubes were re-suspended in 1 mL PBS and 1 µL live/dead marker was added. The tubes were protected from light, incubated at room temperature for 15
minutes and centrifuged (300 RCF, 5 °C, 7 minutes, DEC 9). Supernatant was discarded and cells were washed before staining with Treg antibodies (described in section 4.3.4.2).

**4.3.4.2 REGULATORY T CELL STAINING**

Antibodies are glycoproteins and part of a group of proteins called immunoglobulins (Ig). They are formed by B lymphocytes in response to exposure to a foreign antigen. An antibody is typically Y-shaped and made up of two identical heavy chains (H) and two identical light chains (L) joined by covalent inter-chain disulfide bonds (Boenisch, 2001) (Figure 4.2). The fragment antigen-binding (Fab fragment) region at the tip of the Y-shaped antibody binds to antigens. It is composed of one constant and one variable domain of each of the heavy and the light chain (Boenisch, 2001) (Figure 4.2).

![Figure 4.2](image)

*Figure 4.2. Fab (tip) and Fc (tail) fragments of Y-shaped antibody consist of variable and constant domains of heavy and light chains* (Boenisch, 2001).

Each antibody reacts with a specific site on a particular antigen called the ‘epitope’. An antigen may contain several epitopes. Therefore, one antigen may produce a variety of antibodies (Woychik et al., 1984). Antibodies can be polyclonal or monoclonal. Polyclonal antibodies are produced by different cells and are immunochemically dissimilar (Nakazawa
et al., 2010). Monoclonal antibodies are produced by an individual clone of plasma, are immunochemically identical and react with one specific epitope on the antigen against which they are raised. Polyclonal antibodies are produced in a range of animals, most commonly rabbits, while monoclonal antibodies are almost exclusively produced in mice (Nakazawa et al., 2010).

Treg detection kit (Miltenyi Biotec, Germany) is an optimised antibody cocktail used for staining Tregs. It consists of antibodies for CD45 (clone: 5B1, isotype: mouse IgG2a), CD4 (clone: VIT4, isotype mouse: IgG2a), CD25 (clone: 4E3, isotype: mouse: IgG2b) and CD127 (clone: MB15-18C9, isotype: mouse IgG2a) conjugated to fluorochromes as detailed in Table 4.1.

**Table 4.1. Fluorochromes used in this experiment.**

<table>
<thead>
<tr>
<th>Marker</th>
<th>Expressed by</th>
<th>Expression by Tregs</th>
<th>Antibody-conjugated fluorescent dye</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD45</td>
<td>All leucocytes</td>
<td>High</td>
<td>Vio blue</td>
</tr>
<tr>
<td>CD4</td>
<td>CD4 subgroup of lymphocytes</td>
<td>High</td>
<td>Fluorescein isothiocynate</td>
</tr>
<tr>
<td>CD25</td>
<td>Activated Tregs, natural killer dendritic cells and B cells</td>
<td>High</td>
<td>Allophycocyanin</td>
</tr>
<tr>
<td>CD127</td>
<td>Tregs, natural killer and most of CD8+ cells</td>
<td>Low</td>
<td>R-phycoerythrin</td>
</tr>
</tbody>
</table>

Treg staining was performed by resuspending cells in 100 μL PBS, mixing in 10 μL antibody cocktail and incubating in the dark at 4 °C for 10 minutes as per the manufacturers’ recommendations. After the staining incubation, samples were washed in 1-2 mL PBS and centrifuged (300 RCF, 5 °C, 7 minutes, DEC 9) and supernatant was discarded.

**4.3.4.3 FIXATION**

Fixation is necessary for biosafety reasons and to preserve light scattering characteristics and fluorescence intensities of stained cells for later flowcytometric analysis (Law et al., 2009).
Stained cells were fixed by re-suspension in 250 µL Cytofix fixation buffer (BD Biosciences, USA), vortexed for 30 seconds and incubated for 15 minutes at 4 °C. Cytofix contains neutral pH-buffered saline (Dulbecco’s PBS) with 4 % weight per volume paraformaldehyde. After fixation, cells were washed (300 RCF, 5 °C, 7 minutes, DEC 9). Finally, the supernatant was discarded, cells resuspended in 200 µL of PBS and refrigerated for analysis by flow cytometry.

4.3.5 FLOW CYTOMETER

Flow cytometry is a powerful technique for rapid analysis of multiple quantitative and qualitative parameters of individual cells within heterogeneous populations. Flow cytometry measures optical and fluorescence characteristics of single cells and particles. It is used in a range of applications like immuno-phenotyping to study the proteins expressed by cells, ploidy analysis to predict the behaviour of DNA content in individual cells within a mixed population of cells and cell counting. Flow cytometry can be used to immuno-phenotype whole blood, solid tissues and other biological samples such as urine (Ibrahim and Van, 2007).

The flow cytometer performs analysis by passing thousands of cells per second through a laser beam and capturing the light that emerges from each cell as it passes through (Rowley, 2013). This light signal can be transformed into data by software to report cellular characteristics (Ibrahim and Van, 2007). The primary components of a flow cytometer are the fluidics system (which presents samples to the ‘interrogation point’ and takes away waste), lasers (the light source), optics (direct the light), detectors (receive the light) and electronics and a peripheral computer system which convert signals from the detectors into digital data and perform the necessary analyses (Hedley and Keeney, 2013) (Figure 4.3). For the purpose
of these experiments, a BD FACSCanto II (BD Biosciences, USA) flow cytometer was used to perform multi-colour flow cytometry.

Figure 4.3. Primary components of a flow cytometer, including fluidics, optics, lasers and electronics (Rowley, 2013).

**4.3.5.1 FLUIDICS**

There are two sources of fluid in a flow cytometer: a sheath fluid (usually PBS) and the specimen to be analysed. For accurate analysis, it is important that particles and cells are passed through the laser beam one at a time. In most flow cytometers, this is accomplished by injecting the sample stream containing the cells into the middle of the sheath fluid which then passes through the flow chamber and the ‘interrogation point’ where the laser and sample intersect and the optics collect the resulting signals. The outer sheath fluid ensures that the sample stream is compressed into single cell diameter which is called ‘hydro-dynamic focussing’ (Ibrahim and Van, 2007) (Figure 4.4). The FACSCanto II flow rate can be adjusted between low (10 μL/min) to medium (50 μL/min) to high (120 μL/min). In this experiment, samples were run at high flow rate (120 μL/min). After the sample is passed through the flow cytometer, it exits as waste.
4.2.5.2 LASERS

Lasers are the principle light sources in flow cytometers, providing coherent light at high power in a form that can be easily directed and focused on a sample stream. Most modern flow cytometers have argon- and krypton-ion laser. They emit several monochromatic wavelengths simultaneously, produce high-quality beams with low noise and relatively long lifetimes before requiring gas tube replacement (Telford, 2011). The BD FACSCanto II has three lasers (488 nm solid state, 633 nm He Ne and 405 nm solid state) which can detect up to eight colours/fluorescence signals.

When laser light strikes a cell, light is diffracted around the edges of the cell in the forward direction which is called forward scatter (FSC) or forward angle light scatter (FALS) (Figure 4.5). The magnitude of forward scatter is roughly proportional to the size of the cell and has the same wavelength as the exciting laser light. (Rowley, 2013). A cell travelling through the laser beam scatters light at all angles. Side scatter is light scattered at 90 degrees from the laser path due to the granularity and structural complexity inside the cell. More granular cells have higher side scatter due to internal complexity (Figure 4.5). Samples consist of a large
number of cells and particles. To prevent data collection from particles which have little significance, such as debris and platelets, a threshold is set such that a certain forward scatter pulse size must be exceeded for the instrument to collect the data.

![Diagram of light scattering](image)

**Figure 4.5.** Laser light is diffracted in all directions after striking a cell. Diffraction around the edges of the cell in the forward direction depends on the cell size and is called forward scatter (FSC) or forward angle light scatter (FALS). More granular cells have higher side scatter (Schneider and Grosschedl) due to internal complexity (Rowley, 2013).

Forward and side scatter plots together identify small sized lymphocytes with low internal complexity, medium sized monocytes with slightly more internal complexity and larger neutrophils and other granular leukocytes with high internal complexity (Figure 4.6).
Figure 4.6. Forward versus side scatter plot identify leukocyte populations by cell size and internal complexity. Lymphocytes are identified by smaller size and low internal complexity compared to other leukocytes.

4.3.5.3 FLUORESCENCE

Fluorescent probes, such as antibodies to cellular antigens with covalently-bound fluorescent dyes (fluorochromes), can be used to detect specific cell molecules. When antibody-labelled cells come across a light source, the fluorescent compound absorbs light energy over a certain range of wavelengths which increase the energy level of its electrons for a short period of time. The excited electrons quickly return to their ground state and emit the excess energy as photons of light. This emission of light energy is called ‘fluorescence’ (Figure 4.7).

Fluorochromes with different excitation and emission wavelengths or colours allow analysis of multiple cellular characteristics simultaneously. Table 4.2 summarises the peak excitation and emission wavelengths of fluorescence dyes used in this experiment.
Figure 4.7. Light source excites the antibody-labelled cell electrons to a higher energy level. Excess energy of electrons is emitted as photons on return to their ground state.

Table 4.2. Peak excitation and emission wavelengths of fluorescent dyes used in this experiment.

