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Development of peritoneal endometriosis: Characterisation of immune environment in peritoneal endometriotic lesions

by

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

2015

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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.

Signature

31 March 2015
Abstract

Endometriosis is a common gynaecological condition, defined by the presence of endometrial-like glands and stroma at sites outside the uterine cavity, often causing pain and infertility. There are different kinds of endometriotic lesions, of which peritoneal lesions (superficial lesions on pelvic peritoneal surfaces) are the most common. Peritoneal endometriotic lesions have a range of appearances, which are related to lesion development, starting out as red lesions and progressing to become black and then white scarred.

Endometriosis is an inflammatory condition in which the immune system is thought to play a fundamental role in the establishment and progression of disease. Within peritoneal endometriotic lesions there is evidence of increased immune cell recruitment and activation, however immune cell densities vary greatly. Although the role of the immune system in endometriosis is well established, it is still unclear how the lesion immune environment relates to peritoneal endometriotic lesion stages of development. Therefore, the main aim of this research project was to investigate the development of peritoneal endometriotic lesions by characterising their immune environment according to macroscopic appearances.

Peritoneal endometriotic lesions were prospectively collected (total n=32; red n=12, black n=13 and white n=7). Immunohistochemical staining was performed to identify DC-Sign+ immature and DC-Lamp+ mature dendritic cells; CD4+ helper, CD8+ cytotoxic and Foxp3+ regulatory T cells; CD20+ B cells and CD68+ macrophages. Immune cell densities in and
around peritoneal endometriotic lesion samples were quantified with MetaMorph image analysis software.

All studied immune cell populations were present both in stroma and surrounding tissue of peritoneal endometriotic lesions. While CD8+ cytotoxic T cells, Foxp3+ regulatory T cells and CD68+ macrophages were significantly increased in density in lesion stroma compared to surrounding tissue; DC-Sign+ immature dendritic cells and CD20+ B cells were significantly increased in density in tissue surrounding peritoneal endometriotic lesions. Additionally, there were a number of correlations observed between the densities of different immune cell populations in both stromal and surrounding tissue, indicating relationships and interactions between cell types. However, the density of immune cell populations in and around peritoneal endometriotic lesions was not correlated with the stage of lesion development (lesion appearances).

The recruitment of immune cells to tissue within and around peritoneal endometriotic lesions is likely an attempt to attack the lesion. However, their released products may in fact promote processes such as angiogenesis and fibrosis and thereby promote lesion growth and persistence. Immune-targeted treatment approaches show potential efficacy for endometriosis. This study supports the high variability and complexity of endometriosis. The findings from this thesis have implications for understanding of endometriosis pathogenesis, particularly peritoneal lesion development, and also potentially for development of novel treatment approaches.
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Abbreviations

AFS - American Fertility Society

ASRM - American Society for Reproductive Medicine

Bcl 2 - B cell lymphoma 2 gene

DC - Dendritic cell

DIE – Deep infiltrating endometriosis

ECM - Extracellular matrix

EGF - Epidermal growth factor

ER - Oestrogen receptor

FGF - Fibroblast growth factor

FoxP3 - Forkhead box protein 3

GnRH - Gonadotrophin releasing hormone

HGF - Hepatocyte growth factor

ICAM-1 - Intercellular adhesion molecule-1

Ig - Immunoglobulin

IGF-1 - Insulin-like growth factor-1

IHC - Immunohistochemistry

IL - Interleukin

INF - Interferon

MCP-1 - Monocyte chemotactic protein 1

MCSF - Macrophage colony stimulating factor
MDPA - Medroxyprogesterone acetate

MHC - Major histocompatibility complex

MMP - Matrix metalloproteinase

NGF - Nerve growth factor

NK - Natural killer

NSAIDs - Nonsteroidal anti-inflammatory drugs

NT-3 - Neurotrophin-3

DAB – Diaminobenzidine

E1 - Oestrone

E2 – Oestradiol

EFI - Endometriosis fertility index

OCP - Oral contraceptive pill

PECAM - Platelet endothelial cell adhesion molecule

PDGF - Platelet derived growth factor

PF2 α - Prostaglandin F 2 alpha

PGE₂ - Prostaglandin E₂

PF - Peritoneal fluid

RANTES - Regulated upon Activation, Normal T Expressed and Secreted

SD - Standard deviation

TGF-β - Transforming growth factor-beta

Th Cells - Helper T cells

Th₁ - Helper T cells type 1

Th₂ - Helper T cells type 2
TNF-α - Tumour necrosis factor-alpha

Treg - Regulatory T cell

TrkA - Tropomyosin receptor kinase A

TSP – Thrombospondin protein

TIMP - Tissue inhibitors of metalloproteinase

VEGF - Vascular endothelial growth factor

VEGFR - Vascular endothelial growth factor receptor
Chapter 1

Endometriosis

1.1 Introduction

Endometriosis is a common gynaecological disorder defined by the presence of endometrial-like tissue outside the uterine cavity (Senturk and Arici, 1999). The most common symptoms of this disease are pelvic pain and infertility. The exact prevalence of endometriosis in the general population is not clear, however, in women of reproductive age, it is estimated to range between 10 and 15% (Lebovic et al., 2001). Although the majority of women with endometriosis are of childbearing age, reports have also described infrequent cases in pre-menarchal girls and postmenopausal women (Cramer and Missmer, 2002, Sasson and Taylor, 2009).

Endometriotic lesions can be distinguished as three distinct types: peritoneal, deep infiltrating and ovarian (Amer, 2008, Giudice, 2010). As it is illustrated in Figure 1.1, endometriosis is primarily found on the pelvic peritoneum, ovaries, and rectovaginal septum, and in rare cases on the diaphragm, pleura, and pericardium (Netter, 1954).
Figure 1.1 Anatomical locations of areas that can be affected by endometriosis, usually within the pelvic cavity. Right upper quadrant illustrates a histology stain of the lining of an ovarian endometriotic cyst highlighting endometrial-like glands and stroma (Netter, 1954).

1.2 Pathogenesis

The earliest identification of ectopic endometrial-like tissue was in 1860 by Carl Rokitansky (Rokitansky, 1860). Since then, various theories have been proposed to explain the pathogenesis and aetiology of endometriosis. However, the exact pathogenesis of endometriosis is still not completely understood. In fact, different types of endometriosis may
have different origins, therefore it may not be possible to explain all types and sites of endometriosis with only one theory (Giudice and Kao, 2004, Amer, 2008).

1.2.1 Implantation Theory
The implantation theory, proposed by Sampson (1927b) states that endometrial tissue shed during menstruation passes through the fallopian tubes and can attach to and proliferate at ectopic sites in the peritoneal cavity. Sampson’s implantation theory is generally considered to be the most widely accepted theory of endometriosis pathogenesis (Fauser et al., 2011). There is a range of evidence in support of the theory, such as the phenomenon of retrograde menstruation occurring in most women with patent fallopian tubes (Halme et al., 1984, Liu and Hitchcock, 1986), viability of shed menstrual fragments (Koks et al., 1997, Seli et al., 2003), common anatomic distribution of endometriotic lesions matching with sites accessible by retrograde menstruation (Jenkins et al., 1986) and increased exposure to menstruation leading to increased likelihood of endometriosis (Scott and TeLinde, 1950, D’Hooghe et al., 2006).

1.2.2 Coelomic Metaplasia
Although the implantation theory is well known, the coelomic metaplasia theory for endometriosis is also quite widely held. This theory suggests that endometriotic lesions arise
from transformation or mutations occurring in peritoneal mesothelium or ovarian epithelium (Matsuura et al., 1999, Seli et al., 2003).

The induction theory is an extension of coelomic metaplasia which postulates that menstrual endometrium produces certain factors with the ability to induce metaplasia of the peritoneal mesothelium into endometriosis (Levander and Normann, 1955). Von Recklinghausen (1896) and Russell (1899) similarly proposed the embryonic rests theory suggesting that when cell rests of müllerian origin are stimulated, they may differentiate into endometriosis.

1.2.3 Lymphatic and Vascular Spread

The lymphatic and vascular metastasis theory of endometriosis suggests that the lymphatic and vascular circulation disseminate endometrial cells at menstruation (Halban, 1924, Sampson, 1927a). Shed endometrium enters lymphatic and blood vessels and then can metastatically implant and invade the peritoneum at distant sites to form endometriotic lesions (Javert, 1949). The rare cases of endometriosis found in locations such as the pelvic lymph nodes, pleura, lungs or brain may be explained by lymphatic and vascular metastasis of endometrium (Javert, 1949, Mechsner et al., 2010).
1.2.4 Composite Theories

Endometriosis is not fully explained by any one single theory defined above, therefore, the composite theory of endometriosis proposed by Javert (1951) brings together the theories of implantation, vascular and lymphatic spread and direct extension of endometrial tissue through the myometrium to understand the histogenesis of endometriosis and also to explain different types of endometriosis. Similarly, Nisolle and Donnez (1997) proposed that there is an association between the location and type of endometriotic lesion. The implantation theory explains peritoneal endometriosis while ovarian and deep infiltrating endometriosis can be explained by coelomic metaplasia (Nisolle and Donnez, 1997).

1.3 Eutopic Endometrial Anomalies

There is accumulating evidence suggesting fundamental differences between the eutopic endometrium from women with and those without endometriosis. These biochemical differences include various anomalies in proliferation and apoptosis, immune components, adhesion molecules, proteolytic enzymes and inhibitors, steroid and cytokine production and responsiveness, angiogenesis, lymphangiogenesis, neurogenesis and gene expression and protein production. These endometrial differences may play an important role in the pathogenesis of endometriosis.
1.3.1 Proliferation and Apoptosis

Proliferation is increased in the eutopic endometrium of women with endometriosis compared to endometrium of women without endometriosis (Wingfield et al., 1995, Seo et al., 2004, Burlev et al., 2006). However, some authors found no significant differences in endometrial cell proliferation between women with and those without endometriosis (Jurgensen et al., 1996). There is an increase in the expression of proteolytic markers such as Ki67 and epidermal growth factor, telomerase and telomerase length and transforming growth factor- beta (Bourlev et al., 2006, Sokolov et al., 2005, Park et al., 2009).

Apoptosis is decreased in eutopic endometrium of women with endometriosis compared to endometrium of women without the disease. Apoptosis is a distinct mechanism, which plays a central role in the elimination of cells from tissues without provoking an inflammatory response. Studies have shown the increased expression of anti-apoptotic markers such as B-cell lymphoma/leukemia-2; Bcl-2 and FasL proteins in eutopic endometrium of women with endometriosis (Gebel et al., 1998, Meresman et al., 2000, Dmowski et al., 2001, Szymanowski, 2007, Agic et al., 2009). Increased endometrial proliferation and decreased apoptosis implies that the number of viable shed endometrial cells entering the peritoneal cavity is greater in women who develop endometriosis.
1.3.2 Immune Components

There are alterations in both densities and function of a number of immune cell components in women with endometriosis compared to women without endometriosis (summarised in Table 1.1). It is suggested that immune dysfunction contributes to implantation of endometrial fragments and progression of endometriosis.
Table 1.1 Alterations in endometrial immune cell populations in women with endometriosis.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Density</th>
<th>Function</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dendritic Cells (DCs)</td>
<td>↑ immature DCs in the proliferative phase</td>
<td>Unknown</td>
<td>(Schulke et al., 2009)</td>
</tr>
<tr>
<td></td>
<td>↓ mature DCs in the proliferative phase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T Cells</td>
<td>↓ CD8+cells</td>
<td>↓ Anti-inflammatory action</td>
<td>(Dmowski et al., 1994, Antsiferova et al., 2005, Berbic et al., 2010)</td>
</tr>
<tr>
<td></td>
<td>↑ CD4+, γδ-T cells and Tregs in secretory phase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Macrophages</td>
<td>↑ in proliferative &amp; secretory phases</td>
<td>↓ phagocytosis</td>
<td>(Berbic et al., 2009)</td>
</tr>
<tr>
<td>Natural Killer Cells (NKs)</td>
<td>↓ in the mid-secretory phase</td>
<td>↓ cytotoxicity</td>
<td>(Oosterlynck et al., 1992)</td>
</tr>
</tbody>
</table>

Alterations in these cells may be involved in pain generation and the pathogenesis of endometriosis. The hypothesised role of the immune system is to clear shed and refluxed endometrial tissue and to protect the peritoneal cavity from adhesion and invasion of this tissue, therefore the dysregulated response of the immune system may permit viable endometrial fragments to enter peritoneal cavity and ectopic lesions to develop and survive.

1.3.3 Cell Adhesion Molecules

The expression of cell adhesion molecules is altered in eutopic endometrium of women with endometriosis compared to women without endometriosis. Cell adhesion molecules (CAMs)
are transmembrane receptors that facilitate intercellular binding and cellular interaction with the extracellular matrix (ECM). The establishment of cell-to-cell connections is required by endometrial cells to implant at ectopic sites (Wingfield et al., 1995). In the eutopic endometrium of women with endometriosis, the expression of L1 cell adhesion molecule and intercellular adhesion molecule-1 (ICAM-1) is increased compared to controls (Ota et al., 1996, Viganò et al., 2000, Youssry et al., 2008). This is thought to be associated with the development of endometriosis due to the ability of these cells to promote cell adhesion at ectopic sites.

