The Effect of Macrophages on Fibroblast Activity and Lesion Development in Mouse Models of Endometriosis

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Table of Abbreviations

Abbreviation	Description
αSMA	Alpha smooth muscle actin
β2m	Beta-2-microglobulin
µL	Microlitre
µm	Micrometer
AcLDL	Acetyl low density lipoprotein
BAX	BCL-2 associated X protein
BCL-2	B-cell lymphoma 2
bp	Base pair
BrdU	5-bromo-2'-deoxyuridine
BSA	Bovine serum albumin
CAM	Chorioallantoic membrane
CCL (e.g. CCL17)	Chemokine (C-C motif) ligand
CCR1	Chemokine (C-C motif) receptor 1
COCs/COC	Combined oral contraceptives
CSF-1/Csf-1	Colony stimulating factor-1
CSF-1R	Colony stimulating factor-1 receptor
CXCL (e.g. CXCL13)	Chemokine (C-X-C motif) ligand
CYP	Cytochrome
CYR61	Cysteine-rich, angiogenic inducer, 61
DAB	3,3'-diaminobenzidine
DAPI	4',6-diaminido-2-phenylindole dihydrochloride
dNTPs	Deoxynucleotide triphosphates
DT	Diphtheria toxin
ECM	Extracellular matrix
eGFP	Enhanced green fluorescent protein
EMMPRIN	Extracellular matrix metalloproteinase inducer
EMT	Epithelial-mesenchymal transition
eNOS	Endothelial nitric oxide synthase
FACS	Fluorescence-activated cell sorting

FAK	Focal adhesion kinase
FdU	2’-fluoro-2’-deoxyuridine
Fizz1	Resistin-like molecule alpha 1
Flt1	Fms-related tyrosine kinase 1
FSH	Follicle-stimulating hormone
GFP	Green fluorescent protein
GM-CSF	Granulocyte macrophage colony-stimulating factor
GnRH	Gonadotropin-releasing hormone
H&E	Haematoxylin and eosin
HIF-1α	Hypoxia inducible factor-1α
HLA (e.g. HLA-DR)	Human leukocyte antigen (MHC)
HRP	Horseradish peroxidase
HSV	Herpes simplex virus
ICAM-1	Intercellular adhesion molecule-1
IFN (e.g. IFNγ)	Interferon
Ig	Immunoglobulin
IL (e.g. IL-1)	Interleukin
iNOS	Inducible nitric oxide synthase
IVF	*In vitro* fertilisation
KC	Keratinocyte chemoattractant
LAP	Latency-associated peptide
LH	Luteinising hormone
LPS	Lipopolysaccharide
Ly6C	Lymphocyte antigen 6C
M0	Unpolarised macrophage
M1	Classically activated macrophage
M2	Alternatively activated macrophage
MCP-1	Monocyte chemoattractant protein -1
M-CSF	Macrophage-colony stimulating factor
MDSC	Myeloid derived suppressor cell
MHC (e.g. MHC class II)	Major histocompatibility complex
MIF	Macrophage inhibitory factor
MIP (e.g. MIP-1α)	Macrophage-inflammatory protein

mL	Millilitre
MMP (e.g. MMP-1)	Matrix metalloproteinase
Msr1	Macrophage scavenger receptor 1
MUC1	Mucin 1, Cell surface associated
NC	Negative control
Neo	Neomycin
Neo^r	Neomycin resistance gene
NO	Nitric oxide
NOS (e.g. Nos2)	Nitric oxide synthase
OCT	Optimum cutting temperature compound
OSE	Ovarian surface epithelium
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PFA	Paraformaldehyde
PPARγ	Peroxisome proliferator-activated receptor γ
Prkdc	Protein kinase, DNA-activated, catalytic polypeptide
RANTES	Regulated on activation, normal T cell expressed and secreted (CCL-5)
rASRM	Revised American Fertility Society (AFS) score
SCID	Severe combined immunodeficiency
SR-A	Class A scavenger receptor
TAE buffer	Tris-acetate-EDTA buffer
TAM	Tumor-associated macrophage
TCDD	2, 3, 7, 8-Tetrachlorodibenzo-*p*-dioxin (dioxin)
TCR	T cell receptor
TEM	Tie2-expressing monocytes/macrophage
TGFB/Tgfb (e.g. TGFB1)	Transforming growth factor beta
TNFα	Tumor necrosis factor α
TUNEL	Terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling
uPA	Urokinase-type plasminogen activator
VEGF	Vascular endothelial growth factor
vWF	von Willebrand Factor

Abstract

Endometriosis is a gynaecological disease characterised by the growth of endometrial tissues at ectopic sites. Although this disease affects 10-15% of women worldwide, its pathogenesis is still poorly understood.

Human eutopic endometrial tissues were xenografted into two strains of immunodeficient (SCID) mice with 1) a null mutation for *Tgfb1* gene (*Tgfb1*-/-) and 2) macrophage-restricted expression of GFP (CSF-1R-eGFP/MacGreen). The resulting xenografts were collected at day 10 post-implantation for *Tgfb1*-/- mice and at days 4, 7, 10 and 14 in a time course study using MacGreen mice. Five xenografts collected from *Tgfb1*-/- mice were embedded in paraffin and were compared to *Tgfb1*+/+ tissues for macrophage number, myofibroblast staining (αSMA), proliferating cell number and blood vessel density. Another five xenografts from *Tgfb1*-/- mice and all xenografts from MacGreen mice were frozen in OCT to assess macrophage markers, MHC class II, iNOS, arginase 1 and scavenger receptor A, and collagen type 1.

Using *Tgfb1*-/-/SCID mice, we demonstrated that in the absence of host TGFB1, development of endometriosis-like lesions was suppressed and their glandular area was reduced. We also observed lower numbers of macrophages and a reduced density of myofibroblasts in the lesions from *Tgfb1*-/- mice.

Using MacGreen/SCID mice, we followed the changes in macrophage phenotypes during endometrial xenograft development. Macrophages were phenotypically diverse and pre-dominantly expressed the inflammatory markers MHC class II and iNOS at the early stage of disease development (days 4 and 7). The tissue repair marker, arginase

1, appeared later in lesion development at day 7. Meanwhile, another macrophage marker for tissue healing, scavenger receptor A was higher at day 14 than at the earlier time point. In addition, collagen type 1 staining increased throughout lesion development with its highest intensity evident at day 14.

In the absence of host TGFB1, the number of cells expressing MHC class II was significantly reduced at day 10 compared to the lesions from the wildtype controls. Similarly, iNOS-positive cells were decreased in lesions from *Tgfb1*-/- mice. The number of arginase 1-positive cells was not altered in the lesions from *Tgfb1*-/- mice, suggesting TGFB1 was not critical for arginase 1 expression in these tissues. The abundance of cells expressing scavenger receptor A was significantly reduced in lesions from *Tgfb1*-/- mice. A reduction in the collagen type 1 density was detected in the lesions which developed in a TGFB1-deficient environment.

These studies show that host-derived TGFB1 is critical during endometriosis-like lesion development. The presence of this cytokine altered the abundance of infiltrating macrophages and myofibroblasts. In a time course study, macrophages shifted from inflammatory phenotypes at the early stage to an alternatively activated phenotype associated with tissue healing at later stages. The secretion of collagen type 1 fibres was increased and this was associated with the transition to a remodelling macrophage population. Lesion development appeared to be interrupted when lesions were grown in a TGB1-deficient environment resulting in diminished lesion weight. The understanding behind macrophage activation in endometriotic lesions may provide new information on how endometriosis may be interrupted by moderating macrophage behaviours.

Declaration

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Publications and conference presentations

Publications and conference presentation arising from this thesis

1. Hull, M.L., Johan, M.Z., Hodge, W.L., Robertson, S.A., Ingman, W.V., *Host-Derived TGFB1 Deficiency Suppresses Lesion Development in a Mouse Model of Endometriosis*, The American Journal of Pathology, 2012, vol. 180 (3), p.880-887
2. Johan, M.Z., Ingman, W.V., Robertson, S.A., Hull, M.L., *Lesion Weight and Glandular Development are Suppressed in a TGFB1 Deficient Mouse Model of Endometriosis*, 41st Society of Reproductive Biology Annual Conference, 29th August-1st September 2010, Sydney, Australia (Abstract 133) – Oral presentation
3. Johan, M.Z., Ingman, W.V., Robertson, S.A., Hull, M.L., *Suppression of Endometriosis-like Lesion Development in a TGFB1-/- SCID Mouse Model of Endometriosis*, 11th World Congress on Endometriosis, 4th -7th September 2011, Montpellier, France (Abstract FC5-3) – Poster presentation
4. Johan, M.Z., Ingman, W.V., Robertson, S.A., Hull, M.L., *Activations Status of Macrophages in Lesions from a MacGreen/SCID Mouse Model of Endometriosis*, 60th Annual Meeting Society for Gynecologic Investigation, 20th-23rd March 2013, Orlando, Florida, USA (Abstract T-109) – Poster presentation
5. Johan, M.Z., Ingman, W.V., Robertson, S.A., Hull, M.L., *Altered macrophage phenotypes and collagen level in Tgfb1$^{-/-}$/SCID mouse model of endometriosis*, Society of Reproductive Biology Annual Conference, 25th -28th August 2013, Sydney, Australia (Abstract) – Oral presentation

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Chapter 1

1.1 Introduction

1.1.1 The pathogenesis of endometriosis

Endometriosis is a benign gynaecological disease characterised by the ectopic presence of endometrial-like stroma and glands pre-dominantly in the pelvic cavity, affecting 10-15% of women of reproductive age. The symptoms of endometriosis include pelvic pain, dysmenorrhea, dyspareunia and subfertility [1]. Endometriosis contributes to the subfertility of 45% of couples seeking IVF treatment, [2]. Economically, endometriosis causes productivity loss and is estimated to cost Australian healthcare systems $6 billion annually [3].

The quality of life of women who suffer endometriosis is also dramatically reduced, in terms of opportunities to work productively, social relationships and community participation. Symptomatic endometriosis is associated with mood swings, pain and fatigue which can lead to school and work absenteeism. Important academic and social experiences can be missed when teenagers take time off school. Furthermore, 26% of female school absenteeism is through menstrual-related health issues and 94% of this number is reported to be pain-related [4]. This pain could be associated with the presence of endometriosis as this disease is commonly undiagnosed in young girls.

A retrospective study in 1998 Milwaukee, Wisconsin, USA, reported that on average, there was a delay of 9.28 years from the early onset of symptoms to the actual of diagnosis of endometriosis in 4,000 women [5]. Absenteeism as the results of endometriosis symptoms will significantly reduce the ability of employed women to

develop their career. The mean number of sick days among 6,456 women in the US Army due to endometriosis-related symptoms was 15 days per annum [6]. In Australia, although persistent pain is recognised by the Disability Support Pension (DSP), women who suffer from endometriosis cannot file for disability support. This is despite the disease limits their daily activities including earning income for their family because the pain is considered not assessable (www.painmanagement.org.au). Given that endometriosis affects women on so many levels, any improvement in advancing treatments and diagnosis will have a major benefit for the sufferers to live a healthy normal life.

1.1.1.1 Origin of endometriosis

The origin of endometriosis is still poorly understood, however Sampson's theory [7] is the most widely accepted explanation. According to Sampson, endometrial fragments may pass retrogradely through the fallopian tubes to reach the peritoneal cavity where they implant onto the mesothelial layer of peritoneum. So far, little is known about the peritoneal-endometrial interaction and which factors mediate the implantation of the endometrial tissues into the mesothelial layer.

Given that up to 90% of women experience retrograde menstruation [8], Sampson's theory does not fully explain why only 10% of reproductive women develop endometriosis. Alternative theories have been proposed in attempts to explain the aetiology of endometriosis. The coelomic metaplasia theory by Meyer [9] suggests that metaplastic transformation of coelomic cells to endometrial-like cells can lead to endometriosis. This theory is difficult to prove scientifically since most of the supporting studies are observational and retrospective. The best clinical evidence of

this theory is that 7 men were reported to have endometriotic lesions due to hyperestrogenism on the bladder (n = 3) [10-12], urethral crest (n = 1) [13], lower abdominal wall (n = 1), [14] spermatic cord (n = 1) [15] and testis [16]. In the absence of a menstrual cycle, the incidence of male endometriosis is therefore independent of retrograde menstruation. This theory has been supported in Nakamura *et al*. study. Examination of ovarian endometriotic lesions by Nakamura and colleagues [17] revealed that these lesions consisted of three types of cells, suggesting a serial transformation from mesothelial cells into endometrial-like cells.

An *in vitro* study [18] in isolated human ovarian surface epithelium (OSE) showed that these cells were able to transform into luminal epithelium when exposed to estradiol at a concentration 10-times higher than in the peritoneal fluid. In the same study, co-culture of endometrial stroma with OSE resulted in the formation of an epithelial-mesenchymal arrangement, which suggested that the presence of endometrial stromal cells from retrograde menstruation induced the formation of an endometrial-like cell structure on the ovarian surface.

There are a few limitations with Matsuura's study in showing the metaplastic transformation of coelomic cells. Firstly, the study was only performed *in vitro* with only observational results. As there is no definitive marker for endometriotic lesion, the results obtained were not conclusive. Secondly, although human OSE is considered as part coelomic epithelium, the transformation only involved the arrangement of these cells into forming glandular-like structure with lumen. The use of epithelial marker, epithelial membrane antigen (MUC1) can also be questioned as this marker is not specific to either OSE or endometriotic epithelium, but is shared with ovarian epithelial tumor cells as well (reviewed by [19] and [20]). Thirdly, the higher concentration

of estrogen used (10-fold) *in vitro* study does not represent the peritoneal estrogen level, therefore unlikely to be the key factor that initiates transformation in endometriotic patients. However, this estrogen level abnormality was observed in the rare occurrence of male endometriosis, with most of these patients were on estrogen therapy to treat prostate cancer [12, 13].

Other alternative theories to Sampson's are Müllerian remnant and induction theories. According to the Müllerian remnant theory, endometriosis may arise from the remnants of the Müllerian embryonic tissues [21]. It is thought that these remnants can undergo a metaplastic transformation into endometrial tissues which potentially can be induced by menstrual debris. The induction theory proposes that endometrial fragments of retrograde menstruation release growth factors in the peritoneal cavity that can induce the transformation of the mesothelial layer into the endometrial-like tissues [22]. The induction theory is not inconsistent with Sampson's theory. Both implantation and induction theories share the same attribute: retrograde menstruation may result in endometriosis.

Both theories however, fail to explain why only 10% of reproductive women develop endometriosis when 90% of them experience retrograde menstruation. In addition, the rare endometriosis cases reported in seven men do not support retrograde menstruation as being the only reason of endometriosis incidence. In the reported cases of males with endometriosis [14, 16], abnormally higher estrogen levels and other possible environmental mediators could promote the degenerated Müllerian remnants to become endometriosis-like structure. Thus, the development of endometriosis could be caused by more than a single factor.

1.1.1.2 Other theories of endometriosis pathogenesis

Advancement in scientific knowledge allows an in-depth examination on how endometriosis may arise. In addition to theories mentioned above, four factors have been linked to the pathogenesis of endometriosis. These factors are environment (dioxin exposure) [23], estrogen [24], genetic [25] and immunology [26, 27]. There are a considerable number of studies that explore those risk factors, but no definitive roles for these factors have been identified.

2, 3, 7, 8-Tetrachlorodibenzo-*p*-dioxin (TCDD, also referred to as dioxin in literatures) is a polychlorinated-*p*-dioxin. This toxicant has been listed as one of the environmental factors contributing to endometriosis development as demonstrated in Rhesus monkeys (*Macaca mulatta*) that had received dioxin-containing diet for 10 years [28]. Additionally, endometrial stromal cells exposed to estrogen and dioxin showed an increased invasiveness as measured by the invasion assay [29]. Dioxin exposure may also increase the prevalence of endometriosis by disrupting the activity of immune cells, particularly macrophages. Dioxin treatment has been suggested to promote macrophage tolerance towards ectopic endometrial tissues in cell culture by inducing chemokines RANTES (regulated on activation, normal T cell expressed and secreted) production and secretion [30].

Endometriosis is well established as an estrogen-dependent disease. Aromatase is the primary enzyme in the glandular tissues that converts androstenedione and testosterone to estrogen (reviewed by [31]). The expression of aromatase was reported in the lesions and the endometrium of women with endometriosis. Aromatase mRNA expression was detected in the endometriotic implants (n = 17) and eutopic

endometrium (n = 11) from diseased patients, but not detectable in the endometriosis-free paired tissues and endometrium (n = 7) [32]. Blockades of estrogen activity by inhibition of aromatase reduced the endometriosis lesion volume in a baboon model (*Papio anubis*) [33]. Estrogen and progesterone exhibit a synergistic effect on peritoneal macrophages with a two-fold increase in VEGF production than in untreated control [34].

Genetic polymorphism of reproductive hormones may also predispose women to the risk of developing endometriosis. Estrogen receptor β [35], luteinising hormone β and progesterone receptor gene polymorphisms are among the gonadotropic hormones associated with endometriosis [36, 37]. Other genetic polymorphisms that have been explored include vascular endothelial growth factor (*VEGF*) and cytochrome (CYP) genes. *VEGF*-specific 936T allele was found to be differentially expressed in women with endometriosis compared to disease-free controls [38] in a Spanish study. However, in a North Chinese-based population study, 936C/T allele was not statistically different between women with endometriosis and controls, but other alleles, 1154G/A and 2578C/A did differ in frequencies between two groups [39]. These studies only compared gene polymorphisms within a single race population, making the results significantly varied from one to another. A large scale study involving multiple recruitment centres over different countries is required to better elucidate the genetic polymorphisms which might predispose women to endometriosis.

Cytochrome P450-associated gene, *CYP19* may also be involved in the pathogenesis of endometriosis. In an Italian-based study, *CYP19* gene polymorphism was found to be significantly associated with endometriosis prevalence [40], however in a studied Japanese population this gene was found to be weakly associated with endometriosis [41]. A genome-wide association study has identified some loci that are associated with

the severity of endometriosis [42], however the data were not consistent across population and only limited to the examined ethnic background, which were mainly European and Japanese. In addition to inconsistency and limited racial variation, genetic study also often has low sample sizes and the results have limited direct evidence which make these contributing factors remain controversial and indefinitive [43].

The association between dioxin exposure and genetic factor to the incidence of endometriosis so far is less studied due to the cost involved and the difficulty in finding representative samples. Estrogenic and immunological connections to endometriosis therefore have become a major interest to be explored scientifically and clinically. Natural killer cells, neutrophils, T cells and macrophages are among the immune cells previously found to be associated with endometriosis pathology.

1.1.2 Current treatments for endometriosis

1.1.2.1 Medical therapy

Current management options for patients with endometriosis include hormonal treatments, such as Gonadotropin-releasing hormone (GnRH) agonists, oral contraceptives, danazol and progestins. These endometriosis treatments relieve some of the symptoms but are not curative and can cause undesired side effects such as bone density loss, headaches and increase the risk of developing breast cancer [44].

GnRH agonists cause a flare of FSH and LH release followed by a prolonged suppression. The absence of FSH then causes the failure of egg development and estrogen production. Low estrogen levels inhibit the endometrial tissue growth and

which includes the growth of endometriotic lesions. Treatment with GnRH agonists for endometriosis is normally limited to only six months to minimise the undesired side effects of extended hypoestrogenism, which includes bone loss (reviewed by [45]). Add-back therapy, in which patients receive low doses of steroid hormones, either estrogen, progestins or tibolone along with GnRH agonists, minimises osteoporosis and allows for a longer GnRH regime [46, 47]. Cultured eutopic and ectopic endometrial cells incubated with GnRH agonists had a reduced level of proliferation. In women undergoing 3-6 months of GnRH agonist treatment before surgery to treat endometrioma (n = 25), adenomyosis (n = 20) and uterine myoma (n = 36); a significant decrease in inflammation and angiogenesis was seen in the endometrial tissues (all subjects), myometrium (adenomyosis only) and lesions (endometrioma and uterine myoma) compared to control patients (n = 20, 15 and 20, respectively) [48].

Combined oral contraceptives (COCs) limit endometrial growth and thus the growth of endometriotic tissues which may provide symptomatic benefits. Administering the COC continuously without inducing withdrawal bleed can induce amenorrhea minimising retrograde menstruation and the cyclical hormonal effects on endometriotic tissues that results in tissue breakdown and pain. The COC does not cure endometriosis, but it has been reported to alleviate the dysmenorrhea and non-menstrual pain linked to endometriosis [49]. Side effects associated with the COC include headaches, irregular vaginal bleeding and depression.

Danazol is an androgen which inhibits aromatase activity thereby reducing estrogen production. Danazol has been shown to act locally to improve endometriosis symptoms [50]. Vaginal delivery of danazol has become a promising method of alleviating endometriosis-associated pelvic pain upon completion of 6-12 months

treatment [51]. Danazol still has side effects due its androgenic and anti-estrogenic properties which may include weight gain, voice deepening and acne (reviewed by [52]).

Endometriotic lesions can be surgically removed; although disease recurrence is observed in 18% of women 6 months post-surgery [53]. Laparoscopy is considered the best way to diagnose and excise endometriotic lesions. This surgery is less invasive and leads to shorter hospital stays compared to an open procedure, e.g. laparotomy [54, 55]. However the waiting list for laparoscopy after a consultation with gynaecologist may take up to 12 months, according to Endometriosis Care Centre of Australia (ECCA) (www.ecca.com.au).

1.1.2.2 Conclusion

Women with endometriosis suffer disabling symptoms such as pelvic pain and dysmenorrhea, which limit their daily activities. Understanding the pathophysiology of endometriosis will be valuable in providing an insight into the aetiology of the disease which can then be employed to create a new treatment.