<table>
<thead>
<tr>
<th>Fluorescent dyes used in this experiment</th>
<th>Peak excitation wavelength (nm)</th>
<th>Peak emission wavelength (nm)</th>
<th>BD FACSCanto II excitation laser (nm)</th>
<th>Colour emitted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vio blue</td>
<td>400</td>
<td>452</td>
<td>405</td>
<td>Blue</td>
</tr>
<tr>
<td>Fluorescein isothiocyanate</td>
<td>495</td>
<td>520</td>
<td>488</td>
<td>Green</td>
</tr>
<tr>
<td>Allophycocyanin</td>
<td>652</td>
<td>660</td>
<td>635</td>
<td>Red</td>
</tr>
<tr>
<td>R-phycoerythrin</td>
<td>565</td>
<td>578</td>
<td>488</td>
<td>Yellow</td>
</tr>
</tbody>
</table>

### 4.3.5.4 OPTICS AND DETECTORS

The optics system in the BD FACSCanto II cytometer is composed of both excitation and collection optics. The excitation optics consist of lasers, fibre optic cables, beam-shaping prisms and an achromatic focusing lens which bring light to the flow cell. From the flow cell, laser light is routed to the collection optics, which efficiently gather the signals emitted from each particle.
Light from the collection optics is directed to the appropriate detector which isolates different bands of wavelengths. There are two types of signal detectors in the BD FACSCanto II flow cytometer: the photomultiplier tube (PMT) and the photodiode. The more sensitive PMTs are used to detect the weaker signals generated by SSC and all fluorescence channels. Filters are used to capture peak fluorescence from a fluorochrome and direct it to a specific PMT. The photodioide is used to detect the stronger FSC signals (Lawrence et al., 2008).

Once the light signals or photons strike one side of the PMT or the photodiode, they generate an electrical current by conversion into a proportional number of electrons that are multiplied. The electrical current travels to the amplifier and is converted to a voltage pulse. The highest point of the pulse occurs when the particle is in the centre of the beam. As the particle leaves the beam, the pulse comes back down to the baseline. The voltage pulse is assigned a digital value by an analog-to-digital converter. Data is then transferred to the computer and light signals are then displayed on a data plot.

4.3.5.5 NOISE, SPECTRAL OVERLAP AND COMPENSATION

Noise is an unwanted low-level background signal that occurs within the electronics of a flow cytometer. Noise can appear as collected events even when there are no actual cells or particles in the sample. In this experiment, threshold was set periodically depending on the fluorescent properties of cells. Fluorescent signal from each immune cell differentiated between particles of interest and unwanted debris.

Spectral overlap occurs when portions of fluorochrome emission spectra overlap each other (Figure 4.8). This often occurs when multiple fluorescent-conjugated antibodies are used in the same sample. With spectral overlap, the observed signal in a detector is composed of the
true signal from the fluorochrome of interest and any overlapping signal from other fluorochromes.

Figure 4.8. Spectral properties of two imaginary fluorochromes ‘A’ and ‘B’ are measured in channels FL-1 and FL-2 of the flow cytometer, respectively. Dark blue and dark red shades represent the proportion of spectral overlap by fluorochrome ‘B’ and ‘A’, respectively.

The amount of spectral overlap can be determined by running single colour controls and a mathematical algorithm applied to data to correct for spectral overlap (called ‘compensation’). Compensation particles (BD CompBead anti-mouse Ig, κ; BD Biosciences, USA) were used at regular intervals to calculate the amount of spectral overlap and apply compensation in this project. Beads are ideal for compensation in experiments with rare cell types like Tregs (BD Biosciences, 2009). BD CompBead particles are polystyrene micro-particles coupled to an antibody specific for the kappa light chain of immunoglobulin from mouse. When mixed together with each fluorochrome-conjugated mouse antibody, CompBeads provide distinct positive and negative (background fluorescence) stained populations which can be used to set compensation levels automatically using instrument set-up software. In this experiment, automated compensation was setup by using BD FACSDiva software (BD Biosciences, USA). BD FACSDiva software collects data from each
compensation control tube and automatically calculates accurate compensation values for each fluorochrome combination. All of the spectral overlaps were stored as a flow cytometry standard (FCS) file for immediate use or later recall.

**4.3.5.6 DATA ACQUISITION AND ANALYSIS**

For these experiments, samples were run on the BD FACSCanto II until clear profile of cells was achieved or until the sample was exhausted. FlowJo software (TreeStar, USA) was used to view and analyse flow cytometric data. Each recorded flow cytometry event is visualised as a dot on a dotplot. Two parameters can be displayed simultaneously in a density plot with one parameter displayed on the x-axis and the other on the y-axis. FlowJo allows gating and statistical analyses in table and graphical forms. Gating is the process of selecting subsets of collected events (based on specific parameters) for further analysis. Subsets can be gated to generate further subsets, until a collection has only the cells for which a graphical display or statistics are desired. In order to phenotype cell populations, it is important to create a clear gating strategy. In flow cytometry, this is referred to as ‘gating hierarchy’.

For Treg analyses, initially a gate was applied to select CD45+ leukocytes from other cell populations and debris (Figure 4.9 A). The next gate was applied to leukocyte events to discriminate lymphocytes from granulocytes and monocytes based on cell size (FSC-A) and internal granularity (SSC-A; Figure 4.9.B). The third gate was applied to these events, which comprised of lymphocytes only, to identify the proportion of live lymphocytes (Figure 4.9 C). The fourth gate was applied to the live lymphocyte events to detect CD4+ helper T cells (Figure 4.9 D). Finally, a gate was applied to the CD4+ helper T cell population to identify CD25+CD127\text{dim} Treg cells (Figure 4.9 E). The final gate determined CD4+CD25+CD127\text{dim} Treg numbers as a percentage of total CD4+ cells (Figure 4.9 E).
Figure 4.9. Gating strategy for identification of Tregs. A) Leukocyte population was gated from all recorded events. B) Lymphocytes were separated from other leukocyte sub-groups. C) Live lymphocytes were separated from all lymphocytes. D) Live CD4+ percentage of live lymphocytes was gated. E) The final gate shows CD4+CD25+CD127dim Treg numbers as a percentage of total CD4+ cells.
4.4 IMMUNOHISTOCHEMISTRY

Endometrial Tregs were also studied using immunohistochemistry (IHC).

4.4.1 SAMPLES

Samples for the IHC part of the study included endometrium from participants in flow cytometry experiments (n = 40) and additional samples identified from recent pathology archives at RPAH (n = 48). Women from which samples were identified in archival pathology adhered to the same inclusion and exclusion criteria as flow cytometry participants (detailed in section 4.2.1). For some participants in flow cytometry experiments, endometrial samples suitable for IHC were not available (n = 10).

Samples were staged by an experienced gynaecological histopathologist according to the histological appearance of the endometrium. Of women with endometriosis (n = 61), 5 were in menstrual, 28 in proliferative and 28 in the secretory phase of the menstrual cycle. Of women without endometriosis (n = 27), 4 women were in menstrual, 12 in proliferative and 11 in the secretory phase at the time of sample collection.

4.4.2 TISSUE PROCESSING

4.4.2.1 FIXATION

Tissue samples were fixed in 10 % neutral-buffered formalin to preserve tissue components and morphology (Boenisch, 2001). After fixation, tissue samples were dehydrated by alcohol, cleared of dehydrating agents with xylene and infiltrated with paraffin wax according to a standardised protocol. Fixed samples were embedded into a paraffin block to provide structural support and allow sectioning for visualisation (Miller et al., 2001).
4.4.2.2 TISSUE SECTIONING

Paraffin embedded tissue samples were cooled to -5 °C on a cold plate (Leica EG1150 C, Leica Microsystems Nussloch GmbH, Germany) and cut at 4 μm on a manually operated rotary microtome (Leica RM 2135, Leica Microsystems Nussloch GmbH, Germany). Cut sections were mounted on glass slides (IHC Microscope Slides, FLEX; Dako, Denmark) and dried in a dehydrating oven at 60 °C for 1 hour.

4.4.2.3 DEPARAFFINISATION AND REHYDRATION

Xylol is an organic solvent capable of dissolving wax. Dried slides were allowed to cool after removal from the oven. Slides were deparaffinised with xylol twice (5 minutes each) and rehydrated with decreasing strength of alcohol for two minutes each (100 % alcohol twice, 95 % alcohol and 70 % alcohol) and slow running tap water for two minutes.

4.4.2.4 ANTIGEN RETREIVAL

During tissue fixation and paraffin embedding, partial or complete loss of immune-reactivity can occur for many antigens due to changes in epitope structure which masks the epitope. Antigen retrieval “unmask” or exposes epitopes by breaking down cross-links between proteins formed during fixation and reversing some of the antigen denaturation caused by fixation and paraffin processing (Miller et al., 2001, Ramos-Vara, 2005). Antigen retrieval can be performed by proteolytic digestion or heat treatment in a buffer solution (heat-induced antigen/epitope retrieval, HIER) (Ramos-Vara, 2005).

In this project, HIER was performed on tissue sections for antigen retrieval. A working solution was prepared by diluting 4 mL of the Antigen Target Solution pH 9 (x50 concentrate; Dako, Denmark) in 196 mL distilled water. The working solution was pre-heated
in a water bath to 95-99 ºC.Slides were placed in the preheated antigen retrieval solution and incubated for 20 minutes at 95-99 ºC. After HIER, sections were cooled to room temperature in the retrieval solution (20 minutes) and thoroughly washed under slow-running tap water for 2-3 minutes. Slides were immersed in wash buffer (discussed in section 4.4.3.1) for 10 minutes to reduce the surface tension prior to IHC staining.