### 1.3.4 Proteases and Their Inhibitors

In eutopic endometrium of women with endometriosis, the proteases such as matrix metallaproteases (MMPs) and their inhibitors such as tissue inhibitor of metalloproteinase (TIMPs) are involved in invasion of endometrial cells to the peritoneum and extracellular matrix remodelling (Sillem et al., 1998, Salamonsen et al., 2000). MMP-2 (degrades type IV collagen) and MMP-9 (involved in vascular growth and possible role in tumour metastasis) are increased, while the expression of their tissue inhibitors which are TIMP-2 and TIMP-1 respectively is decreased in the eutopic endometrium of women with endometriosis compared to controls (Chung et al., 2002, Collette et al., 2004). Increased proteolytic activity of proteases involved in extracellular matrix remodelling in the eutopic endometrium of women with endometriosis explains the invasive properties of viable endometrial cells at ectopic sites.
1.3.5 Steroid Production and Responsiveness

There is a decrease in steroid production and responsiveness in eutopic endometrium in women with endometriosis. Since endometriosis is defined as a hormone-dependent disorder, the mitogenic action of oestrogen in endometriosis leads to endometriotic lesion development whereas; progesterone antagonises the mitogenic effects of oestrogen. Eutopic endometrium from women with endometriosis has been found to contain high levels of aromatase, which converts androstenedione and testosterone to oestrone (E1) and oestradiol (E2) in various tissues including the ovary and placenta, however normally it is not present in the endometrium. (Nisolle et al., 1994, Noble et al., 1997, Winer et al., 2002). The oestrogen and progesterone receptor status of eutopic endometrium from women with and without endometriosis does not vary (Chamie et al., 2011), hence the ability to produce oestrogen in eutopic endometrium from women with endometriosis may contribute to promotion of their growth in ectopic locations. Progesterone is the main stimulator of steroid enzymes and plays a protective role to balance the actions of oestradiol (E2). The eutopic endometrium of women with endometriosis is partially resistant to the action of progesterone, since there is a decrease in the expression of progesterone receptor-B (PR-B) (Overton, 2007, Chamie et al., 2011). This leads to impaired secretory transformation, decreased apoptosis and an increase in proliferation of endometrial cells in the peritoneal cavity (Overton, 2007)
1.3.6 Angiogenesis

Higher expression of angiogenic factors has been observed in eutopic endometrium of women with endometriosis compared to women without endometriosis (Oosterlynck et al., 1993b, Donnez et al., 1998, Mahnke et al., 2000, Kim et al., 2001, Taylor et al., 2002, Hayrabedyan and Kyurkchiev, 2005, Bourlev et al., 2006, Hur et al., 2006). The primary angiogenic growth factor in the endometrium is considered to be vascular endothelial growth factor-A (VEGF-A), with its receptors VEGFR-1, VEGFR-2, and neuropilin-1 (NRP-1). The expression of potent angiogenic factors VEGF-A, angiopoietin-1 (Ang-1), and Ang-2, and their receptors VEGFR-2 and Tie2 was increased in the eutopic endometrium of women with endometriosis compared to women without endometriosis (Figure 1.2) (Donnez et al., 1998, Khan et al., 2003, Takehara et al., 2004, Bourlev et al., 2006, Hur et al., 2006, Gilabert-Estelles et al., 2007).
Figure 1.2 Expression of VEGF-A in eutopic endometrial tissue during the proliferative phase. VEGF-A immunoreactivity is weaker in the gland of eutopic endometrium of women without (A) than with (B) endometriosis (magnification 400x) (Takehara et al., 2004).

Although angiogenic activity is clearly increased in eutopic endometrium of women with endometriosis (Tan et al., 2002, Khan et al., 2003, Bourlev et al., 2006, Hur et al., 2006), the total uterine blood vessel density is not different between women with and without endometriosis (Hey-Cunningham et al., 2010). Importantly, however, density of newly forming blood vessels is increased in eutopic endometrium of women with endometriosis. Over-expression of these angiogenic factors in the eutopic endometrium allows shed endometrial fragments to attract a blood supply once they reach the peritoneum, ensuring their survival.
1.3.7 Lymphangiogenesis

In addition to the increased angiogenesis, it is becoming more apparent that there are some changes in local lymphangiogenesis (lymphatic growth) in eutopic endometrium of women with endometriosis. However, little is known regarding lymphangiogenesis in the endometrium of women with and without endometriosis.

There is an increase in the expression of a large range of growth factors, which also play a role in angiogenesis such as angiopoietins, fibroblast growth factor and hepatocyte growth factor and a decrease in IGF-1, IGF-2 and VEGF-C expression (Sbracia et al., 1997, Takehara et al., 2004). The evidence of altered lymphangiogenesis in eutopic endometrium of women with endometriosis is supported by the finding of locally increased lymphatic micro-vessel density in these women. The lymphatic vessel density within the basal-layer endometrium in women with endometriosis during the menstrual cycle is increased in comparison to women without the disease (Figure 1.3) (Hey-Cunningham et al., 2010).
Figure 1.3 Comparison of mean density of lymphatic micro-vessels in the basal layer of the endometrium in women with and without endometriosis during the menstrual cycle. The lymphatic micro-vessels density is increased in eutopic endometrium of women with endometriosis compared to women without endometriosis during the proliferative and secretory phases (P = proliferative phase, S = secretory phase, M = menstrual phase; * p<0.05) (Hey-Cunningham et al., 2010).

The increased expression of lymphangiogenic factors and density of lymphatic vessels are associated with the theory of lymphatic spread of endometriosis which is the dissemination of the fragments of endometrial tissue into the lymphatic circulation at menstruation and forming endometriotic lesions (Sampson, 1927a, Javert, 1949).
1.3.8 **Neurogenesis**

In eutopic endometrium of women with endometriosis, there are some alterations in neurogenesis, which is the process of nerve fibre development within tissues, occurring in endometrium and endometriotic lesions.

The expression of neurotrophins, their receptors and other neuronally active molecules is increased in eutopic endometrium of women with endometriosis compared to women without the disease. Specifically, expression of NGF and its receptors TrkA and p75 is increased, particularly in the functional layer of the endometrium (Tokushige et al., 2008). Additionally, some of the important angiogenic and lymphangiogenic factors and receptors are also neuronally active. The expression of these parameters is known to be mostly increased in the eutopic endometrium from women with endometriosis compared to control endometrium. For example, VEGF-A, which is increased in eutopic endometrium from women with endometriosis, has neurotrophic qualities (Sondell et al., 1999, Sondell et al., 2000, Tucker and Mearow, 2008). Other eutopic endometrial disturbances in endometriosis related to neurogenesis include increased densities of neuroendocrine and immune cells that produce neurotrophins.

Furthermore, the eutopic endometrium from women with endometriosis contains small, unmyelinated nerve fibres in the functional layer which are most likely sensory C and
autonomic nerve fibres (Figure 1.4) (Tokushige et al., 2006a, Al-Jefout et al., 2007, Al-Jefout et al., 2009, Bokor et al., 2009, Tokushige et al., 2007). The nerve fibre densities in basal endometrium of women with endometriosis and myometrium are also significantly increased compared to women without the disease (Figure 1.4) (Tokushige et al., 2006a). The presence of nerve fibres and expression of NGF implies that the eutopic endometrium may be involved in pain generation and contribute to various pain symptoms in women with endometriosis (Zhang, 2009, Fraser, 2010a).

A variety of anomalies in proliferation and apoptosis, immune components, adhesion molecules, proteolytic enzymes and inhibitors, steroid and cytokine production and responsiveness, and angiogenesis, lymphangiogenesis and neurogenesis occur in eutopic endometrium from women with endometriosis. Although some of the data in the literature mandate further investigation, these differences appear to contribute the pathogenesis of endometriosis and development of endometriotic lesions.
Figure 1.4 Density of nerve fibres in the functional and basal layers of the endometrium from woman with and without endometriosis. (A) Endometrium from the functional layer of a woman with endometriosis stained for PGP9.5 (magnification ×400) indicating multiple small nerve fibres. (B) Endometrium from the functional layer of a woman without endometriosis stained for PGP9.5 (magnification ×400) showing no nerve fibres identified. (C) Endometrium from the basal layer of a woman with endometriosis stained for PGP9.5 (magnification ×200) showing thick nerve fibre trunks stained red. (D) Endometrium from the basal layer of a woman without endometriosis stained with PGP9.5 (magnification ×200) showing small nerve fibres (Tokushige et al., 2006a).
1.4 Symptoms

The main presenting symptoms of endometriosis are pain and infertility (Fraser, 2010a).

1.4.1 Pelvic Pain

The most common presenting symptom of endometriosis is pelvic pain, which can include dysmenorrhoea (pain during menstruation), dyspareunia (pain on sexual intercourse), dyschezia (bowel motion pain), dysuria (pain on urination), mid-cycle pelvic pain or in extreme cases, pain during all times of the menstrual cycle (Mahmood et al., 1991, Mounsey et al., 2006, Fraser, 2008). The prevalence of pelvic pain amongst women with endometriosis is estimated to be between 40 to 90% (Murphy, 2002). Studies indicate that 90% of women suffer from dysmenorrhoea, 42% from deep dyspareunia and 39% from non-menstrual pelvic pain (Jamieson and Steege, 1996, Eskenazi and Warner, 1997, Zondervan et al., 1999, Cramer and Missmer, 2002, Schindler, 2004, Mounsey et al., 2006, Vercellini et al., 2007).

Endometriosis pain has an enormous social and psychological impact on the lives of women across several domains such as productivity at work, income and relationships with partners and family (Denny, 2004, Dancet et al., 2012).

Numerous researchers have found that there is no significant association between documented symptoms of endometriosis and the extent of disease upon surgical examination (Vercellini, 1996, Hurd, 1998, Fauconnier et al., 2002, Fauconnier and Chapron, 2005). Currently, pain generation mechanisms and pain perception in women with endometriosis are
incompletely understood. However, there is evidence that nociceptive, inflammatory, or neuropathic mechanisms contribute to endometriosis-associated pelvic pain. It is suggested that the inconsistencies of the relationships of the extent of disease upon surgical examination and the presence or severity of pain are likely due to variable roles of different pain mechanisms in endometriosis (Howard, 2009).

1.4.2 Infertility

The second most common presenting complaint of women with endometriosis is infertility. Infertility is defined by the failure to achieve a successful pregnancy after 12 months or more of timed unprotected intercourse or therapeutic donor insemination (Practice Committee of American Society for Reproductive Medicine, 2008). The estimated prevalence of infertility in women diagnosed with endometriosis is between 30 to 50% (Kistner, 1979, Muse, 1988, Giudice et al., 2002, Ozkan et al., 2008).

The biologic mechanisms, which may be responsible for reduced fertility in endometriosis, remain poorly understood. However, a number of factors have been proposed which may explain the link between endometriosis and infertility. These mechanisms include aberrant folliculogenesis (Toya et al., 2000), ovulatory dysfunction (Donnez and Thomas, 1982, Cahill et al., 1997), sperm phagocytosis (Oral et al., 1996, Pillai et al., 1998), decreased fertilisation and altered tubal motility (Ayers, 1982, Wardle et al., 1985), implantation
defects (Arici et al., 1996), immunological factors (Lachapelle et al., 1996) and poor-quality embryonic development (Garrido et al., 2000).

1.4.3 Other Associated Symptoms

Other symptoms described by women with endometriosis include menstrual bleeding and gastrointestinal disturbances. Uterine or menstrual bleeding disturbances reported by women suffering from endometriosis include premenstrual spotting, heavy menstrual bleeding, inter-menstrual bleeding and prolonged menstruation (beyond eight days) (Jansen, 1993, Vercellini et al., 1997, Ballard et al., 2008, Fraser, 2008). Gastrointestinal symptoms include painful abdominal bloating, pain and discomfort on bowel movements, diarrhoea, constipation and in rare cases, cyclical rectal bleeding (Muse, 1988, Fraser, 2008, Seaman, 2008, Luscombe, 2009). These gastrointestinal symptoms may or may not be associated with endometriotic lesions involving the bowel (Muse, 1988).

1.5 Diagnosis

1.5.1 Surgical Diagnosis

The diagnosis of endometriosis via direct surgical visualisation of the pelvic cavity is the current “gold standard” to identify the presence of endometriotic lesions (Kennedy et al., 2005, Banerjee et al., 2006). Surgical diagnosis can be done via laparoscopy (keyhole surgery in which operations in the abdomen are performed through small incisions and
insertion of a camera and other instruments) or laparotomy (an open operation requiring a larger cut in the skin) (Hershlag and Markovitz, 2005, Anna-Sofia et al., 2013). Histopathological confirmation of biopsied tissues to microscopically identify endometrial-like glands and stroma should also be performed as the visualisation of peritoneal lesions alone has limited accuracy (Wood et al., 2002, Winkel, 2003, Fernando et al., 2013, Dunselman et al., 2014). The recognition of the wide range of endometriotic lesion types (peritoneal, ovarian and deep infiltrating) and colours (clear, red, black and white) is important to accurately diagnose endometriosis (Jansen and Russell, 1986, Albee et al., 2008). There is an undeniable delay, which is approximately six to 10 years in diagnosis of endometriosis, which is associated with the diverse range and non-specific nature of symptoms presented by women suffering from endometriosis (Kuohung et al., 2002, Ballard et al., 2006, Sinaii et al., 2008).

**1.5.2 Imaging**

Imaging techniques currently used in diagnosis of endometriosis are ultrasound and magnetic resonance imaging (MRI). Imaging has limited efficacy in diagnosing endometriosis as it lacks adequate resolution to identify adhesions or superficial peritoneal implants (Schenken, 1996, Wellbery, 1999). Endometriomas, bladder lesions, and deep nodules such as those in the rectovaginal septum can be diagnosed by transvaginal ultrasound. MRI is of value in identifying the presence and the extent of deeply infiltrating lesions and it may also help in
detecting bowel and ureteric involvement (Umaria and Olliff, 2001, Kinkel et al., 2006, Mounsey et al., 2006).

1.6 Classification

A number of attempts have been made to categorise endometriosis by “stages” of disease severity (Cullen, 1920, Acosta et al., 1973). In 1979, the American Fertility Society (AFS) released a classification of endometriosis which was amended in 1985 and again revised in 1996 and is now known as the American Society for Reproductive Medicine (ASRM) classification (ASRM, 1997). This classification system of endometriosis is used to determine the disease stage (ranging from I, indicating minimal disease, to IV, indicating severe disease) on the basis of the type, location, appearance, and depth of invasion of lesions and the extent of disease and adhesions (ASRM, 1997, Giudice, 2010).

Although the ASRM endometriosis classification is widely used, it does not predict fertility or pregnancy rates following treatment (Palmisano et al., 1993, Guzick et al., 1997) recurrence of dysmenorrhoea symptoms or recurrence of disease (Vercellini, 1996). Recently, the endometriosis fertility index (EFI) was proposed by Adamson and Pasta (2010) to predict non-assisted-pregnancy rates after surgical procedures in women with a surgical diagnosis of endometriosis. It combines ASRM scores with other information such as a “least function score” (determines the level of dysfunction of the fallopian tube, fimbria and ovary), age, years of infertility and prior pregnancies, to provide a numeric score from 0 (lowest
probability of fertility) to 10 (highest probability of fertility) (Adamson and Pasta, 2010). Whether the different appearance/age/stage of endometriosis is related to the development of endometriosis is still unclear, this will be better understood at the end of this study.