1.1.3 Ectopic endometrial implantation in endometriosis

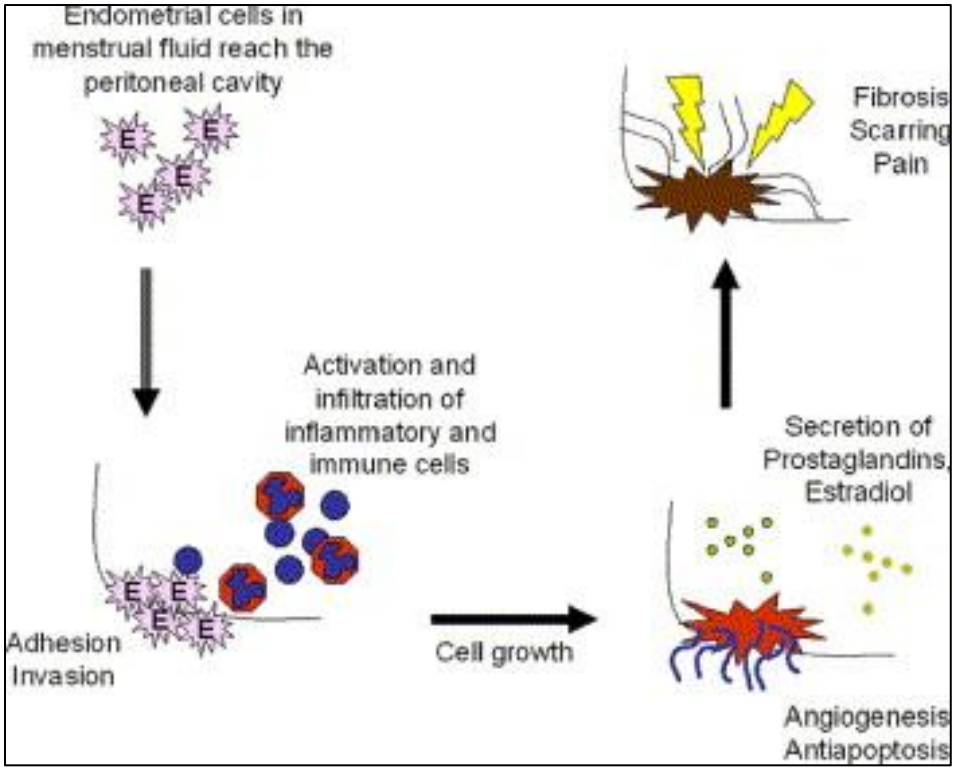


Figure 1 A: Theoretical mechanisms of endometriosis pathogenesis.

Briefly, the formation of endometriotic lesion requires several steps, which includes adhesion, invasion and angiogenesis. The aberrant expression of anti-apoptosis molecules by the endometriotic lesion may protect the cells from being cleared. From Flores *et al*. [56].

An important aspect of disease progression is the interaction between endometrial tissues and the site of implantation, where ectopic endometrial tissues grow into the peritoneal layer [56]. A number of animal studies have helped to model the events taking place in endometriosis development. *In vitro* culture of endometrial cells suggested that endometriosis is initiated by the adhesion of these cells into the mesothelial layer lining the peritoneal cavity [57]. In a primary co-culture study, endometrial stromal and epithelial cells had high affinity to bind to the mesothelial cells of peritoneal layer [58]. The shed endometrial fragments from retrograde menstrual fluid are not supported by a vasculature; therefore some of the cells become necrotic in the peritoneal cavity as

demonstrated by an *in vitro* culture of primary cells [59]. These necrotic cells attract an inflammatory response, initiated by the infiltration of neutrophils and followed by monocytes (reviewed in [60]). Ingestion of necrotic cells by macrophages increased the production of TGFB, but reduced the production of inflammatory-associated cytokines, including IL-1β and TNFα [61]. Macrophages and TGFB therefore could be important in initiation of endometriosis.

The adhesion and invasion of the endometrial tissues require an extracellular matrix (ECM) remodelling stage, which involves tissue breakdown followed by the proliferation of glands and generation of the extracellular matrix [56]. Early evidence shows that VEGF-A [62, 63], CYR61 [64], urokinase plasminogen activator (uPA) and matrix metalloproteinase-3 (MMP-3) [65] are among the ECM-related proteins that are elevated in the tissues or peritoneal fluid of endometriotic women. Thus, it appears that tissue degradation and remodelling take place during endometriosis establishment. Following the attachment of ectopic endometrial tissues to the mesothelium, a vascular supply then develops to provide nutrients and oxygen to the healing tissues, as reported in a mouse model [66]. This step allows reorganisation and growth of endometrial tissues at these ectopic sites.

As described above, the presence of necrotic and apoptotic cells in endometrial tissues attracts neutrophils and macrophages to the peritoneal environment. For example, in a baboon model, injection of menstrual effluent which contained endometrial tissues increased the total number of peritoneal leukocytes and also the number of leukocytes positive for TNFα, TGFB1 and HLA-DR [67]. Following inflammatory activation, macrophage digestion of the necrotic and apoptotic cells further inhibits the production of pro-inflammatory cytokines. A culture of mouse macrophages , J774

cells after digestion of apoptotic Jurkat cells showed reduction in inflammatory cytokines TNFα and chemokines, including macrophage-inflammatory protein 2 (MIP-2), MIP-1α and keratinocyte chemoatrractant (KC) [68]. This reduction in pro-inflammatory cytokines and chemokines production was reportedly to be mediated by TGFB1. In addition, macrophages are suggested to be the major source of neutrophil chemoattractants and the reduction in cytokine production may impair the inflammatory cell recruitment into the inflamed tissues [68]. A subsequent reduction in the production of pro-inflammatory cytokines and neutrophil chemoattractants, and an increase in anti-inflammatory cytokines cause the inflammatory response to subside and the extracellular matrix to remodel.

Most of the data highlighting the steps involved in endometriosis development were obtained from *in vitro* and animal model studies. The important caveat of these studies is they have not been tested in humans and how these results could translate into human disease is still unknown.

1.1.3.1 Escaping immune surveillance

In addition to necrotic cells, the presence of endometrial tissues in the peritoneal cavity is known to provoke an inflammatory response. The concentration of cytokines, such as TNFα and TGFB1, and also peritoneal leukocyte numbers were significantly increased following peritoneal injection of endometrial tissues [67]. Endometrial tissues must escape immune surveillance in order to survive. An aberrant peritoneal immune response has been suggested as a predisposing factor in the pathogenesis of endometriosis. Although many studies showed increase macrophage number in peritoneal fluid from women with endometriosis, Chuang *et al*. [69] showed that many of

these macrophages were deficient in CD36 marker and had reduced phagocytic activity. Similarly, the functional capacity of peritoneal natural killer cells [70] to exhibit cytotoxicity against endometrial cells was also defective in women with endometriosis. This suggests that there might be a reduction in the time to clear the endometrial fragments from the peritoneal cavity and this may allow ectopic endometrial tissue to implant.

In addition to alterations in immune cell function, cytokines appear to promote immune tolerance towards the ectopic endometrial tissues. Interleukin-8 (IL-8) enhances endometriotic cell proliferation [71] and promotes an immune-tolerant environment for endometrial tissues. IL-8 was reported to increase the apoptotic rate of T cells *in vivo* [72]. Thus IL-8 could be indirectly suppresses immune system activity, promoting immune tolerance towards ectopic endometrial tissues.

Ectopic endometrial tissues are also characterised by their anti-apoptotic protection mechanisms, making them less vulnerable to clearance by immune cells. In women with endometriosis, eutopic endometrial tissues have lower TUNEL-positive cells in both the stromal and epithelial compartments compared to controls, and a lower expression level of the pro-apoptotic *BAX* gene only in the late secretory phase [73]. Meresman *et al*. [74] also found a reduction in apoptosis in eutopic endometrium of endometriotic women, however in their study it was not cycle phase dependent. Furthermore, endometriosis did not appear to alter apoptosis-associated BAX and BCL-2 protein levels.

In summary, endometriosis could be caused by the abnormalities in the immune cells, cytokines, and apoptosis in clearing the self-tissues. These abnormalities might

together provide conditions that permit the attachment and growth of endometrial tissues at the ectopic sites. Targeting molecules responsible for these events could provide a new therapeutic strategy to treat endometriosis, however clearly a better understanding of the precise underlying immune changes is required to achieve this.

1.1.3.2 Formation of blood vessels

The formation of blood vessels is important for the survival of ectopic endometrial tissues. Many factors have been shown to be important for vascular development in the endometriotic lesions. The mechanisms behind this process however are still poorly understood and need further research. Cytokine such as vascular endothelial growth factor (VEGF) has been reported in the literature to be important in mediating blood vessel development in the endometriotic lesions [75]. Higher VEGF has been reported in the peritoneal fluid of women with endometriosis (n = 62) compared to controls (n = 66) [76]. The application of neutralising antibody against human VEGF-A significantly reduced the number of mice that developed endometriosis like lesions after grafted with human endometrial tissues [66]. Moreover in this study, exposure of the mouse model to the inhibitor of VEGF receptor, Flt1, significantly reduced the number of endothelial cells and lesion formation compared to the control.

The source of capillary buds in the formation of blood vessels is unknown. The shed endometrial tissues could have progenitor endothelial cells, which upon stimulation get recruited to form a new vascular network. Blood vessels from the peritoneal layer could also infiltrate into the ectopic endometrial tissues following tissue adherence and attachment.

Macrophages appear to have critical roles in this vascular development. A study by McLaren *et al*. showed macrophages are one of the sources of VEGF in peritoneal fluid. VEGF secretion by primary human peritoneal macrophages was enhanced in the presence of estradiol and progesterone [34]. The macrophage subtype expressing the pro-angiogenicTie2+ marker were identified to play important roles for assisting neovascularisation and establishment of ectopic endometrial tissues [77]. This series of events are similar to the tissue injury and repair mechanisms [78]. Many factors have been implicated to be involved in the ECM degradation and remodelling; among them are TGFB1, macrophages and fibroblasts (myofibroblast).

1.1.4 Macrophage activity in endometriosis

1.1.4.1 Inflammatory macrophages and initiation of endometriosis

Macrophages are white blood cells of myeloid lineage which participate in the innate immune system as well as in the adaptive immune response. In response to antigenic stimuli, monocytes from peripheral blood differentiate into resident tissue macrophages. Macrophages phagocytose these antigens, process and present them to T cells via the Class II major histocompatibility complex (MHC) receptor [79]. Antigen on MHC class II receptor is recognised by the T cell receptor (TCR) on T cells. Recognition of these presented antigens by T helper (CD4+) or T cytotoxic (CD8+) leads to an adaptive immune response toward these specific antigens.

Using mouse models of endometriosis, the presence of ectopic endometrial tissue was shown to trigger an inflammatory reaction which includes the initial recruitments of neutrophils followed by macrophages [80-82]. A large number of neutrophils and macrophages were recruited in the early stages of endometriosis development. In a

syngeneic mouse model of endometriosis, there was a dramatic increase in neutrophil and F4/80-macrophage numbers from day 2 to day 5 in the lesion [81]. Similarly in baboons (*Papio anubis*, *Papio cynophalus*), following intrapelvic injections of endometrial tissues, there was an increase in the numbers of leukocytes, TNFα-positive cells, T lymphocyte-specific CD3-positive cells and HLA-DR-positive cells [67]. The levels of macrophage inflammatory protein (MIP) chemokine increased in association with the influx of these immune cells [81]. Prolonged neutrophil activity can cause cell damage due to the release of microbicides, which include reactive oxygen species (reviewed by [83]). It has also been suggested in a cervical cancer model and other systems that recruited macrophages are capable of inhibiting further neutrophil chemotaxis, resulting in a rapid resolution of inflammation [84], which could apply to endometriosis [85].

In women with endometriosis it has been pointed out that some of these activated macrophages had reduced phagocytic ability [69, 86]. These macrophages might promote endometriosis development, as opposed to clearing the ectopic endometrial tissues. As will be explained later on, macrophage plasticity could be one the factors that may account for this anti-inflammatory property.

1.1.4.2 Macrophage recruitment into endometriosis tissue

Macrophage infiltration into an endometriotic lesion is one of the key events manifesting macrophage roles in disease progression. Macrophages displaying different activation markers, such as CD68, CD163 and CD206 were present in the endometriotic lesions [82]. However, there is a lack of study describing the initial recruitment of macrophages into the lesion. Wound healing and graft studies reported

the influx of Ly6C-expressing cells into the tissue representing the early stage of macrophage activity [87]. As this has never been reported in endometriosis, this could be an early initiation of macrophage infiltration into endometriotic lesion. Ly6C is a myeloid differentiation antigen expressed on neutrophils, eosinophils and monocytes. In a peritoneal foreign body response, expression of this marker was short lived and co-expressed by macrophages [87]. Given Ly6C expression is specific to murine cells and its equivalent has not been identified in human cells, more studies are needed to provide the link between this marker and endometriosis.

Human tissues and mouse models provided evidence that the presence of endometrial tissues ectopically could be the necessary step for macrophage recruitment at the early stage of the disease. Monocyte chemotactic protein-1 (MCP-1) is the critical chemokine for monocyte recruitment into the peritoneal cavity of a peritonitis mouse model [88]. Macrophage-derived MCP-1 was significantly higher within 4 hours when mouse endometrial stromal and epithelial cells were intraperitoneally injected into experimental mice compared to control groups and later time points [89]. The initial increase of MCP-1 in this mouse model suggests that this chemokine may play important role in early monocyte chemotaxis following an encounter with ectopic endometrial tissues. MCP-1 protein was found at higher concentration in the peritoneal fluid of women with endometriosis, compared to controls [90] and the expression was localised in both glandular epithelium and stroma of an endometriotic lesion [91]. In addition to MCP-1, RANTES has also been suggested to be involved in the macrophage chemotaxis into the lesions. Peritoneal fluid from women with moderate to severe endometriosis had increased chemotactic property than disease-free control and this was mediated by RANTES [92]. Therefore there is a clear indication of the influx

of inflammatory-type macrophages occurs in the early stage of endometriosis and their recruitment is mediated by chemokines.

1.1.4.3 Macrophage plasticity

It is known that macrophages have functional plasticity, with the capability to fluctuate between different phenotypes depending on local microenvironmental signals. Non-polarised macrophages can be stimulated to have phagocytic phenotypes. Immature and undifferentiated macrophages, often referred to "M0" in literature can be induced into an inflammatory phenotype, designated as "M1". Murine M0 macrophages are derived from bone marrow precursors [82]. Human resting macrophages (M0) can be derived *in vitro* from M-CSF (macrophage colony stimulating factor)-treated primary monocytes [93, 94]. M0 cells are differentiated to M1 phenotype by culturing them in IFNγ for mouse macrophages [82], or for human cells, induced by either IL-1β, TNFα or LPS [95] or a combination of IFNγ and LPS [94] or a combination of LPS and polyinosinic acid [93]. This process is called classic macrophage activation. M1 macrophages are characterised experimentally by cytokine secretion (IL-1β, IL-6, TNFα and IFNα) or surface marker expression (CD86 and CD40) [93]. These pro-inflammatory macrophages are also characterised by the expression of MHC class II and inducible NO synthase (iNOS) [82, 96]. Classically activated macrophages attack intracellular pathogens by producing nitric oxide which causes oxidative damage and engulfing these pathogens by endocytosis.

Mouse macrophages M0 also can be differentiated *in vitro* to M2 phenotype by culturing the cells in a combination of M-CSF and IL-10 [82]. Meanwhile in human cell culture, M2 can be induced from M0 by exposure to IL-4 and IL-13 [94, 97]. This M2

subtype is termed as alternatively activated macrophages. M2 macrophages release growth factors which stimulate proliferation, differentiation and tissue growth [98, 99]. These polarised M2 macrophages are differentiated from M1 inflammatory macrophages by their surface marker expression and their cytokine profiles [82, 93].

Among markers expressed by the alternatively activated macrophages are arginase 1, scavenger receptor (SR), IL-4 receptor, peroxisome proliferator-activated receptor gamma (PPARγ), resistin-like molecule alpha 1 (Fizz1), mannose receptor (CD206) and chinitase-like molecule (Ym1/Ym2) (reviewed by [96]). Arginase 1 is believed to be a marker for the alternatively activated macrophage phenotype and has been suggested to be involved in collagen synthesis; although its exact role remains unknown [100]. Another role of arginase 1 as reported in helminth infection models is promoting Th2-mediated inflammation and fibrosis as measured by total collagen staining [101].

There are several subtypes of M2 macrophages described in human and mouse cells, namely M2a, M2b and M2c, as determined by the combination of inducing agents and also the macrophage cytokine expression. In mouse, these subtypes have been shown to be present in a remodelling tissue such as in a burn injury model. M2a was present immediately after a burn injury followed by M2c, identified by CCL17 and CXCL13, respectively and both types expressed mannose receptor [102]. M2b on the other hand, was characterised by the expression of CCL1 and IL-10, and did not appear until day 15 of injury and persisted longer than both subtypes. Meanwhile, for human cells, M2a subset was derived from M0 monocytes after exposure to IL-4 or IL-13 [93, 94], M2b was obtained by a combination of immune complexes (IC) and toll-like receptor (TLR) agonists or IL-1R [103], while M2c was derived after IL-10, TGFB or glucocorticoid exposure [93]. In humans, M2c macrophages exert a more potent anti-inflammatory

response and regulation of tissue remodelling (reviewed by [97]). M2a and M2c are characterised by the expression of arginase 1, mannose receptors and scavenger receptors. Meanwhile M2b is identified by the surface marker IL-10 and CCL1 [103, 104]. In endometriosis however, there are limited data on the different subsets of M2 macrophages and how they may influence disease progression.

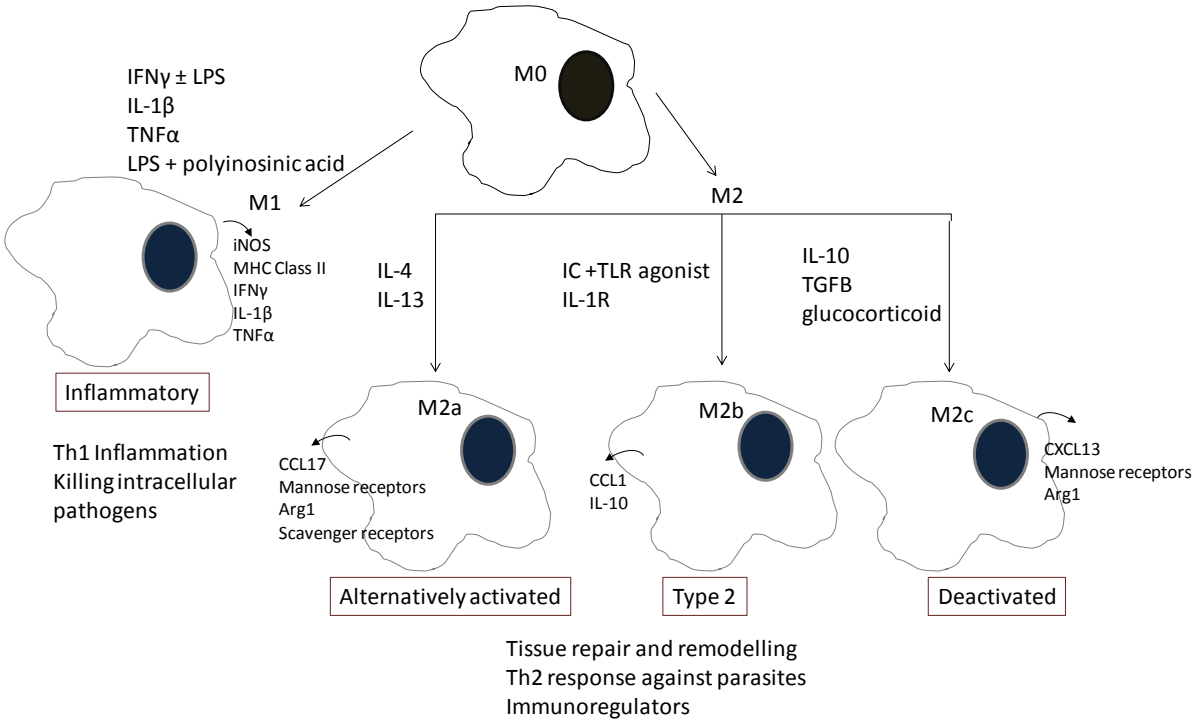


Figure 1 B: A simplistic view of macrophage activation and their associated functions.

In brief, M0 macrophages can be differentiated into multiple subtypes for different functions after exposure to different microenvironments. Macrophages with M1 phenotypes exhibit pro-inflammatory properties and are effective in killing intracellular microbes. Among markers used to identify this population of macrophages are iNOS, MHC class II and pro-inflammatory cytokines, such as TNFα and IL-1β. On the other hand, M2 macrophages are less inflammatory and involved in tissue repair. These macrophages express markers which include arginase 1, mannose receptors and scavenger receptors. Abbreviations: IFN, interferon; IL, interleukin; TNF, tumor necrosis factor; iNOS, inducible nitric oxide synthase; Arg1, arginase 1; CCL, chemokines (C-C motif) ligand; IC, immune complexes; TLR, Toll-like receptor; IL-1R, interleukin 1 receptor; TGF, transforming growth factor; CXCL, chemokines (C-X-C motif) ligand. (References: [93, 94, 97, 102, 103])

More than one marker is needed to determine the phenotypes of polarised macrophages. Lolmede *et al*. [93] used scavenger receptors, CD36 and CD163 to assess

for the markers of M2 macrophages. In terms of secreted cytokines and growth factors, M1 was more potent over M2a and M2c in producing IL-6, TNFα, IFNγ, IL-1β, IL-12, nitrites and VEGF. Lolmede and colleagues [93] also found only IL-10 was secreted at a significantly higher level by M2c macrophages.

M1 macrophages are present following cytokine stimulation or elicitation with microbial product, such as LPS. In endometriosis development, M1 macrophages could be the major immune cell during the early stage of endometriosis. For example, D'Hooghe *et al*. demonstrated that intrapelvic injection of endometrial tissues into the baboons, led to an increase in inflammatory cells [67]. However the leukocytes were not clearly specified as M1 macrophages in the D'Hooghe study, because the markers were not well established in the literature at the time. Nevertheless M1 macrophages could be a macrophage subtype in the peritoneal cavity, since a high percentage of peritoneal cells are positive for the MHC class II molecule, human leukocyte antigen (HLA-DR) and inflammatory cytokine, TNFα after endometriosis induction.

In endometriosis, macrophage activation has not been well documented. The mechanism by which M1 macrophages may transform into the M2 phenotype during endometriosis, is largely unknown. There is limited published evidence showing this *in vivo* of mouse and primate models. Evidence of M2 macrophages in endometriosis lesions which was published by Smith *et al*. [105], showed that macrophages expressing the M2 macrophage marker, CD163 (scavenger receptor) were 1.8-fold higher in the endometriosis lesions compared to eutopic endometrium from Rhesus Macaque monkeys. This study however only looked at a tissue bank from already deceased animals, thus it only had limited data pertaining to the stage of endometriosis lesion development.

As demonstrated in a mouse model of endometriosis, when inflammatory macrophages predominated in the peritoneal cavity, fewer endometriosis-like lesions developed and these lesions were much smaller than the control group [106]. In contrast, the less inflammatory macrophage subtype (M2) [82], favoured the establishment of endometriosis. Signals from the ectopic tissues or regression in the inflammatory cytokine environment are the possible reasons for this change in macrophages.