4.4.3 AUTOSTAINER

In this project, an automated slide processing system Dako Autostainer Plus Universal Staining System (Dako, Denmark) was used. It consists of a slide processor and dedicated desktop computer and can stain 48 slides simultaneously. The reagent, reagent dispense volume and reagent dispense location were individually programmed for each slide with the autostainer. For all staining runs, the dispense volume was set at 100 μL per dispense location. The specific reagent dispense locations were individually selected for each slide to ensure the reagent covered the total tissue area.

4.4.3.1 WASH BUFFER

Wash buffers remove non-specifically bound proteins in specimens to reduce or eliminate background. In this project, buffer was prepared by diluting Wash Buffer concentrate (20x; Dako, Denmark), a Tris-buffered saline solution containing Tween 20 with pH 7.6 (+ 0.1), in deionised water. Buffer rinse steps were programmed on the Autostainer between each reagent step.

4.4.3.2 BLOCKING

Positive staining that is not due to antigen-antibody binding is called ‘non-specific’ background staining. This non-specific staining can be eliminated or reduced by blocking
steps. Endogenous enzymatic activity is strongly present on a number of cells and tissues. The presence of endogenous peroxidase and alkaline phosphatase (AP) can obscure specific staining of the target antigen (Gao et al., 2008). Dual Endogenous Enzyme Block (DEEB; Dako, Denmark) was used to inhibit endogenous peroxidase and AP. After an initial buffer wash, the Autostainer was programmed with a 10 minute DEEB incubation period.

4.4.3.3 ANTIBODY

In this project, a monoclonal mouse anti-human Foxp3 antibody (clone 236A/E7, Abcam Cambridge, UK) at a dilution of 1:100 was used to identify Tregs. The antibody was diluted in Antibody Diluent (Dako, Denmark) which comprised of Tris HCL buffer with a stabilising protein and a preservative. The Autostainer was programmed to incubate the slides with Foxp3 antibody for 30 minutes.

4.4.3.4 AMPLIFIER

EnVision FLEX+ Mouse LINKER (Dako, Denmark) was used as an amplifying agent. It amplifies the signal from mouse antibodies 4-5 fold compared to regular staining with EnVision alone. Following primary antibody incubation, amplifier was applied for 30 minutes.

4.4.3.5 DETECTION SYSTEMS

EnVision+ Dual Link System- Horseradish Peroxidise (HRP) (Dako, Denmark) was used as the detection system. This system is based on a dextran back-bone to which a large number of HRP molecules are conjugated to secondary antibodies. The goat anti-mouse and anti-rabbit Igs are purified and coupled with peroxidise-labelled polymers in Tris-HCl buffer and stabilising protein and anti-microbial agents are added. The anti-mouse antibodies in the
system reacted with the immunoglobulins used in this project. EnVision was applied for 30 minutes.

4.4.3.6 VISUALISATION

Diaminobenzidine+ (DAB+) chromogen was used for visualisation with this staining. The DAB working solution was prepared by thoroughly mixing 1 drop of DAB+ Chromogen per 1 mL of Substrate Buffer (Dako, Denmark). DAB+ was applied for 10 minutes and produced brown end-product at the site of the target antigen.

After immunohistochemical staining was complete, slides were rinsed thoroughly with tap water.

4.4.3.7 PROTOCOL

Staining was performed on the Autostainer as follows:

1. Wash buffer rinse
2. DEEB - 10 minutes
3. Wash buffer rinse
4. Mouse monoclonal anti-human Foxp3 antibody - 30 minutes
5. Wash buffer rinse
6. EnVision FLEX+ Mouse LINKER - 30 minutes
7. Wash buffer rinse
8. EnVision+ Dual Link System-HRP - 30 minutes
9. Wash buffer rinse
10. Wash buffer rinse
11. DAB - 10 minutes
12. Wash buffer rinse
4.4.3.8 POSITIVE AND NEGATIVE CONTROLS

Positive and negative endometrial controls were used as part of each staining run. Positive controls were endometrial tissue known to contain Foxp3+ cells. Antibody diluent was used, instead of the primary antibody, for negative controls in each Autostainer run. All staining with diluent controls was negative.

4.4.4 COUNTERSTAINING AND COVER SLIPPING

Slides were counterstained with Mayers haematoxylin, a blue nuclear stain, for assessment of tissue morphology. Slides were quickly dipped in Mayers haematoxylin (approximately 1 second) and washed in tap water until the water ran clear. Slides were blued in hot tap water for 15 seconds.

After counterstaining, slides were dehydrated with increasing concentrations of alcohol for 2 minutes each (70 % alcohol, 95 % alcohol and 100 % alcohol twice), placed in xylol (2 x 3 minutes) and coverslipped with Ultramount (Fronine Pty Ltd, Australia).

4.4.5 SLIDE ANALYSIS

Slides were examined initially under low magnification with the Olympus BX51 microscope (Olympus, Japan). Cell counting was performed under 400x magnification. Fields of view were captured using an Olympus DP70 digital camera with Image Pro Plus Discovery Software (Media Cybermetics Japan). Where possible, 20 fields of view were analysed for each sample. In samples where the tissue was not large enough, as many fields of view as could fit within the tissue area were captured (overall mean number of fields of view captured = 11; range 5 to 20).
Tregs were identified by mid to dark brown staining of round, oval or bean-shaped nuclei which ranged from 3 to 4 µm in size (Figure 4.10). Treg counting was performed blinded to the presence or absence of endometriosis and the gynaecological histopathology endometrial dating report. Treg counting was validated by two investigators performing blinded counts on all fields of view from 10 tissue specimens. The Intraclass correlation coefficient for the two investigators counts was 0.95 (95% confidence interval 0.93–0.96; n = 325; p = < 0.001).

Figure 4.10. Foxp3 were identified by mid to dark brown staining of round or oval shaped Treg nuclei under 400x magnification.

4.4.6 STATISTICAL METHODS

Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) 21.0 software. One-Sample Kolmogorov-Smirnov Test was used to determine distribution of variables. Age was normally distributed in all groups and was analysed using the independent sample t-test. Data was either skewed or calculated as percentages for other variables, therefore, non-parametric statistical testing was performed (Mann–Whitney U-test denoted by W U z). Statistical analysis using Kruskal–Wallis test (denoted by H) was
conducted to determine Treg densities across the menstrual cycle. The correlation between immune cell densities and age of participants was determined by applying Pearson correlation test and Treg counting by two blind investigators by using Intraclass correlation coefficient. Differences between groups were considered to be statistically significant at p-values of less than 0.05.
CHAPTER 5: RESULTS

5.1 FLOW CYTOMETRY

This chapter presents the results of multicolour flow cytometric analysis of peripheral blood and endometrial leukocytes from women with and without endometriosis.

5.1.1 AGE DISTRIBUTION OF PATIENTS

Initial analysis was conducted to investigate age distribution and variation between women with and without endometriosis and women with and without infertility. No significant variation was observed in age between women with and without endometriosis (mean age ± SD; 34.5 ± 5.3 yrs versus 37.3 ± 6, respectively). Similarly, there was no statistically significant difference in age between fertile (35.3 ± 8.8) and infertile (36.1 ± 4.6) women.

The participants’ age did not appear to correlate to the numbers of circulating or endometrial Tregs, CD4+ cells and live lymphocytes.

5.1.2 BLOOD IMMUNE CELL CHANGES IN WOMEN WITH AND WITHOUT ENDOMETRIOSIS

Peripheral blood Tregs, CD4+ lymphocytes and percentages of live lymphocytes were assessed between women with and without endometriosis, throughout the menstrual cycle (Table 5.1).
Table 5.1. Median densities and interquartile ranges of blood Tregs, CD4+ cells and live lymphocytes in the menstrual cycle in women with and without endometriosis.

<table>
<thead>
<tr>
<th></th>
<th>Tregs</th>
<th>CD4+</th>
<th>Live lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control vs Endo</td>
<td>Control vs Endo</td>
<td>Control vs Endo</td>
</tr>
<tr>
<td><strong>Overall</strong></td>
<td>4.7 (2.3) vs 4.4 (3.0)</td>
<td>43.9 (14.1) vs 39.8 (13.3)</td>
<td>76.3 (20.2) vs 81.9 (17.0)</td>
</tr>
<tr>
<td><strong>P</strong></td>
<td>5.7 (3.2) vs 4.9 (2.2)</td>
<td>43.4 (18.9) vs 43.8 (13.0)</td>
<td>76.1 (28.5) vs 89.7 (22.4)</td>
</tr>
<tr>
<td><strong>S</strong></td>
<td>4.0 (2.6) vs 5.0 (4.1)</td>
<td>43.7 (14.7) vs 35.7 (18.0)</td>
<td>79.8 (24.1) vs 87.5 (23.8)</td>
</tr>
</tbody>
</table>

Results represented as median (IQR). Menstrual phase results not shown due to insufficient numbers for statistical analysis. P = Proliferative, S = Secretory.

**Tregs**

In women without endometriosis, Treg median densities showed little variation throughout the menstrual cycle (Figure 5.1 A), with numbers being lowest in the secretory phase of the normal cycle.

In endometriosis, Treg densities also showed limited variation throughout the cycle. The density of Tregs, however appeared to be lowest during the menstrual phase. Treg density gradually increased through the proliferative phase and peaked in the secretory phase (Figure 5.1 A). However these changes were not found to be statistically significant.