1.7 Treatment

1.7.1 Surgical

Endometriosis can be treated pharmacologically or surgically. Common surgical treatments include laparoscopy or laparotomy during which endometriotic lesions, cysts, nodules and adhesions may be excised (cut out) or diathermied (burnt out). In some severe cases, surgical treatment may also involve colorectal excision or hysterectomy (removal of the uterus). With hysterectomy, one or both of the ovaries and fallopian tubes may also be removed. The generally preferred approach to excise lesions and restore anatomy is laparoscopic surgery as it is less invasive (Catalano, 1996). However, a highly skilled and experienced endoscopic surgeon is required for the laparoscopic excision of endometriotic lesions.

Surgical therapeutic procedures for endometriosis are effective in significantly reducing pain in about 65% of women with severe disease (Sutton et al., 1997, Banerjee et al., 2006) and improving fertility outcomes for 18%, particularly in women with minimal to mild endometriosis (Marcoux et al., 1997, Adamson and Pasta, 2010, Pahlajani and Falcone, 2010). However, there is recurrence of pain symptoms and requirement for further surgery in 20 to 50% of women following conservative surgery (the removal of as many endometrial
implants and cysts as possible without causing surgical scarring and subsequent adhesions) (Vercellini et al., 2006, Guo, 2009, Vercellini et al., 2009).

### 1.7.2 Medical

Medical management of endometriosis usually involves the use of analgesics for pain relief and/or hormonal suppression of endometriotic lesion activity.

#### Analgesics

Analgesic therapies are used to manage various pain symptoms in endometriosis. Nonsteroidal anti-inflammatory drugs (NSAIDs) in conjunction with intermittent stronger analgesic alternatives such as ibuprofen, codeine and tramadol are first choice for management of pain in endometriosis. In extreme pain, stronger analgesics such as oxycodone, intramuscular injections of pethidine or morphine may be recommended for acute use (Fraser, 2008, Ferrero et al., 2010).

#### Hormone Therapy

Various hormonal treatments have been demonstrated to reduce the severity of endometriosis pain symptoms safely and efficaciously by suppressing the growth of endometrial cells and endometriotic lesions (Table 1.2). Hormone therapies may be used as a treatment in mild endometriosis or as an added or combined therapy prior to, or after surgery, especially in moderate to severe forms of endometriosis. Use of the oral contraceptive pill is suitable as an
initial approach for medical treatment. Progestogens offer the most highly effective suppression with minimal side effects (Fraser, 2008, Fraser, 2010b). Highly effective hormonal suppression is also provided by gonadotrophin-releasing hormone (GnRH) analogues and danazol (Fraser, 2008, Vercellini et al., 2008, Brown et al., 2010). Additionally, selective oestrogen receptor modulators (Stratton et al., 2008), progesterone receptor modulators (Chwalisz et al., 2005) and aromatase inhibitors (Takayama et al., 1998) are alternative approaches for which good therapeutic outcomes have been reported.
### Table 1.2 Hormonal treatments for endometriosis.

<table>
<thead>
<tr>
<th>Medication</th>
<th>Indications</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Combined OCPs</td>
<td>Pain relief</td>
<td>Common usage in primary care</td>
</tr>
<tr>
<td>Danazol (Danocrine)</td>
<td>Pain relief</td>
<td>Significant androgenic side effects</td>
</tr>
<tr>
<td>Depot MDPA (Depo-Provera)</td>
<td>Pain relief</td>
<td>Common usage in primary care</td>
</tr>
<tr>
<td>Gestrinone</td>
<td>Pain relief</td>
<td>Hot flashes</td>
</tr>
<tr>
<td>Gonadotropin – releasing hormone analogues (e.g., goserelin [Zoladex], leuprolide [Lupron], triptorelin [Trelstar Depot])</td>
<td>Pain relief</td>
<td>Expensive; significant side effects (hypoestrogenic symptoms)</td>
</tr>
<tr>
<td>Levonorgestrel intrauterine system (Mirena)</td>
<td>Pain relief after surgery</td>
<td>Can be placed easily in primary care setting</td>
</tr>
<tr>
<td>MDPA (Provera)</td>
<td>Pain relief</td>
<td>Common usage in primary care</td>
</tr>
<tr>
<td>Nafarelin (synarel)</td>
<td>Pain relief</td>
<td>Expensive; significant side effects</td>
</tr>
</tbody>
</table>

MDPA = medroxyprogesterone acetate; OCPs = oral contraceptive pills. Adapted from (Mounsey et al., 2006).
Chapter 2
Peritoneal Endometriotic Lesions

2.1 Introduction

Endometriotic lesions can be primarily defined as peritoneal, ovarian and deep infiltrating endometriosis (DIE) (Nisolle and Donnez, 1997, Donnez et al., 2003).

2.1.1 Peritoneal Endometriosis

Peritoneal endometriosis is simply superficial lesions on peritoneal surfaces. The peritoneum is a membrane lining the interior of the abdominal cavity and surrounding its organs. These lesions are most commonly found on the pelvic side walls, the uterosacral ligaments (Giudice, 2010) and will be covered in detail in the remainder of this chapter.

2.1.2 Ovarian Endometriosis

Ovarian endometriotic lesions, as the name suggests, is endometriosis on the ovaries. There is superficial endometriosis on the ovary and endometrioma or ‘chocolate’ cyst. Superficial ovarian endometriosis occurs when the lesion penetration is less than four mm on the surface of the ovaries. However, the ‘chocolate cyst’ is an accumulation of a thick brown fluid caused by old cyclical “menstrual” bleeding (Figure 2.1) and it can grow to more than 10 cm in diameter (Redwine, 1987, Nisolle and Donnez, 1997, Fraser, 2008). Chocolate cysts may
be a progression of superficial peritoneal endometriosis with localised scarring, invagination of the ovarian cortex and formation of a pseudo-cyst (Brosens et al., 1993, Brosens, 1997).

Figure 2.1 Laparoscopic appearance of a chocolate cyst (Fraser, 2008).

2.1.3 Deep-infiltrating Endometriosis

Another form of endometriotic lesion phenotype is DIE, defined as endometriotic lesions that penetrate greater than 5 mm below the peritoneal surface (Figure 2.2) (Koninckx and Martin, 1992, Chamie et al., 2011). Between 5% and 12% of women with endometriosis will have
DIE. The most frequent locations of DIE are the uterosacral ligaments, pouch of Douglas (cul-de-sac), rectum and bladder (Chapron et al., 2002, Koh et al., 2012). Smooth muscle cells and endometrial glands aggregation leads DIE lesions to form nodules that are fibrous and “adenomatous” (Donnez et al., 1995). These nodules can invade the muscularis wall of the rectum or other pelvic organs (Koninckx and Martin, 1992). DIE can be severe and destroy the posterior cul-de-sac with extensive adhesions (Sampson, 1922).

Figure 2.2 Laparoscopic appearance of deep infiltrating endometriosis (Chamie et al., 2011).
2.2 Lesion Establishment

According to the retrograde menstruation theory, fragments of endometrium refluxed through the fallopian tubes into the peritoneal cavity then attach to and grow on peritoneal surfaces (Sampson, 1927b). Shed endometrial cells were first found to be able to create endometriotic lesions in 1950 with inversion of the uterus and diversion of menstrual flow into the peritoneal cavity in monkeys, where 50% of the animals developed endometriosis (Scott and TeLinde, 1950). Similar outcomes are described in baboons with development of endometriosis after injection of menstrual endometrium into the retroperitoneal space (D’Hooghe et al., 1995, D’Hooghe et al., 2001a, D’Hooghe et al., 2009) and in women with formation of endometriotic lesions after injection of menstrual effluent into abdominal fat (Ridley and Edwards, 1959). These findings demonstrated that viable endometrial cells in menstrual effluent are able to develop into endometriotic lesions.

In the establishment of peritoneal endometriotic lesions, five critical steps have been postulated: attachment of endometrial cells to the peritoneal surface, invasion of these cells into the mesothelium, endometrial cellular proliferation, recruitment of inflammatory cells and angiogenesis (Oosterlynck et al., 1993b, Sharpe-Timms, 2001, Witz et al., 2002, Taylor et al., 2002, Ulukus et al., 2006).

Cell attachment is thought to be mediated by integrins at the peritoneal surface and inflammatory cytokines such as IL-8, which foster endometrial adhesion to mesothelial cells.
Findings suggest that increased metalloproteinase activity in and around endometriotic lesions may facilitate endometrial cell invasion of the extracellular matrix and growth of lesions (Bruner et al., 1997, Brooks et al., 1997).

After the endometrial tissue adheres to the peritoneum and invades the mesothelium, proliferation of endometrial cells and establishment of a blood supply are necessary. Persistence of lesions is supported with the local macrophage, lymphocyte and mesothelial cell production of inflammatory cytokines and growth factors (such as interleukins [IL-1, IL-6, IL-8], TNF-α and RANTES) with proliferative and angiogenic enhancing properties (Giudice, 1994, Cohen et al., 1996, Arici, 2002). Eventually, establishment of new blood vessels occur involving the secretion of number of angiogenic factors including VEGF, angiogenin, fibroblastic growth factor (FGF), hepatocyte growth factor (HGF), transforming growth factors (TGF) to support survival and growth of lesions (Donnez et al., 1996, McLaren, 2000, Olive, 2005). Peritoneal fluid immune cells, mainly macrophages, secrete many of these angiogenic growth factors (McLaren et al., 1996a, Gazvani and Templeton, 2002, Lin et al., 2006).

### 2.2.1 Lesion Appearances

Peritoneal endometriotic lesions appear in a range of macroscopic appearances, which reflect different stages in development (Figure 2.3) (Ueki, 1991, Overton, 2007). Serial laparoscopies and correlation of lesion appearance and women’s age have shown the
development of endometriosis from clear to pigmented lesions of different types (Jansen and Russell, 1986, Redwine, 1987). Clear vesicles and red lesions represent the early stages. Red lesions are rich with blood vessels which is a main reason for them to be considered the most active peritoneal lesion type (Bloom, 1978). The lesions undergo cycles of partial shedding and regrowth, inducing an inflammatory reaction and scarification process that encloses them. The enclosed lesion becomes “black” because of the build-up haemosiderin from repeated “menstrual” breakdown. In some cases, the inflammatory process and subsequent fibrosis totally devascularise the lesion, and white plaques of old collagen are all that remain, giving a white appearance (Dan, 1990). White opacifications are considered latent stages of endometriotic lesions; they probably are inactive and could be quiescent for a long time (Jansen and Russel, 1987, Nisolle and Donnez, 1997).
Figure 2.3 Laparoscopic appearances of peritoneal endometriotic lesions; (A) red lesion, (B) black lesions and (C) white lesion (Overton, 2007).
2.2.2 Peritoneal Fluid and its Roles in Lesion Development

Studies have demonstrated functional changes in several immunological and related components of the peritoneal fluid of women with endometriosis (Oral et al., 1996, Ho et al., 1997b, Senturk and Arici, 1999, Gazvani and Templeton, 2002). Women with endometriosis also have a greater peritoneal fluid volume than fertile controls, patients with tubal disease, or those with unexplained infertility (Syrop and Halme, 1987, Oral et al., 1996). Peritoneal fluid contains cellular and soluble constituents, including macrophages, lymphocytes, natural killer cells, prostaglandins, cytokines and growth factors. Studies also demonstrated that the number of peritoneal fluid immune cells significantly increased in women with endometriosis, implying that these cells may be chemotactically attracted to the peritoneal cavity in response to the disease, or their increased presence could represent the primary abnormality (Harada et al., 2001, Gazvani and Templeton, 2002, Bedaiwy and Falcone, 2003).

Peritoneal fluid macrophages, in addition to being increased in number, are more activated in endometriosis (Halme J et al., 1983, Oral et al., 1996, Ho et al., 1997b, Gazvani and Templeton, 2002). Their released products such as cytokines, prostaglandins (PGs), complement components and hydrolytic enzymes regulate and alter the function of neighbouring immune and tissue cells (Khan et al., 2004a). Peritoneal fluid macrophages play important roles in modulating the growth and inflammatory behaviour of endometriotic
lesions by stimulating implantation and proliferation of misplaced, and possibly altered, endometrial cells (Dmowski et al., 1994).

Approximately 30 to 50% of peritoneal fluid cells are lymphocytes (Oosterlynck et al., 1992) and their total number is higher in women with endometriosis (Badawy et al., 1984, Gallinelli et al., 2004). Increased numbers of CD4+ and CD8+ T cells have been reported in the peritoneal fluid of women with endometriosis with the proportion of CD4+ T cells lower than CD8+ T cells 40% and 69% respectively (Hill et al., 1988). In addition to increased numbers, CD4:CD8 (T-helper to T-suppressor) ratio was noted to be increased in peritoneal fluid in endometriosis (Steele et al., 1984, Badawy et al., 1987, Witz et al., 1994, Dmowski et al., 1994, Szyllo et al., 2003, Gallinelli et al., 2004). There is also an alteration in anti-inflammatory cytokine production by T cells in the peritoneal fluid of women with endometriosis. There is a decrease in Th1 cytokine production; IL-2, IL-12, interferon (IFN)-γ and TNF-α, whereas Th2 (helper T cells type 2) cytokine production; IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13 were increased in the peritoneal fluid (Giudice and Kao, 2004) which potentially promote endometriotic lesion growth, and pathogenesis through the activating cell mediated immunity in the peritoneal cavity. Decreased cytotoxicity and altered cytokine production by T cells may promote survival of shed fragments and endometriotic lesion development (Halis and Arici, 2004). Besides the alterations of T cell functions, many recent findings have shown alterations in B-cell function in endometriosis patients as evidenced by
abnormal antigen-antibody reaction and increased B-cell function (Bedaiwy and Falcone, 2003).