Halme *et al*. [107] in 1987, suggested that macrophages recruited into and surrounding the lesion can further differentiate into different phenotypes, which could be important for endometriotic tissue maintenance. In contrast to lesions from an animal model, human lesions can only be evaluated at one point in time, which therefore makes it difficult to study the progression of macrophage phenotypes.

HLA-DR antigen, a marker for antigen presenting cells, was identified in 11 endometriotic lesion biopsies [108]. Bacci *et al*. [82] on the other hand, showed the preferential expression of alternative activated markers, scavenger receptors, CD206 and CD163 in endometriotic lesions (n = 28) and peritoneum, but not disease-free peritoneum. M2-specific marker, PPARγ was also present in endometriotic lesions and expressed by glandular epithelial and stromal cells, suggesting this marker is not unique to macrophages [109]. Both studies however did not localise M1-specific markers, such as CD86 and MHC class II in the lesions, as these markers could also present in these tissues. Heterogeneity of endometriotic lesions, due to disease severity, menstrual cycle and other gynaecological disorders can also alter the macrophage profile in lesions, makes studying macrophage phenotype difficult in these tissues.

Peritoneal fluid collected during laparoscopy could provide an insight into how microenvironments may influence macrophage phenotypes. Higher expressions of IL-6, IL-8 and MCP-1 were reported in the peritoneal fluid of women with endometriosis (n = 21) compared to the disease-free patients (n = 23) [110]. While IL-6 and IL-8 have been shown to be highly expressed by murine M1 macrophages [93], MCP-1 was significantly higher in the CD14-positive peripheral macrophages of women with advanced stage of endometriosis (stage 3 and 4) compared to the mild stage (stage 1 and 2) [111]. Lolmede *et al*. [93] also showed the average MCP-1 level secreted by murine M2 macrophages to be higher than M1 and M2a subsets, although this is not statistically significant.

The classification of macrophages into M1 and M2 types is a way to simplify the heterogeneity of macrophages. However, in endometriosis it is difficult to determine the early and late stage as the macrophage phenotype could subtly change depending on tissues and microenvironment. One of the hypotheses in this study is that macrophage activity is dynamic as endometriosis develops. This can be assessed in mouse model but is difficult to evaluate in women.

The current understanding about the phenotypic changes of macrophage in the development of endometriosis is very limited. It has been functionally tested by Bacci and colleagues [82] but the activation markers have not been described over time. This gap will be addressed in this project by using the mouse model of endometriosis, evaluating tissues at different time points post-endometriosis induction.

1.1.4.4 Importance of macrophage in tissue remodelling

Macrophages have been demonstrated to be important during the early establishment and also for vascularisation of the ectopic tissues in the syngeneic mouse model [77, 82].

Similarly, in a wound healing model, selective macrophage depletion resulted in a massive reduction of collagen synthesis, re-epithelialisation, angiogenesis and cellular proliferation [112].

The importance of macrophages during extracellular matrix remodelling has been established in wound healing experiments. Tumor associated macrophage (TAM) activation is essential for the initiation of neo-angiogenesis [113] and a source of pro-angiogenic molecules, such as VEGF. Similarly, Capobianco *et al*. [77] showed pro-angiogenic macrophages that were Tie-2 positive were critical for normal vascularisation in ectopic endometrial growth. Mice deficient in these macrophages demonstrated an increase in apoptotic endothelial cells and a reduced vascular density [77].

During the initiation of tissue repair process, inflammatory macrophages recruited to the site of injury downregulate their production of NO, moderating their inflammatory activity to reduce tissue damage. This was demonstrated in a mouse model of skeletal injury using mice which cells expressing chemokine receptor CX_3CR1 were labelled with GFP ($CX_3CR1^{GFP/+}$). All circulating macrophages positive for F4/80 and CD11b were $CX_3CR1^{GFP/+}$ cells [114]. Induction of skeletal injury in this $CX_3CR1^{GFP/+}$ mouse model resulted in the recruitment of CX_3CR1^{lo} and $Ly6C^+$ cell population which noted for their secretion of pro-inflammatory cytokines IL-1β and TNFα [115]. The authors found that this inflammatory ($CX_3CR1^{lo}Ly6C^+$) subset peaked at 24 hours and decreased by day 2, replaced by anti-inflammatory ($CX_3CR1^{hi}Ly6C^-$) monocytes/macrophages, which increased until day 7. This population of cells expressed a higher amount of TGFB1 and IL-10 than the initially recruited cells. TGFB1 and IL-10 are typically associated with an anti-inflammatory macrophage phenotype. The phenotype of macrophages appeared

to be dynamically altered throughout tissue repair, presumably reflecting the adjustment in their functions as the skeletal muscle undergoes tissue repair and recovery [115].

There are subtle differences between tumor-associated macrophages (TAMs) and Tie2-expressing macrophages/monocytes (TEMs). Microarray study demonstrated murine TAMS have increased in expressions of alternatively activated marker-related mRNA, *Arg1* (2.2-fold increase) and scavenger receptors, *Cd163* (15.6) and *Msr2* (3.5) and reduced pro-inflammatory markers, *Nos2*, *Tnf* and *Cxl10* (3.9-, 3.1- and 2.8-fold decrease) compared to TEMs [116]. These data suggest that TEMs have a greater ability to support the angiogenesis associated with tissue remodelling, than TAMs.

1.1.4.5 Conditional macrophage ablation studies

The functional roles of macrophages have been extensively studied *in vivo* and *in vitro* primarily using complete and conditional depletion mouse models. Macrophage ablation has been achieved using 1) macrophages expressing diphtheria toxin (DT) receptor; 2) specific antibodies against macrophage receptor (e.g. anti-F4/80), 3) clodronate-containing liposomes, 4) loxP recombinant insert and 5) transgenic knockout (e.g. *Csf*$1^{op/op}$ and F4/$80^{-/-}$) [117-121].

Of these, techniques 1 and 3 successfully depleted 70-90% of the macrophage population [118, 122], however technique 3 is not cell specific and other cells phagocytosing the liposomes get depleted as well. Bacci *et al*. [82] had used anti-F4/80 and clodronate-containing liposomes to specifically knock out macrophages in studying endometriosis. The depletion of macrophage reduced the weight of ectopic endometrial tissue and the number of endothelial cells responsible for blood vessel

formation. An application of anti-F4/80 *in vitro* on macrophage culture reduced *Tnfa* and *Il12* mRNA expressions and the secretion of IFNγ in response to heat-mediated *Listeria monocytogenes* [123]. Cre/loxP system targeting lysozyme promoter in the myeloid cells achieved 83-98% depletion of mature macrophages [117]. In the peritoneal cavity, the number of F4/80-positive cells was reduced by 30.5% in the colony stimulating factor 1-knockout (*Csf*$1^{op/op}$) compared to the wildtype mice [124]. F4/80-knockout mice on the other hand did not exhibit any macrophage abnormality (normal CD11b and SR-A expressions), although peripheral tolerance was altered in these knockout animals [121].

1.1.5 Myofibroblast differentiation during endometriosis establishment

1.1.5.1 The origins and characteristics of myofibroblasts

Myofibroblasts are mesenchymal cells with the features of fibroblasts and smooth muscle cells [125]. The name "myo-"means muscle. Alpha smooth muscle actin (αSMA) is the primary marker used to identify myofibroblasts, however other markers are also used to characterise them. These include fibroblast specific protein-1 (Fsp-1), collagen $1\alpha_1$ and fibronectin [126]. As the majority of these myofibroblasts are of mesenchymal origin, these cells could also express vimentin [127] and the smooth muscle marker, desmin [128]. Myofibroblasts have been implicated as one of the major contributors to tumor metastasis and fibrosis [129]. During tumor metastasis, myofibroblasts play an important role at the invasion stage as the increased motility of these cells is vital to the invasion of the extracellular matrix.

1.1.5.2 Fibroblast-to-myofibroblast differentiation is mediated by growth factors

Fibroblasts can transdifferentiate into myofibroblasts via cell-cell contact mechanisms or under the influence of growth factors, such as TGFB1 [130, 131] or granulocyte-macrophage colony-stimulating-factor (GM-CSF); with the latter requiring macrophage recruitment prior to the appearance of the alpha smooth muscle actin-positive cells [132]. The precise role of these recruited macrophages in transforming the fibroblast remains unknown. GM-CSF appears to prime these macrophages to release growth factors required to stimulate fibroblasts to myofibroblast transformation.

TGFB1-mediated transition from fibroblasts to myofibroblasts has several mechanistic pathways which include hyaluronan (HA) and its receptor, CD44 and Rho/Rok. *In vitro* HA inhibition significantly reduced the αSMA expression of TGFB1-stimulated fibroblasts [130]. Acharya *et al*. [127] also showed that CD44 is required for the TGFB1-mediated migration of fibroblasts (7-10% were αSMA-positive) in the wound healing process. Additionally, in the tissue repair process, most of the fibroblasts were remodelled to become myofibroblasts [133]. Therefore, CD44 expression appears to be important in both fibroblast migration and differentiation into myofibroblasts.

Several *in vitro* studies have shown that cell-cell contact has a critical role transforming myofibroblasts from fibroblasts. The lack of cellular contacts due to the cell injury activated Rho/ROK pathway, which subsequently enhanced nuclear translocation of αSMA-related transcription factors, by activating *cis*-promoter elements of αSMA [134]. This mechanism also directly promotes actin polymerisation and contributes to the increased motility of fibroblasts. In cornea, the differentiation of fibroblasts to

myofibroblasts was mediated through epithelial-stromal interactions, which was induced by EMMPRIN (Extracellular Matrix Metalloproteinase Inducer) during cornea healing [135]. In this study, EMMPRIN co-localised with αSMA protein, indicating its importance in myofibroblast development. However, the exact mechanism of EMMPRIN's involvement in promoting acquisition of myofibroblast properties is still poorly understood. EMMPRIN expression has been detected in the glandular and stromal components of ovarian endometriotic lesions [136], although the exact relationship of this protein to myofibroblasts needs further study.

1.1.5.3 Epithelial-mesenchymal transition (EMT) as the source of myofibroblast

Epithelial-mesenchymal transition (EMT) is another event that could transform non-mesenchymal cells into myofibroblasts/fibroblasts. The transition of epithelium to mesenchyme is defined as an event in which cells possessing the typical epithelial markers (i.e. cytokeratin and E-cadherin), lose these markers and acquire classical mesenchymal markers (e.g. vimentin). This event is important during embryogenesis [137] and organogenesis, in kidney [138] and heart [139] development. In adults, EMT has been associated with tumor metastasis, fibrosis and other pathological problems. As demonstrated by Mariasegaram *et al*. [140], TGFB was able to stimulate epithelial cell transition to αSMA-positive fibroblasts. In rat renal tubular epithelial cells, IL-1 stimulated epithelial cell transformation into mesenchymal myofibroblasts [141]. There is evidence that menstrual shedding induces the epithelial layer of the mesothelium to transdifferentiate into motile mesenchymal myofibroblasts during the ectopic implantation of endometrial tissues [142].

1.1.5.4 Possible macrophage-myofibroblast transformation

The possibility of myofibroblast derivation from macrophages was reported as early as 1983 by Campbell and Bryan [143]. This occurrence was also described by Bhawan *et al*. [144] in a juvenile xanthogranuloma case; a condition in which yellowish lesions characterised microscopically by an excessive infiltration of tissue macrophages (histiocytosis). Cells with an intermediate phenotype of macrophages that contained myofilament bundles were identified by electron microscopy suggesting transdifferentiation. While this technique was considered valid at that time, at the present time, immunohisctochemical staining against αSMA and F4/80 is required to validate the observation.

The transdifferentiation of macrophages into αSMA-positive myofibroblasts was also observed in the transgenic mice (MacGreen) that were generated to have macrophages that displayed green fluorescent protein (GFP). In this study which aimed to examine the peritoneal immunological response to boiled egg cube, 12-50% of macrophages differentiated morphologically and phenotypically into myofibroblasts (GFP-positive cells that were also αSMA positive) at 14 to 28 days post-implantation [87]. This macrophage-myofibroblast transformation indicates a macrophage transition to a remodelling function during the resolution phase of inflammatory response and the expression of αSMA coincides with the reorganisation of extracellular matrix. This healing mechanism not only conserves the resources of the host tissues but also provides a quick repair of the injured cells.

1.1.5.5 Myofibroblasts in endometriosis

Myofibroblasts are present in the endometriotic lesions and they appear to participate in the tissue repair stage of this disease. These cells began to disappear in an apoptotic process as the tissue healed [145]. The dissolution of myofibroblasts was limited because in endometriotic tissues apoptosis was impaired and αSMA was maintained throughout disease progression. In a nude mouse model of endometriosis, myofibroblasts were demonstrated to originate from the murine host [146]. This suggests that in human endometriotic tissues, myofibroblasts may be derived from the site of implantation. The transdifferentiation of an infiltrating macrophage population could be the source of macrophage-associated myofibroblasts.

Myofibroblasts could also be derived from peritoneal mesothelial cells via EMT [147]. Tumor necrosis factor-alpha (TNFα) has been identified as the factor potentially regulating this EMT. As TNFα activity is predominant in the early invasion stage of endometriosis, mesothelial EMT may be limited to the early invasive stage of lesion development. Chae *et al*. [148] compared the sera of women with and without endometriosis and found that the levels of circulating soluble TNF-receptor (sTNFR) and not TNFα itself correlated more closely with the development of endometriosis. As Chae and colleagues only measured the sera of women with existing endometriosis, this study does not exclude the possibility that high TNFα serum levels promote early endometriosis development whereas the TNFα's decoy receptor subsequently neutralises the effect of TNFα, to maintain the growth of the endometriotic lesions.

In addition to TNFα stimulation, there are a few alternate explanations regarding the origin of myofibroblasts in endometriosis. The first possible explanation is metaplasia

of endometrial stromal cells into myofibroblasts, as demonstrated in ovarian endometriosis [149, 150]. The second explanation is that myofibroblasts could originate from the local peritoneal mesothelial layer, either from the resident epithelial or fibroblast cells. Lastly, local remnants of the Müllerian duct system have been suggested to be myofibroblast progenitors [151]. In deep infiltrating endometriosis, myofibroblasts were highly expressed in the stromal component [145]. van Kaam *et al*. suggested that the presence of these cells may be a result of the mechanical stress experienced by these ectopic tissues to their deep infiltrating locations. However, since this study did not have control lesions (e.g. peritoneal endometriosis) and αSMA expression is known to be present in peritoneal endometriotic lesions, myofibroblasts could be critical in endometriotic lesion regardless of locations.

1.1.5.6 Conclusion

Although many publications report the presence of myofibroblasts in endometriosis, the function of these cells is still poorly studied. As endometriosis is a heterogeneous disease, there is a considerable variation in myofibroblast presence between endometriotic lesions and patients. The presence of these cells in endometriotic lesions provides a novel area of interest for researchers and could be a new target for endometriosis therapies.

1.1.6 TGFB1 is the main mediator in endometriosis development

TGFB, predominantly TGFB1 is one of the main mediators believed to be involved in endometrial-peritoneal interactions. TGFB superfamily proteins consist of 33 multi-functional growth factors (reviewed in [152]). At low concentrations, TGFB1 has a

stimulatory role and it becomes inhibitory at higher doses. For an example, at low doses (25 pg/ml to 2.5 ng/ml) TGFB1 increased rat skin fibroblast proliferation, while at higher doses (25 ng/ml to 250 ng/ml) it inhibited cell proliferation [153].

Briefly, TGFB1 mediates signalling via binding to the TGFB Type II receptor (TGFBR2), which phosphorylates the TGFB Type I receptor (TGFBR1). This phosphorylated TGFBR1 in turn activates SMAD2 and SMAD3 promoting downstream signalling effects, such as binding to transcriptional co-activators [154]. TGFB signalling can be negatively regulated by SMAD6 and SMAD7 [155]. SMAD6 competes with SMAD4, preventing it from binding to phosphorylated SMAD1 [156]. Phosphorylated SMAD1-SMAD4 complex is needed for signalling effects, such as gene expression. As depicted in Figure 1 C (page 53), SMAD7 inhibits phosphorylation of SMAD2 and SMAD3 upon TGFBR1 activation, thus antagonistically regulates TGFB1 pathway [157]. TGFB1 regulation of motility and cytokine synthesis in myofibroblasts is focal adhesion kinase (FAK)-dependent through two pathways; the major pathway is ERK-dependent and the minor pathway requires p38 MAPK [158, 159].

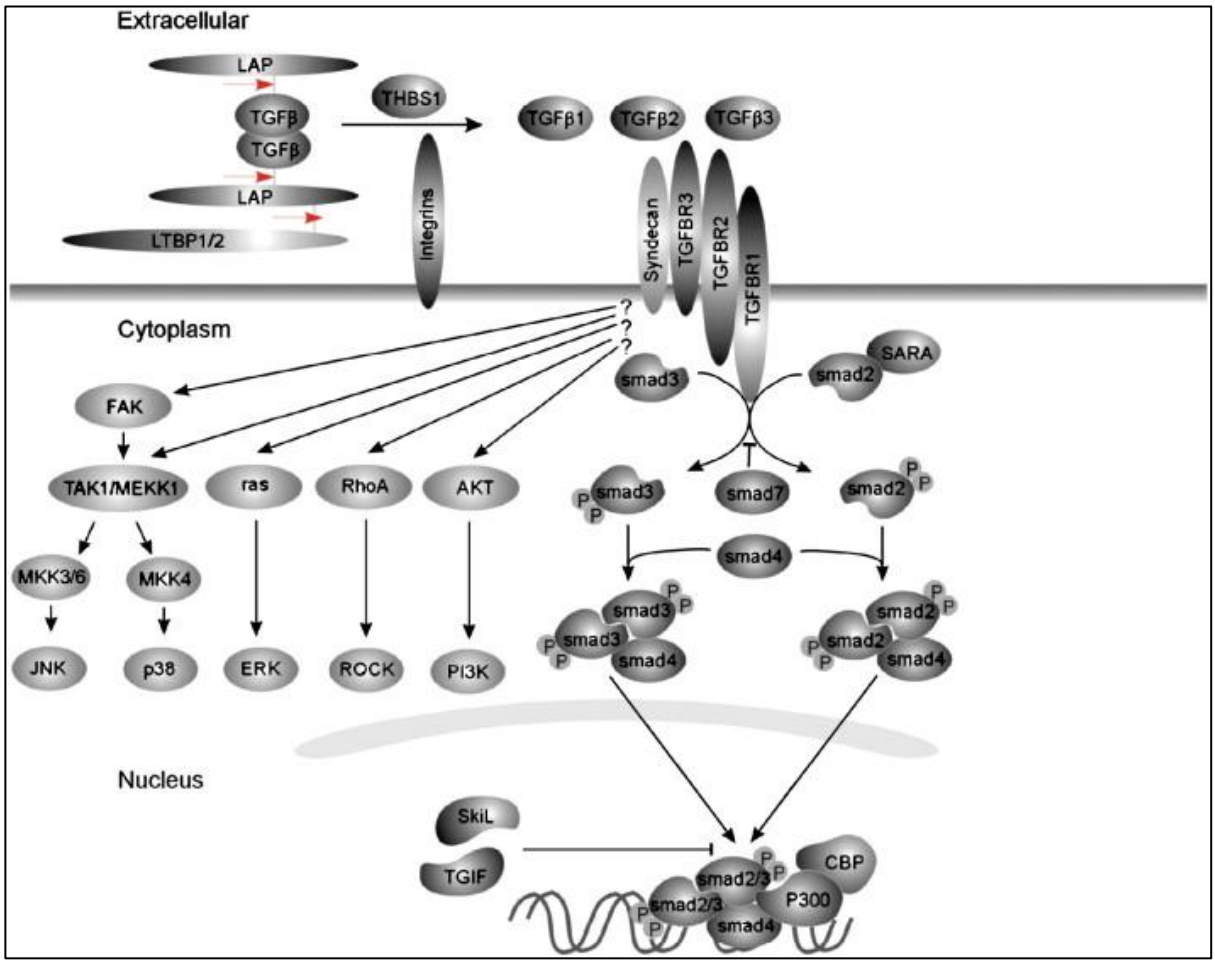


Figure 1 C: TGFB molecular pathways, which involve the release of mature TGFB proteins from LAP.

Active TGFB proteins bind to receptors, which in turn activate SMAD2 and SMAD3 proteins via phosphorylation [154]. SMAD7 is an inhibitor protein in TGFB-regulated pathways.

1.1.6.1 Latent TGFB activation

TGFB is secreted as part of an inactive latent complex. Dissociation from its Latency-Associated Peptide (LAP) is necessary prior binding to its receptor [160, 161]. LAP non-covalent linkages to TGFB can be interrupted proteolytically through binding to αvβ6 integrins followed by cleavage by MMP-9, MMP-12 or by thrombospondin-1; or non-proteolytically by reactive oxygen species or a mild acidic environment [161]. An *in vitro* study using macrophage cell line showed that nitric oxide (NO) secreted by

macrophages is able to liberate active TGFB from its latent complex [162, 163], demonstrating macrophage ability to regulate TGFB activity. Therefore TGFB1 activation is necessary for its biological roles, including upregulation of αSMA in fibroblasts [164].

1.1.6.2 TGFB1 involvement in endometriosis pathogenesis

Many clinical studies have demonstrated the involvement of TGFB1 in endometriosis. Higher concentrations of TGFB were found in the peritoneal fluid, serum and endometriotic lesions from women with endometriosis when compared to disease-free patients [165-167]. TGFB1 protein was found to be localised in the stromal and glandular cells in eutopic endometrium [167].

Similarly, in the endometriotic lesions, TGFB1 is localised in the stromal compartment and increased expression has been noted in the epithelial cells and nerve fibres [168, 169]. The presence of TGFB1 in the nerve fibres of the endometriotic tissues was positively correlated with the severity of dysmenorrhea [169]. TGFB-downstream signalling molecules SMAD2, 3 and 7 are expressed in both epithelial and stromal cells [170]. Type 1 and 2 TGFB receptor and phosphorylated SMAD2 proteins are prominently expressed in the glandular epithelium and the proximate stromal area, suggesting epithelium is the central TGFB1 signalling site in endometriotic lesions [145].

Investigation of endometriosis using a nude mouse model implicates TGFB1 as important during disease development. In this model *TGFB1* mRNA from both human transplants and murine host tissues was the central transcript in a molecular pathway identified as essential for tissue remodelling and collagen deposition in endometriosis-like lesion development [146].

In addition to protein and mRNA expressions, *TGFB1* gene polymorphisms were also studied and found to be associated with endometriosis pathogenesis. *TGFB1* gene polymorphic allele -509T was found to be associated with the severity of endometriosis [171] in Chinese women, however two following studies in Korean and European women did not find any association between a single allele -509T with the risk of endometriosis or its severity [172-174].