**CD4+ cells**

Significant differences were not observed in the densities of CD4+ cells throughout the menstrual cycle or between women with and without endometriosis (Figure 5.1 B).
**Percentages of live lymphocytes**

Slight variations were observed in the percentages of live lymphocytes throughout the menstrual cycle. In women without endometriosis, the median density of live lymphocytes appears to be reduced in the menstrual and proliferative phases. In women with endometriosis, median density of circulating live lymphocytes followed a similar pattern, although the density of live lymphocytes appeared to be higher in women with endometriosis compared to controls across all phases of the menstrual cycle (Figure 5.1 C).

![Graph comparing Tregs, CD4+ cells, and live lymphocytes](image)

**Figure 5.1.** Comparison of median numbers of blood Tregs, CD4+ cells and live lymphocytes in all phases of the menstrual cycle between women with and without endometriosis (M = Menstrual, P = Proliferative, S = Secretory).
5.1.3 ENDOMETRIAL IMMUNE CELL CHANGES IN WOMEN WITH AND WITHOUT ENDOMETRIOSIS

Table 5.2 demonstrates Tregs, CD4+ cells and live lymphocytes proportions in women with and without endometriosis across different phases of the menstrual cycle.

Table 5.2. Median densities and inter-quartile ranges of endometrial Tregs, CD4+ cells and live lymphocytes in the menstrual cycle in women with and without endometriosis.

<table>
<thead>
<tr>
<th></th>
<th>Tregs</th>
<th>CD4+</th>
<th>Live lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control vs Endo</td>
<td>Control vs Endo</td>
<td>Control vs Endo</td>
</tr>
<tr>
<td>Overall</td>
<td>8.1 (4.6) vs 9.6 (8.2)</td>
<td>22.5 (7.5) vs 24.2 (9.1)</td>
<td>71.6 (30.4) vs 77.7 (17.4)</td>
</tr>
<tr>
<td>P</td>
<td>8.2 (27.4) vs 9.9 (8.3)</td>
<td>23.4 (4.6) vs 28.4 (10.1)</td>
<td>58.5 (31.3) vs 83.9 (17.1)</td>
</tr>
<tr>
<td>S</td>
<td>6.7 (7.3) vs 7.6 (7.8)</td>
<td>21.3 (9.1) vs 21.8 (7.6)</td>
<td>79.5 (21.8) vs 76.9 (12.7)</td>
</tr>
</tbody>
</table>

Results represented as median (IQR). Menstrual phase results not shown due to small number of participants. P = Proliferative, S = Secretory.

**Tregs**

In women without endometriosis, density of endometrial Tregs did not significantly change during the menstrual cycle. During the proliferative phase of the normal cycle, Treg density greatly varied (Median = 8.8 – 45.1). In women with endometriosis, Treg density was observed to decline from menstrual through to secretory phase, although these differences were not found to be statistically significant (Figure 5.2 A).

**CD4+ cells**

In both women with and without endometriosis, CD4+ cells appeared to follow a similar pattern throughout the phases of the menstrual cycle. An increase in CD4+ cells occurred from menstrual to the proliferative phase, followed by a decline in the secretory phase. A
strong trend suggests that endometrial CD4+ cell declined from proliferative to the secretory phase in women with endometriosis (W U z = -1.9; p = 0.06). In women without the disease, this reduction from proliferative to secretory phase was not so marked (Figure 5.2 B).

Percentages of live lymphocytes

While there were no statistically significant differences in the percentages of live lymphocytes throughout the menstrual cycle, it was interesting to note that in women without endometriosis, percentages of live lymphocytes were lowest in the proliferative phase. However, in endometriosis, percentages of live lymphocytes gradually declined from menstrual through to secretory phase of the menstrual cycle (Figure 5.2 C).
Figure 5.2. Comparison of median numbers of endometrial Tregs, CD4+ cells and live lymphocytes in all phases of the menstrual cycle between women with and without endometriosis (M = Menstrual, P = Proliferative, S = Secretory).

5.1.4 BLOOD IMMUNE CELL CHANGES IN FERTILE AND INFERTILE WOMEN

Peripheral blood Tregs, CD4+ lymphocytes and percentages of live lymphocytes were compared between fertile and infertile women throughout the phases of the menstrual cycle (Table 5.3).
Table 5.3. Median densities and interquartile ranges of blood Tregs, CD4+ cells and live lymphocytes in the menstrual cycle in fertile and infertile women.

<table>
<thead>
<tr>
<th></th>
<th>Tregs</th>
<th>CD4+</th>
<th>Live lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fertile vs Infertile</td>
<td>Fertile vs Infertile</td>
<td>Fertile vs Infertile</td>
</tr>
<tr>
<td>Overall</td>
<td>5.5 (1.5) vs 3.8 (3.2)</td>
<td>45.1 (16.3) vs 42.2 (14.9)</td>
<td>76.3 (21.3) vs 81.9 (18.0)</td>
</tr>
<tr>
<td>P</td>
<td>5.6* vs 4.4 (2.7)</td>
<td>44.1* vs 43.8 (10.0)</td>
<td>63.4* vs 89.9 (19.4)</td>
</tr>
<tr>
<td>S</td>
<td>4.2* vs 4.8 (3.5)</td>
<td>45.1* vs 41.2 (18.0)</td>
<td>82.1* vs 88.2 (19.6)</td>
</tr>
</tbody>
</table>

Results represented as median (IQR). Menstrual phase results not shown due to small number of participants. P = Proliferative, S = Secretory, * IQR missing due to small number of participants.

**Tregs**

In fertile and infertile women, densities of circulating Tregs showed little variation across the menstrual cycle (Figure 5.3 A).

**CD4+ cells**

Significant differences were not observed in CD4+ cells throughout the menstrual cycle or between fertile and infertile women (Figure 5.3 B).

**Percentages of live lymphocytes**

There was a strong trend for decrease in percentages of live lymphocytes in fertile women during the proliferative phase of the cycle compared to infertile women (W U z = -1.9; p = 0.05; Figure 5.3 C). This reduction also occurred during the menstrual and secretory phases in fertile patients compared to infertile women, although level of significance was not reached. In infertile patients, percentages of live lymphocytes showed very little variation throughout the menstrual cycle.
5.1.5 ENDOMETRIAL IMMUNE CELL CHANGES IN FERTILE AND INFERTILE WOMEN

Table 5.4 summarises endometrial Tregs, CD4+ lymphocytes and percentages of live lymphocytes in fertile and infertile women throughout the phases of the menstrual cycle.
Table 5.4. Median densities and interquartile ranges of endometrial Tregs, CD4+ cells and live lymphocytes in the menstrual cycle between fertile and infertile women.

<table>
<thead>
<tr>
<th></th>
<th>Tregs Control vs Infertile</th>
<th>CD4+ Control vs Infertile</th>
<th>Live lymphocytes Control vs Infertile</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Overall</strong></td>
<td>11.8 (7.6) vs 8.0 (6.0)</td>
<td>22.5 (4.8) vs 3.8 (9.0)</td>
<td>67.5 (20.8) vs 83.3 (18.4)</td>
</tr>
<tr>
<td><strong>P</strong></td>
<td>17.7* vs 8.3 (3.1)</td>
<td>22.5* vs 27.7 (8.7)</td>
<td>57.0* vs 84.2 (22.7)</td>
</tr>
<tr>
<td><strong>S</strong></td>
<td>11.6* vs 6.8 (9.9)</td>
<td>23.9* vs 19.1 (7.7)</td>
<td>76.5* vs 76.8 (18.4)</td>
</tr>
</tbody>
</table>

Results represented as median (IQR). Menstrual phase results not shown due to small number of participants. P = Proliferative, S = Secretory, * IQR missing due to small number of participants.

**Tregs**

A significant increase in endometrial Treg populations were observed in fertile women, compared to infertile women during the proliferative phase (W U z = -2.3, p = 0.02). In fertile women, Treg density appeared to vary throughout the cycle with a peak in the proliferative phase of the cycle. In infertile women, very little variation was observed in Treg populations throughout the menstrual cycle (Figure 5.4 A).

**CD4+ cells**

CD4+ cells were significantly reduced in fertile compared to infertile women during the proliferative phase of the cycle (W U z = -2.3, p = 0.02). In infertile women, there was a very strong trend for CD4+ populations to vary throughout the menstrual cycle (H = 5.8; p = 0.05). CD4+ numbers in these women peaked in the proliferative phase but reached their lowest levels in the secretory phase (Figure 5.4 B).
Percentages of live lymphocytes

A significant reduction in the percentages of live lymphocytes occurred in fertile patients during the proliferative phase of the cycle compared to infertile participants ($W \ U \ z = -2.1; \ p = 0.03$). No differences were noted between fertile and infertile patients during other phases of the cycle. In fertile women, endometrial live lymphocyte numbers decreased from menstrual to the proliferative phase, before rising again in the secretory phase. In infertile women, percentages of live lymphocytes were relatively constant throughout the menstrual cycle (Figure 5.4 C).
Figure 5.1.4. Comparison of median endometrial numbers of Tregs, CD4+ cells and live lymphocytes in all phases of the menstrual cycle between fertile and infertile women (M = Menstrual, P = Proliferative, S = Secretory, * p value < 0.05).

5.1.6 BLOOD IMMUNE CELL CHANGES IN ENDOMETRIOSIS-ASSOCIATED INFERTILITY

Circulating Tregs, CD4+ lymphocytes and percentages of live lymphocytes were analysed in fertile and infertile women with and without endometriosis during the menstrual cycle (Table 5.5).
Densities and IQRs of circulating Tregs, CD4+ cells and live lymphocytes in infertile women without endometriosis and fertile and infertile women with endometriosis are summarised in Table 5.5. Data from the menstrual phase is not shown due to extremely small sample size during this time of the cycle.