The number of peritoneal fluid NK cells is unchanged in women with endometriosis (Oosterlynck et al., 1991). However, there is a significant decrease in cytotoxicity of these cells in peritoneal fluid in endometriosis (Oosterlynck et al., 1992, Wilson et al., 1994, Ho et al., 1995, Bedaiwy and Falcone, 2003). This is thought to facilitate survival of refluxed endometrial cells at ectopic sites (Koninckx et al., 1998, Harada et al., 2001). Locally decreased NK-mediated cytotoxicity in peritoneal fluid might be implicated in pathogenesis of endometriosis by failure to clear refluxed endometrial cells and allowing their proliferation and subsequent formation as lesions. This is thought to be associated with the concentration of soluble ICAM-1 (sICAM-1), which is present in the peritoneal fluid and thought to originate from ectopic endometrial stromal cells and peritoneal mesothelial cells (Somigliana et al., 1996). Soluble ICAM-1 is able to bind leukocytes, inhibiting leukocyte – epithelial cell adhesion and scavenger function (Becker et al., 1993). It has been shown to be associated with evasion of the immune system, suggesting that this may also allow endometriotic lesion progression (Becker et al., 1991). Thus, shedding of ICAM-1 may be a mechanism by which ectopic endometrial cells can escape immune surveillance and may account for the reduced NK cell activity observed in women with endometriosis.
There is evidence that the expression of prostaglandin concentrations is increased in the peritoneal fluid of women with endometriosis (Oral et al., 1996, Oral and Arici, 1996). Additionally, it has been shown that significantly more prostaglandins (especially prostaglandin E2 and prostaglandin F2a) are released by peritoneal macrophages from women with endometriosis in comparison to that of macrophages from women without the disease (Karck et al., 1996). Prostaglandins are also involved in the regulation of the production and function of cytokines, and they have been implicated in endometrial cell proliferation (Orlicky et al., 1986, Graham et al., 1994, Hickey et al., 2014). Additionally, prostaglandins and specifically prostaglandin E2 stimulate activity of aromatase, which promotes the growth of endometriotic lesions via local oestrogen production (Giudice and Kao, 2004, Velasco et al., 2006). Therefore the elevated concentrations of prostaglandins in peritoneal fluid may play an important role in the development and persistence of peritoneal endometriotic lesions.

Cytokines and growth factors are mainly produced by macrophages in the peritoneal cavity in response to a variety of inflammatory stimuli but lymphocytes, endometriotic lesions and mesothelial cells of the peritoneum also release a variety of cytokines (Harada et al., 2001). The concentration of cytokines and growth factors such as TNF-α, interleukins such as IL-6 and IL-1 and RANTES (Regulated on Activation, Normal T-Cell Expressed and Secreted) is elevated in peritoneal fluid of women with endometriosis (Khorram et al., 1993, Koyama et al., 1993, Ortiz et al., 1996, Harada et al., 1997, Ho et al., 1997b). Other cytokines have been
identified including IL-4 (Hsu et al., 1997), IL-5 (Koyama et al., 1993), IL-8 (Iwabe et al., 1998), (Bedaiwy et al., 2002), IL-10 (Ho et al., 1997a), IL-12 (Mazzeo et al., 1998, Bedaiwy et al., 2002), IL-13 (McLaren et al., 1997), interferon-γ 10, monocyte chemotactic protein-1 (MCP-1) (Arici et al., 1997), macrophage colony stimulating factor (MCSF) (Fukaya et al., 1994) and transforming growth factor (TGF)-α (Oosterlynck et al., 1994). All these cytokines found increased in the peritoneal fluid of women with endometriosis compared to without are believed to facilitate implantation and progression of displaced endometrial cell in the peritoneum.

As a conclusion, peritoneal fluid may have an important role in peritoneal endometriotic lesion development and persistence through its components, which can promote inflammation, adhesion and proliferation of endometrial cells in the peritoneal cavity.

### 2.2.3 The Role of the Peritoneum in Lesion Establishment

There is increasing evidence that the pelvic peritoneal mesothelium may make important contributions to the development and progression of peritoneal endometriotic lesions (Witz et al., 1999, Giudice and Kao, 2004, Stegeman et al., 2013, Jin, 2014). It has been found that endometriotic lesions are associated with peritoneal cell-cell disruption, which allow for sites of adhesion and invasion for endometrial cells (Witz et al., 2002, Nair et al., 2008, DeSancho, 2014).
Mesothelial cells lining the pelvis could be damaged after exposure to menstrual effluent and these results in an altered morphology leading to gaps between the cells and loss of tight junctions (Dunselman et al., 2001). Studies found that attachment and invasion of menstrual cells were described only to damaged areas in peritoneum (Witz et al., 2002). Additionally, presence of mesothelial layer prevents endometrial cells going into the invasion chambers (Nair et al., 2008). This was supported with another study demonstrated that human peritoneal mesothelial cells from women without endometriosis resist invasion, whereas those from women with endometriosis cannot resist invasion. Therefore, loss of mesothelial monolayer or adhesion between the mesothelial cell monolayer in peritoneum may facilitate invasion of attached ectopic endometrial cells, implying that the peritoneal mesothelium may play an essential role in development of endometriotic lesions.

2.3 Structure and Function of Peritoneal Endometriotic Lesions

In this section, structure and function of peritoneal endometriotic lesions will be discussed in terms of immune cell populations, blood vessels, lymphatic vessels and nerve fibres.

2.3.1 Immune Cell Populations

It has been repeatedly shown that there are changes in immune cell populations in peritoneal endometriotic lesions compared to eutopic endometrium, including T and B cells, DC, NK cells and macrophages.
Dendritic cells (DCs) are a heterogeneous population of antigen presenting cells involved in
the initiation and modulation of immune responses (Banchereau and Steinman, 1998). While
the density of CD1a+ immature DCs was higher in endometriotic lesions than the eutopic
endometrium, significantly lower numbers (very few or none) of CD83+ mature DCs were
detected in lesions (Schulke et al., 2009). Additionally, CD1a DCs were most abundant at the
lesion side and as the distance from lesion increased, their density in the peritoneum
progressively decreased (Schulke et al., 2009). CD1a and CD83 DC populations were not
detected at all in normal peritoneum of women without endometriosis (Schulke et al., 2009).
Thus, DCs might be recruited to lesions in order to initiate an immune response against the
invasive abnormal tissue.

T cells are a family of lymphocytes which are essential for the establishment and
maintenance of immune responses, primarily through the secretion of cytokines and directly
binding and destructing foreign antigens (Piccinni, 2005). CD8+ T cells are cytotoxic killer
cells which directly destroy abnormal cells, CD4+ T cells are helper cells which transmit
signals from antigen presenting cells to stimulate the immune system (Andersen et al., 2006,
Waisman and Becher, 2014) and FoxP3+ T cells are regulatory T cells which control and
suppress a range of immune responses (Fehervari and Sakaguchi, 2004). The density of
activated cytotoxic T cells and helper T cells is higher in endometriotic lesions than in the
eutopic endometrium, regardless of the phase of the menstrual cycle (Witz et al., 1994, Oral
et al., 1996, Jones et al., 1998, Paul Dmowski and Braun, 2004, Seeley et al., 2005, Ganewatta et al., 2010). Additionally, FoxP3+ regulatory T cells are detected in the stroma of some peritoneal endometriotic lesions and a great variation in the density of these cells was observed between lesions (Figure 2.4) (Berbic et al., 2010).

B cells are also lymphocytes and a component of the adaptive immune response, which act to synthesise antibodies against antigens following activation (Batista 2008). The population of activated B cells in the endometriotic lesions was found to be significantly higher than in the eutopic endometrium, although no differences were detected in inactivated B-cells or other B-cell subsets (Antsiferova et al. 2005; Dmowski et al. 1994). These altered immune conditions might suggest that B cells are involved in the development of endometriosis and endometriotic lesions; however, it is important to consider that T cells are regulators of B cell maturation, and these changes might be a consequence of increased T cell populations and activity (Fagarasan and Honjo 2000).
Figure 2.4 The variation in the density of FoxP3+ regulatory T cells within peritoneal endometriotic lesions (Berbic et al., 2010).

Density of macrophages was found to be increased and show great variations in peritoneal endometriotic lesions (and interestingly also in unaffected peritoneum) from women with endometriosis compared to normal peritoneum from women without endometriosis (Table 2.1) (Oosterlynck et al., 1993a, Khan et al., 2004a, Khan et al., 2004b, Berbic et al., 2009, Tran et al., 2009). The accumulation of activated macrophages and their products may have
important roles in the development of endometriotic tissues. IL-6 and TNF-a both elevated in peritoneal endometriotic lesions in comparison with eutopic endometrium (Sokolov et al., 2005, Birt et al., 2013) and released by activated peritoneal macrophages, can promote aromatase activity in endometriotic stromal cells and increase the production of oestrogen, a key hormone for the growth of lesions (Velasco et al., 2006). Macrophages are also capable of producing a number of angiogenic factors; including fibroblast growth factor, angiogenin and vascular endothelial growth factor (VEGF) (Sunderkötter et al., 1994, McLaren et al., 1996b); with the capacity to stimulate formation of new blood vessels in the implanting endometriotic tissue (Lin et al., 2006). The expression of FGF and VEGF are up-regulated in peritoneal endometriotic lesions in comparison with eutopic endometrium (McLaren, 2000, Hayrabedyan and Kyurkchiev, 2005). Macrophages show significantly less phagocytic activity in endometriotic lesions than macrophages in normal peritoneum (Dmowski et al., 1998). Therefore, these cells may play roles in the growth and development of endometriotic lesions.
Table 2.1 The variations between the densities of macrophages with the mean number (per mm²) in peritoneal endometriotic lesions. Group I, normal peritoneum from women without endometriosis; Group II peritoneal lesions from women with endometriosis; Group III unaffected peritoneum from women with endometriosis. Adapted from (Tran et al., 2009).

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Minimum</th>
<th>Maximum</th>
<th>Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>18</td>
<td>0</td>
<td>11</td>
<td>1.4</td>
<td>3.6</td>
</tr>
<tr>
<td>Group II</td>
<td>24</td>
<td>3.7</td>
<td>275.7</td>
<td>67.7</td>
<td>67.9</td>
</tr>
<tr>
<td>Group III</td>
<td>14</td>
<td>3.7</td>
<td>99.3</td>
<td>32.8</td>
<td>31.4</td>
</tr>
</tbody>
</table>

P value
- P<0.001 between Groups I and II
- P<0.001 between Groups I and III
- P=0.12 between Groups II and III

Natural killer (NK) cells are the effector cells of the innate immune system, which usually recognise and destroy abnormal cells via killer inhibitory and killer activating receptors that inhibit or direct cytotoxic activity, respectively (Maeda et al., 2002, Vivier et al., 2008). Although a statistically significant increase in NK cell numbers were found in lesions compared to normal peritoneum by a recent study (Ganewatta et al., 2010), not much is known about NK cells in peritoneal endometriotic lesions.

Immune cell activation results in release of a cascade of cytokines from various cells (Iwabe et al., 2002). 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 2.2 summarises the expression and roles of key cytokines in endometriotic lesions. Pro-inflammatory cytokines from endometriotic lesions and associated immune cells contribute to the enhanced inflammatory reaction associated with endometriosis that promotes the survival of these lesions instead of leading to their demise (Senturk and Arici, 1999, Lebovic et al., 2001).
Table 2.2 Important cytokines and their possible roles and levels in endometriotic lesions compared to eutopic endometrium.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Produced by</th>
<th>Function/s</th>
<th>Levels</th>
<th>Possible Roles</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interleukin-1 (IL-1)</td>
<td>Macrophages</td>
<td>Pro-inflammatory; induces angiogenesis and expression of adhesion molecule</td>
<td>↑</td>
<td>Promotes blood supply to lesions and endometrial cell-peritoneal adhesion</td>
<td>(Bedaiwy et al., 2002, Voronov et al., 2003, Hudelist et al., 2005)</td>
</tr>
<tr>
<td>Interleukin-1β (IL-1β)</td>
<td>Monocytes</td>
<td>Stimulate interleukin-6 secretion</td>
<td>↑</td>
<td>Facilitates neo-vascularisation of lesions</td>
<td>(Wu and Ho, 2003)</td>
</tr>
<tr>
<td>Interleukin-4 (IL-4)</td>
<td>T helper 2, natural killer and mast cells</td>
<td>Regulates Th2 cell proliferation by induces Growth factor independent-1</td>
<td>↑</td>
<td>Stimulates proliferation of endometriotic stromal cells, facilitates lesion establishment</td>
<td>(Antsiferova et al., 2005, Zhu et al., 2002)</td>
</tr>
<tr>
<td>Interleukin-6 (IL-6)</td>
<td>T cells, macrophages and endometriotic lesions themselves</td>
<td>↑ production of Endo-1 protein, mediate VEGF expression, possibly facilitate in neo-vascularisation</td>
<td>↑</td>
<td>↑ lesion establishment through involvement in impaired immunity</td>
<td>(Wu and Ho, 2003, Ulukus et al., 2006)</td>
</tr>
<tr>
<td>Interleukin-8 (IL-8)</td>
<td>Macrophages and endometriotic lesions themselves</td>
<td>Potent growth and differentiation factor for activated B cells</td>
<td>↑</td>
<td>Endometrial cell-peritoneal adhesion, improves blood supply and proliferation within lesions</td>
<td>(Burkman, 1991, Rafet Gazvani et al., 1998, Harada et al., 2001)</td>
</tr>
</tbody>
</table>
In summary, the increases in immune cell densities in the stroma of peritoneal endometriotic lesions may suggest targeting of the immune response at the core of lesions in an attempt to inhibit further development. Changes in immune cell populations and their released products in endometriosis appear to increase angiogenesis, growth of lesions, inflammatory cell recruitment, adhesions and oestrogen production. These changes may result in a failure of the immune system to clear peritoneal endometriotic lesions and instead enhance their development and persistence.

### 2.3.2 Blood Vessels

A number of studies have shown that the density of blood vessels and other angiogenic parameters are increased within peritoneal endometriotic lesions compared to normal
peritoneum, and that they show variations between different types of lesions (Figure 2.5) (Donnez et al., 1998, Tan et al., 2002, Bourlev et al., 2006, Reichelt et al., 2012). Endometriotic lesions have a greater micro-blood vessel density compared to normal endometrium (Young et al., 2013). Furthermore, peritoneal endometriotic lesions have higher blood vessel density compared to ovarian endometriosis, whereas DIE lesions have the highest blood vessel density of any lesion type (Tan et al., 2002, Machado et al., 2008). Interestingly, the amount of vascularisation in and around endometriotic lesions correlates with the mitotic activity within the lesion (Nisolle et al., 1993).