1.1.6.3 Macrophage activation by TGFB1

During the tissue repair process, TGFB1 is a potent chemoattractant of monocyte infiltration [175]. TGFB1 also has been suggested to be involved in monocyte differentiation into activated macrophages, although no conclusive evidence has been established [176]. During the inflammatory process, an *in vitro* study suggested that TGFB1 could be one the mediators to promote the migratory behaviour of macrophages [177]. In cervical cancer, TGFB induced the activation of macrophages via upregulation of colony-stimulating factor-1 (CSF-1) [178]. CSF-1 receptor (CSF-1R) is also important in the proliferation, activation and differentiation of macrophages [179].

However, prolonged exposure to TGFB1 deactivated macrophage migration [177] and reduced the expression of the pro-inflammatory cytokines [68]. Therefore, the effect of TGFB1 on macrophages is a context dependent and it could regulate macrophages from both directions. This is likely to be altered by the activation state of the macrophages and other components of the tissue microenvironment.

1.1.6.4 TGFB secretion by macrophages

Macrophages are not only highly responsive to TGFB1, but are also a possible source of TGFB1 as demonstrated by the infiltrating macrophages in the human endometriotic cysts [168]. This study however only relied on the immunohistochemical staining and did not demonstrate the secretion of cytokine by macrophages. In baboons, induction of endometriosis led to a pelvic environment which contained high levels of TGFB1 and macrophages [67]. In an *in vitro* study, macrophages increased secretion of TGFB1 and decreased secretion of the pro-inflammatory cytokines, interleukin-1β (IL-1β) and transforming growth factor-α (TNFα) following phagocytosis of apoptotic cells [61]. The increase of TGFB1 levels in the peritoneal fluid of endometriotic women could be the result of phagocytosis by macrophages.

Hypoxia-associated molecules have been identified to be important in the endometriotic tissues [180] and in a mouse model of endometriosis; hypoxia occurs at the early stage of tissue implantation [181]. Hypoxia inducible factor-1α (HIF-1α) is also partly responsible for bone marrow-derived macrophage recruitment following the release of CXCL12 and VEGF by tumor cells [182]. Besides VEGF, in hypoxic environments, macrophages also induce the expression of TGFB1 in surrounding cells [183]. Therefore, hypoxic condition could be one of the stimuli that leads to TGFB1 secretion by macrophages.

1.1.6.5 TGFB in ECM remodelling

Secreted TGFB1 appears to act through different cellular pathways to mediate extracellular matrix (ECM) remodelling in endometriosis. TGFB1 signalling is important

in regulating the production of ECM proteins, including fibronectin, osteopontin and collagen [184-186], which were found to be increased in endometriotic stromal cells [187]. In addition to ECM protein expression, TGFB also increases the invasiveness of endometrial cells, which may be important during the adhesion step of ectopic endometrial implantation [188].

Through the expression of integrins and their ligands, cell adhesion and migration are also regulated by TGFB1. Examples of integrins upregulated by TGFB1 in fibroblasts include integrins α2β1, α3β1, αvβ3 and αvβ5, which all are important in myofibroblast differentiation [189]. The expressions of these integrins were associated with the high cell motility in the cultured ovarian carcinoma stromal cells [190]. The α2β1 and α3β1 integrins are expressed by mesothelial cells, although they do not appear to be critical for the attachment of endometrial cells onto mesothelium *in vitro* [191]. The exact role of TGFB1 in modulating ECM remodelling during endometriotic lesion establishment needs to be further explored particularly how TGFB1 interacts with the endometrial stromal cells and host tissues.

1.1.6.6 *In vivo Tgfb1*-null mouse model

Targeted disruption of the *Tgfb1* gene has been achieved by homologous recombination to create *Tgfb1*-null mutant mice (*Tgfb1*-/-). Newly born *Tgfb1*-/- mice are indistinguishable from their littermates, however by the time of weaning (21 days old); the mutant mice are noticeably smaller than their age-matched siblings. At this age, these mutant mice start to develop a wasting syndrome, exhibiting inflammation and tissue necrosis, which then lead to multiple organ failure and death [192].

To prevent these auto-immune complications an immune suppressive condition was induced either by 1) the administration of dexamethasone [193], 2) anti-CD11 [194], 3) creating a β2-microglobulin-deficiency in the animals or 4) back-crossing mice onto homozygous background of spontaneous mutation severe combined immune deficiency (*Prkdc*^{scid}) (SCID) [194, 195]. Although an immuno suppressed condition enabled the mice to live up to 10 weeks, on SCID genetic background for example, *Tgfb1*-/- mice remain physically smaller and more susceptible to stress than their littermate controls.

Tgfb1-/- mice have reduced fertility in both males and females. Pregnancy in successfully mated *Tgfb1*-/- females failed to continue due to impaired haematopoiesis and vasculogenesis in homozygous knockout offspring [196]. The frequency of knockout to wildtype (*Tgfb1*+/+) offspring deviates from the expected Mendelian ratio, suggesting some *Tgfb1*-/- mice failed to develop during embryogenesis (reviewed by [197]).

Although a mouse carrying a null mutation in *Tgfb1* gene has reduced fertility and reproductive function due to ovarian defects, mutant females have a relatively normal uterine structure, with no detectable abnormality in the glandular abundance and structure [198]. Therefore, murine endometrial development is not highly dependent on the presence of TGFB1 and makes it suitable to study the growth of ectopic endometrial tissues.

1.1.6.7 Macrophages in *Tgfb1*-null mutant mice

The absence of *Tgfb1* gene in the null mutant mice causes multi-focal inflammation in major organs which then leads to a wasting syndrome and death.

Immunohistochemistry against 1a antigen of MHC class II showed an increased expression in the 8 days old *Tgfb1*-/- pups in pulmonary venous endothelium section; similarly FACS analysis of *Tgfb1*-/- spleen nodes had higher MHC class II-positive cells than the littermate controls (54.3 vs. 31%) [199]. These data are supported by observations in homozygous knockout mice for both *Tgfb1* and MHC class II alleles, which did not develop autoimmunity or inflammation [200]. MHC class II-positive cells and hence systemic inflammation are also reduced in β2-microglobulin-deficient *Tgfb1*-/- mice compared to *Tgfb1*-/- mice with normal β2-microglobulin [195]. Therefore, multi-focal inflammation observed in the *Tgfb1*-/- mice requires the action of MHC class II cells.

TGFB1 is known to suppress iNOS expression *in vitro* and *in vivo* [163, 201]. In *Tgfb1*-/- heart tissues, iNOS mRNA and protein expressions are more abundant than the wild type mice [202]. The activation of iNOS is regulated via transcription factors Stat1α and IFNγ-regulatory factor-1 (IR-1), which are upregulated in *Tgfb1*-/- mice [202]. Similar observation was made in the *in vitro* culture of primary murine macrophages, in which TGFB1 suppressed the iNOS activation by reducing iNOS mRNA level and increasing iNOS protein degradation [201]. As mentioned before, iNOS production of nitric oxide (NO) induces TGFB1 via activation of LAP in a wound healing tissue [163], suggesting indirect functional regulation of TGFB1 and iNOS.

1.1.6.8 Other isoforms of TGFB1

Compared to TGFB1, the importance of other TGFB isoforms in endometriosis is far less studied. The expressions of TGFB2 and 3 have been highlighted in the human tissues and rat model studies. *TGFB*2 mRNA transcript was significantly higher in

ectopic tissues (n = 13) when compared to eutopic tissues from patients without endometriosis (n = 6) [203]. In human endometriotic lesion, TGFB2 protein was immunolocalised in the glandular epithelial cells and the stromal area surrounding these cells [168].

In an autotransplant rat model of endometriosis, expression of the *Tgfb*2 transcript peaked at day 14 post endometriosis induction, followed by a decrease at day 21 (n = 8 in each group) compared to intact endometrium (n = 6) [203]. In another rat model of endometriosis, Chegini *et al*. demonstrated that TGFB2 protein was mainly localised in the stromal compartment of endometriosis-like lesion [204]. Although TGFB2 was suggested to be highly expressed by the inflammatory cells in this lesion, Chegini did not mention the marker for these cells.

In endometriotic tissues, TGFB3 protein was strongly localised to the epithelium, which was surrounded by the CD68-positive stromal cells [168]. Similarly, in autotransplant rat models, *Tgfb3* mRNA was 2.38-fold higher in the endometriosis-like lesions compared to eutopic tissue [205] and the protein form of this growth factor was expressed by the epithelial cells [204].

1.2 Conclusion

Our imperfect understanding of endometriosis pathophysiology hinders the development of new treatments. Following retrograde menstruation, it is well established that the misplaced endometrial tissues need to attach and implant for the tissue to grow. However the cellular mechanisms that lead to growth and survival of the ectopic tissues remain poorly understood.

Macrophages are likely to be one of the key cells that participate in endometriosis development. Early evidence demonstrates that macrophages are able to acquire tissue healing properties and thus could be important for extracellular matrix (ECM) development in endometriotic lesions. ECM remodelling involves a macrophage-fibroblast interaction via secretion of growth factors to modulate their activities. This study aims to dissect the interactions between macrophages and fibroblasts and their roles in endometriotic lesion development. We hypothesise that the cytokine TGFB1 is a key signalling protein that modulates macrophage activity and fibroblast differentiation in endometriosis progression.

1.3 Hypothesis

TGFB1 is important in endometriosis-like lesion development because of its role in regulating macrophage function and myofibroblast activity.

The aims of this study were:

3.1 To develop a *Tgfb1*-/- mouse model of endometriosis and to determine the effects of TGFB1 deficiency on endometriosis-like lesion development.

3.2 To create a macrophage-restricted GFP expression mouse model of endometriosis and to determine longitudinal changes in functional markers of macrophages and fibroblasts in endometriosis-like lesions.

3.3 To determine if the phenotypes of macrophages are altered in the host TGFB1 deficiency of endometriosis-like lesions from *Tgfb1*-/- mutant and *Tgfb1*+/+ mice.

Chapter 2

2.1 Background

2.1.1 Models to study endometriosis

Endometriosis is still poorly studied and one of the reasons for this is that this disease is only specific to human and some primates. In 1958, Ridley and Edwards successfully induced endometriosis in women by injecting menstrual tissue intraperitoneally [206]. However, animal models are clearly needed to progress the study of this disease. Furthermore, any new treatment for endometriosis would need to undergo extensive animal study and Phase 1 trials. Therefore, animal models for endometriosis have been developed and used to test the efficacy and side effects of newly developed pharmaceutical compounds [207, 208]. There are several *in vivo* models of endometriosis which have been used over the past two decades including primates [28, 209], Balb/c mice [210], Wistar rats [205, 211, 212], immunocompromised mice [146, 213] and the chicken chorioallantoic membrane (CAM) [214].

2.1.1.1 Primate models

Although endometriosis is a gynaecological condition unique to humans, some female primates will spontaneously develop this disease if they are removed from natural habitat and prevented from getting pregnant. Examples of primates reported to develop endometriosis either spontaneously or through induction are Kenyan baboons (*Papio doguera*) [215], Mandrills (*Mandrillus sphinx*) [216], cynomolgus monkeys (*Macaca fascicularis*) [217] and Rhesus monkeys (*Macaca mulatta*) [28]. The advantage of primate models, such as Rhesus monkeys and baboons is that they are a natural model of

spontaneous endometriosis. In addition to this, unlike other models, primates have menstrual cycles and therefore best represent human conditions. Unlike in humans, the symptoms of endometriosis in these primates were loss of appetite, constipation and anorexia [217, 218] due to the blockage of the intestinal tract. Due to the cost of maintaining the animal colony, ethical and conservation issues, the use of non-human primates is prohibitive for most laboratory groups. These limitations discourage the full utilisation of this model in studying endometriosis [207].

2.1.1.2 Immunocompetent rodent models

Rodents are another tool to study endometriosis, to understand the pathogenesis of endometriosis and to test new pharmaceutical agents. Laboratory rodents are abundant, easy-to-manipulate and cost effective. Thus these criteria open up new directions to study endometriosis in a laboratory setting. Immunocompetent rodents are wildtype mice or rats with a fully functional immune system. Induction of endometriosis-like lesions in these mice requires transplantation of uterine fragments from genetically or immunologically compatible donors to reduce the risk of tissue rejection.

There are several disadvantages of these models such that they do not well represent endometriosis *in vivo*. Firstly, in most of the syngeneic murine models, the uterine fragments transplanted into recipients will include myometrium, which is not part of normal endometriotic lesions. To overcome this issue, the endometrial fragments can be carefully scraped from myometrium [219], although this results in tissue breakage, thus increasing the clearance of these tissues by phagocytic cells [106]. Secondly, the estrous cycle in rodents is different to the menstrual cycle in humans. This can be

resolved by a timely injection of estrogen and progesterone to mimic the human menstrual cycle (Refer to 2.1.1.6 section). Finally, some studies using immunocompetent mice have used sutures [81] or extracellular matrix-containing media (e.g. Matrigel) [220] to increase the rate of implantation. These techniques are not consistent with the implantation theory by Sampson, which suggests that endometrial tissue spontaneously adheres to the peritoneal cavity; therefore they do not represent the biological process that normally takes place during endometrial fragment attachment. In the rat model of endometriosis, a lower inflammatory score was observed when endometriosis was induced by attaching endometrial fragments with fibrin glue [221]. However these methods to fix the endometrial graft to the implantation sites do not represent the actual endometriosis pathology. Presumably these interventions would impair upregulation of molecules, such as MMPs that are necessary for the adherence of tissues to the ectopic sites. Similarly, by supplying ECM molecules in the media, the necessary pathways may not be activated, thus compromising the data obtained. These effects can be negated by using PBS [219], saline [77] or ECM-free media [146] as the injections of endometrial tissues in these media do not affect the induction of endometriosis. Similarly, Grummer *et al*. [222] found no morphological difference between sutured endometrial tissues and freely-implanted tissues. Due to the invasiveness of endometrial cells and the expression of VEGF *in vivo*, the endometrial tissues do not require any non-biological fixation (such as gluing or suturing) to the peritoneal wall of a host mouse.

2.1.1.3 Chicken chorioallontoic membrane (CAM)

Another *in vivo* model that has been used to study endometriosis utilises chicken chorioallontoic membrane (CAM). CAM is a vascular membrane of a fertilised egg that is important for gas exchange for the developing chick embryo. At this stage, the chicken embryo has not developed a competent immune system; therefore the graft of human tissues is feasible (reviewed by [223]). Human endometrial cells or tissue fragments are implanted on membrane after a small portion of egg shell is cut open, between 6 [224] to 11 days [225] of incubation. CAM has been utilised as an endometriosis model to study the invasiveness of primary stromal cells [224] and endometrial fragments [214, 226]. The success rate for the endometrial tissues to form lesions in CAM is variably between 68% [225] and 83% [214]. The formation of endometriosis-like lesions in this model requires invasion and angiogenesis regulated by donor-derived factors such as VEGF, MMP-1, MMP-2, MMP-9 and intercellular adhesion molecule 1 (ICAM-1) [214, 224, 226]. The use of this model has been useful in demonstrating the initial critical steps that might be involved in endometriosis development.

In contrast to the primate and rodent models, the use of CAM has several advantages owing to its simplicity and it is also cheaper to set up and operate [223]. Although this model has been extensively used as an angiogenesis model, the ability of the membrane to support the growth of the endometrial tissues can be problematic. Proliferative cells were almost absent 72 hours post grafting onto the membrane, suggesting the membrane might not have the essential growth factors to support cell proliferation [225]. Secondly, as the graft is normally placed on the membrane which has existing blood vessels, the formation of new vessels can be hard to distinguish

(reviewed by [223]). In addition, studying endometriosis-like lesions in CAM cannot be done for a longer period of time as the maximum incubation is limited to 10 days post transplantation [227]. This model has also been suggested to be unsuitable for immunological study as non-specific inflammatory response was reported after grafting (reviewed by [223]). For these reasons, CAM seems to be only applicable for studying angiogenesis and invasion stages of endometriosis-like lesions. It is unlikely to be suitable for study of macrophage contributions to endometriosis-like lesion development, due to the lack of mediators of inflammatory reactions. Compared to rodents, the avian immune system is far less studied. Similarly, the expressions of integrins, adhesion molecules and cytokines are less defined in chicken compared to rodent models. Taking these reasons together, CAM is not suitable to study the macrophage behaviours over an extended period of time.

2.1.1.4 Immunodeficient mouse models

A second mouse model which has been successfully utilised to study endometriosis *in vivo* is the immunodeficient mouse. Several strains of immunodeficient mice have been used in endometriosis models, such as severe combined immunodeficiency (*Prkdc*^{scid}) [228], Recombinant Activating Gene 2-yc-knockout (*Rag2*$^{-/-}$ *yc*$^{-/-}$) [229] and nude (*Foxn1*^{nu} mutation) mice [230]. These mutant mice lack humoral immune cells and therefore do not reject human endometrial xenografts, which enables researchers to iatrogenically grow ectopic endometrial tissues [231]. These mouse models however, do differ from human endometriotic disease because their immune system is compromised [232] and the inter-species interaction between murine host cells and human xenograft cells may not completely represent the true endometriotic disease

process [228]. Several studies have implanted human xenografts and followed the ectopic growth of the endometrial fragments for up to 28 days in SCID and nude mice [222, 228, 230] and the tissue morphology was maintained. Although human-mouse interactions may not represent the true endometriotic conditions in human, the use of immunodeficient mice allows researchers to study human tissues *in vivo*.

2.1.1.5 Genetically manipulated mouse models

In 2001, the first paper that utilised genetically modified mice in a model of endometriosis was published and so far, only a few others studies employed these models [77, 82, 220]. Genetic manipulation in mice is the only way to definitively test the effect of an absence or overexpression in the host of specific genes or proteins in endometriosis. For example, the importance of beta-2-microglobulin (β2m) in cell clearance via MHC class I expression was shown when endometriosis was induced in a genetically manipulated syngeneic mouse model [210]. Induction of endometriosis in β2m-knockout mice resulted in 3.6-fold decrease in weight and surface area of the lesions compared to wildtype mice; 3 weeks post endometrial tissue inoculation. Recent advances in gene technology have enabled researchers to use GFP-expressing transgenic mice to study endometriosis development more closely. Implantation of donor wildtype endometrial tissues into a syngeneic GFP-expressing host mouse and vice versa revealed the interaction between the host and endometrial components of ectopic lesions and the importance of host cells in the formation of blood vessels [233].

In addition to conventional gene knockout developed through gene deletion or substitution, there is also a conditional gene targeting technology which regulates gene expression in a time- and cell-specific way. Mice with these genes can be

manipulated to create *in vivo* endometriosis models. For example, a suicide gene promoter has been exploited to study the roles of macrophages during endometriosis development as demonstrated by Capobianco *et al*. [77]. In this study, a transgenic mouse expressing Herpes simplex virus (HSV) type 1 thymidine kinase gene under a promoter for pro-angiogenic angiopoietin receptor -Tie2 was used [234]. Tie2-HSV macrophages were harvested from the transgenic mice and transplanted into lethally irradiated wildtype mice to obtain bone marrow chimeric mice. After an induction of endometriosis using syngeneic uterine transplant, the anti-viral ganciclovir (GCV) was injected to deplete Tie2-positive macrophages, which resulted in a reduction of vascular areas in endometriosis-like lesions. The advantage of using genetically modified macrophages to evoke a deficiency in Tie-2 macrophages over traditional liposome clodronate phagocytic immune cell depletion method (where immune cells phagocytose toxic liposome-containing clodronate, resulting in their death) is that it is Tie-2 macrophage specific and results in more effective elimination [77]. These modifications allowed specific functional studies to be conducted to further our understanding of endometriosis.

Another example of conditional mouse model is a *Cre/loxP* transgenic system. This system is useful to inactivate genes which are critical in the early development and if knocked down conventionally will cause an embryonic lethality. Similarly, the *Cre/loxP* system can also be used to create a transgenic mouse with an overexpression of genes via a removal of STOP codon [235], and if was done ubiquitously will result in embryonic death [236]. To remove the target sequence, *loxP* is inserted upstream and downstream of the gene of interest to create a floxed gene. This transgene is injected into embryonic stem cells and selected for the presence of this *loxP*-flanked sequence using

selectable markers, such as fluorescent protein [237]. Mice positive for *loxP* transgene are then crossed with cre-recombinase mice. Cre-recombinase catalyses the excision, inversion or translocation of *loxP* sequence, depending on the orientation and location of this transgene [238]. To determine the efficiency of Cre-mediated catalysation, *β-gal* gene was inserted in a way that upon cre removal of floxed gene, β-gal sequence was restored and this can be detected using lacZ assay [220, 237]. The use of *Cre*-positive mice is also useful in development of conditional knockout/activation mice to create a cell-specific target. Therefore, the *Cre/loxP* conditional knockout strategy is useful to regulate gene expression in a time and cell-specific manner.

The use of this system has been demonstrated in knocking down immune cell functions, in particular macrophages. Mouse with myeloid-restricted Cre expression by inserting *Cre* gene into lysozyme M (*LysMcre*) locus crossed to floxed β polymerase mouse [117]. The peritoneal exudates were collected and purified using FACS analysis. Upon Cre activation, 99% of peritoneal neutrophils and 95% of $F4/80^{+}$ macrophages had β polymerase gene knocked down. This experiment however was only performed *ex vivo* and the consequence of this deletion on macrophage functions *in vivo* has not been published. A second example utilising the *Cre/loxp* system is a conditional macrophage-specific arginase 1 knockout, which was achieved by crossing a floxed arginase 1 ($Arg1^{-/flox}$) mouse to *LysMcre* [101]. This macrophage-specific arginase 1 knockout reduced inflammation following parasitic infection, *Schistosoma mansoni*.

In addition to knocking down genes, *Cre*/*loxP* also has been used to conditionally express genes which in normal tissues, are not normally activated. Oncogene *K-ras* has been manipulated in mouse models to be activated in specific tissues to encourage tumor development. For example, Cheng *et al*. [220] used the Cre/*loxP* system to activate

a gene, *K-ras* specifically in the endometrium rather than ubiquitously. The donor mouse was genetically manipulated with insertion of *K-ras* gene under the Ah-Cre promoter. The induction of Cre recombinase via Ah promoter activated *K-ras* gene which was expressed in the donor endometrial glandular and stromal cells. Menstrual-like endometrium was generated using a mouse model following withdrawal of hormonal treatment [220]. This menstrual-like endometrium was intraperitoneally injected into an immunocompetent host mouse to mimic endometriosis. Donor cells with the switched on *K-ras* gene promoted the growth of the lesions in a wildtype host mouse, indicating that *K-ras* is important in endometriosis. These findings are consistent with the Dinulescu's finding [239], which showed that *K-ras* activation at specific site, such as at the ovarian surface epithelium, induced the formation of endometriosis-like glandular morphology.