Table 5.5. Median densities and interquartile ranges of blood Tregs, CD4+ cells and live lymphocytes in the menstrual cycle in fertile and infertile women with and without endometriosis.

<table>
<thead>
<tr>
<th></th>
<th>Proliferative</th>
<th>N</th>
<th>Secretory</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tregs</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No endo, Infertile</td>
<td>4.7*</td>
<td>3</td>
<td>4.1 (2.6)</td>
<td>7</td>
</tr>
<tr>
<td>Endo, Fertile</td>
<td>5.7*</td>
<td>2</td>
<td>4.2*</td>
<td>3</td>
</tr>
<tr>
<td>Endo, Infertile</td>
<td>4.2 (1.5)</td>
<td>11</td>
<td>5.6 (5.9)</td>
<td>12</td>
</tr>
<tr>
<td><strong>CD4+</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No endo, Infertile</td>
<td>43.9*</td>
<td>3</td>
<td>43.5 (14.7)</td>
<td>7</td>
</tr>
<tr>
<td>Endo, Fertile</td>
<td>47.6*</td>
<td>2</td>
<td>45.1*</td>
<td>3</td>
</tr>
<tr>
<td>Endo, Infertile</td>
<td>42.5 (15.3)</td>
<td>11</td>
<td>34.4 (14.7)</td>
<td>12</td>
</tr>
<tr>
<td><strong>Live lymphocytes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No endo, Infertile</td>
<td>71.9*</td>
<td>3</td>
<td>79.0 (14.7)</td>
<td>7</td>
</tr>
<tr>
<td>Endo, Fertile</td>
<td>62.1*</td>
<td>2</td>
<td>82.1*</td>
<td>3</td>
</tr>
<tr>
<td>Endo, Infertile</td>
<td>89.2 (11.5)</td>
<td>11</td>
<td>86.1 (23.8)</td>
<td>12</td>
</tr>
</tbody>
</table>

Data represented as median (IQR). P=Proliferative, S=Secretory, * No inter-quartile ranges due to small number of participants.

**Tregs**

Overall, Treg density showed very little variation between fertile and infertile women with endometriosis and fertile and infertile women without endometriosis, when all phases of the menstrual cycle combined (Figure 5.5 A). During the proliferative and secretory phases, little variation was noted between fertile and infertile endometriosis patients (Table 5.5; Figure 5.6 A).
**CD4 + cells**

In endometriosis, circulating CD4+ numbers did not differ markedly between fertile and infertile women throughout the cycle (Figure 5.5 B). CD4+ cells did not show any marked difference between proliferative and the secretory phase, although CD4+ cells were higher in fertile women with endometriosis compared to infertile endometriosis patients in the secretory phase of the cycle (Figure 5.6 B).

**Percentage of live lymphocytes**

Percentages of live circulating lymphocytes appeared to be lower in women with endometriosis, who were fertile, compared to those who were infertile (Figure 5.5 C). While this difference was observed in both proliferative and secretory phases, it was significant only in the proliferative phase of the cycle (W U z = -2.1; p = 0.03; Figure 5.6 C). No significant differences were observed between the percentages of live lymphocytes between fertile and infertile controls.
Figure 5.5. Overall median densities of blood Tregs, CD4+ cells and live lymphocytes and their comparison between fertile and infertile women with and without endometriosis.
Figure 5.6. Median densities of blood Tregs, CD4+ cells and live lymphocytes in fertile and infertile women with endometriosis in the proliferative (P) and secretory (S) phases (* p value < 0.05).

5.1.7 ENDOMETRIAL IMMUNE CELL CHANGES IN ENDOMETRIOSIS-ASSOCIATED INFERTILITY

Median endometrial densities and IQRs of live lymphocytes, CD4+ cells and Tregs were analysed in fertile and infertile women with endometriosis and infertile women without the disease which are summarised in Table 5.6.
Table 5.6. Median densities and interquartile ranges of endometrial Tregs, CD4+ cells and live lymphocytes in the menstrual cycle in fertile and infertile women with and without endometriosis.

<table>
<thead>
<tr>
<th></th>
<th>Proliferative</th>
<th></th>
<th>Secretory</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td></td>
<td>N</td>
<td></td>
</tr>
<tr>
<td><strong>Tregs</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No endo, Infertile</td>
<td>8.1*</td>
<td>3</td>
<td>6.5 (7.3)</td>
<td>6</td>
</tr>
<tr>
<td>Endo, Fertile</td>
<td>17.8*</td>
<td>2</td>
<td>11.6*</td>
<td>3</td>
</tr>
<tr>
<td>Endo, Infertile</td>
<td>8.9 (6.0)</td>
<td>6</td>
<td>7.1 (14.3)</td>
<td>6</td>
</tr>
<tr>
<td><strong>CD4+</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No endo, Infertile</td>
<td>24.2*</td>
<td>3</td>
<td>21.3 (9.1)</td>
<td>6</td>
</tr>
<tr>
<td>Endo, Fertile</td>
<td>26.1*</td>
<td>2</td>
<td>23.9*</td>
<td>3</td>
</tr>
<tr>
<td>Endo, Infertile</td>
<td>28.8 (11.3)</td>
<td>6</td>
<td>18.7 (11.2)</td>
<td>6</td>
</tr>
<tr>
<td><strong>Live lymphocytes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No endo, Infertile</td>
<td>58.9*</td>
<td>3</td>
<td>79.5 (21.8)</td>
<td>6</td>
</tr>
<tr>
<td>Endo, Fertile</td>
<td>51.4*</td>
<td>2</td>
<td>76.5*</td>
<td>3</td>
</tr>
<tr>
<td>Endo, Infertile</td>
<td>86.9 (6.8)</td>
<td>6</td>
<td>78.5 (15.7)</td>
<td>6</td>
</tr>
</tbody>
</table>

Data represented as median (IQR). P=Proliferative, S=Secretory, * No inter-quartile ranges due to small number of participants.

**Tregs**

There was no significant difference in Treg cells populations between fertile and infertile women with endometriosis or between the infertile women without endometriosis. Tregs appeared to be increased in fertile controls compared to the other groups (Figure 5.7 A). In women with endometriosis who were fertile, Treg density appeared to be higher during the proliferative phase in comparison to infertile endometriosis patients. However this difference did not reach statistical significance (Figure 5.8 A). Similarly, no significant difference was noted between fertile and infertile endometriosis patients during the secretory phase of the cycle.
**CD4+ cells**

Overall endometrial density of CD4+ cells showed little variation between fertile and infertile women with endometriosis (Figure 5.7 B). In infertile endometriosis patients, CD4+ cells were significantly reduced from proliferative to the secretory phase (W U z = -2.2; p = 0.03; Figure 5.8 B). While a similar pattern was noted in fertile women with endometriosis, this reduction did not reach level of statistical significance.

**Percentage of live lymphocytes**

Overall percentages of live endometrial lymphocytes did not significantly vary between fertile and infertile controls or in fertile endometriosis patients; however they appeared to be increased in infertile women with endometriosis (Figure 5.7 C). During the proliferative phase of the cycle, percentages of live lymphocytes were significantly increased in infertile endometriosis patients, compared to fertile women with endometriosis (W U z = -2.1; p = 0.03). This difference was not observed in the secretory phase (Figure 5.8 C).
Figure 5.7. Overall median densities of endometrial Tregs, CD4+ cells and live lymphocytes and their comparison between fertile and infertile women with and without endometriosis.
Figure 5.8. Median densities of endometrial Tregs, CD4+ cells and live lymphocytes in fertile and infertile women with endometriosis in the proliferative (P) and secretory (S) phases (* p value < 0.05).
5.2 IMMUNOHISTOCHEMISTRY

Foxp3+ Treg counts were analysed between fertile and infertile women with and without endometriosis.

5.2.1 AGE DISTRIBUTION

Age distribution did not statistically differ between women with endometriosis (mean age ± SD = 35.7 ± 7.0 yrs) and those without the disease (33.8 ± 8.0). Similarly, no differences in age distribution were noted between fertile (35.7 ± 8.0) and infertile (35.5 ± 7.0) women. Furthermore, the age of participants did not appear to significantly correlate with Foxp3 density in the endometrium.

5.2.2 FOXP3+ CELLS IN WOMEN WITH AND WITHOUT ENDOMETRIOSIS

Median densities of Foxp3+ cells in women with endometriosis and controls were analysed across different phases of the menstrual cycle. Results (presented as median and interquartile ranges of Foxp3+ cells per mm²) are shown in Table 5.7.

Table 5.7. Median densities and interquartile ranges of Foxp3 in the menstrual cycle in women with and without endometriosis.

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>N</th>
<th>Endometriosis</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Menstrual</td>
<td>0.3 (5.2)</td>
<td>4</td>
<td>1.5 (8.3)</td>
<td>5</td>
</tr>
<tr>
<td>Proliferative</td>
<td>1.4 (3.8)</td>
<td>12</td>
<td>1.4 (4.8)</td>
<td>28</td>
</tr>
<tr>
<td>Secretory</td>
<td>1.5 (5.5)</td>
<td>11</td>
<td>2.1 (8.1)</td>
<td>28</td>
</tr>
</tbody>
</table>

Data represented as median (IQR), * IQR not shown due to small sample size in the group.
**Foxp3+ cells during the normal menstrual cycle**

No significant differences were observed in Foxp3+ cell density during the phases of the menstrual cycle.