**Figure 2.5** Red flare lesion with extensive vascularisation surrounding peritoneal endometriosis (Overton, 2007).
Several angiogenic growth factors; such as VEGF-A, endoglin, epidermal growth factor (EGF), transforming growth factor-α (TGF-α), fibroblast growth factor (FGF), platelet endothelial cell adhesion molecule (PECAM) and integrin-αvβ3 and their receptors VEGFR-1 and VEGFR-2; are increased in expression in endometriotic lesions compared to normal endometrium (Healy et al., 1998, Kim et al., 2001, Van Langendonckt et al., 2004, Print et al., 2004, Bourlev et al., 2006, Pupo-Nogueira et al., 2007).

Red lesions have higher expression of VEGF-A, its VEGFR-2 receptor (Nisolle et al., 1993, Bourlev et al., 2006) and other angiogenic cytokine levels compared to older black or white scarred lesions (Donnez et al., 1998, Tan et al., 2002); and express lower levels of angiogenesis inhibitors such as thrombospondin protein 1 (TSP-1) (Tan et al., 2002). This suggests more active angiogenic processes in red lesions (Nisolle et al., 1993). Red, black and white endometriotic lesions have different mitotic activity, which appears to correlate with the content of VEGF in the peritoneal fluid (Khan et al., 2004b).

2.3.3 Lymphatic Vessels

A few studies have recently demonstrated high lymphatic vessel density and expression of lymphatic growth factors in peritoneal endometriotic lesions. Lymphatic vessel density is increased in the stroma of peritoneal endometriotic lesions compared to the surrounding sub-peritoneal tissue but not statistically significantly different to normal peritoneum (Hey-
Cunningham et al., 2011). On the other hand, density of lymphatic vessels in DIE is significantly higher than corresponding healthy tissues (Keichel et al., 2011). Lymphatic micro-vessels play an important role in immune surveillance and increased stromal lymphatic vessel density parallels immune cell distribution, which is also increased in the stroma of endometriotic lesions (as reviewed above in section 2.3.1). The increases in immune cell and lymphatic vessel densities in the stroma of peritoneal endometriotic lesions may suggest targeting of the immune response at the core of lesions in an attempt to inhibit further development.

Expression of a range of lymphangiogenic growth factors and their receptors are also increased in endometriotic lesions compared to normal peritoneum and eutopic endometrium from women with and without endometriosis. Expression of VEGF-C and VEGF-D are elevated in peritoneal endometriotic lesions (Figure 2.6) (Takehara et al., 2004, Reichelt et al., 2012) and VEGF-A and VEGF-C in ovarian endometriosis (Takehara et al., 2004). In addition, other growth factors known to promote lymphangiogenesis, such as IGF-1 and IGF-2, are increased in endometriotic lesions (Khan et al., 2003, Milingos et al., 2006, Milingos et al., 2011).
Figure 2.6 The variations of lymphatic vessel density with standard error bars in peritoneal endometriotic lesion stroma (A) and surrounding (B) throughout the menstrual cycle. Data are represented as mean ± SEM (P= proliferative phase, S= secretory phase, M= menstrual phase) (Hey-Cunningham et al., 2011).

In summary, studies demonstrate that lymphatic vessels are present in peritoneal endometriotic lesions and that this may be induced by lymphangiogenic growth factors expressed by lesions. Although little is currently known about the roles of lymphatic vessels or lymphangiogenesis in the establishment and progression of endometriotic lesions, they most probably support local maintenance as well as dissemination of endometriotic cells. This would occur in close interaction with immunological factors in the peritoneal cavity.
2.3.4 Nerve Fibres

Several researches have demonstrated the presence of nerve fibres in endometriotic lesions (Anaf et al., 2000, Tulandi et al., 2001, Tamburro et al., 2003, Berkley et al., 2004, Tokushige et al., 2006b, Mechsner et al., 2007). Significantly more nerve fibres are present in peritoneal endometriotic lesions compared to normal peritoneum (Tokushige et al., 2006b, Mechsner et al., 2007). Furthermore, the density shows great variation with the mean densities of 16.3 ± 10.0 within peritoneal endometriotic lesions (Figure 2.7) (Tokushige et al., 2006b). Nerve fibre density is also increased in ovarian endometrioma compared to ovarian cortex from women with ovarian endometriosis and those without endometriosis (Tokushige et al., 2010, Zhang et al., 2010). Furthermore, it also has been shown that there is a great variation in density of nerve fibres between peritoneal endometriotic lesions. Additionally, DIE lesions have substantially greater density of nerve fibres than peritoneal lesions (Wang et al., 2009a). A mixture of sensory A-δ, sensory C, adrenergic and cholinergic fibres is found in peritoneal lesions (Tokushige et al., 2006a). These nerve fibres are pain conducting and the presence of functional nerve fibres in peritoneal lesions suggests a critical role in pain processing and perception, although exact pathways remain unclear.

Researchers have also shown that a wide range of neurotrophins (NGF and NT-3), their receptors (TrkA and p75) and other neuronally active molecules are present in endometriotic glands and stroma of peritoneal lesions, as well as ovarian and DIE lesions (Anaf et al., 2002, Tokushige et al., 2006a, Mechsner et al., 2007, Odagiri et al., 2009, Wang et al., 2009a,
Wang et al., 2009b, Tokushige et al., 2010). In addition, there is evidence that recruited immune cell sub-populations in peritoneal lesions contribute to increased local neurotrophic factors (Torcia et al., 1996, Kerschensteiner et al., 1999, Noga et al., 2007). In particular, more nerve fibres were found in lesions with increased numbers of activated macrophages (which are likely secreting neuroattractant cytokines and providing a suitable environment for nerve in-growth in lesions; Figure 2.7) (Anaf et al., 2006, Kalu et al., 2007, Berbic et al., 2009, Hassa et al., 2009, Tran et al., 2009).

Figure 2.7 The correlation between macrophage and nerve fibre densities in peritoneal endometriotic lesions, showing variation in both densities (Tran et al., 2009).
In summary, immune cells, blood vessels, lymphatic vessels and nerve fibres play a range of important roles in the structure and function of peritoneal endometriotic lesions. Additionally, it is apparent that there is variation in immune cell density, lymphatic vessels, blood vessels and nerve fibres within peritoneal endometriotic lesions. The reasons for and the implications of this are currently largely unclear. However, the variation is likely to be associated with the age or stage of lesion development (red, black, white peritoneal endometriotic lesions), as is the case for angiogenic parameters. Improved understanding of how lesion appearance (which correlates with lesion age or stage of development) exactly relates to the microscopic structure and function of lesions is required.
Chapter 3
Aims and Hypotheses

3.1 Introduction

Previous studies have shown that densities of immune cell populations are increased within peritoneal endometriotic lesions in comparison to the surrounding peritoneum and normal peritoneum. Additionally, the immune cells have shown great variations in density between lesions. While these observations are considered to be important for endometriotic lesion development, exactly how these cells relate to lesion appearance, age or stage of development is currently unclear.

3.2 Primary Hypothesis

It is hypothesised that the variations in immune cell environment in and around endometriotic lesions are associated with the stage of lesion development.

3.3 Secondary Hypotheses

Following from primary hypothesis, it is specifically hypothesised that:

- The density of immune cell populations is higher in the stroma of peritoneal endometriotic lesions compared to the surrounding tissue.
• The density of immune cell populations is higher in red lesions (early stage of development), implying that the immune response in these lesions is more active, compared to black and white lesions (later stages of development).
• Black lesions have higher density of immune cell populations than white lesions (earlier versus later stages of development, respectively).

3.4 Aims

To test the hypotheses stated above, the main aim of this research project was to investigate the development of peritoneal endometriotic lesions by characterising their immune environment by their macroscopic appearance.

The specific aims of this study were to determine the density and distribution of immune cell populations; specifically, dendritic cells, T cells, B cells and macrophages; in and around red, black and white peritoneal endometriotic lesions.
Chapter 4
Methodology

4.1 Ethics Approval

This study has been approved by the Ethics Review Committee (Royal Prince Alfred Hospital Zone) of the Sydney Local Health District (protocol number: X11-0270 & HREC/II/RPAH/419).

4.2 Participant Recruitment and Sample Collection

Participants were prospectively recruited from gynaecology operating theatres at Royal Prince Alfred Hospital (n=32; mean age=31.5, age range=20-45). The study included participants who were non-pregnant, required laparoscopic excision of endometriosis, of a reproductive age or pre-menopausal and regularly menstruating, understood the conditions of the study and gave informed consent. Women who were pregnant or post-menopausal or unable to consent to involvement were excluded from this study. Women who provided informed consent were asked to provide information regarding their symptoms; gynaecological, obstetric and, past medical history including autoimmune disorders; family history of endometriosis, and current medications.

Collection of peritoneal endometriotic lesion samples involved wide laparoscopic excision with minimal use of diathermy. Appearance of lesions prior to excision was categorised
during the procedure by surgeons as red, black or white and laparoscopic pictures captured before the excision.

4.3 Sample Processing

4.3.1 Fixation

Tissue samples were fixed in 10% neutral-buffered formalin to preserve tissue components and morphology as close to its natural state as possible (Boenisch, 2001). After the tissue samples were fixed, they were dehydrated by alcohol, cleared of dehydrating agents using xylene and infiltrated with paraffin wax to replace xylene according to a standardised protocol. Paraffin embedding provides structural support and allows sectioning for visualisation (Boenisch, 2001, Miller et al., 2001).

4.3.2 Sectioning

Paraffin embedded tissue samples were cooled to -5°C on a cold plate (Leica EG1150 C, Leica Microsystems Nussloch GmbH, Germany; Figure 4.1 (A)) before cutting as it improves sectioning. Cooled tissue blocks were cut at 4μm on a manually operated rotary microtome (Leica RM 2135, Leica Microsystems Nussloch GmbH, Germany; Figure 4.1 (B)). Cut sections were mounted on glass slides (IHC Microscope Slides, FLEX; Dako, Denmark) and dried in an oven at 60°C for 60 minutes.
Figure 4.1 (A) Leica EG1150 C cold plate and (B) manually operated microtome used for cutting tissue sections. Source: leica.microsystems.com
4.3.3 Deparaffinisation and Rehydration

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

4.3.4 Haematoxylin and Eosin Staining (H&E)

Haematoxylin and eosin (H&E) staining was performed for all samples to assess tissue morphology and locate the lesion within the tissue block. Deparaffinised and rehydrated slides were H&E stained using the following protocol:

1. Harris Haematoxylin 4 mins
2. Rinse well in tap water 1 min
3. Differentiate in acid alcohol and stop action by washing quickly in running tap water
4. Blue in Scott’s Bluing Solution 30 secs - 1min
5. Rinse in tap water (examine microscopically)
6. Wash well in tap water 1 min
7. 70% alcohol 1 min
8. Counterstain with Alcohol Eosin 15 - 60 secs
9. 95% alcohol 1 min
10. 100% alcohol 1 min
11. 100% alcohol 1 min
12. Xylene 2 mins
13. Xylene 2 mins
14. Coverslip with Ultramount (Fronine Pty Ltd, Riverstone, Australia).

4.4 Immunohistochemistry Concept and Protocol

4.4.1 Antigen Retrieval

Antigen retrieval is a method to unmask antigens which have been heavily modified by fixation and/or paraffin processing. Cross-links between proteins formed during fixation are broken down and some of the antigen denaturation caused by fixation and paraffin processing is reversed with this method (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 study, HIER was performed on tissue sections prior to immunohistochemical (IHC) staining. HIER involved immersing tissue slides in a preheated buffer solution prepared by diluting 4 mLs of Antigen Target Solution pH 9 (x50 concentrate; Dako, Glostrup, Denmark) in 196 mLs distilled water. The working solution was preheated in a water bath to 95-99°C. Slides were immersed in the preheated solution and incubated for 20 minutes at 95-99°C. Retrieved sections were cooled to room temperature in a cold-water bath, rinsed in water and incubated in TRIS buffer for 10 minutes to break the surface tension prior to staining.
4.4.2 Antibodies

Antibodies belong to a group of proteins called immunoglobulins (Ig). There are five major classes of immunoglobulin: IgG, IgA, IgM, IgD, and IgE. Each immunoglobulin structure is composed of four identical chains including two heavy (that determine the class and subclass of the molecule) and two light chains (that found as kappa or lambda types differ across the different classes). The Y shaped antibody has a specific recognition site on its light chains that reacts with a site on antigen called epitope (Buchwalow and Ebrary, 2010). The antibody-antigen binding complex is commonly used in IHC. Figure 4.2 below represents this specific binding interaction.
Figure 4.2 The antibody-antigen binding interaction.

Antibodies used in this project were monoclonal primary antibodies, which are the product of an individual clone of plasma cells. Monoclonal antibodies bind to a single epitope on the antigen and currently, are exclusively produced in mice (Boenisch, 2001). Antibodies used in this research are detailed in Table 4.1.
Table 4.1 Antibodies used in this study.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Stains</th>
<th>Species</th>
<th>Dilution</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>DC-Lamp</td>
<td>Mature dendritic cells</td>
<td>Monoclonal mouse (clone 1010E1.01)</td>
<td>1:50 with Amp Link</td>
<td>Dendritics, Lyon, France</td>
</tr>
<tr>
<td>DC-Sign</td>
<td>Immature dendritic cells</td>
<td>Monoclonal mouse (clone 102E11.06)</td>
<td>1:100</td>
<td>Dendritics, Lyon, France</td>
</tr>
<tr>
<td>CD4</td>
<td>Effector T cells</td>
<td>Monoclonal mouse (clone 4B12)</td>
<td>1:60 with Amp Link</td>
<td>Dako, Glostrup, Denmark</td>
</tr>
<tr>
<td>CD8</td>
<td>Cytotoxic T cells</td>
<td>Monoclonal mouse (clone C8/144B)</td>
<td>1:100</td>
<td>Dako, Glostrup, Denmark</td>
</tr>
<tr>
<td>FoxP3</td>
<td>Regulatory T cells</td>
<td>Monoclonal mouse (clone 236A/E7)</td>
<td>1:50 with Amp Link</td>
<td>Abcam, Cambridge, UK</td>
</tr>
<tr>
<td>CD20</td>
<td>B cells</td>
<td>Monoclonal mouse (clone JCB117)</td>
<td>1:400</td>
<td>Dako, Glostrup, Denmark</td>
</tr>
<tr>
<td>CD68</td>
<td>Macrophages</td>
<td>Monoclonal mouse (clone PG-M1)</td>
<td>1:300</td>
<td>Dako, Glostrup, Denmark</td>
</tr>
</tbody>
</table>

4.4.3 Amplifier

EnVision FLEX+ Mouse (LINKER) (Dako, Glostrup, Denmark) was used as an amplifying agent as required. Initial staining with some antibodies including DC-Lamp, CD4 and FoxP3 gave weak, unsatisfactory results even at high concentrations and the use of the amplifier gave four-five fold increases in signal.
4.4.4 Detection System

EnVision+ Dual Link System-HRP (Dako, Glostrup, Denmark) was used as the detection system. This system is based on a horseradish peroxidise (HRP) labelled polymer, which is conjugated with secondary antibodies directed against mouse. The reagent contains goat secondary antibodies with anti-mouse Ig specificity coupled with peroxidise-labelled polymers in Tris-HCl buffer with stabilising protein and anti-microbial agents. It is a signal enhancing detection system.