2.1.1.6 Estrogenised rodent models

One of the limitations in using the rodent as an endometriosis model is the lack of menstrual cycle. Endometrium proliferates in estrogenic environment in both humans and mice. In the mouse with natural cycle, endometrium is subjected to a 4-5 days of estrous cycle of the host mouse (reviewed by [240]). To mimic the human condition, 14 days of estrogen supplementation is required. A few studies have been performed without ovariectomy and estrogen supplementation, but relied only on the estrous cycle of the rodents as the source of estrogen. Human endometrial tissues implanted in ovariectomised mice had reduced cellular proliferation [81], indicating that estrogen supplementation is more reflective of human disease.

Another way to replicate human disease is to develop a menstrual model using mice that have been ovariectomised and given steroid hormone injections. A mouse model of menstruation was developed by exposing female mice to progesterone and estrogen in a time-specific manner to induce decidualisation. Briefly, an adult female mouse was ovariectomised and allowed to recover for 7-10 days. After the recovery period, the mouse was treated with 17β-estradiol for 2 days (day 1 and 2) [220] to 3 days (day 1, 2, 3) [241], followed by 3-day of no treatment. At day 6 or day 7 both progesterone and estradiol were administered for the next two days, followed by oil injection into the lumen on the next day to induce decidualisation. Cheng *et al*. [220] managed to generate a menstrual-like tissue at day 10 after progesterone withdrawal.

Therefore, an exposure to progesterone requires an estrogen injection as progesterone is known to regress the endometrial tissue growth in mouse models. For example, ovariectomised mice treated with progesterone, either alone or following estrogen priming had reduced stromal cell densities than the mice treated with estrogen alone [242]. In this study, progesterone alone however was shown to increase the proliferation of stromal cells, as determined by CD31/BrdU double immunostaining. Although progesterone is important in endometriosis progression, it is not essential for a 14 day short-term study.

2.1.2 Rationale of the methods

In this study, we chose to implant human endometrial fragments subcutaneously, rather than intraperitoneally. The reasons for this are first, subcutaneous injection of human endometrial fragments ensures the tissues will be encapsulated by the skin and peritoneal layer, which enhances tissue adherence and the establishment of a blood

supply. When Burns *et al.* [243] injected endometrial tissues intraperitoneally; they did not vascularise and became visibly necrotic containing no detectable proliferating cells. Secondly, subcutaneous implantation of endometrial fragments has been shown to increase the recovery rate of the lesions (63-100%) [244] compared to intraperitoneal injection (33-66%)[222]. Finally subcutaneous injection confers a reduced risk of damage to internal organs as the peritoneum is kept intact. For these three reasons, subcutaneous injection of human endometrial tissues was utilised in our study.

2.1.3 Time-point study in endometriosis models

Laparoscopic confirmation for the presence of endometriosis provides information on the disease severity, but does not determine the state of disease development. Studying endometriosis development in human would require multiple surgeries and is therefore unethical. For this reason, *in vivo* animal models are required to study the longitudinal development of an endometriotic lesion.

Time-course studies have been performed in both immunocompromised [222] and immunocompetent mice [81]. Adhesion of human endometrial fragments occurred from 2 days post-tissue injection onwards [222]. Angiogenesis was reported to begin in endometriosis-like lesions 4 days post-tissue inoculation [81, 222]. Gr-1 (Ly6G)-positive neutrophils and F4/80+ macrophages infiltrated the tissue at day 1 and neutrophils lasted to day 5, persisting longer than macrophages and the levels of these immune cells returned to their original numbers at day 14 [81]. Macrophage depletion at days 4 and 8 in a syngeneic mouse model disrupted the growth and vascularisation of endometriotic lesions [82]. Although the duration of most of the studies was up to two weeks, Grummer *et al*. [222] allowed human endometrial xenografts to grow

intraperitoneally in immunodeficient mice up to 28 days and the glandular morphology was maintained.

Using non-human primates, which are more similar to human than rodents, researchers were able to study endometriosis for up to 15 months. Endometriosis may develop in primates from controlled mating and exposure to menstrual effluent [245]. The use of non-human primates allows researchers to do laparoscopic examination of the lesions *in vivo* [246].

The development of endometriosis in a primate model takes a longer time than in rodents. Induction of endometriosis by injection of menstrual fluid into the peritoneal cavity of baboons takes up to 30 days before endometriotic lesions are observed [247]. The time frame to study endometriosis progression in primates was longer than rodents as endometriosis developed progressively in these models [209]. In some primate models, endometriosis was induced by injecting endometrial tissues obtained using curettage. These curettage samples however contained cell layers which are normally not present in the menstrual shedding (reviewed by [207]). Endometriosis induction in these primate models often resulted in a very low success rate. For example, only 27.6% of experimental baboons developed endometriosis lesions and 12 months later, these lesions disappeared in 2 animals [247]. Performing study in baboons requires years of work and very specific skills. In addition, only a small percentage of animals develop the disease, considering the cost and time taken to do the experiments.

2.1.4 Conclusion

Animal models in endometriosis research are invaluable tools to study this disease. The use of primate and rodent models allows researcher to follow disease progression

over time. This provides a better understanding and allows development of new therapies. To address the hypothesis for this study, a model of endometriosis is required to study macrophage and fibroblast roles in endometriosis development at different time-points. For this reason, a mouse model was chosen as they are more easily available and extensive knowledge of the mouse immune system exists. We chose an immunocompromised mouse model by implanting SCID mice with human endometrial fragments to mimic the condition of endometriosis. As our group had access to human endometrial tissues, we used immunocompromised mice to study ectopic endometrial tissue behaviour in these models. The protocols, advantages and disadvantages of these models have been reviewed and described in literature [213, 222].

2.2 Materials and Methods

2.2.1 Endometrium biopsy

The use of human endometrial tissues for this study was approved by the Children, Youth & Women's Health Service (CYWHS) Human Research Ethics Committee and the Human Ethics Committee, The University of Adelaide.

2.2.1.1 Collection of human endometrial tissues

Human endometrial tissues were collected from women undergoing laparoscopy for abdominal pain and/or unexplained infertility with informed consent. Women were recruited from the Women's and Children's Hospital gynaecological outpatient clinics, when a decision had been made to undertake diagnostic surgery. Inclusion criteria included women aged 18-45 with a regular ovulatory menstrual cycle. The use of hormonal medications within 3 months prior to surgery was an exclusion criteria. Eutopic endometrium was collected using a pipelle suction curettage (Pipelle de Cornier, Laboratoire CCD, France) while the patients were under anaesthesia and these tissues were immediately placed in phenol-free, serum-free Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (Sigma-Aldrich Co., MO, USA) supplemented with 1% Insulin-Transferrin-Selenium-X Supplement (ITS) (GIBCO™, Invitrogen Corporation, Grand Island, NY), 1% Penicillin/Streptomycin, 0.1% EX-CYTE® Growth Enhancement Supplement (Celliance, Kankakee, IL) and 10 nM of 17β-estradiol (Sigma-Aldrich). This media was freshly made and used within a week. Tissues were transported on ice to the Medical School North, The University of Adelaide, Frome Road laboratory and prepared for *in vivo* injection within two hours of collection.

2.2.1.2 Endometrial tissue preparation

The tissues were rinsed in phenol-free, serum-free media and cut into 1 mm^3 using a single-edged razor blade under the laminar flow cabinet. Fifteen to eighteen tissue fragments were placed into each well of a 4-well culture plate containing 500 µL phenol-free, serum-free medium. The tissue culture plates were stored at 37°C until ready for use. Endometrial fragments from each well were loaded into a 1 ml syringe and capped with a 19G needle for *in vivo* injection of the endometrial tissues into the experimental mice. The cycle stage of endometrial tissue samples did not influence the development of endometriosis-like lesions in mouse models.

2.2.2 Animal models

The use of animals in this study was approved by Animal Ethics Committee (Medical), The University of Adelaide and CYWHS Animal Ethics Committee, Women's and Children's Hospital. GMO Dealing Authorisation was obtained from Institutional Biosafety Committee, The University of Adelaide (IBC Identifier: 10029).

2.2.2.1 eGFP-CSF-1R (MacGreen) mouse

The colony stimulating factor 1 (CSF-1) receptor promoter is encoded by *c-fms* gene and expressed exclusively by the mononuclear phagocyte lineage and their precursors. A MacGreen mouse contains a transgenic gene, which codes for an enhanced green fluorescent protein (eGFP) under the CSF-1 receptor promoter. Therefore, in this mouse all macrophages and their progenitors exclusively expressed a green fluorescent protein that can be detected by confocal microscopy. In order to introduce human tissues and avoid graft versus host rejection, immunocompromised mice need

to be used in xenograft mouse models of endometriosis. Therefore, male MacGreen mice were bred to female SCID (*Prkdc*^{scid}) mice to generate first filial cross (first generation or F1) progeny, which were heterozygous for the MacGreen transgenic gene and the immunocompromised SCID mutation. F1 progeny were then cross bred to produce a second filial cross (second generation or F2). These mice harboured the CSF-1R-eGFP construct insert and were homozygous for the SCID mutation. The genotypes of F2 pups were determined using PCR amplification of the transgene (MacGreen). F2 pups positive for MacGreen were either heterozygous or homozygous. Initial genotyping for the SCID mutation was not successful so the presence of the SCID mutation was determined using flow cytometry which confirmed the absence of lymphocytes (Refer to 2.2.2.3.6).

Since this gene construct is autosomal dominant, progeny that are heterozygous for MacGreen will have the same phenotype as their homozygous littermates as all cells with one copy of CSF-1R-eGFP gene construct will express eGFP protein and appear positive during confocal microscopy. Differences in the intensity of eGFP expression in heterozygotes and affected homozygotes have not been documented and both heterozygotes and homozygotes positive for the CSF-1R-eGFP mutation are likely to have been used in experiments.

2.2.2.1.1 *Tgfb1*-null mutant

Tgfb1-null mutant (*Tgfb1*-/-) mice were originally generated using a gene targeted disruption technique by homologous recombination of embryonic stem cells [192] at the University of Cincinnati College of Medicine, Ohio, USA. In brief, a construct with a modified *Tgfb1* gene containing *neo*^{r} (neomycin-resistance) sequence at exon 6 was

electroporated into D3 embryonic stem (ES) cells. The cells with *neo*^{r} gene was positively selected using antibiotic G418, thus cells with no antibiotic-resistance gene were eliminated. Two of the three successful ES cell clones with one copy of the modified *Tgfb1* allele were electroporated into C57BL/6J blastocysts. These blastocysts were implanted into pseudopregnant females with C3H/HeNxC57BL/6J background to obtain chimeric progeny with agouti coat. Five of male mice with the high amount of agouti coat colour were mated to CF1 females and produced 2 male progeny. These males were used for subsequent breeding and passed the disrupted *Tgfb1* gene to 50% of offspring. The presence of this *neo*^{r} transgene was used to distinguish *Tgfb1*-null mutant (*Tgfb1*-/-) and heterozygous (*Tgfb1*+/-) mice from homozygous wildtype (*Tgfb1*+/+) mice.

2.2.2.2 Animal husbandry and handling

All animals used for this study were housed at the Laboratory Animal Services, Medical School South, The University of Adelaide. The *Tgfb1* mutant and MacGreen colonies were maintained under controlled light (12L:12D photoperiod) and temperature, pathogen-specific free conditions, and were fed *ad libitum* a standard diet and sterilized water. All husbandry and experimental procedures in mice were performed in a laminar flow hood using sterile instruments and sterile gloves. The maximum number of mice housed in one cage was 6.

Mice were placed together for breeding at 6-8 weeks of age. The ratio of male to female for breeding was 1:1, unless a low number of males were available and then 1:2 or 1:3 breeding ratios were used. With 1:2 and 1:3 breeding ratios, females were separated when one of them became pregnant. *Tgfb1*-/- mice have impaired

fecundity; therefore heterozygous (*Tgfb1*+/-) mice were used for breeding which produced progeny with wildtype (*Tgfb1*+/+), heterozygous (*Tgfb1*+/-) and knockout (*Tgfb1*-/-) genotype (**Figure 2.1**).

Immunodeficient MacGreen mice on a SCID background were generated by cross-breeding MacGreen males with SCID females. The F1 population was crossbred to produce second filial cross (F2) mice that were double positive MacGreen and SCID mutations. MacGreen SCID (*eGFP-csf1r*$^{+}$*/Prkdc*^{scid}) F2 progeny were then mated to produce the third filial cross (F3). Some female (n = 10) F3 progeny were used in the first experiment, while the rest were kept for breeding. The breeding stock from the resulting F3 and F4 progeny were selected to have both the MacGreen and SCID mutations. The pain and discomfort from blood sampling to determine the SCID phenotype could therefore be avoided as all F3 mice were homozygous for both mutations.

2.2.2.3 pEGFP-N1 and *Tgfb1* genotyping and SCID phenotyping

2.2.2.3.1 Tail digest

Approximately 0.5 cm of tail tip was snipped from newly weaned mice (3 weeks old) and placed in sterile 1.5 ml Eppendorf tubes. This sample was digested with 100 µg Proteinase K (Sigma-Aldrich), freshly added into 0.5 ml of digest buffer (17 mM Tris, 17 mM EDTA, 170 mM NaCl, and 0.85% SDS) and incubated at 55°C for 4 to 5 hours. Five µl of digested DNA was added to 95 µl of sterile water and heated to 95°C to inactivate Proteinase K. The DNA sample was kept at 4°C until PCR analysis was performed.

2.2.2.3.2 PCR conditions

The PCR reaction mixtures contained 1 X DNA polymerase reaction buffer, 2.5 mM $MgCl_2$, 0.55 U Taq polymerase (Fisher Biotec, WA, Australia), 200 µM dNTPs (Pharmacia Biotech), 2 µM of each primer (forward and reverse) (Geneworks Pty Ltd, Hindmarsh, SA, Australia) and 2 µl of extracted DNA in a 25 µl volume.

PCR for the *Tgfb1* gene was carried out using OmniGene TRSM2 thermal cycler (Hybaid Limited, Middlesex, UK). The PCR profile was an initial 94°C denaturation step for 3 minutes followed by 35 cycles consisting of 94°C, 61°C and 72°C (45 seconds each), then a hold at 72°C for 3 minutes.

The PCR reaction to detect the MacGreen (pEGF-N1) insert and SCID mutation (*prkdc*) included an initial denaturation step at 94°C for 5 min, followed by 94°C, 60°C and 72°C, for 1 min each then 72°C for 7 minutes. Thirty cycles of amplification were carried out for pEGF-N1 using GeneAmp PCR System 9700 thermal cycler (Applied Biosystem) and 40 cycles for *prkdc* were carried out on OmniGene TRSM2 thermal cycler.

2.2.2.3.3 PCR primers

Table 2 A: PCR primers for mouse genotyping

Gene	Nt position	Primer sequence	Product length (bp)	Genbank
pEGFP-N1	742 (fwd)	5'GAC GTA AAC GGC CAC AAG TT	78	CVU55762
	819 (rev)	5'GAA CTT CAG GGT CAG CTT GC		
prkdc	189422 (fwd)	5'GTC AGT CTC ATG TTG CCA ATG	241 (normal)	AB030754
	190635 (rev)	5'AGT TAT AAC AGC TGG GTT GGC	211 (impaired)	
Tgfb1 intact	231 (fwd)	5'GAG AAG AAC TGC TGT GTG CG	134	L42461
	364 (rev)	5' GTG TCC AGG CTC CAA ATA TAG G		
Tgfb1 neo	231 (fwd)	5' GAG AAG AAC TGC TGT GTG CG	545	L42461 & U43611
	929 (rev)	5' CTC GTC CTG CAG TTC ATT CA		

2.2.2.3.4 Detection of *Tgfb1* and GFP PCR products

PCR products of *Tgfb1* (**Figure 2.2**) and *pEGFP-N1* (**Figure 2.3**) were separated on 2% agarose gel (Promega, WI, USA) with 1 x loading buffer for 40 minutes. Agarose was dissolved in TAE buffer mixed with either 0.1-0.2 µg/ml Ethidium Bromide (Invitrogen Life Technologies, Carlsband, CA, USA) or 0.01% GelRed™ (Biotium, Hayward, CA, USA) as intercalating agents to detect DNA. The gel was photographed using either DC120 (Kodak) digital camera for Ethidium Bromide-stained gels or Gel Doc™ EZ Imager (Bio-Rad Laboratories Inc., Hercules, CA, USA) for GelRed™ under UV light to detect bands of PCR products.

2.2.2.3.5 *Prkdc* restriction digest

A thymidine (T) to adenosine (A) transversion mutation in SCID mouse creates a restriction site for Alu 1 endonuclease in an exonic region of *prkdc* gene [248, 249], which is not present in the wildtype gene. This base pair change was used to detect the presence of the SCID mutation in the mouse DNA.

Two micrograms of *prkdc* PCR products were digested with 2 U Alu1 (Sigma-Aldrich) in 1 x digestion buffer for 3 hours at 37°C, and stored at -20°C until further detection analysis. Digested *prkdc* products were run on 3% agarose gel for two hours. Despite different incubation conditions, the expected bands were not detected (**Figure 2.4**). For this reason FACS analysis was used to determine the SCID phenotype as a more reliable method.

2.2.2.3.6 Fluorescence-activated cell sorting (FACS)

A horizontal incision was made about 5 cm from the tip of tail of a fully anaesthetised mouse and approximately 100 µl of tail blood was collected into a tube containing 100 µl of heparin (Sigma-Aldrich). The blood-heparin mixture was washed in FACS buffer [0.1% bovine serum albumin (BSA)/0.5% Sodium Azide in 1x phosphate-buffered saline (PBS)] and spun at 4°C to collect the cell pellet. Red blood cell lysis was performed by resuspending the pellet in distilled water for 10 seconds before adding 10xPBS. The suspension was further washed and pelleted before being resuspended in FACS buffer for T cell staining.

2.2.2.3.7 T cell-specific CD3 staining

The lymphocyte suspension was incubated for 30 minutes at room temperature with phycoerythrin (PE)-conjugated T-cell specific CD3e antibody (1:50; BD Biosciences). Cells were then washed and resuspended in 500 µl of FACS buffer. CD3e-labelled cells were detected using the FACS Canto (BD Biosciences) with FACS Diva software (BD Bioscience). CD3e-positive cells were counted by gating on the lymphocyte population and excluding debris and dead cells. The negative control for each sample was prepared in the same way, except that the cell suspension was incubated with FACS buffer only, in the absence of antibody. Mice were classified as homozygous for the *Prkdc* mutation when no CD3e-positive T cells were detected in the peripheral blood (**Figure 2.5C**).

2.2.3 Mouse preparation

2.2.3.1 Ovariectomy

Female mice were ovariectomised at the age of six weeks. These procedures were performed under sterile conditions and the mice were anaesthetised by isoflurane/oxygen mixture inhalation. The dorsal mid-lumbar area was shaved and swabbed with an alcohol wipe. A transverse incision was made halfway between rib cage and the base of the tail. Using two pairs of forceps, the skin was carefully lifted and separated from the muscle wall. Left ovary was located under a triangular fat pad beneath the muscle wall. Through an incision made in the peritoneum, the ovary was extracted using forceps and dissected from its position adjacent to the uterine horn. The uterine horn was placed back into the body cavity and the muscle wall was brought together. The ovary on the other side was removed in a similar manner.

Through the transverse incision, a small pocket was made on the lower left plank and a 1.5 mg (60-days release) estradiol pellet (Innovative Research of America, Sarasota, FL, USA) was inserted subcutaneously. A few drops of 0.02% Mepivacaine (Nature Vet Pty Ltd, Glenorie, NSW, Australia) were administered over the incised muscle wall and the skin incision was clipped using stainless wound clips. In Chapter 3, ovariectomised mice received 0.1 mg/two days of β-estradiol 17-valerate (Sigma-Aldrich) in peanut oil (Sigma-Aldrich), instead of estrogen pellets.

2.2.3.2 *In vivo* injection of human endometrial tissues

Depending on the availability of human tissues, ovariectomised mice were anaesthetised and subcutaneously injected at ventral midline site with 15-18 pieces of endometrial fragments one to four weeks after ovariectomy. Each mouse received one injection of human endometrial fragments, unless when there were extra tissues and/or low numbers of mice, then two injections (of 15-18 pieces of tissue fragments) were given for one mouse at two separate locations. If the mouse received two injections, the locations of tissue fragments were noted in the cage card. The wellbeing of the animals was monitored throughout the experiments.

2.2.3.3 Harvesting endometriosis-like lesions from experimental mice

2.2.3.4 *Tgfb1* experiment

Ten days after the injection of endometrial tissue fragments, the *Tgfb1*-/- and *Tgfb1*+/+ control mice were injected intraperitoneally with 100 µl mixture of 10 mg/ml 5-bromo-2'-deoxyuridine [BrdU, 90% (v/v)] and 2'-fluoro-2'-deoxyuridine [FdU, 10% (v/v)] (Sigma-Aldrich). An hour following this injection, the mice were euthanised and

any visible lesions were identified. These subcutaneous lesions were carefully excised from the skin and peritoneal layer. The uterus and peritoneum were collected to serve as control tissues. All tissues were rinsed in cold phosphate-buffered saline (PBS) to remove blood and prepared for either paraffin or frozen sections. Tissues destined for paraffin sections (Chapter 3) were fixed in 4% paraformaldehyde (PFA) at 4°C overnight. Tissues were then rinsed in 70% ethanol the next morning to remove fixative and stored in fresh 70% ethanol until further processing. Tissues intended for frozen sections (Chapter 5) were immediately embedded in Tissue-Tek optimum cutting temperature (OCT) compound on dry ice (ProSciTech, Thuringowa, QLD, Australia). OCT blocks wrapped in foil were stored at -80°C until ready for cryosectioning.