**Foxp3+ cells in endometriosis**

Large variations in Foxp3+ cell density were noticed in the secretory phase (0-18.7 per mm²). No significant differences were noted in Foxp3 cell density during the menstrual cycle of women with endometriosis. Similarly there were no significant differences between women with and without endometriosis (Figure 5.9).

![Figure 5.9. Comparison of median densities of Foxp 3+ cells between women with and without endometriosis (M = Menstrual, P = Proliferative, S = Secretory).](image)
5.2.3 FOXP3+ CELLS IN FERTILE AND INFERTILE WOMEN

Median densities of Foxp3+ cells in fertile and infertile women were analysed across different phases of the menstrual cycle. The results are summarised in Table 5.8.

Table 5.8. Median densities and interquartile ranges of Foxp3+ cells in the menstrual cycle in fertile and infertile women.

<table>
<thead>
<tr>
<th>Phase</th>
<th>Fertile</th>
<th>N</th>
<th>Infertile</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Menstrual</td>
<td>0*</td>
<td>3</td>
<td>1.8*</td>
<td>3</td>
</tr>
<tr>
<td>Proliferative</td>
<td>2.3 (2.4)</td>
<td>12</td>
<td>1.4 (4.8)</td>
<td>26</td>
</tr>
<tr>
<td>Secretory</td>
<td>1.3 (3.1)</td>
<td>11</td>
<td>3.2 (7.7)</td>
<td>22</td>
</tr>
</tbody>
</table>

Data represented as median (IQR), * IQR not shown due to small sample size.

**Foxp3+ cells in fertile and infertile women**

Foxp3+ cell numbers did not appear to vary markedly in the menstrual cycle in fertile and infertile women. However, the range in cell densities greatly varied in infertile women during the proliferative (0 - 8.6 per mm²) and secretory phase (0 – 11.8 per mm²) and such variations were not noted in the menstrual phase. No significant differences were seen between fertile and infertile women during the menstrual cycle (Figure 5.10).
Figure 5.10. Comparison of median densities of Foxp3+ cells between fertile and infertile women in the menstrual cycle (M = Menstrual, P = Proliferative, S = Secretory).

### 5.2.4. FOXP3+ CELLS IN ENDOMETRIOSIS-ASSOCIATED INFERTILITY

Cell densities were analysed across different phases of the menstrual cycle in fertile and infertile women with and without endometriosis. These results are summarised in Table 5.2.2.

Table 5.2.2. Median densities and interquartile ranges of Foxp3+ cells in the menstrual cycle in fertile and infertile women with and without endometriosis.

<table>
<thead>
<tr>
<th></th>
<th>Menstrual</th>
<th>N</th>
<th>Proliferative</th>
<th>N</th>
<th>Secretory</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infertile, Endo</td>
<td>1.7 (5.2)</td>
<td>4</td>
<td>1.3 (6.3)</td>
<td>17</td>
<td>2.6 (10.2)</td>
<td>15</td>
</tr>
<tr>
<td>Fertile, Endo</td>
<td>*</td>
<td>0</td>
<td>1.9 (2.6)</td>
<td>9</td>
<td>1.2 (3.3)</td>
<td>12</td>
</tr>
<tr>
<td>Infertile, Control</td>
<td>*</td>
<td>0</td>
<td>0.9 (3.8)</td>
<td>8</td>
<td>6.1 (8.3)</td>
<td>6</td>
</tr>
<tr>
<td>Fertile, Control</td>
<td>1.7*</td>
<td>2</td>
<td>2.0 (9.7)</td>
<td>4</td>
<td>*</td>
<td>0</td>
</tr>
</tbody>
</table>

Data represented as median (IQR), * Data not shown due to small sample size.
**Foxp3+ cells in infertile women without endometriosis**

Foxp3+ cell numbers appeared to increase from proliferative to the secretory phase in infertile women without endometriosis (Figure 5.11). This difference, however, was not found to be statistically significant. In fertile women without endometriosis, no large variations in Treg counts were observed between menstrual and the proliferative phase.

**Foxp3+ cells in fertile and infertile women with endometriosis**

Median density of Foxp3+ cells in infertile women with endometriosis was slightly higher in the secretory phase compared to menstrual and the proliferative phases. Range in cell densities varied greatly in the secretory phase in infertile women with endometriosis (0 – 21.2 per mm²). Overall, Foxp3+ cells did not show marked variations throughout the menstrual cycle in fertile and infertile women with endometriosis.

![Figure 5.11. Density of Foxp3+ cells in fertile and infertile women with and without endometriosis throughout the menstrual cycle (M = Menstrual, P = Proliferative, S = Secretory).](image-url)
CHAPTER 6: DISCUSSION

6.1 INTRODUCTION

This project aimed to characterise Treg populations in blood and the endometrium in women with endometriosis and associated infertility during the menstrual cycle. In order to characterise Treg populations, this thesis also investigated CD4+ T cells and live lymphocytes during the normal cycle, in endometriosis and infertility. The results of this project show that density of endometrial and circulating live lymphocyte populations are increased in endometriosis, infertility and endometriosis-associated infertility compared to controls. The numbers of proliferative phase Tregs in the endometrium are also increased in fertile compared to infertile women.

6.2 NORMAL MENSTRUAL CYCLE

6.2.1 DECREASED SURVIVAL OF LYMPHOCYTES FROM THE PROLIFERATIVE PHASE

During the normal cycle, the numbers of endometrial live lymphocytes appeared to show significant differences, decreasing from menstrual to the proliferative phase, before rising again in the secretory phase. This pattern was also noted amongst the fertile group of patients, suggesting that reduced viability of lymphocytes in the proliferative phase may also play a role in uterine receptivity. The numbers of immune cells show day-to-day variations in the endometrium which are important to carry out normal functions such as menstruation, endometrial remodelling and preparation for embryo implantation (Salamonsen and Lathbury, 2000). Previous studies show generally decreased numbers of immune cells in the proliferative phase (Mettler et al., 1996, Salamonsen and Lathbury, 2000). Reduced numbers
of endometrial immune cells in the early and late parts of the proliferative phase may serve different purposes. In the early proliferative phase, decreased immune cell numbers may allow regeneration of the endometrial glandular and stromal components after menstrual shedding (Finn, 1986) while decreased numbers of immune cells in the late proliferative phase may be in preparation for embryo implantation (Arruvito et al., 2007, Guerin et al., 2009).

The findings of the present study indicate that lymphocyte viability may also be reduced in blood during the normal proliferative phase. This study is the first to date to demonstrate leukocyte viability differences during the menstrual cycle. Current evidence suggests that lymphocyte count in blood is reduced in proliferative compared to the menstrual and the secretory phases (Rosemary et al., 2014). Earlier, Mathur et al. (1979) had performed day-to-day analysis of blood lymphocytes and concluded that lymphocyte count in blood negatively correlates with serum oestradiol levels (Mathur et al., 1979). Since oestradiol levels peak in the late proliferative phase, it was suggested that reduced numbers of lymphocytes at the time of ovulation may be in preparation to suppress immuno-reactivity during embryo implantation (Mathur et al., 1979).

Our findings of generalised reduced viability of lymphocytes in the proliferative phase are an important observation. These changes may be vital not only to allow for normal immune cell turnover, but play active roles in preparation for implantation of the embryo, should pregnancy occur.
6.2.2 INFLUX OF IMMUNE CELLS AND INCREASED LYMPHOCYTE VIABILITY
IN THE SECRETORY PHASE

During the normal cycle, the viability of lymphocytes appeared to increase again in the secretory phase. Previous studies have noted marked endometrial influx of most of the immune cell types in the secretory phase (Mettler et al., 1996, Salamonsen and Lathbury, 2000). Recruitment of immune cells in the secretory phase may be essential to prepare for possible implantation and menstruation if implantation does not occur (Salamonsen, 1998, Salamonsen and Lathbury, 2000). Although the numbers of T lymphocyte sub-populations fluctuate in the secretory phase (Klentzeris et al., 1995, Mettler et al., 1996), endometrial total T lymphocyte count shows little variation in the menstrual cycle between late proliferative and the secretory phase (Mettler et al., 1996). Since the viability of lymphocytes has not been analysed previously, it can be suggested that recruitment of live immune cells in the secretory phase may be in preparation to target shed fragments during menstruation. Increased numbers of live lymphocytes during the normal secretory phase were also observed in blood. These findings of increased numbers of live lymphocytes in the secretory phase may be part of a generalised immune response required in preparation for menstruation, during which time immune cells play important roles in targeting shed fragments, allowing for regeneration and repair of the endometrium (Salamonsen, 1998).

6.2.3 VARIATIONS IN CD4+ CELL COUNT

The findings from this study showed that during the normal cycle, endometrial CD4+ T cell numbers vary minimally between the proliferative and secretory phases. Similar findings were observed in fertile participants of the study. Low variability in the numbers of endometrial CD4+ cells during the peri-implantation period is in line with a previous finding of little variation in endometrial CD4+ cell count between the proliferative and secretory
phases (Mettler et al., 1996). Despite no large variations in numbers, cytokine production by CD4+ sub-populations is altered in early pregnancy (Veenstra et al., 2003) in preparation for implantation. Since the increased numbers of endometrial CD4+ cells during the peri-implantation period have been linked to pregnancy loss (Lachapelle et al., 1996, Bartha et al., 2000), minimal variations in CD4+ cell count during the menstrual cycle appear to support implantation of embryo.