4.4.5 Visualisation

Liquid Diaminobenzidine+ (DAB+) Substrate Chromagen (Dako, Denmark) was used as the visualisation system. This is a high-sensitivity DAB system suitable for use in peroxidase-based IHC. DAB forms a brown end-product at the site of the target antigen upon oxidation. A DAB working solution was prepared by adding one drop (20 μL) of DAB Chromagen per 1 mL of substrate buffer according to the volume required. Since it has a carcinogenic effect, DAB+ was prepared with caution and disposed of in a safe way.

4.4.6 Autostainer

An automated slide processing system, Dako Autostainer Plus Universal Staining System (Dako, Glostrup, Denmark), was used for IHC staining in this study (Figure 4.3). The Autostainer is made up of a slide processor, dedicated desktop computer, printer and a labelling system. A maximum of 48 slides can be processed in one run and the reagent, dispense volume and dispense location were individually programmed for each slide. The
drop zones for each slide were also selected individually according to the tissue location to ensure full coverage with a dispense volume total of 200 μL (two dispense locations of 100 μL).

![Image of Dako Autostainer Plus Universal Staining System](https://www.dako.com.au)

**Figure 4.3** Dako Autostainer Plus Universal Staining System (Dako, Glostrup, Denmark). Source: www.dako.com.au

### 4.4.7 Wash Buffer

Wash buffers remove non-specifically bound proteins in specimens to reduce or eliminate background. The buffer was prepared by diluting Wash Buffer concentrate (10x; Dako, Denmark), a Tris-buffered saline solution containing Tween 20 with pH 7.6 (+ 0.1), in deionised water. Buffer rinse steps, which are a buffer wash and air blow cycle, were programmed on the Autostainer between each reagent step.
4.4.8 Blocking

Blocking steps eliminate and reduce non-specific background staining which is a result of positive staining that is not due to antigen-antibody binding (Gao et al., 2008). Dual Endogenous Enzyme Block (DEEB; Dako, Glostrup, Denmark) inhibits the activity of endogenous peroxidise, pseudoperoxidase and alkaline phosphatase, which are frequently observed in IHC procedures resulting in non-specific staining. The presence of endogenous peroxidase and alkaline phosphatase can obscure specific staining of the target antigen (Gao et al., 2008). Following an initial buffer wash to ensure reagent spread, the Autostainer was programmed with a 10 minute DEEB blocking step.

4.4.9 Protocol

The following protocol was performed for tissue staining on the Autostainer:

1. Wash buffer rinse
2. DEEB block 10 mins
3. Wash buffer rinse
4. Primary antibody 30 mins
5. Wash buffer rinse
6. EnVision FLEX+ Mouse (LINKER) (as required) 15 mins
7. Wash buffer rinse
8. EnVision+ Dual Link System-HRP 30 mins
9. Wash buffer rinse
10. Wash buffer rinse

11. DAB+ 10 mins

12. Wash buffer rinse

4.4.10 Counterstaining and Cover-Slapping

The protocol for counterstaining and cover-slapping of IHC stained slides was as follows:

1. Rinse with deionised water

2. Tap water 30 secs

3. One quick dip in Mayer's Haematoxylin

4. Rinse in running tap water until water runs clear

5. Blue in hot tap water 15 secs

6. 70% alcohol 2 mins

7. 95% alcohol 2 mins

8. 100% alcohol 2 mins

9. 100% alcohol 2 mins

10. Xylool 3 mins

11. Xylool 3 mins

12. Coverslip in Ultramount (Fronine Pty Ltd, Riverstone, Australia).

4.5 Quantification of Immune Cell Populations

All stained slides were observed using an Olympus BX51 microscope (Olympus, Tokyo Japan). All peritoneal endometriotic lesion areas were captured under x10 magnification
objective using an Olympus DP70 digital camera (Olympus, Tokyo Japan). Quantification of positive cells was performed using MetaMorph software (Molecular Devices, Downington, PA, USA).

4.5.1 Regions

Immune cell populations were examined in endometriotic stroma of peritoneal lesions as well as in the tissues immediately surrounding lesions. Three different regions were drawn around each peritoneal endometriotic lesion area: (1) immediately inside the glandular epithelium (gland region), (2) at the edge of the endometriotic stroma (stroma region) and (3) 250 μm from the edge of the stroma (surrounding region). An example of the lesion regions is shown in Figure 4.4.
**Figure 4.4** Region areas in a captured peritoneal endometriotic lesion (CD4 effector T cell staining; image captured under 10x magnification).

### 4.5.2 Thresholding and Measurement

Thresholds were manually created for each marker using the ‘Set Colour Threshold’ function of MetaMorph. This was done using the ‘Set by Example’ option and clicking on positive (brown) pixels until all shades of brown staining were included in the threshold. Thresholds were tested on multiple images (at least five) for every marker to ensure all positive cells were included within the threshold before being finalised. An example of thresholding with MetaMorph is shown in Figure 4.5.
Figure 4.5 Thresholding of CD8+ cytotoxic T cells in and around a peritoneal endometriotic lesion area with the ‘Set Colour Threshold’ function in MetaMorph. Positive pixels included within the threshold are indicated with a red overlay.

After thresholding was performed, the next step was setting up scoring parameters (setting the ‘State’) for every marker based on morphometric characteristics (size). This was done in the ‘Integrated Morphometry Analysis’ (IMA) function from the MetaMorph tool-bar (Figure 4.6). Minimum and maximum size limits (in pixels) were determined for each cell type based on positively stained cell morphology.
Figure 4.6 Measurement of CD8+ cytotoxic T cells in and around a peritoneal endometriotic lesion area with the ‘Integrated Morphometry Analysis’ function in MetaMorph. Within the regions, the green overlay indicates the positive cells detected by the threshold and within the size limits for the cell type which are therefore measured.

4.5.3 Data Processing

All MetaMorph measurements were directly logged to Microsoft® Excel. For each image, the number of positive cells and region area measurements were obtained. Raw measurements included all counts and area within entire regions (including smaller regions contained within) so exact measurements for endometriotic stroma only and surrounding tissue only were obtained by subtracting smaller embedded region measurements. Stromal tissue cell counts and region areas were calculated by subtracting gland region measurements from stroma region measurements. Similarly, surrounding tissue cell counts and region areas
were calculated by subtracting stroma region measurements from surrounding region measurements.

Area measurements from MetaMorph were provided in pixels, so were converted to mm with the following formula (derived as 250 µm = 450 pixels):

\[
\text{Area mm}^2 = \frac{\text{Pixel area}}{3240000}
\]

Measurements for different areas of samples (images) were summed to give the total positive cell counts and mm\(^2\) area per lesion sample (for both stroma and surrounding). The density per mm\(^2\) of immune cell populations in the stroma and surrounding regions was calculated by the following:

\[
\text{Density per mm}^2 = \frac{\text{Positive cell count}}{\text{Area mm}^2}
\]

### 4.6 Statistical Analyses

Statistical analyses were performed using IBM Statistical Package for the Social Sciences (SPSS) version 21.0 software. One-Sample Kolmogorov-Smirnov test was used to determine distribution of variables. To compare marker densities in stromal versus surrounding tissue, either paired sample t tests or the non-parametric equivalent, the Wilcoxon Signed Ranks Test (z score) were performed. To compare marker densities between lesion types (red, black
or white) within either stromal or surrounding tissue either one-way analysis of variance (ANOVA with contrasts for pairwise comparisons, or for non-normally distributed data, the Kruskal-Wallis chi-square test, with Mann-Whitney U z tests for pairwise comparisons, were used. Correlations between marker densities within either stromal or surrounding tissue were examined using either the Pearson’s correlation coefficient if both variables were normally distributed or the Spearman’s correlation coefficient (r_s) if data were skewed. Statistics were considered to be statistically significant with P-values of less than 0.05.
Chapter 5
Results

5.1 Density of Immune Cell Populations in and around Peritoneal Endometriotic Lesions

Immune cell populations were present in and around peritoneal endometriotic lesions, with variations in numbers and distribution. Representative images of staining are provided for all dendritic cell (Figure 5.1), T cell (Figure 5.2), B cell (Figure 5.3) and macrophage (Figure 5.4) populations. The staining on negative controls was always negative (Figure 5.5).
Figure 5.1 Immunohistochemical staining for dendritic cell populations in and around peritoneal endometriotic lesions. DC-Sign+ immature dendritic cells at 100x magnification (A) and 400x magnification (B); and DC-Lamp+ mature dendritic cells at 100x magnification (C) and 400x magnification (D) stained brown with DAB+ chromagen.
Figure 5.2 Immunohistochemical staining for T cell populations in and around peritoneal endometriotic lesions. CD4+ effector T cells at 100x magnification (A) and 400x magnification (B); CD8+ cytotoxic T cells at 100x magnification (C) and 400x magnification
(D); and FoxP3+ regulatory T cells at 100x magnification (E) and 400x magnification (F) stained brown with DAB+ chromagen.

Figure 5.3 Immunohistochemical staining for CD20+ B cells at 100x magnification (A) and 400x magnification (B) in and around peritoneal endometriotic lesions, stained brown with DAB+ chromagen.
Figure 5.4 Immunohistochemical staining for CD68+ macrophage populations at 100x magnification (A) and 400x magnification (B) in and around peritoneal endometriotic lesions, stained brown with DAB+ chromagen.

Figure 5.5 A representative image of negative control at 100x magnification (A) and 400x magnification (B).

5.1.1 Lesion Stroma Compared to Surrounding Tissue

In peritoneal endometriotic lesions, densities of immune cell populations showed significant variations between stromal and surrounding tissues (Figure 5.6).
Figure 5.6 Graphs showing variations in the densities per mm² of DC-Sign+ immature dendritic cells (A), DC-Lamp+ mature dendritic cells (B), CD4+ effector T cells (C), CD8+ cytotoxic T cells (D), Foxp3+ regulatory T cells (E), CD20+ B cells (F) and CD68+ macrophages (G) showing variations in the densities of populations between stroma and surrounding tissue of peritoneal endometriotic lesions. The solid line in the box plots represent the median density, the length of the box denotes the interquartile range (IQR; 25th to 75th centiles) and the whiskers indicate the minimum and the maximum values of the data that are not outliers. The circles represent outliers 1.5-3 IQRs from the end of the box. The asterisk represents an extreme outlier defined as a value >3 IQRs from the end of the box. In bar chart, data represent the mean ± SD density per mm².

Dendritic cells

Density of DC-Sign+ dendritic cells was significantly lower in lesion stroma compared to surrounding tissue (p<0.001, Mann Whitney U z=3.903, Figure 5.6 [A]), while there was no
statistically significant difference for DC-Lamp+ dendritic cells between stromal and surrounding tissue.

**T cells**

Density of CD8+ and FoxP3+ T cells were significantly higher in stromal compared to surrounding tissue of peritoneal endometriotic lesions (p=0.014, df=31, t=2.60, Figure 5.6 [D] and p=0.035, U z=2.213, Figure 5.6 [E]; respectively). However, there was no statistically significant difference in CD4+ T cell density between lesion stroma and surrounding tissue. There were CD4+ and CD8+ T cell aggregates in the tissue surrounding peritoneal endometriotic samples for 9 of the 32 samples. These aggregates ranged in size range from 40 µm diameter to 100x300 µm in size (Figure 5.7).

![Figure 5.7 Examples of CD4+ (A) and CD8+ (B) T cell aggregates in the tissue surrounding peritoneal endometriotic lesions.](image)
**B cells**

CD20+ B cells showed significantly higher density in surrounding tissue in comparison to lesion stroma \((p=0.033, \ U \ z=2.129, \ \text{Figure} \ 5.6\ F)\). There were CD20+ B cell aggregates in tissue surrounding lesions for 9 of 32 lesion samples (same samples with CD4+ and CD8+ T cell aggregates), ranging in size between 40 µm diameter to 100x300 µm in size (Figure 5.8).

![Figure 5.8](image.png)

**Figure 5.8** Example of a CD20+ B cell aggregate in surrounding tissue of a peritoneal endometriotic lesion.

**Macrophages**

Stromal density of CD68+ macrophages was significantly higher than the surrounding density \((p=0.012, \ U \ z=-2.499, \ \text{Figure} \ 5.6 \ [G])\).
5.1.2 Lesion Appearance

There was no correlation observed between participant age and lesion type. None of the immune cell populations studied differed significantly in density between the three lesion types (red, black and white) either in the stroma or surrounding tissue (Figures 5.9 and 5.10).
Figure 5.9 The densities of immune cell populations in stroma of peritoneal endometriotic lesions for red, black and white lesion appearances. DC-Sign+ immature dendritic cells (A), DC-Lamp+ mature dendritic cells (B), CD4+ effector T cells (C), CD8+ cytotoxic T cells (D), Foxp3+ regulatory T cells (E), CD20+ B cells (F) and CD68+ macrophages (G). Data represent the mean ± SD densities per mm².
Figure 5.10 The densities of immune cell populations in tissue surrounding peritoneal endometriotic lesions for red, black and white appearances. DC-Sign+ immature dendritic cells (A), DC-Lamp+ mature dendritic cells (B), CD4+ effector T cells (C), CD8+ cytotoxic T cells (D), Foxp3+ regulatory T cells (E), CD20+ B cells (F) and CD68+ macrophages (G). Data represent the mean ± SD densities per mm².