2.2.3.5 MacGreen/SCID experiment

MacGreen/SCID mice were randomly divided into four groups destined for euthanasia at different time points after injection of endometrial tissue on days 4, 7, 10 and 14 in each experiment. Fifty-one mice were used in five experiments with 10 mice in the first experiment (2 mice were euthanised at day 4 post-injection, 3 at day 7, 3 at day 10 and 2 at day 14), 10 in the second experiment (3 mice were euthanised at day 4, 3 at day 7, 2 at day 10 and 2 at day 14), 6 in the third (one mouse was euthanised at each time point for day 4 and day 7, and two mice were euthanised at each time point for day 10 and day 14), 11 in the fourth (2 mice were euthanised at day 4 and 3 mice were euthanised at each time point for day 7, 10 and 14) and 14 mice in the last experiment (3 mice for day 4, 4 for day 7, 3 for day 10 and 4 for day 14). The mice were sacrificed at the allocated time, after an injection of BrdU/FdU as described above. Visible lesions, uteri and peritoneal layers were collected and rinsed in cold PBS to remove

blood. Following an overnight fixation in 4% PFA, the endogenous GFP proteins in tissues was preserved in an overnight incubation of 18% sucrose in PBS at 4°C. On the following day, sucrose-impregnated tissues were frozen in OCT compound on dry ice and stored as described above.

2.2.4 Histology and immunohistochemistry

2.2.4.1 Tissue preparations

Paraffin-embedded endometrial xenografts were serially sectioned and 5 µm tissue sections were mounted on SuperFrost slides and dried at 37°C overnight. OCT-embedded tissues were cut into 5 µm sections and mounted on SuperFrost slides. These cryosections were stored at -20°C with silica gel if used within two weeks or kept at -80°C if longer storage was required.

2.2.4.2 Haematoxylin and eosin staining

Haematoxylin and eosin (H&E) staining was performed on every 10^{th} section from each lesion. Paraffin and cryosections were stained using a standard haematoxylin and eosin protocol before being mounted in DPX mounting medium.

2.2.4.3 Antibodies and lectins

2.2.4.3.1 Paraffin sections

All primary antibodies listed are monoclonal, unless otherwise stated. Immunohistochemistry on paraffin sections was performed using the following primary antibodies: rat anti-mouse F4/80 (Caltag Laboratories, Burlingame, CA, USA and eBioscience) which detects murine macrophages, mouse anti-alpha smooth

muscle actin (Clone 1A4, Sigma-Aldrich) identifies myofibroblasts and polyclonal rabbit anti-human von Willebrand Factor (vWF) (Chemicon International Inc., Temecula, CA, USA) that detects endothelial cells. BrdU In-situ Detection Kit (BD Pharmingen, San Diego, CA, USA) was used to localise BrdU-labelled proliferative cells. Isotype-matched mouse and rat IgGs were used as negative controls for the monoclonal antibodies (both Sigma-Aldrich), whereas normal rabbit IgG served as a control for the polyclonal rabbit VWF antibody (Chemicon).

Detection of primary antibodies was performed using the following biotinylated secondary antibodies: rabbit anti-rat IgG, rat anti-mouse IgG and goat anti-rabbit IgG (all from Chemicon). Streptavidin/HRP was from DakoCytomation (Glostrup, Denmark).

2.2.4.4 Frozen sections

The following primary antibodies were used on frozen sections to detect markers of macrophage phenotype: rat anti-mouse MHC class II (1-a/e) from hybridoma culture of TIB-120 (ATCC, Rockville, MD), polyclonal rabbit anti-mouse iNOS (NOS2) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), rat anti-mouse scavenger receptor type I/II (CD204; clone 2F8) (AbD Serotec, Oxford, UK) and polyclonal goat anti-arginase 1 (Abcam, Cambridge, UK). In addition to detecting macrophage markers, the following primary antibodies were also used: rabbit anti-mouse collagen 1 (AB765P) (Chemicon) which detects type 1 collagen fibres and polyclonal chicken anti-TGFB1 which identifies mouse and human active TGFB1 (R&D Systems, Minneapolis, MN, USA).

Visualisation of primary antibodies was done using following secondary antibodies: Alexa Fluor 594-conjugated goat anti-rabbit IgG (A11012) and rabbit anti-rat IgG, and biotinylated rabbit anti-chicken IgY (Invitrogen); biotinylated rabbit anti-goat IgG

(Chemicon). Streptavidin Alexa Fluor 594 conjugate (S11227) was obtained from Invitrogen. All sections were counterstained with 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI) (D9542; Sigma-Aldrich).

Table 2 B: Primary antibodies for paraffin sections

Reactivity	**Antigen**	**Manufacturer**	**Isotype (Clonality)**	**Concentration**
Mouse	F4/80	Caltag Laboratories	Rat IgG2a	4 µg/ml
		eBioscience	Rat IgG2a	0.6 µg/ml
Mouse/ human	αSMA (Clone 1A4)	Sigma Aldrich	Mouse IgG2a	4 µg/ml
Human	von Willebrand Factor	Chemicon	Rabbit IgG (polyclonal)	1.7 µg/ml
Synthetic nucleoside	BrdU	BD Biosciences	Mouse monoclonal (Not disclosed)	Not disclosed

Table 2 C: Primary antibodies for PFA/sucrose-fixed frozen sections

Reactivity	Antigen	Manufacturer	Isotype (Clonality)	Concentration
Mouse	MHC class II (1-a/e)	Hybridoma supernatant (TIB-120, ATCC, Rockville, MD)	Rat IgG2a kappa	Neat overnight
Mouse/Human	iNOS (NOS2)	Santa Cruz Biotechnology	Rabbit IgG (Polyclonal)	2 µg/ml overnight
Mouse	Liver Arginase (Arginase 1)	Abcam	Goat IgG (Polyclonal)	10 µg/ml for an hour
Mouse	CD204 Scavenger receptor type I/II	AbD Serotec, Raleigh, NC	Rat IgG2b (2F8)	10 µg/ml overnight
Mouse (< 0.1% reactivity with human antigen)	Collagen type 1	Chemicon	Rabbit IgG (Polyclonal)	12.5 µg/ml for an hour
Human	TGFB1	R&D Systems	Chicken IgY (Polyclonal)	4 µg/ml overnight

Table 2 D: Primary antibodies for frozen sections

Reactivity	Antigen	Fixation	Concentration
Mouse	F4/80	100% acetone	10 µg/ml overnight
Mouse/Human	iNOS (NOS2)	100% acetone	1.6 µg/ml overnight
Mouse	MHC class II (1-a/e)	96% ethanol	Neat overnight
Mouse	CD204 Scavenger receptor type I/II	96% ethanol	5 µg/ml overnight
Mouse	Liver Arginase (Arginase 1)	100% acetone	10 µg/ml for an hour
Mouse (< 0.1% reactivity with human antigen)	Collagen type 1	100% acetone	12.5 µg/ml for an hour

2.2.4.5 Immunohistochemistry and immunofluorescent conditions

2.2.4.5.1 Immunohistochemical staining on paraffin sections (Chapter 3)

Representative tissue sections were dewaxed and rehydrated in descending concentrations of ethanol and washed in PBS or tap water. Proteinase K (20 µg/ml) antigen retrieval was carried out for vWF staining for 15 minutes at 37°C. Endogenous hydrogen peroxidase was blocked with 3% hydrogen peroxide in either 50% methanol (for F4/80 staining) or PBS. Serum blocking was done to reduce background staining before primary antibody application using serum from the secondary antibody host species (20 minutes at 5-10% concentrations). Anti-F4/80 was incubated at 1:50 dilution (Caltag) or 1:800 (eBioscience) and anti-vWF at 1:600 overnight at 4°C. Anti-

αSMA antibody (at 1:500) was incubated for 30 minutes at room temperature. After the incubation with the primary antibody, the slides were rinsed in three changes of PBS for 5 minutes. Biotinylated secondary antibodies were then applied: 1:1000 rabbit anti-rat IgG (anti-F4/80 staining), 1:800 goat anti-rabbit IgG (anti-vWF) and 1:14000 goat anti-mouse IgG (αSMA), for 40 minutes, an hour and 30 minutes, respectively. Following a PBS wash, the tissue sections were then exposed to Streptavidin/HRP (45 minutes; 1:500). Chromogen DAB substrate was applied to localise HRP activity by development of brown product. Nuclear counterstaining was done using haematoxylin solution, for approximately 1 minute. Slides were then dehydrated in serial dilutions of ethanol and Safsolvent before being mounted using DPX mounting medium. Sections of mouse uterus and human endometrium were used as species-specific tissue controls for each antibody.

2.2.4.5.2 Immunofluorescent staining on PFA-sucrose fixed frozen sections (Chapter 4)

Fluorescent staining experiments were performed at room temperature in the dark to minimise photo-bleaching. Five µm tissue sections were air dried for 5-10 minutes at room temperature. Prior to the antibody incubation, sections were blocked in 10% secondary host serum diluted in PBS/0.5% BSA for 20 minutes. Undiluted anti-MHC class II antibody and anti-iNOS (NOS2, H-174) at 1:100 were incubated overnight at 4°C. Anti-arginase 1 (1:50) and anti-collagen 1 (1:80) antibodies were incubated for an hour at room temperature. After primary antibody incubations, sections were rinsed in PBS three times.

All secondary antibody and streptavidin incubations were done for one hour at room temperature. Antibodies against MHC class II and CD204 were detected using AlexaFluor 594-conjugated rabbit-anti rat IgG diluted at 1:500 and 1:1000, respectively, in PBS/BSA. Anti-NOS2 antibodies were detected using AlexaFluor594-goat anti-rabbit IgG, diluted at 1:250. Biotinylated rabbit anti-goat IgG (1:500) was applied to detect anti-arginase 1 antibody, followed by a Streptavidin/AlexaFluor-594 (1:200) incubation with a PBS wash in between. After another rinse in PBS, sections were counterstained with 3µM DAPI diluted in PBS (Sigma-Aldrich) for 5 minutes. Sections were coverslipped in DAKO fluorescence mounting medium and slides were stored in the dark before being visualised within a week.

Active TGFB1 staining was carried out using a three-layer detection method. Sections were air dried and blocked in 10% rabbit serum as described above before an overnight incubation at 4°C with anti-TGFB1 diluted at 1:50 in PBS/BSA with 2% rabbit serum. After PBS rinses, primary antibodies were detected with biotinylated secondary rabbit anti-chicken IgY (1:400 dilution) for an hour at room temperature. After a wash in PBS, Streptavidin/Alexa Fluor 594, diluted at 1:200 in PBS, was applied and incubated for another hour. After a final rinse in PBS, nuclear counterstaining using DAPI was carried out and sections were mounted as described previously.

2.2.4.5.3 Immunofluorescent staining on frozen sections (Chapter 5)

Immunostaining for different macrophage activation markers was performed in adjacent sections of endometriosis-like lesions. Five µm tissue sections were fixed for 10 minutes in cold fixative at 4°C. Slides were fixed in 96% ethanol for anti-MHC class II and anti-CD204 staining or 100% acetone for anti-arginase 1, anti-iNOS and anti-

collagen 1, followed by two rinses in PBS. Prior to the antibody incubation, sections were blocked in 10% secondary host serum, diluted in PBS/0.5% BSA for 20 minutes. Primary antibodies against iNOS at 1:127 dilution, anti-MHC class II (1:10) dilution and anti-CD204 (1:200) were incubated overnight at 4°C. Anti-arginase 1 (1:50) and anti-collagen 1 (1:80) antibodies were incubated for an hour at room temperature. After primary antibody incubations, sections were rinsed in PBS three times.

All secondary antibody and streptavidin reagents were incubated for an hour at room temperature. Antibodies against MHC and CD204 were detected using AlexaFluor 488-conjugated rabbit-anti rat IgG diluted at 1:1200 and 1:1000, respectively, in PBS/BSA. Anti-NOS2 antibodies were detected using goat anti-rabbit IgG, diluted at 1:800. Anti-arginase 1 antibody binding were detected using biotinylated rabbit anti-goat IgG (1:500), followed by a Streptavidin/AlexaFluor-488 (1:200) incubation with a PBS wash in between. After another rinse in PBS, sections were counterstained with 3µM DAPI diluted in PBS (Sigma-Aldrich) for 5 minutes to detect nuclei. Sections were coverslipped in DAKO fluorescence mounting medium and stored in the dark before being visualised within a week.

2.2.4.6 Image acquisition

Histochemistry and immunohistochemistry slides were imaged using Nanozoomer Software (Hamamatsu Photonics, Hamamatsu City, Japan). Immunofluorescent images were captured using Nikon Eclipse TE2000 and Nikon Eclipse Ni fluorescence microscopes and viewed on computer using IPlab 3.6 software (Scanalystics, Inc., Fairfax, VA, USA) and NIS Elements Imaging Software (Nikon Instruments Inc., Tokyo,

Japan). Fluorescent images were merged and analysed using ImageJ software (Wayne Rasband, National Institutes of Health).

2.2.4.7 Morphometric analyses

All morphometric analyses were made by an assessor blinded to genotype. Five sections stained with haematoxylin and eosin were selected for morphometric analysis. The glandular area fraction was determined by measuring the total area of the glands (epithelium and lumen), and this value was divided by the total area of the tissue in these 5 sections from each lesion. Using the total area of glands, the average gland size was determined by for each lesion. The total size of lumen inside the gland was measured using the same technique and this value was used to determine the area of epithelial cell layer.

The abundance of stained cells was quantified in 5 randomly chosen fields of 2 sections from each lesion at 10x magnification. Only cells with a visible nucleus were included in the analysis. Macrophage density was determined by manually counting F4/80 positive cells in tissue within a 100 μm depth from the surface of the lesion. The macrophage density from these 5 areas was then averaged for each lesion. Images for αSMA and vWF staining were analysed using ImageJ software. Each microscopy field was converted to 8-bit gray images and binary images were adjusted using the threshold option. The area comprised of HRP-positive cells in the entire stromal tissue region was measured and was divided by the total stromal area to obtain the percentage area that was αSMA-positive or vWF-positive. Individual blood vessels were traced to obtain the area of vessels per field divided by the total stromal area in each field to calculate the area of vessels/μm^2. BrdU positive cells were manually

counted in 5 randomly selected fields within the epithelial and stromal compartments, and expressed as a percent of the total number of cells in these compartments per microscopic field.

The immunofluorescent images were captured at 20x magnification from six different fields of the sections for all antibody staining. Three fields were chosen from the periphery and three fields were from the middle of the tissue sections. In eGFP immunostained slides, three images from the same field were taken using three filters (blue, green and red) and overlayed using ImageJ to generate a three-colour merged picture. Similarly, the immunofluorescent slides of *Tgfb1*-/- and *Tgfb1*+/+ frozen lesion sections were captured using two filters, blue and green. The DAPI-stained individual macrophages which co-localised with the specific markers were counted using ImageJ software (Plugins>Analyze>Cell Counter). The intensity of collagen type 1 fibres was assessed using a method similar to αSMA staining measurement. The number of macrophage-specific markers and collagen percentage area were averaged from these 6 microscopy fields. The average numbers were plotted using GraphPad Prism on dot plot graph.

2.2.4.8 Statistical analyses

Data from histological and immunohistological analyses were averaged to provide a single result for each lesion developed from an injection of human endometrial tissues. In the weight and morphometric analyses, median and ranges were calculated. A non-parametric Mann Whitney U test (GraphPad Prism 5, GraphPad Software, Inc., La Jolla, CA and IBM SPSS Statistics Version 20, Somers, NY, USA) was used to compare medians from the *Tgfb1*+/+ and *Tgfb1*-/- groups with significance inferred at $p<0.05$.

In comparing data from multiple groups (Chapter 4), data from each lesion were analysed as specified. The medians were analysed using non-parametric Kruskal-Wallis test (GraphPad Prism 5 and IBM SPSS Statistics) with significance inferred at $p<0.05$, followed by Dunn's multiple comparisons test ($p<0.05$ was considered significant) to compare the medians between two groups.

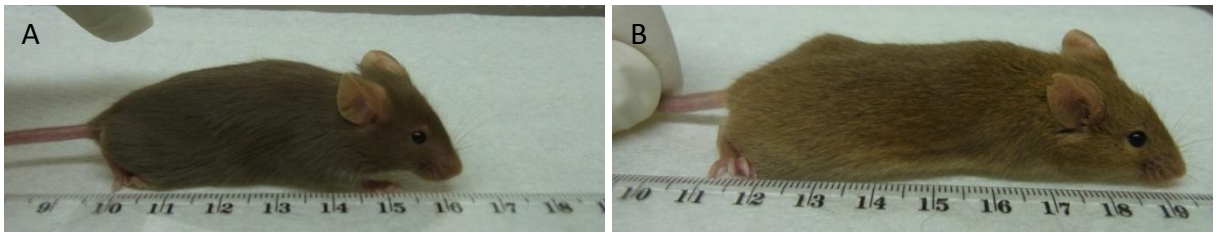


Figure 2. 1: The physical difference between a *Tgfb1*-/- female (A) and a wildtype (*Tgfb1*+/+) littermate (B) at the age of 6 weeks.

In this study, the progeny with knockout (*Tgfb1*-/-) and wildtype (*Tgfb1*+/+) genotype were derived from heterozygous breeding pairs.

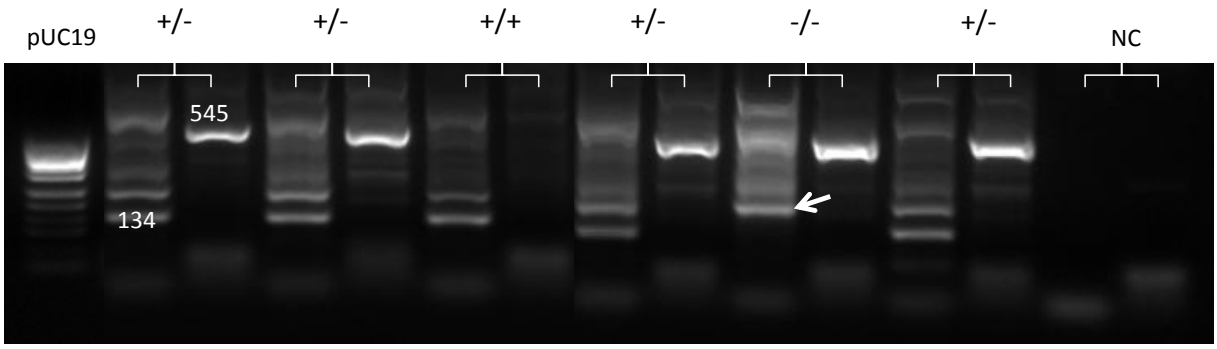


Figure 2. 2: Gel electrophoresis for *Tgfb1* genotyping.

DNA from each mouse was amplified for intact wildtype (*Tgfb1*+/+) (left lane) and transgene *Neo* (right), yielding PCR products of 134 bp and 545 bp, respectively. White arrow indicates a non-specific band, which presents in all samples, except negative control (NC).

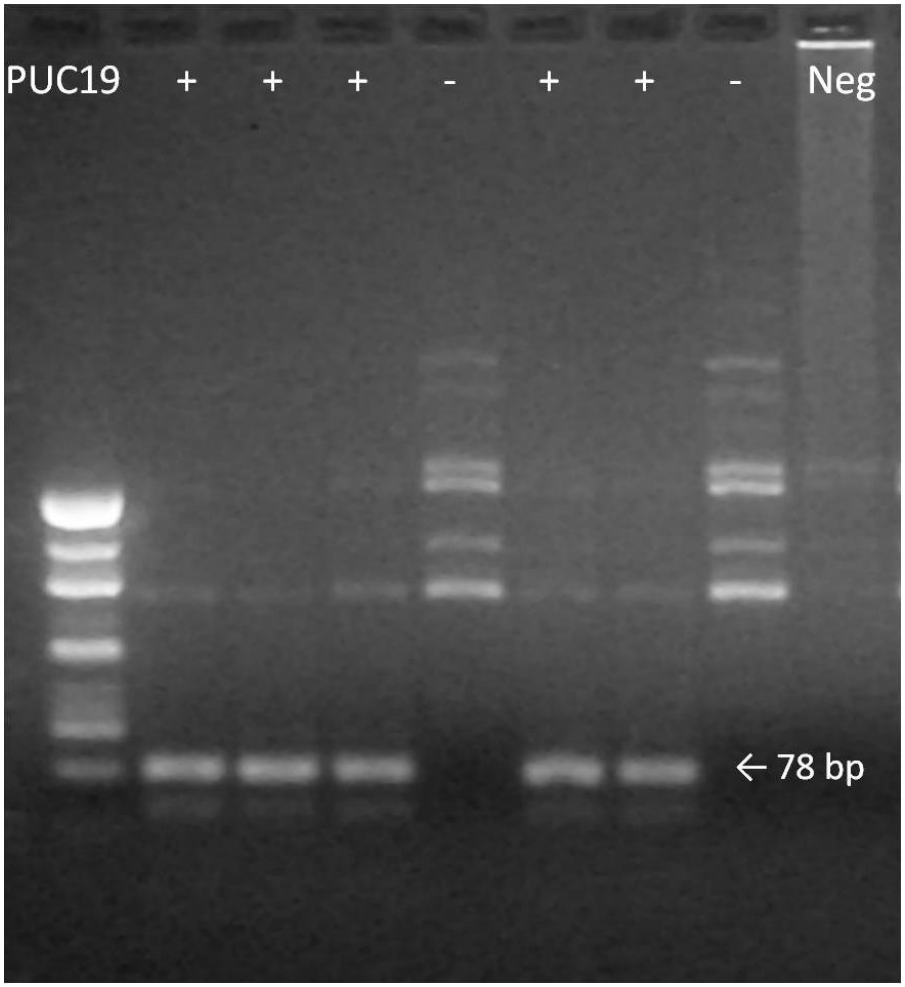


Figure 2. 3: Genotyping eGFP transgene by PCR.

PCR products amplified by pEGFP-N1 primers from 30 cycles, yielding a product of 78bp (transgene). Each lane represents DNA of a single mouse, determined to be positive (+) and negative for transgene (-). This method only detects the presence of one allele of transgene; therefore positive mouse could be either homozygous or heterozygous.