6.2.4 Tregs IN THE SECRETORY PHASE
Flow cytometric analysis demonstrated a decrease in the numbers of Tregs during the secretory compared to the proliferative phase of the normal cycle. Similar but more pronounced differences were observed in the endometrium of fertile women. These findings are in accordance with previously published data, which has shown that normally, endometrial Tregs numbers dramatically decrease in secretory compared to the proliferative phase of the menstrual cycle (Berbic et al., 2010). It has been suggested that higher numbers of Tregs in the proliferative phase may suppress other immune cells in preparation for possible implantation, while their decline in the secretory phase may be essential in the lead up to menstruation to allow other immune cells to effectively target the shed fragments (Berbic et al., 2010, Basta et al., 2010). Although there is no other study that compares the numbers of proliferative and secretory phase Tregs in the endometrium, it has been shown that women with low numbers of endometrial Tregs are less likely to mediate immune-tolerance of the fetus and can result in spontaneous pregnancy-loss (Sasaki et al., 2004).

Increased numbers of Tregs were also evident in blood during proliferative compared to the secretory phase. These findings were also observed amongst women with proven fertility. Previous findings have shown that numbers of Tregs positively correlate with circulating
oestradiol levels in fertile women (Arruvito et al., 2007). Serum oestradiol levels peak in the late proliferative phase, just before ovulation, which is considered to be important for preparation of the endometrium for implantation of the embryo (Groothuis et al., 2007). Apart from the endometrial structural changes under the influence of oestradiol, increase in Treg count may facilitate implantation of embryo by suppressing pro-inflammatory immune cells (Arruvito et al., 2007, Tai et al., 2008).

6.3 ENDOMETRIOSIS

6.3.1 INCREASED SURVIVAL OF LYMPHOCYTES

In women with endometriosis, a number of significant differences in immune cell viability were noted during the menstrual cycle. In these women, an increase in the live lymphocyte count occurs in the proliferative phase, during which time leukocyte viability is low in women without the disease. It has previously been shown that in endometriosis, the glandular and stromal components of the endometrium are more resistant to apoptosis compared to women without the disease (Dmowski et al., 2001, Harada et al., 2004, Nishida et al., 2005, Nasu et al., 2009). Generally, increased survival of endometrial cells in endometriosis may help more viable endometrial cells reach the peritoneal cavity and hence the establishment of endometriotic lesions (Vinatier et al., 2000, Sharpe-Timms, 2001, Ulukus and Arici, 2005). It is unknown if, in addition to the glandular and stromal cells, immune populations are also likely to be more resistant to apoptosis in women with endometriosis compared to controls. Increased survival of endometrial live lymphocytes in the proliferative phase in women with endometriosis may interfere with endometrial receptivity.
Interestingly, the current results indicate that viability of lymphocytes may be increased across all phases of the menstrual cycle in blood of women with endometriosis compared to women without the disease. It is unclear why there may be increased survival of immune cells in blood and how it may influence the endometrial populations. It can be hypothesised that there is increased survival of immune cells in women with endometriosis as a result of the generalised increased resistance to apoptosis. There is some evidence that certain pro-inflammatory cytokines, such as IL-8 and TNF-alpha, can promote survival of immune cells in women with endometriosis (Kwak et al., 2002, Kyama et al., 2003). Increased levels of these cytokines in blood of women with endometriosis (Gazvani and Templeton et al., 2002) may contribute to a generalised increase in resistance to apoptosis of immune cells in women with endometriosis.

6.3.2 CD4+ CELL COUNT

In endometriosis, a strong trend suggested that endometrial CD4+ cell proportions are increased in the proliferative compared to secretory phase of the cycle. Bulmer et al (1998) have previously shown that numbers of endometrial CD4+ lymphocytes do not show large variations in endometriosis. In endometriosis, cytokine secretion is altered by CD4+ cells that may affect macrophage function (Nasu et al., 2009). It can be hypothesised that an increase in numbers of endometrial live CD4+ cells is part of a generalised increase in live lymphocyte count that may create a hostile environment interfering with successful implantation and contribute to endometriosis-associated infertility.

6.3.3 Tregs IN THE SECRETORY PHASE

In women with endometriosis, endometrial Foxp3+ cell numbers appeared to decrease in the secretory compared to the proliferative phase. However, endometrial Treg proportions
detected by flow cytometry appeared to be higher across all phases of the menstrual cycle in women with endometriosis compared to controls. The results from flow cytometric analysis are in concordance to previously published data showing that in women with endometriosis, endometrial Treg count normally decreases from the proliferative to secretory phase during which time Tregs are persistently elevated in endometriosis (Berbic et al., 2010). It was previously suggested that failure of the immune system to clear refluxed endometrial tissue due to increased suppression by Tregs in the late secretory phase may facilitate their implantation in the peritoneal cavity. In addition to changes in Treg count, certain endometrial Foxp3 mRNA genetic polymorphisms are over-expressed in endometriosis compared to controls that can alter numbers of Tregs and contribute to pathogenesis of the disease (André et al., 2011, Barbosa et al., 2012). The findings of the present study did not reach statistical significance. This may be explained by smaller numbers of samples in the present study. Furthermore, immune cell numbers show day-to-day variations in the menstrual cycle and due to small sample size, the present study could not investigate in-phase variations (early, mid and late phase).

As in the endometrium, Treg proportions appeared to increase in blood of women with endometriosis compared to controls in the secretory phase. Although the numbers of Tregs in blood of women with endometriosis have not been studied previously during the secretory phase, it has been reported that overall Treg count is increased in women with endometriosis compared to controls (Jarosław et al., 2007). It is known that Treg count positively correlates with serum oestradiol levels and normally decreases in the secretory phase (Arruvito et al., 2007). Since endometriosis is an oestrogen-dominant disease, blood Treg proportions in endometriosis may appear to increase secondary to increased levels of oestradiol in the secretory phase.
6.4 INFERTILITY

6.4.1 INCREASED SURVIVAL OF LYMPHOCYTES FROM INFERTILE WOMEN

In infertile women, there was a significant increase in endometrial proportions of live lymphocytes in the proliferative phase compared to fertile controls. Previously it has been reported that lymphocyte count is increased in infertile women compared to fertile controls in the mid-secretory phase (Stewart-Akers et al., 1998). No such evidence was found in the proliferative phase in another study that reported only minimal variations in endometrial lymphocyte counts in infertile women (Klentzeris et al., 1994) but this used less advanced techniques and did not consider lymphocyte viability. While the viability of endometrial lymphocytes has not been determined in infertility previously, it has been shown that peri-implantation increase in the endometrial immune cell count is associated with an increased release of pro-inflammatory cytokines in infertile women (Stewart-Akers et al., 1998). Pro-inflammatory cells undergo apoptosis in early pregnancy under the influence of cytokines. Therefore, disturbances in the intricate balance of pro- and anti-inflammatory cytokines may result in rejection of early pregnancy (Reinhard et al., 1998).

In blood, a strong trend suggested increase in proportions of live lymphocytes in infertile women in the proliferative phase. The numbers of circulating lymphocytes were previously shown not to vary between infertile women and fertile controls (Vujisić et al., 2004). However, viability of lymphocytes was not assessed in their study and study participants included women undergoing artificial reproductive techniques with hormonal preparations which may have altered the cell counts.
On the basis of these results, it can be hypothesised that increased survival of blood and endometrial lymphocytes in the proliferative phase may contribute to creation of a hostile environment for embryo implantation. However, the underlying mechanisms that may contribute to increased survival of immune cells in infertility still remain to be further investigated.

6.4.2 CD4+ CELLS IN THE PROLIFERATIVE PHASE

In infertile women, the endometrial CD4+ cell counts were significantly increased during the proliferative phase compared to fertile participants. This corresponds with a previous finding of increase in the endometrial CD4+ cell count in infertile women in the proliferative phase (Klentzeris et al., 1994). An intricate balance of pro-inflammatory (Th-1) and anti-inflammatory (Th-2) immune cells and cytokines is essential to avoid fetal rejection in early pregnancy (Sykes et al., 2012) as increase in the numbers of endometrial CD4+ cells before implantation may interfere with early pregnancy. As CD4+ cells include Th-1 and Th-2 immune cell populations, further classification of CD4+ populations would be helpful in order to elucidate exact implications in infertile women.

6.4.3 Tregs IN INFERTILITY

In the endometrium, flow cytometric results showed a significant decrease in the numbers of Tregs in infertile women during the proliferative phase compared to fertile participants. In unexplained infertility, endometrial Treg Foxp3 mRNA expression is decreased in the mid-secretory phase compared to fertile controls (Jasper et al., 2006). It has been suggested that altered Foxp3 expression in infertile women may result in impaired endometrial recruitment of Tregs or differentiation of uterine T cells into Tregs (Jasper et al., 2006, André et al., 2011). Since a pre-ovulatory surge of endometrial Tregs in the proliferative phase is thought
to facilitate immune-tolerance for successful implantation (Guerin et al., 2009), a decrease in Treg count before implantation may lead to embryo-rejection. Evidence suggests that Tregs have more inhibitory effect on Th-1 cells compared to Th-2 cells (Cosmi et al., 2004) and in early pregnancy, there is a decrease in Th-1/Th-2 ratio (Doria et al., 2006). Therefore, an increase in the numbers of Th-1 immune cells may result in rejection of pregnancy (Guerin et al., 2009) as women with early pregnancy loss have reduced numbers of endometrial Tregs compared to fertile controls (Sasaki et al., 2004, Arruvito et al., 2007, Yang et al., 2008).

In addition to previous reports of decreased Foxp3 expression during the secretory phase, this study indicates that an endometrial immune environment unfavourable to implantation may be established earlier in the cycle than previously recognised. Un-moderated immune responses in the endometrium during this time would contribute to establishment of an endometrial environment unfavourable for implantation.