5.2 Correlations between the Densities of Immune Cell Populations

Stroma

In stromal tissue of peritoneal endometriotic lesions, statistically significant positive correlations were observed between CD4+ and CD8+ T cell densities (p<0.001, Spearman’s rho r_s=0.647; Figure 5.11 [A]) and between CD4+ T cell and CD68+ macrophage densities (p=0.029, r_s=0.386; Figure 5.11 [B]). There was a strong trend for positive correlation between DC-Sign+ dendritic cell and CD4+ T cell densities (p=0.050, r_s=0.349; Figure 5.11 [C]).
The correlations between the densities of different immune cell populations in the stroma of peritoneal endometriotic lesions. CD8+ cytotoxic T cells with CD4+ effector T cells (A) and CD68+ macrophages with CD4+ effector T cells (B), DC-Sign+ dendritic cells with CD4+ effector T cells (C).

**Surrounding**

In tissue surrounding peritoneal endometriotic lesions, there were 11 different positive correlations between immune cell population densities (Figure 5.12). There was correlation
between the densities of DC-Sign+ dendritic cells with CD4+ T cells (p<0.001, rs=0.608, Figure 5.12 [A]); and CD20+ B cells (p=0.003, rs=0.508; Figure 5.12 [B]) and DC-Sign+ dendritic cells with CD68+ macrophages (p=0.002, rs=0.522; Figure 5.12 [C]) DC-Lamp+ dendritic cells with both CD4+ T cells (p=0.001, rs=0.543, Figure 5.12 [D]) and CD68+ macrophages (p=0.037, rs=0.371; Figure 5.12 [E]). Density of CD4+T cells was correlated with all other studied cell populations (CD8+ T cells p=0.008, rs=0.463, Figure 5.12 [F]; Foxp3+ p=0.045, rs=0.357, Figure 5.12 [G]; CD20+ p=0.037, rs=0.370, Figure 5.12 [H]; and CD68+ p<0.001, rs=0.704, Figure 5.12 [I]). There was correlation between the densities of CD8+ T cells with both CD20+ B cells (p=0.002, rs=0.527, Figure 5.12 [J] and CD68+ macrophages (p=0.30, r=0.384, Figure 5.12 [K]).
Figure 5.12 The positive correlations between the densities of different immune cell populations in tissue surrounding peritoneal endometriotic lesions. DC-Sign+ immature dendritic cells correlated with CD4+ T cells (A), DC-Sign+ immature dendritic cells with CD20+ B cells (B), DC-Sign+ immature dendritic cells with CD68+ macrophages (C), DC-Lamp+ mature dendritic cells with CD4+ effector cells (D), DC-Lamp+ mature dendritic cells with CD68+ macrophages (E), CD4+ effector T cells with CD8+ cytotoxic (F), CD4+ effector T cells with Foxp3+ regulatory T cells (G), CD4+ effector T cells with CD20+ B cells (H), CD4+ effector cells with CD68+ macrophages (I), CD8+ cytotoxic T cells with CD20+ B cells (J) and CD8+ cytotoxic T cells with CD68+ macrophages (K).
Chapter 6
Discussion

6.1 Introduction
This study has observed the presence of all studied immune cell populations; including dendritic cells, T cells, B cells and macrophages; in and around peritoneal endometriotic lesions. Some of these cell populations have been examined in endometriotic lesions for the first time in this study. It has been demonstrated that while CD8+ cytotoxic T cells, Foxp3+ regulatory T cells and CD68+ macrophages were significantly higher in density within lesion stroma; DC-Sign+ immature dendritic cells, CD4+ helper T cells and CD20+ B cells were significantly increased in density in tissue surrounding peritoneal endometriotic lesions. Additionally, this study has for the first time shown correlations between different immune cell population densities in lesion stroma and surrounding tissue, which suggest functional interactions in terms of peritoneal lesion pathogenesis. Due to the great variations in immune cell densities in peritoneal endometriotic lesions, it was initially hypothesised that densities of immune cell populations correlate with stage of lesion development. Subsequent results from this study found no relationship between lesion appearance and immune environment.

Evidence from this thesis indicates immune cell populations present have a range of possible roles in and around peritoneal endometriotic lesions. The recruitment of immune cells to tissue within and around peritoneal endometriotic lesions, likely in an attempt to attack the
lesion but their released products may in fact promote processes such as angiogenesis and fibrosis and thereby promote lesion growth and persistence. The results suggest that the numbers and function of immune cells are altered at some point in endometriosis to allow further lesion growth and persistence through their released products. In addition, functional interactions between different immune cell populations might suggest cooperation between these cells in order to promote the development of lesions.

6.2 Immune Environment within Peritoneal Endometriotic Lesions

This study has shown that all the immune cell populations studied were present in the stroma of peritoneal endometriotic lesions. These cells include dendritic cells, T cells, B cells and macrophages. CD8+ T cells, Foxp3+ T cells and CD68+ macrophages were significantly higher in density within lesions compared to the surrounding tissue. In addition, there was a correlation between the densities of some of these immune cell types, indicating possible functional relationships and interactions within lesion stroma. These cells may be recruited to stroma in an attempt to clear the lesion, however, actually promote the growth and persistence of peritoneal endometriotic lesions through their released products.

For the first time, this study has shown the presence of DC-Sign+ immature and DC-Lamp+ mature dendritic cells in the stroma of peritoneal endometriotic lesions. These dendritic cells were observed at low density and they were not present in all lesion sections. A previous study has also confirmed the presence of CD1a+ immature and CD83+ mature dendritic cells
in peritoneal endometriotic lesions and that these cells were not detected at all in normal peritoneum of women without endometriosis (Schulke et al., 2009). DC-SIGN+ cells regulate adhesion processes, such as DC trafficking and T-cell synapse formation, as well as antigen capture (Geijtenbeek et al., 2000, Teunis et al., 2002). DC-LAMP+ cells function in the processing and presentation of antigens by transferring of peptide-MHC class II molecules to the surface of DC (de Saint-Vis et al., 1998). Thus, dendritic cells might be recruited to endometriotic lesions in order to initiate an immune response and subsequent clearance of ectopic antigens. However, these cells may influence other leucocyte populations through their released cytokines and chemokines (such as IL-6, IL-10, IL-12, TNF-α, RANTES and MCP-1), which also promote lesion growth (Nagorsen et al., 2004). Supporting this, immature dendritic cells were found to infiltrate peritoneal lesions and to encourage angiogenesis and lesion growth a murine model (Fainaru et al., 2008).

Alterations in DC populations may directly contribute aberrant T cell function in women with endometriosis (Schulke et al., 2009). Similarly, a positive correlation was observed between DC-Sign+ immature dendritic cells and CD4+ helper T cell densities in the stroma of peritoneal endometriotic lesions. This might be because of the fact that DC-Sign+ cells present antigens to CD4+ T cells to initiate an immune response (Garcia-Vallejo et al., 2013). CD4+ T cells produce Th1 and Th2 cytokines (such as IL-2, IL-12, interferon-γ and TNF-α; and IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13; respectively), which potentially promote endometriotic lesion growth in endometriosis. Interestingly, decreased Th1 and increased
Th2 cytokine levels; indicating a switch to the Th2 immune response which implies endometriosis pathogenesis through the activating cell mediated immunity in the peritoneal cavity, were observed in the peritoneal fluid of women with endometriosis (Giudice and Kao, 2004). Therefore correlated DC-Sign+ and CD4+ populations in peritoneal endometriotic lesions potentially promote endometriotic lesion growth and pathogenesis by secretion of cytokines and growth factors (Halis and Arici, 2004). Even though DC-Sign+ cells initially might be attacking the lesion stroma to inhibit lesion growth, their altered cytokine production and by activated T cells may actually promote survival of shed fragments and endometriotic lesion development.

This study has shown increased density of CD8+ cytotoxic T cells in stroma of lesions compared to surrounding tissue. This study has also detected a relationship (positive correlation) between CD8+ and CD4+ T cell densities in lesion stroma. It has been previously found that both CD8+ and CD4+ T cells are increased, and the CD4:CD8 ratio decreased in peritoneal endometriotic lesions (compared to normal peritoneum and eutopic endometrium) and the peritoneal fluid of women with endometriosis (Witz et al., 1994, Oral et al., 1996, Jones et al., 1998, Paul Dmowski and Braun, 2004, Seeley et al., 2005, Ganewatta et al., 2010). CD8+ T cells are cytotoxic killer cells, which directly destroy abnormal cells, while CD4+ are helper T cells, which transmit signals from antigen presenting cells to stimulate the immune system (Andersen et al., 2006, Waisman and Becher, 2014). There is evidence that activated CD8+ T cells can stimulate CD4+ T cells by
direct interaction and that CD4+ cells help in primary responses by CD8+ T cells (Castellino and Germain, 2006). Therefore, CD8+ T cells and functional interactions of CD8+ T cells with CD4+ T cells might support endometriotic lesion growth (although these cells are likely recruited to lesion stroma to attack the abnormal cells and inhibit the lesion growth).

The current study found increased density of FoxP3+ regulatory T cells in the stroma of peritoneal endometriotic lesions compared to surrounding tissue, similar to a previous study (Berbic et al., 2010). FoxP3+ regulatory T cells control and suppress a range of immune responses (Fehervari and Sakaguchi, 2004), including T cell, dendritic cell, B cell, macrophage and natural killer cell proliferation and cytokine release (Thornton, 2005, Sakaguchi et al., 2008, Giatromanolaki et al., 2008, Nandakumar et al., 2009). Therefore, Foxp3+ T cells might be recruited to lesion stroma in an attempt to control the locally recruited immune cells and inflammation.

This study has for the first time shown higher density of macrophages within the stroma of peritoneal endometriotic lesions in comparison to the surrounding tissue. Previous studies have shown an increase in the numbers of macrophages in peritoneal endometriotic lesions compared to normal peritoneum and eutopic endometrium (Oosterlynck et al., 1993a, Khan et al., 2004a, Khan et al., 2004b, Berbic et al., 2009, Tran et al., 2009). Since macrophages generally act as scavengers of the body, they are thought to be recruited to endometriotic lesions to phagocytose the ectopic tissue. However, they can secrete cytokines and growth
factors such as IL-6, IL-8, IL-10 and TNF-α; and a number of angiogenic factors, including fibroblast growth factor, angiogenin and VEGF (Sunderkötter et al., 1994, McLaren et al., 1996b); which stimulate the growth and persistence of endometriotic lesions (Iwabe et al., 2000, Sokolov et al., 2005, Velasco et al., 2006, Birt et al., 2013, Lin et al., 2006).

A positive correlation was detected between the density of CD68+ macrophages and CD4+ T cells in this study. T helper cells help activate macrophages at the sites of infection and both T helper cells and macrophages are known to produce a range of cytokines and growth factors which facilitate the development of endometriotic lesions in endometriosis (Telser, 2002, Lin et al., 2006). Therefore, the increased numbers of macrophages and the positive correlation with CD4+ T cells within stroma may stimulate the growth of peritoneal endometriotic lesions.

In conclusion, this study suggests that immune cells are recruited to the stroma in an attempt to clear peritoneal endometriotic lesions. However, their presence may in fact promote lesion growth due to the factors they are known to produce. Other factors are also involved in endometriotic lesion pathogenesis, including the surrounding sub-peritoneal tissue.

6.3 Immune Environment around Peritoneal Endometriotic Lesions

This study also examined the presence of all studied immune cells in the sub-peritoneal tissue immediately surrounding peritoneal endometriotic lesions. Many of these particular cell
types had not specifically been studied around peritoneal endometriotic lesions previously, including dendritic cells, CD4+ T cells, B cells and macrophages. The densities of DC-Sign+ immature dendritic cells, CD4+ helper T cells and CD20+ B cell populations were higher in the tissue surrounding peritoneal endometriotic lesions compared to lesion stroma. The densities of a number of immune cell populations in the surrounding tissue were also significantly correlated with each other, suggesting local interactions between these cells. Another interesting observation of the study was the immune cell aggregates, containing CD4+ and CD8+ T cells and CD20+ B cells, in the tissue surrounding lesions in around a third of samples. These immune cells might promote tissue fibrosis and lesion persistence through their released products in surrounding tissue, even though they may initially recruited the area to suppress the lesion growth.

There is evidence that peritoneal endometriotic lesions are surrounded by thickened fibrotic areas made up smooth muscle metaplasia which is innervated and quite different compared to normal peritoneum (Anaf et al., 2000, Konno et al., 2003, Giudice and Kao, 2004, Odagiri et al., 2009). During the development and progression of endometriotic lesions, tissue fibrosis may lead to scarring, altered tissue function and contribute to chronic pain (Nisolle and Donnez, 1997, Matsuzaki et al., 1999). Inflammatory cells such as macrophages, fibroblasts and lymphocytes have an important role in generating fibrotic tissue via production of fibrogenic cytokines (Kisseleva and Brenner, 2008, Higashi-Kuwata et al., 2009, Prokop et al., 2011).
DC-Sign+ and DC-Lamp+ dendritic cells were observed for the first time in the tissue surrounding peritoneal endometriotic lesions. Additionally, the density of DC-Sign+ immature dendritic cells was higher in surrounding tissue compared to lesion stroma. Conversely, a previous study showed increased numbers of CD1a+ immature dendritic cells in stroma of peritoneal endometriotic lesions compared to surrounding tissue (Schulke et al., 2009). These apparently differing results may be because of the markers used. DC-Sign+ immature dendritic cells mediate MHC-II dependent antigen presentation pathways, have adhesion receptors and play important roles in inflammation and T cell activation (Teunis et al., 2002, Zhou et al., 2006). By contrast, CD1a is a cell surface glycoprotein that is structurally related to the MHC molecules and mediates MHC-independent antigen presentation pathways (Krenács et al., 1993). This study has also detected the presence of DC-Lamp+ dendritic cells in low numbers in lesion surrounding tissue. These cells may be important in tissue surrounding peritoneal endometriotic lesions to recognise and target ectopic “endometrial” cells and initiate appropriate immunological responses.