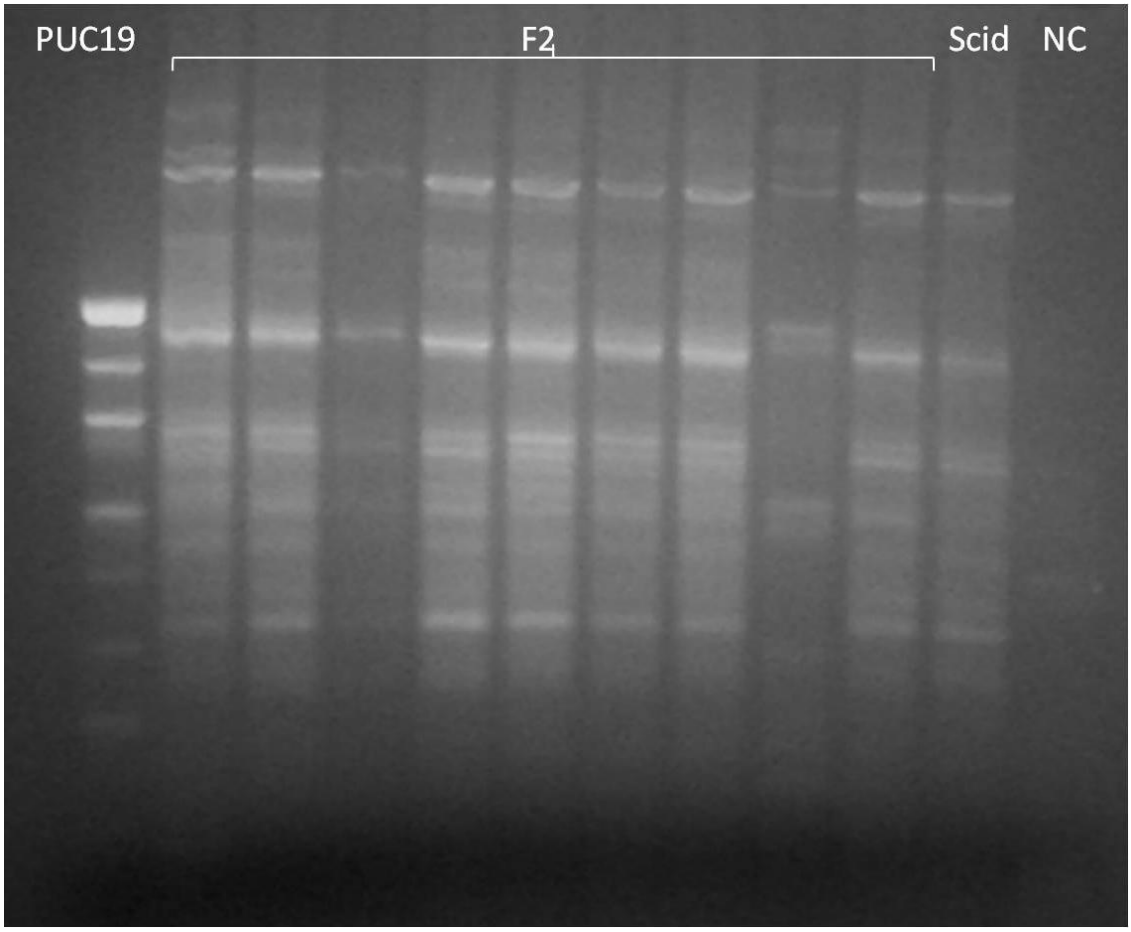
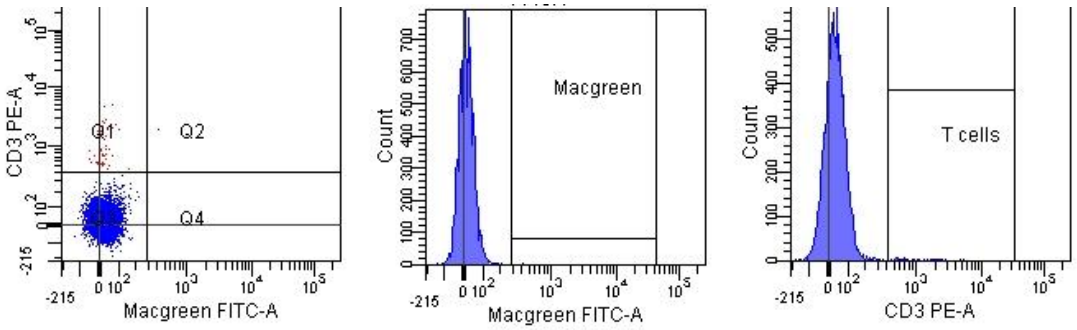


Figure 2. 4: Gel electrophoresis of *Alu1*-digested *prkdc* products of SCID x MacGreen mice to detect for the presence of SCID-point mutation.

Prkdc PCR products were subjected to Alu1 digest for two hour at 37°C. The digested products were separated on 3% agarose gel for 2 hours.

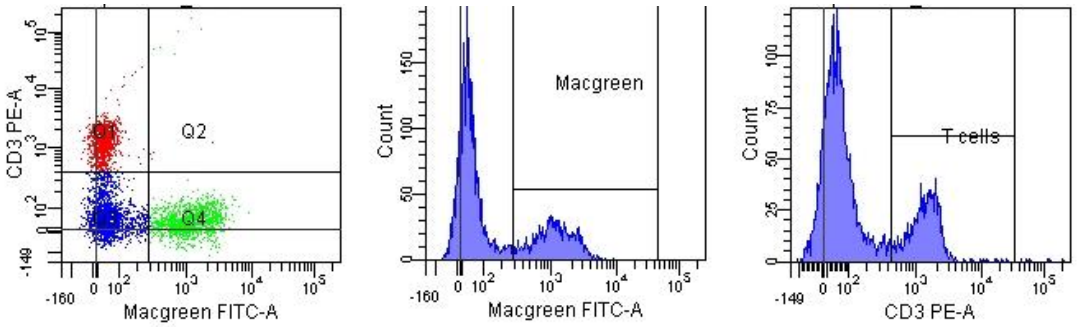
Prkdc^{Scid}/*c-fms-eGFP*$^{-/-}$

A



Prkdc$^{+/+}$/*c-fms-eGFP*$^{+/\pm}$

B



Prkdc^{Scid}/*c-fms-eGFP*$^{+/\pm}$

C

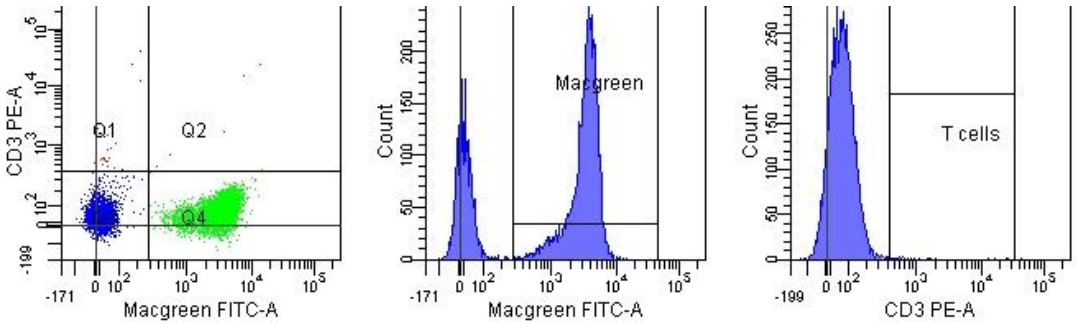


Figure 2. 5: The percentage of T lymphocyte-specific CD3e-positive cells of *Prkdc*^{Scid} mouse (A) and SCID x MacGreen progeny (C).

Blood samples obtained from SCID x MacGreen progeny and *Prkdc*^{Scid} mice were labelled with PE-conjugated CD3e antibody and analysed by flow cytometry. The cell population was gated to exclude debris and red blood cells. The absence of red-labelled T cells in SCID mouse (A) is noticeable in the Q1 of quadrant plot and in the T cell histogram. This mouse was also negative for GFP-macrophage (MacGreen) as indicated in Q4 of the plot and MacGreen histogram. This strain of mouse was crossed to MacGreen mice with a wildtype level of T cell population (B) to produce progeny with MacGreen cells and no/low CD3e T cells (C).

Chapter 3

3.1 Introduction

3.1.1 Transforming growth factor beta 1

Transforming growth factor beta 1 (TGFB1) is a multi-functional growth factor that promotes cellular proliferation, immune regulation and wound healing. There is evidence that TGFB1 is a key growth factor associated with endometriosis development. TGFB1 protein has been found to be elevated in the peritoneal fluid, serum and lesions of women with endometriosis than in disease-free controls [165, 166, 250]. The bioavailability of TGFB1 in serum and peritoneal fluid showed an association with the severity of disease, as women with rASRM Stage II endometriosis (n = 10) had higher TGFB1 levels than those with rASRM Stage I disease (n = 10) and control women (n = 5). Furthermore, women (n = 6) with Stage III endometriosis had higher TGFB1 protein levels than Stage II women (n = 10) in both serum and peritoneal fluid [165].

Additionally, immunoreactivity against the secreted form of TGFB1 protein was increased in the epithelium of patients with ovarian endometriotic cysts (n = 11) compared to the epithelium of endometriosis-free endometrium (n = 13) [168]. High levels of TGFB1 protein in peritoneal fluid and lesions indicate that this cytokine is likely to be important in endometriosis and could influence the severity of this disease.

There is evidence that TGFB1 is highly involved in endometriotic lesion development. A gene array study using a mouse model of endometriosis showed that the *Tgfb1* mRNA was upregulated and a central molecule in a regulatory molecular network that

appeared to be highly significant in the cross talk between ectopic endometrial tissue and the host peritoneum [146]. A model of the pathophysiological events that lead to endometriosis has been formulated using data from animal and human studies. Flores *et al*. [56] proposed that there was an initial activation and infiltration of inflammatory cells, followed by the adhesion and invasion of endometrial cells, angiogenesis, inhibition of apoptosis and finally fibrosis [56]. The inflammatory cells, including macrophages could be one of the possible sources of TGFB1 [251] during endometriosis development. TGFB1 has been postulated to be involved in cell adhesion, extracellular matrix remodelling and increased cell survival [56, 73].

TGFB1 mediates its cellular functions via ligation to TGFB type II receptor (TGFBR2). *Tgfbr2* conditional knockout mice with an induced disruption of exon 4 displayed a similar phenotype to that of the*Tgfb1* null mice [252]. A fatal wasting syndrome developed in mice carrying floxed exon 4 (*loxP*-flanked exon 4) following administration of interferon inducer polyI:polyC, which activated *Cre*-recombinase transgene and in turn excised exon 4 of *Tgfbr*2 [252]. Further histological examination revealed extensive multi-focal inflammation in major organs, including heart and lungs characterised by massive infiltration of lymphocytes and granulocytes.

Macrophage-derived TGFB1 could be responsible for the initiation of endometrial adhesion onto the surface of the peritoneum. *TGFB1* mRNA silencing using an anti-sense gene sequence noticeably reduced gene expression of α3 and α6 integrins in culture of U937 macrophage cells [251]. The authors speculated that TGFB1 protein expression by macrophages may influence endometriosis development, although no endometrial cell experiments were undertaken.

3.1.2 *Tgfb1*-null mutant mice

TGFB1 is a multifunctional cytokine and some of these functions overlap with other isoforms, TGFB2 and TGFB3. The non-redundant functions of TGFB1 in diseases can be determined using null mutant mice. *Tgfb1*-/- mutant mice were originally developed by Shull *et al*. [192]. Only 50% of *Tgfb1*-/- embryos survived post-natally, while the rest exhibited developmental retardation in embryonic haematopoiesis and disrupted vascularisation of the yolk sac at the pre-implantation stage [196, 253]. Homozygous *Tgfb1*-knockout mice usually have a shorter lifespan compared to their wildtype littermates, suffering from a wasting syndrome marked by the inflammation of major organs. Kulkarni *et al*. [199] did not detect an overcompensatory expression of either *Tgfb2* or *Tgfb3* mRNA in *Tgfb1*-null mutant pups.

One of the TGFB1 functions that was not detected when the other TGFB isoforms were deleted in mice is its role in regulation of immune cells [254-256]. Histological analyses of *Tgfb1*-/- pups revealed massive infiltration of lymphocytes and macrophages into multiple organs, including heart and lungs [199]. Inflammatory exacerbation seen in *Tgfb1*-/- pups clearly demonstrates the important roles of TGFB1 in protecting major organs against autoimmune disease.

The lifespan of *Tgfb1*-/- mice can be increased when their immune system is suppressed by either breeding into a severe combined immunodeficiency (*Prkdc*^{Scid}) background or neutralising their immune cells. For this reason, in our study *Tgfb1*-/- mice were bred onto an immunocompromised SCID background to extend their life span.

3.1.3 Tissue remodelling in endometriosis

TGFB1 is an important cytokine which promotes tissue remodelling by mediating myofibroblast differentiation and neo-angiogenesis. Myofibroblasts are activated fibroblasts expressing alpha smooth muscle actin (αSMA). In human fetal lung fibroblasts, TGFB1 initiated the transdifferentiation of fibroblasts to myofibroblast *in vitro* and *in vivo* [257]. TGFB1 was shown to induce the upregulation of αSMA through TGFBR2 [258]. A higher number of myofibroblasts is present in the proliferative phase human endometrium when compared to the endometrium at the secretory phase [259]. Myofibroblasts were also seen in higher abundance in deep-infiltrating endometriosis [145]. An overexpression of the *ACTA2* gene, encoding for αSMA protein was detected in peritoneal endometriotic lesions (n = 18) when compared to paired normal endometrium [259]. These findings suggest that myofibroblasts have a role in ectopic and eutopic endometrial remodelling and regeneration.

Our understanding of endometriotic lesion development is impaired by the poverty of naturally occurring animal models to study this disease. Mouse models have been developed to study endometriosis and proven to be useful to elucidate the critical events taking place during endometriosis development. As TGFB1 appears to be important during the critical stages of endometriosis development, we chose to study the functional roles of TGFB1 using *Tgfb*1 null mutant mice. The aim of the study was to examine the effects of host derived TGFB1 deficiency on the endometriotic lesion development.

3.2 Materials and methods

Materials and methods for this chapter are described in detail in Chapter 2. Briefly, eutopic endometrial fragments from pre-menopausal (n = 7) patients were subcutaneously injected into ovariectomised and estrogen supplemented *Tgfb1*-/- mice (n = 8), as well as littermate *Tgfb1*+/+ (n = 19) and heterozygous (*Tgfb1*+/-) (n = 4) controls. Ten days following tissue injection, the resulting lesions were collected and measured.

3.3 Results

3.3.1 Patient demographics

Endometrial tissues were obtained from seven pre-menopausal patients, aged 22 to 45 years (median = 38 years) at the time of surgery. All patients had a normal 28-day cycle, with no hormonal medications for 3 months prior to tissue collection. Five patients had laparoscopy surgery and two patients demonstrated no symptoms of endometriosis. Of five patients, three patients did not have any visible endometriotic lesions, one patient had Stage I endometriosis and one patient was at Stage II (**Table 3.1**).

3.3.2 Endometriosis-like lesion development

Endometriosis-like lesions were evident in comparable proportions of recipient mice irrespective of genotype, including 13 of 19 *Tgfb1*+/+ mice (**Figure 3.1A**; 68%), 4 of 4 *Tgfb1*+/- mice (100%) and 5 of 8 *Tgfb1*-/- mutant mice (63%). Although there was some variation in the number of mice that developed lesions in each experiment, in

both the control (n = 1-3) and *Tgfb1*-/- groups (n = 0-1), the lesions were relatively evenly distributed across the experiments.

The median weight of lesions that developed in *Tgfb1*-/- mice was significantly reduced by 11-fold compared to lesions that developed in *Tgfb1*+/+ mice [median (range) = 0.0028 g (0.0024-0.0156 g) and 0.0313 g (0.0013-0.12 g) respectively; p = 0.031]. The median lesion weight in the *Tgfb1*-/- demonstrated a 6-fold reduction compared to lesions that developed in *Tgfb1*+/- mice [median (range) = 0.016 g (0.013-0.019 g)] (**Figure 3.1B**). A 2-fold decrease in median lesion weight in *Tgfb1*+/- mice compared with *Tgfb1*+/+ mice was also shown. These results suggest a gene dose response effect, although the small sample size in the *Tgfb1*+/- group precluded statistical analysis. The median lesion size (**Figure 3.1C**) was not affected by the genotype of host mice [median (range) = 10.5 (127.2-0.036 mm^3) for *Tgfb1*+/+, median (range) = 3.0 (0.018-8 mm^3) for *Tgfb1*+/- and median (range) = 3.8 (0.0020-14.2 mm^3) for *Tgfb1*-/-].

3.3.3 Morphometric analyses

To examine endometriosis-like lesions morphometrically, five sections from *Tgfb1* +/+ (n = 19) and *Tgfb1* -/- (n = 5) mice were stained with haematoxylin and eosin (**Figure 3.2**). In *Tgfb1* +/+ mice the percentage of glandular and stromal areas each comprised approximately half of the total endometriotic lesion area [**Figure 3.2E**; median (range) = 52.2% (30.5-66.7%) and 47.8% (33.3-69.5%) respectively]. In contrast, the median glandular area in lesions from *Tgfb1*-/- mice was reduced to 35.4% (14.1-64.0%), with a corresponding increase in the stromal area to 64.6% (36.0-86.0) ($p<0.05$).

Glandular morphology was examined to determine the effects of TGFB1-deficiency on glandular development in the lesion. The absence of host TGFB1 did not impair the

number of glands present in the endometriosis like lesions [median (range) = 17 (6-32) for lesions from *Tgfb1*-/- mice and 11 (2-24) for lesions from *Tgfb1*+/+ mice; p = 0.083; **Figure 3.3C**]. In average, glands in lesions harvested from *Tgfb1*-/- mice were 86.8% smaller than the glands in lesions from *Tgfb1*+/+ mice [**Figure 3.3D**; median (range) = 0.029 mm^2 (0.0082-0.17 mm^2) and 0.22 mm^2 (0.0025-1.0 mm^2), respectively; p = 0.019]. The total lumen area was also reduced by 75.7% in the lesions from *Tgfb1*-/- mice compared to those from *Tgfb1*+/+ mice [**Figure 3.3E**; median (range) = 0.45 mm^2 (0.070-0.91 mm^2) and 1.9 mm^2 (0.080-5.2 mm^2), respectively; p = 0.031]. The size of lumen and glands were proportionally similar in both groups, since the epithelial cell layer was not altered in the absence of host TGFB1 [**Figure 3.3F**; median (range) = 0.22 mm^2 (0.056-0.63 mm^2) for *Tgfb1*+/+ lesions and 0.13 mm^2 (0.064-0.49 mm^2) for *Tgfb1*-/- lesions; p = 0.23].

3.3.4 Macrophage abundance in periphery

An antibody against F4/80 was used to localise mouse-derived macrophages present in the human endometriosis-like lesions in *Tgfb1*+/+ (**Figure 3.5C**) and *Tgfb1*-/- (**Figure 3.5D**) mice. In both groups, murine macrophages were observed predominantly in the peripheral regions of the lesions, and sparsely within the central part of the tissues. Macrophage abundance up to a 100 µm depth into the lesion from the periphery was reduced by 66% in lesions from *Tgfb1*-/- mice compared to *Tgfb1*+/+ mice [**Figure 3.5E** ; median (range) = 1801 cells/mm^2 (528-3072 cells/mm^2 in *Tgfb1*+/+ mice compared to 613 cells/mm^2 (148-1532 cells/mm^2) in *Tgfb1*-/- mice; $p<0.05$)]. F4/80 antibody detects macrophages of mouse origin only, as demonstrated by the positive staining in mouse uterine tissue (**Figure 3.4B**) and the absence of staining in human endometrium (**Figure**

3.4C). Non-specific rat IgG2a antibodies did not produce specific staining in xenograft tissue (**Fig 3.4A**).

3.3.5 Myofibroblast abundance

Immunostaining with αSMA antibody examined the abundance of myofibroblasts in the stromal compartment of endometriosis-like lesions from *Tgfb1*+/+mice (**Figure 3.6C**) and *Tgfb1*-/- mice (**Figure 3.6D**). The area comprised by myofibroblasts throughout the lesion was reduced by 47% in lesions from *Tgfb1*-/- mice compared to *Tgfb1*+/+ mice [**Figure 3.6E**; median (range) = 15.0% (4.3-28.1%) positivity in *Tgfb1*+/+ compared to 8.0% (0.66-14.9%) positivity in *Tgfb1*-/- mice; $p<0.05$]. Staining was not demonstrated by mouse IgG2a negative control antibodies in xenograft tissue (**Figure 3.4D**).

3.3.6 Cell proliferation and blood vessel density

The incorporation of a synthetic nucleoside, BrdU into dividing cells during S-phase of cell cycle was used to assess the proliferative activity of the endometriosis-like lesions. Proliferating cells were evident in both the stromal and epithelial compartments in lesions from *Tgfb1*+/+ mice (**Figure 3.7B, C**) and *Tgfb1*-/- mice (**Figure 3.7E, F**). BrdU-positive cells were counted manually in five fields in a blind manner. The number of BrdU positive epithelial cells [median (range) = 7.4% (0.050-10.7%) for *Tgfb1*+/+ and 5.6% (0.63-15.9%) for *Tgfb1*-/- mice] and stromal cells [median (range) = 3.4% (0.0030-9.4%) for *Tgfb1*+/+ mice and 2.1% (0.56-7.8%) for *Tgfb1*-/- mice] was not altered by genotype (**Figure 3.7I**). No positive BrdU staining was identified in an unlabelled mouse uterine section (**Figure 3.7H**).

Blood vessel distribution in endometriosis-like lesions was examined by von Willebrand Factor (vWF) immunostaining. Blood vessels were observed throughout lesions from *Tgfb1*+/+ and *Tgfb1*-/- (**Figure 3.8C** and **D**, respectively) mice, and neither the average area of the vessels [**Figure 3.8E** ; median (range) = 398 μm^2 (158-1202 μm^2) for *Tgfb1*+/+ and 333 μm^2 (243-471 μm^2) for *Tgfb1*-/-] nor the percent positive staining with the lesions [**Figure 3.8F**; median (range) = 0.92% (0.28-2.1%) for *Tgfb1*+/+ and 0.93% (0.089-1.2%) for *Tgfb1*-/-] were affected by genotype. vWF antibody detection of both mouse and human vessels can be observed in mouse uterine (**Fig 3.4H**)) and human endometrium tissues (**Figure 3.4I**). Specific staining of xenograft tissue was not identified using normal polyclonal rabbit IgGs (**Figure 3.4G**).