In blood, Treg numbers appeared to decrease in infertile women compared to fertile participants during the proliferative phase. Treg numbers have not been studied in blood in infertile women previously but experiments in a mouse model suggest that Treg dysregulation in blood may lead to embryo-rejection by Th1-mediated exaggerated maternal immune response (Aluvihare et al., 2004). Our results suggest a generalised decrease in the numbers of Tregs in the proliferative phase, and thus inefficient immune-suppression, may allow for persistent elevation of other immune-cell populations that may in turn create a hostile environment, interfering with successful embryo implantation.
6.5 ENDOMETRIOSIS-ASSOCIATED INFERTILITY

6.5.1 INCREASED SURVIVAL OF LYMPHOCYTES FROM ENDOMETRIOSIS-ASSOCIATED INFERTILITY

The results from this thesis also reveal a number of interesting differences in immune-mediated responses in women with endometriosis-associated infertility. In these women, the numbers of proliferative-phase endometrial live lymphocytes increased significantly compared to women with endometriosis who were fertile. Roles of endometrial lymphocytes and their subgroups have not been studied in endometriosis-associated infertility previously. It can be hypothesised that increased numbers of live lymphocytes before implantation in infertile women with endometriosis alter the endometrial conditions, making them less favourable for embryo implantation. As in the endometrium, survival of lymphocytes was significantly increased in blood of infertile women with endometriosis compared to fertile women with the disease in the proliferative phase. Previously, increased numbers of lymphocytes have been observed in blood of infertile women with endometriosis (Badawy et al., 1987).

In endometriosis, immune cells may adversely affect fertility by altered cytokine production (Dimitriadis et al., 2006, Vassiliadis et al., 2005). Decidualisation is a pre-requisite for embryo implantation and endometrial stromal cells have reduced capability to decidualise in endometriosis (Klemmt et al., 2006). IL-11 enhances decidualisation of endometrial stromal cells normally (Dimitriadis et al., 2002) but its levels are reduced in endometriosis-associated infertility (Dimitriadis et al., 2006) which may interfere with implantation of embryo. Similarly, production of leukaemia inhibitory factor (LIF) - a marker of endometrial
receptivity produced by lymphocytes - is decreased in endometriosis-associated infertility (Achache and Revel, 2006, Dimitriadis et al., 2006).

The results of this project suggest a generalised increase in the proportions of live lymphocytes in infertile women with endometriosis in the proliferative phase compared to fertile women with the disease. Since not all women with endometriosis are infertile, it appears that women with endometriosis, who have increased viability of immune cells in the proliferative phase, are more likely to be infertile.

6.5.2 CD4+ CELLS

A significant increase in endometrial CD4+ count was observed in the proliferative phase in infertile women with endometriosis compared to the secretory phase. These changes in CD4+ numbers were not observed in fertile women with endometriosis. Similar but less marked findings were observed in blood of infertile women with endometriosis. Although the numbers of CD4+ lymphocytes or their sub-populations in endometrium and blood have not been studied previously in infertility in endometriosis, it has been reported that Th-1 and Th-2 blood cytokine profiles do not differ between women with endometriosis-associated infertility and controls (Podgaec et al., 2010). As the function of CD4+ cells is altered in endometriosis (Mettler et al., 1996) and infertility before implantation (Sykes et al., 2012), increased survival of live immune cells in infertile women with endometriosis before implantation may alter the favourable endometrial conditions, which may be due to increased resistance to apoptosis or secondary to reduced suppression by Tregs in the proliferative phase.
6.5.3 Tregs

Treg density appeared to increase in infertile women with endometriosis in the proliferative phase compared to fertile controls with endometriosis. Although less marked, a similar trend was also observed in blood of infertile participants with endometriosis compared to fertile women with the disease. An increase in Treg density in fertile women with endometriosis in the proliferative phase may suppress other immune cells and increase chances of embryo implantation.

The current findings build on previous indications of Foxp3 and Treg disturbances in endometriosis-associated infertility. André et al (2011) have previously studied various endometrial Foxp3 genetic polymorphisms in infertile women with endometriosis compared to women with unexplained infertility and fertile controls. They demonstrated that the expression of certain Foxp3 polymorphisms is related to the presence of infertility in endometriosis. Endometrial Treg numbers and function in endometriosis and associated infertility may be determined by the presence or absence of certain Foxp3 polymorphisms which may relate to the fact that not all women with endometriosis are infertile and infertility in endometriosis is independent of the stage of the disease (Koch et al., 2012).

6.6 LIMITATIONS OF THE STUDY

The power calculation indicated that more samples would be ideal but analyses were restricted by small sample sizes, particularly in the menstrual phase. Unfortunately it was not feasible to increase numbers within the scope of this research degree candidature and it should be noted that the study participants represent a year of full-time recruitment and thorough review of tissue archives. Some other limitations of this study were:
Immune cells show day-to-day variations in the menstrual cycle. Analysis in the early, mid and late parts of each phase was not able to be performed due to limited numbers of participants in each phase, which may have affected the results.

IHC was performed on a cohort of patients, which included participants for whom samples were not available for flow cytometry (although most participants in flow cytometry experiments were also included in IHC).

Treg sub-populations were not analysed separately. Individual Treg subpopulations, which may have different proportions in the total Treg pool, may play individual roles during the normal menstrual cycle and in endometriosis and infertility.

Functional analyses were not performed in our study. Function of Tregs is reported to be altered in endometriosis and infertility. Tregs may have disturbed function in these conditions despite normal numbers.

6.7 CONCLUSIONS

Overall, this project showed important differences in the numbers of blood and endometrial lymphocyte populations in women with endometriosis and between fertile and infertile women. The most interesting finding, which has not been reported before, was the increased survival of live lymphocytes in the endometrium and blood in patients with endometriosis, infertility and endometriosis-associated infertility. This finding was most evident in the proliferative phase of the menstrual cycle. Increased numbers of live immune cells in the proliferative phase may alter the endometrial environment before implantation of the embryo.

This thesis also demonstrated decreased numbers of endometrial Tregs in infertile women in the proliferative phase compared to fertile women. It appears that increased survival of immune cells before implantation in infertile women may be directly related to decreased
suppression of immune response by Tregs. This study also indicates that in endometriosis, infertility and endometriosis-associated infertility, an unfavourable endometrial immune environment for implantation may be established much earlier (in the proliferative phase) in the menstrual cycle than was previously recognised. Altered immune responses in the endometrium during this period may contribute to the establishment of an endometrial environment unfavourable for implantation.

The specific conclusions of this study with respect to the original aims and hypotheses are as follows:

- Overall, the numbers of circulating and endometrial Tregs appear to vary throughout the menstrual cycle, with some subtle differences evident between fertile and infertile women and in participants with and without endometriosis.
- No dramatic changes occur in circulation or the endometrium of women with endometriosis in the secretory phase compared to women without the disease. However, Treg numbers in circulation and blood appear to increase in the secretory phase in women with endometriosis compared to controls.
- Endometrial numbers of Tregs appear to decrease in infertile women compared to fertile women in the menstrual cycle which is significant in the proliferative phase. No dramatic changes occur in Treg density in circulation of infertile women compared to fertile participants throughout the menstrual cycle.
- The numbers of endometrial Tregs appear to decrease in infertile women with endometriosis compared to fertile women with the disease in the proliferative and secretory phases. Similarly, Treg numbers in blood do not vary markedly between fertile and infertile women with endometriosis. Treg analysis in blood in women without endometriosis was restricted by small number of participants.
6.8 FUTURE DIRECTIONS

The results of this project indicate important alterations in Tregs and other lymphocyte populations in endometriosis and infertility, which has given rise to some interesting questions that need to be answered. Future studies could include the following:

- Day-to-day analysis of immune cell populations in blood and the endometrium during the menstrual cycle in endometriosis, infertility and endometriosis-associated infertility.
- Mechanisms governing apoptosis in immune cells in blood and the endometrium in endometriosis, infertility and endometriosis-associated infertility.
- Assessment of viability of shed endometrial fragments and immune cell populations in endometriosis and infertility using cell culture techniques.
- Identification of specific cell markers for Treg sub-group analysis in endometriosis and infertility.
- Functional analyses of Tregs and sub-groups in endometriosis and infertility.
- Investigation of whether specific Treg subsets are recruited into the endometrium at various phases of the menstrual cycle.
- Analysis of migration of immune cell populations between blood, peritoneal cavity and the endometrium in the normal menstrual cycle, endometriosis and infertility in all phases.
- Studies to determine Treg genetic polymorphisms in endometriosis, infertility and endometriosis-associated infertility.

These studies are likely to improve our understanding of the immunological mechanisms that underlie endometriosis, infertility and endometriosis-associated infertility. Better understanding of the immunological alterations in these reproductive pathologies may help
develop new strategies to prevent them in high risk populations, improve implantation rates and reduce sub-clinical pregnancy loss.
APPENDICES

APPENDIX A

PREPARATION OF BUFFER

Buffer solution was prepared by mixing 0.5 gram of bovine serum albumin and 1 tablet of phosphate buffered saline (Dulbecco ‘A’ Tablets, Oxoid limited, UK) per 100 mL of distilled water until they were completely dissolved.

APPENDIX B

PREPARATION OF PHOSPHATE BUFFERED SALINE

Phosphate buffered saline (PBS) solution was prepared by adding one tablet of PBS (Dulbecco ‘A’ Tablets, Oxoid limited, UK) per 100 mL of distilled water.
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