Positive correlations were observed between the densities of DC-Sign+ dendritic cells with CD4+ T cells (similar to stroma) and also both DC-Sign+ and DC-Lamp+ dendritic cells with CD68+ macrophages in lesion surrounding tissue. Macrophages appear to be able mediate the inflammatory potential of dendritic cells (Pulendran et al., 2007) and additionally, interactions of these cells can promote an acute cytokine response (Zhang et al.,
Therefore, since their released products are known to promote lesion growth and persistence, these functionally related cells in lesion surrounding tissue may contribute to the development of peritoneal endometriotic lesions.

This study has for the first time found increased CD4+ T cell density in surrounding tissue compared to stroma of peritoneal endometriotic lesions. Through chemotaxis, CD4+ T cells are recruited to sites of inflammation where they promote secretion of fibrogenic cytokines (for example, TGF-β1, PDGF, IL-1, IL-4, IL-5, IL-10, and IL-13) (Kisseleva and Brenner, 2008, Wynn, 2004) and can activate fibroblasts to produce collagen (Wynn, 2004), ultimately resulting in tissue scarring. CD4+ T cell density was correlated with Foxp3+ regulatory T cell density but given Foxp3 is be expressed in a sub-set of CD4+ T cells (Hori et al., 2003), this correlation is unsurprising. In addition, there were correlations between the densities of both CD4+ and CD8+ T cells with CD68+ macrophages. Macrophages are activated by CD4+ T cell-derived cytokines to release mediators that induce the proliferation of fibroblasts and the synthesis of collagen (Wynn, 2004, Laskin et al., 2011). In addition, an increase in the number of activated macrophages has been observed in fibrotic diseases (Higashi-Kuwata et al., 2009, Prokop et al., 2011) indicating that these cells themselves are a potential source of fibrogenic cytokines. Therefore, these cells with functional correlations might contribute to the development of fibrotic tissue surrounding peritoneal endometriotic lesions.
For the first time, this study has detected an increase in the density of CD20+ B cells in surrounding tissue of peritoneal endometriotic lesions compared to lesion stroma. Since B cells enhance T cell activity in immune responses (Nelson, 2010), promote the survival and proliferation of activated CD8+ T cells (Deola et al., 2008), and function together with CD8+ T cells to promote the survival of tumours (Qin et al., 1998); increased density of CD20+ B cells might be associated with increased numbers of T cells (with the capacity to produce fibrogenic cytokines) in lesion surrounding tissue. Supporting this, correlations between the densities of CD20+ B cells with CD4+ and CD8+ T cell densities were observed in lesion surrounding tissue. There was also a correlation between CD20+ B cell and DC-Sign+ immature dendritic cell densities in lesion surrounding tissue. It has previously been shown that dendritic cells signal to naive B cells to initiate antigen-specific antibody responses (Wykes and Macpherson, 2000). Therefore, increased numbers of CD20+ B cells in surrounding tissue might reflect specific immune responses against the adjacent lesions but ultimately promote fibrosis and the persistence of peritoneal endometriotic lesions along with T cells.

This study has for the first time detected lymphoid aggregates in tissue surrounding peritoneal endometriotic lesions. The aggregates were observed to contain CD4+ and CD8+ T cells and CD20+ B cells. Previous studies have shown T and B cell aggregates in uterine endometrium, and that these aggregates were small or absent during the early proliferative phase, significantly larger at mid cycle and during the secretory phase but absent in post-
menopausal women (Yeaman et al., 1997, Marshall and Jones, 1988). Therefore, it was suggested that the development of endometrial aggregates is hormonally influenced and that they develop during the menstrual cycle largely by trafficking of cells to the endometrium (Yeaman et al., 1997). At this stage what immune cell aggregates are doing in the tissue surrounding peritoneal endometriotic lesions is unclear; however, they might be trafficking to the tissue surrounding lesion in attempt to attack the adjacent lesions, but promote fibrosis and persistence of peritoneal endometriotic lesions via their released products.

In conclusion, immune cell populations present in tissue surrounding peritoneal endometriotic lesions, some with higher density than within the lesion stroma, likely contribute to lesion persistence and function. Although these cells may initially be recruited to attack the adjacent lesions, their released fibrogenic products promote tissue fibrosis and scarring and may ultimately facilitate lesion persistence.

6.4 Immune Environment in Different Lesion Appearances

Contrary to what was hypothesised, none of the immune cell populations studied differed significantly in density between the three peritoneal endometriotic lesion appearances (red, black and white) either in the stroma or surrounding tissue. As outlined in the literature review, different macroscopic appearances of lesions reflect different stages in development (Jansen and Russell, 1986, Redwine, 1987). Red lesions represent early stages, followed by black and then white peritoneal endometriotic lesion appearances due to progressive scarring
(Bloom, 1978, Dan, 1990). Previous studies have shown great variations in the densities of immune cell populations in peritoneal endometriotic lesions, as has the current study. It was hypothesised that these variations might be related to the stage of the lesion development, with higher density in red lesions than black and white lesions and higher density in black lesions compared to white lesions.

While lesion appearances were not found to be linked to immune cell densities in and around lesions, great variations in density were observed between lesion samples. At this stage, it is unknown what these variations in immune environment are due to. Other possible reasons for these variations include individual differences, menstrual cycle phase effects and impact of hormonal treatment. It was examined whether there was a correlation between participant age and immune cell densities (data not reported) but there was no evidence that age was linked to variations in densities.

Immune related parameters vary between individuals. Many individual-related factors are known to effect immune cell populations within the body, including stress and depression. Psychological stress has been associated with suppressed cellular immune function in some, but not all, individuals; and CD8+ cytotoxic T cells were found to be increased in circulation, after exposure to stress (Manuck et al., 1991). Previous studies have also suggested that depression is associated with immune changes such as increased release of peripheral cytokines (Hodes et al., 2014). Currently, there is also accumulating evidence suggesting that
genetic variants are associated with how the innate immune response and cytokines vary across individuals (Lee et al., 2014, Ferraro et al., 2014). These factors likely contribute to and may explain the immune variations between lesions.

Menstrual cycle phase could also account for the immune variations observed. Endometrial leukocytes are known to undergo significant changes through the menstrual cycle and have important roles in menstruation, endometrial remodelling, implantation and decidualisation (Salamonsen and Lathbury, 2000, Salamonsen et al., 2002). While endometriotic lesions do not show exactly the same cyclical changes as the eutopic endometrium, they are hormonally responsive (Berbic et al., 2010, Schulke et al., 2009). There is no strong evidence for major changes in immune cell densities within endometriotic lesions during the cycle; however, there may be subtle cyclical changes in immune cell populations such as CD1a+ immature dendritic cells (Schulke et al., 2009). At this stage, it cannot be excluded that the menstrual cycle phase of women included in this study may be associated with the variations in lesion immune cell populations. This was not considered in analyses, as accurate menstrual cycle dating was not available for all participants. Participants were asked about their last menstrual period (LMP) date but LMP self-reports are known to be unreliable because of errors of recall and inaccuracies for determining menstrual cycle phase (Waller et al., 2000, Wegienka and Baird, 2005, Wideman et al., 2013). Unfortunately endometrium and/or blood samples were not available for all participants to confirm cycle dating, as their collection is not part of routine endometriosis surgery at Royal Prince Alfred Hospital.
Furthermore, hormonal treatment could be another contributor to variations of density of immune cells in peritoneal endometriotic lesions. Initially it was planned that only women not on hormonal treatment be eligible for inclusion in the study; however, in order to be able to recruit sufficient numbers within the limited time frame, a small sub-group of participants on oral contraception was included. Hormonal treatment is commonly used for management of endometriosis-associated symptoms (Crosignani et al., 2006). Very few participants were on hormonal treatment in this study (oral contraception pill n= 4; 2 red, 1 black and 1 white lesion). It is unknown whether or how oral contraceptive pills affect the immune system, however the 4 samples from participants on oral contraception did not show any obvious differences in immune cell densities in and around lesions compared to the rest of the cohort.

It is also possible that sample number limitations meant that the study was not able to demonstrate links between lesion appearance and immune environment in and around peritoneal endometriosis lesions. Due to time limitations in this prospective study, only a moderate number of samples were able to be collected. Initially, the total number of samples recruited was 61 but then some of the samples were excluded because of reasons such as combined colour appearances or lesion unable to be located within the paraffin tissue block. Even though statistical analysis has shown no significant relationships between lesion appearance and the density of immune cell populations, there appear to be some patterns in lesions of different appearances. For example, density of a range of populations such as DC-Sign+, CD4+ and CD8+ cells appeared to be higher in white lesions. It may be that with
greater sample numbers, significant differences between lesion appearances would be demonstrated.

6.5 Summary and Perspective
Altered immune responses in endometriosis are thought to play fundamental roles in establishment and progression of peritoneal lesions. The findings from this thesis have implications for understanding of endometriosis pathogenesis, particularly peritoneal lesion development, and also potentially for future treatment approaches. The presence of all studied immune cell populations and increased densities of these cells in around peritoneal endometriotic lesions suggest that they may be recruited to lesion sites in an attempt to attack the ‘foreign’ ectopic tissue. However, through their released products, specifically cytokines and growth factors, they may in fact support lesion development and persistence. This may particularly be via promotion of fibrosis by immune cells in tissue surrounding peritoneal lesions, which this study has considered for largely the first time.

While there was high variation in studied cell densities between samples, there did not appear to be a relationship between the densities of immune cell populations in and around peritoneal endometriotic lesions and lesion appearances (stage of development). In fact, endometriosis is known to be a complex and highly variable disease in terms of the age of symptom onset, the delay to diagnosis, types of symptoms, the anatomical sites of ectopic lesions and response to treatment (Olive et al., 2004, Fraser, 2010a). This study further
supports complexity and variability in endometriosis. Thus, although exactly how these cells promote lesion growth and why there are variations between the densities of immune cells are still unclear, this study has provided clues for better understanding of the immune environment in and around peritoneal endometriotic lesions.

Individualised treatment approaches may provide more effective management of endometriosis due to the complexity and high variability of the disease (Olive et al., 2004). Current management for endometriosis generally includes medical (analgesic and hormonal suppression) and/or surgical approaches (Fraser, 2008). Individualised treatment for endometriosis should be tailored according to the desired treatment outcome, whether it is relief of pain, improvement of fertility, or the prevention of recurrence (Özkan and Arici, 2009).

Some current medical modalities for endometriosis may have immune-related effects, with danazol and GnRH-analogues suggested to suppress the ability of peritoneal immune cells to secrete inflammatory cytokines, regulating endometriotic lesion growth and establishment (Nothnick, 2001). Further to this, the current study provides a better understanding of the immune environment in and around peritoneal endometriotic lesions and may contribute to the basis for development of immune-targeted treatment options. For instance, treatments based on anti-cytokine antibodies or cytokine-receptor antagonists such as TNF-α inhibitors, angiogenesis inhibitors and matrix metalloprotease inhibitors, which may be effective by
targeting specific immune cells altered in peritoneal endometriosis (Olive et al., 2004, Bedaiwy and Falcone, 2004). One such example is human TNF binding protein-I which inhibits the production of TNF-α by macrophages and has been shown to significantly reduce endometriosis in a baboons model (D’Hooghe et al., 2001b).

While this study has provided new insights into the immune environment in and around peritoneal endometriosis lesions, the work does have some challenges and limitations in terms of sample collection and technical components. Firstly, as discussed in section 6.4, there was difficulty recruiting the desired numbers of suitable participants within the time frame. Obtaining suitable samples, which were lesions of only one appearance, was another difficulty as lesions can also have mixed appearances such as red-black and black-white or even red-black-white (Redwine, 1987, Dan, 1990).

Another challenge was demonstrating natural killer (NK) cells in peritoneal endometriotic lesions. Initially, the study included characterisation of 8 different immune cells, including NK cells. However, even though extensive optimisation was performed; such as use of different antibodies (CD56 [clone 123C3] and CD57 [clone TB01], Dako, Glostrup, Denmark), different retrieval methods (pH 6 and pH 9, retrieval time 20-30 minutes) and a range of dilutions (between 1:50 and 1:400, with and without the addition of an amplifying link); unfortunately staining was unsatisfactory with strong detection of nerve fibres but very weak labelling of NK cells. This staining pattern is known to be associated with NK cell
markers (Giuliani et al., 2014). Thus, staining for NK cells had to be abandoned because of the limited time for this study.

6.6 Conclusions

In response to the hypotheses and aims, the specific conclusions of this study are:

- Variations in immune cell environment in and around peritoneal endometriotic lesions are not associated with lesion appearance (the stage of lesion development)
  - There are no statistically significant differences between immune cell densities in and around peritoneal endometriotic lesions of red compared to black compared to white appearances.

- The densities of CD8+ T cells, Foxp3+ T cells and CD68+ macrophages are significantly higher in the stroma of peritoneal endometriotic lesions compared to the surrounding tissue (p=0.014, df=31, t=2.60, p=0.035, U z=2.213, p=0.012, U z=2.499, respectively).

- The densities of DC-Sign dendritic cells and CD20+ B cells are significantly higher in the tissue surrounding peritoneal endometriotic lesions compared to the stroma (p<0.001, U z=3.903, p=0.033, U z=2.129, respectively).

- The density of CD4+ T cells was increased in the tissue surrounding peritoneal endometriotic lesions compared to stroma, however there was no statistically significant difference.
6.7 Future Directions

The results obtained in this study provide interesting insights into the immune environment in and around peritoneal endometriotic lesions. However, there are many aspects of lesion development and immunology that are not yet understood. Key questions that should be addressed in future studies of immune involvement in peritoneal endometriotic lesion establishment include the following:

- Further investigating immune cell densities in and around peritoneal endometriotic lesions in a larger cohort of well characterised participants and samples, documenting factors such as
  - lesion appearance and location
  - the phase of the menstrual cycle (by eutopic endometrial dating)
  - endometriosis history (including symptoms and previous surgeries)
  - hormonal and other treatment status
  - personal situation including current stress, depression.

- Functional studies of individual immune cell populations in and around peritoneal endometriotic lesions, including production of fibrotic and other cytokines.

- Investigating the functional interactions between different immune cell populations in stroma and tissue surrounding peritoneal endometriotic lesions to understand their cooperation in promoting lesion growth.
• Determining if the peritoneal lesion stroma and fibrotic surrounding tissue themselves release cytokines and growth factors to aid lesion progression and how these factors interact with the recruited immune cells.

• Examination of immune environment in and around other lesion types (ovarian and deep infiltrating).

• Using in vitro and animal models to develop more specific and appropriate immune based treatment approaches.
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