Table 3. 1: Clinical data of the recruited patients for eutopic endometrial tissue collection

Experiment/Patient	Age at the time of surgery	Stage of cycle	Revised AFS score
1	30	Proliferative	No (S)
2	45	Proliferative	No (S)
3	22	Proliferative	Stage II
4	38	Late Secretory	Stage I
5	45	Proliferative	No (S)
6	34	Proliferative	Asymptomatic
7	42	Mid Secretory	Asymptomatic

No (S) = surgically proven no endometriosis

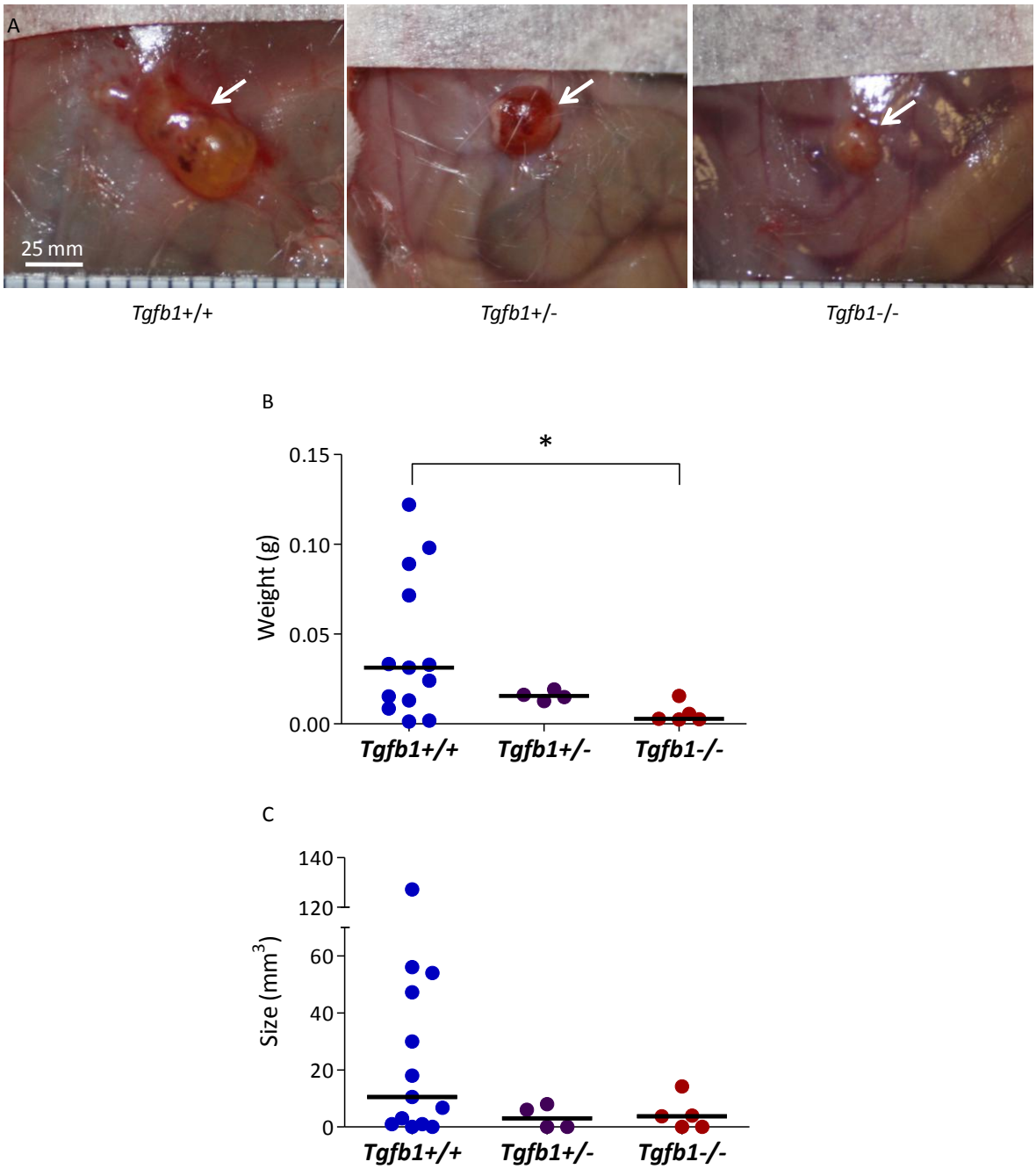


Figure 3. 1: Gross morphology of endometriosis-like lesions photographed at day 10 after tissue injection.

Human endometrial fragments were subcutaneously implanted into *Tgfb1*-wildtype, heterozygous and knockout mice. The resulting lesions (black arrow) were photographed at day 10 after implantation. Data were analysed using the Mann Whitney U test, with significance inferred at $p<0.05$. Each symbol represents data from one lesion, with median percent shown as a bar.

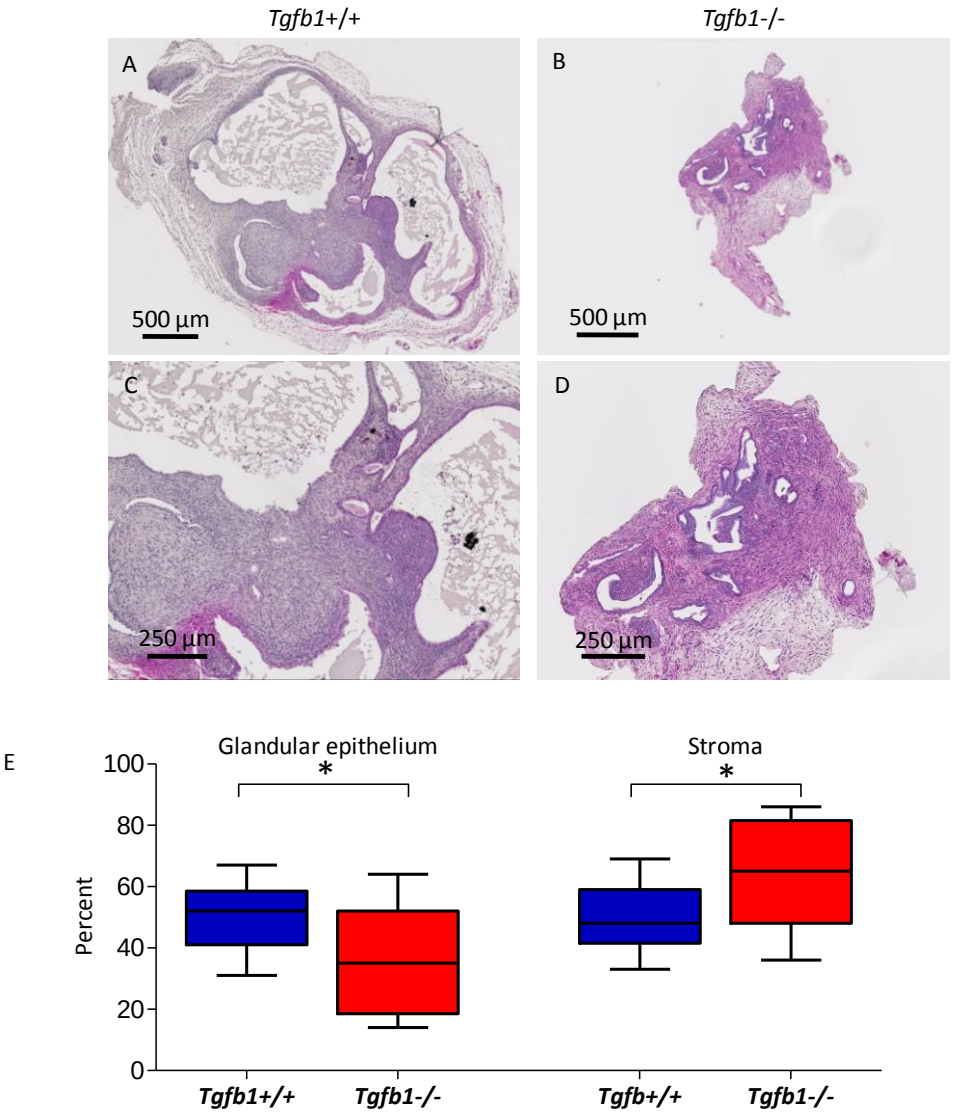


Figure 3. 2: Morphology of endometriosis-like lesions as stained using Haematoxylin and Eosin.

Day 10 lesion sections from *Tgfb1*+/+ (A and C) and *Tgfb1*-/- (B and D) contain glandular epithelium and stroma, characteristics of human endometrial tissues. (E) The percent of glandular area and stromal area was determined from each lesion and the data were plotted for each mouse. The medians (horizontal bars) were analysed using Mann Whitney U test (* $p<0.05$). Boxes represent the 25^{th} to 75^{th} percentile, with median line; error bars represent minimum and maximum values.

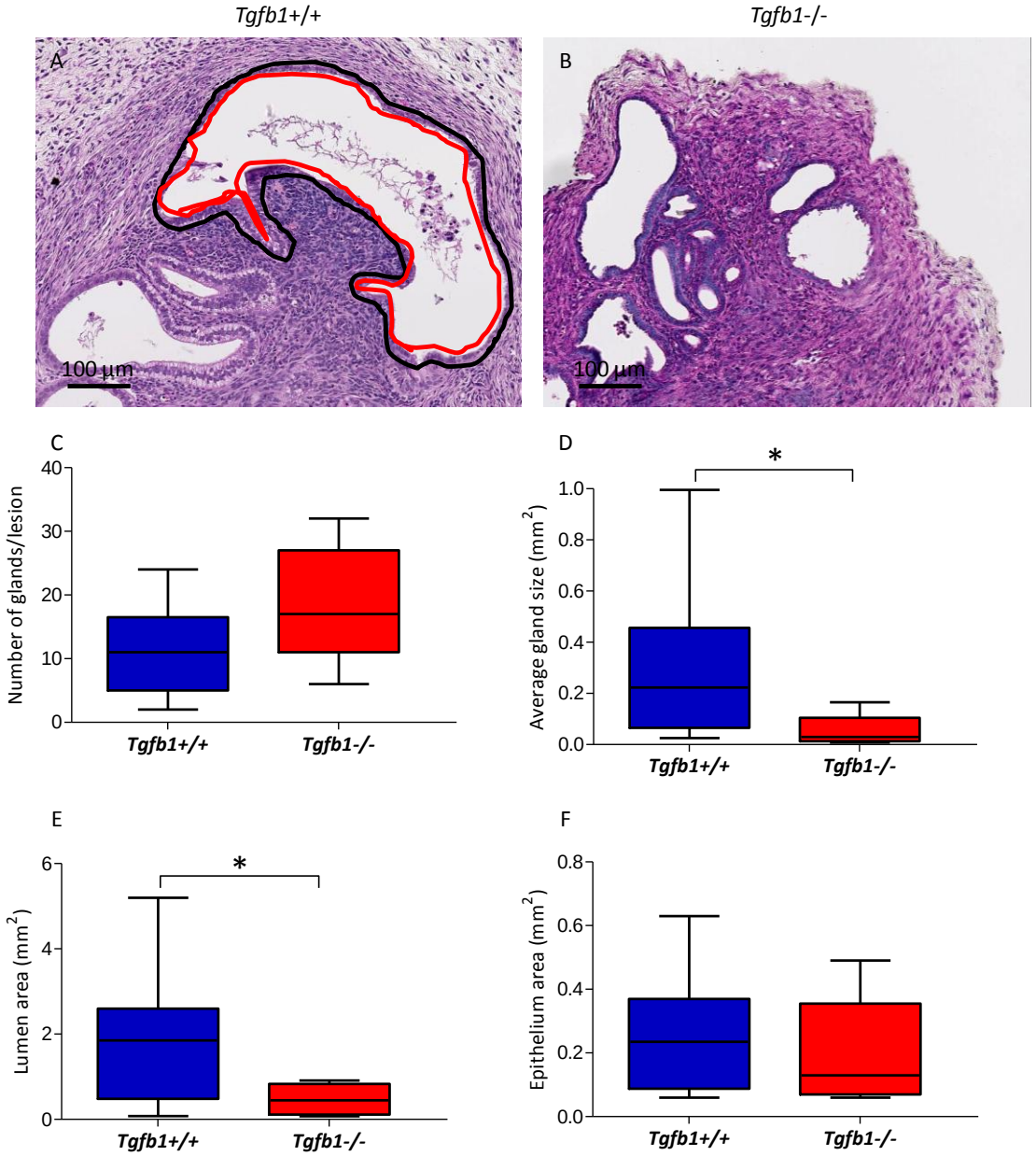


Figure 3. 3**:** Glandular morphology of endometriosis-like lesions from *Tgfb1*+/+ and *Tgfb1*-/- mice.

(A and B) The glands of lesions from both genotypes consisted of epithelial cell layer and lumen (red line). (C) The number of glands present in each lesion was comparable between both groups. (D) The average size of the glands however was lower in the lesions from *Tgfb1*-/- mice. (E) The total lumen area per lesion was also reduced in *Tgfb1*-/- lesions compared to *Tgfb1*+/+. (F) The total epithelial cell area was not altered in the absence of host TGFB1. The medians (horizontal bars) were analysed using Mann Whitney U test (* $p<0.05$). Boxes represent the 25th to 75th percentile, with median line; error bars represent minimum and maximum values.

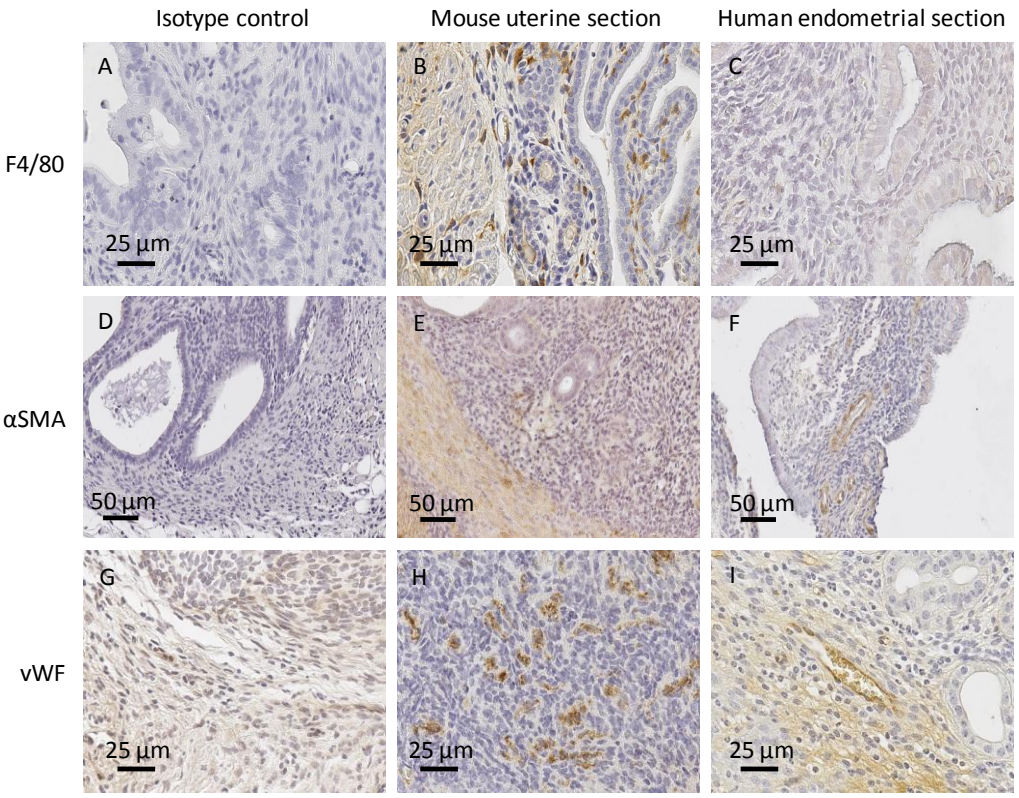


Figure 3. 4: Isotype and species-specific controls for each immunostaining condition.

The specificity of each primary antibody was confirmed by substituting the primary antibody with an isotype-matched IgG (A, D and G). Endometriosis-like lesion sections were incubated with non-immunised rat IgG for F4/80 staining (A), mouse IgG2a for αSMA (D) and rabbit IgG for vWF (G). Species specificity of the primary antibodies was tested on both murine (B, E and H) and human tissues (C, F and I).

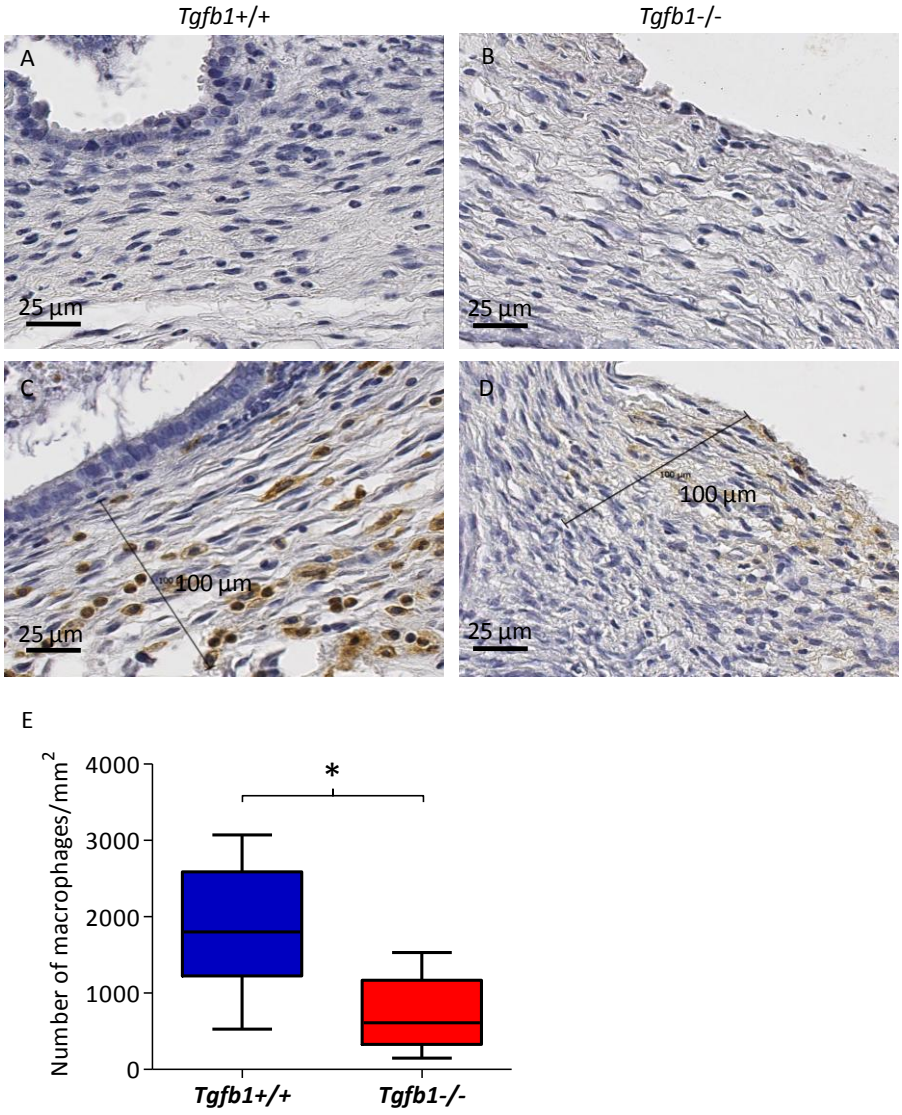


Figure 3. 5: F4/80 immunostaining to localise murine macrophages in *Tgfb1*-wildtype (C) and -knockout (D) lesions.

Omission of the primary antibody (PBS only) did not demonstrate specific staining (A and B). The number of F4/80-positive cells was counted from five different fields within 100 µm peripheral region (E). Data were analysed using the Mann Whitney U test (* $p<0.05$). Boxes represent the 25th to 75th percentile, with median line; error bars represent minimum and maximum values.

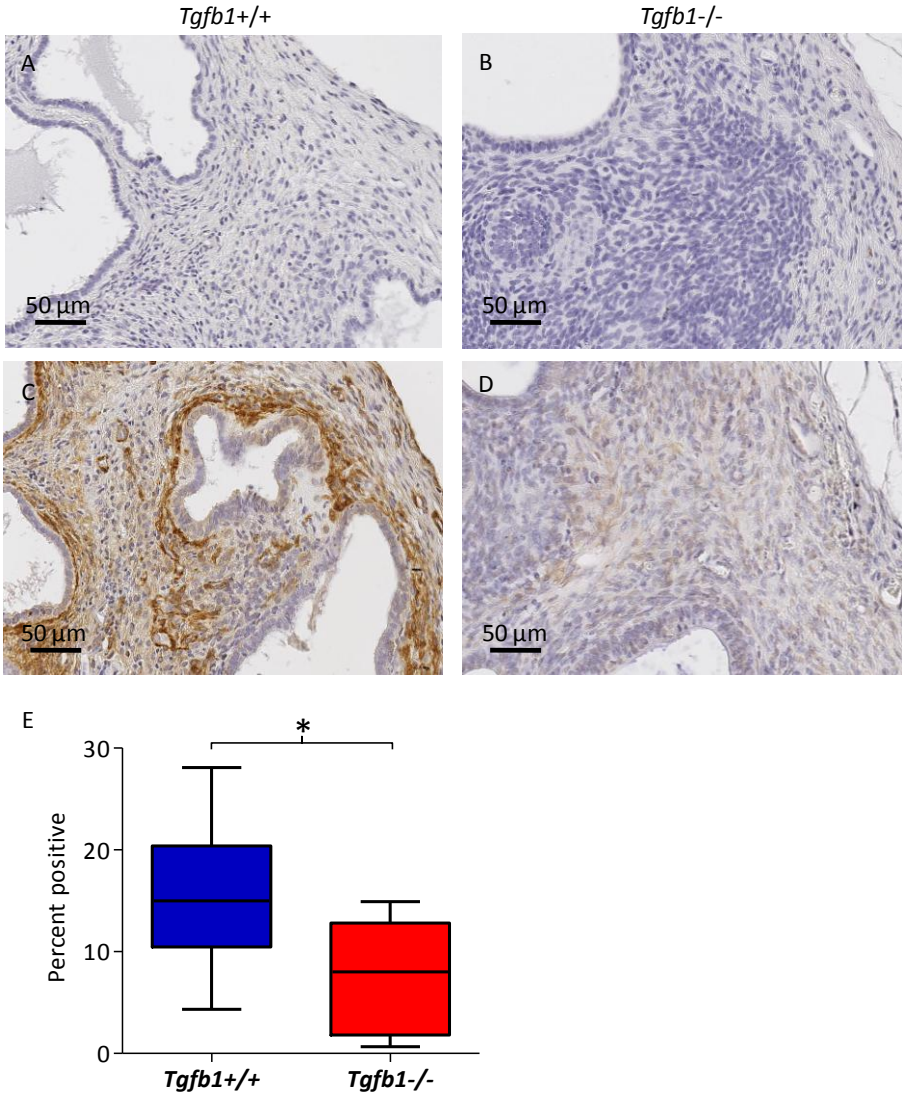


Figure 3. 6: Alpha smooth muscle actin (αSMA) immunostaining to localise myofibroblasts in *Tgfb1*-wildtype (C) and -knockout (D) lesions.

Omission of the primary antibody (PBS only) did not demonstrate specific staining (A and B). The percent area for αSMA-positive cells was determined from five different fields using ImageJ software (E). Data were analysed using the Mann Whitney U test (* $p<0.05$). Boxes represent the 25^{th} to 75^{th} percentile, with median line; error bars represent minimum and maximum values.

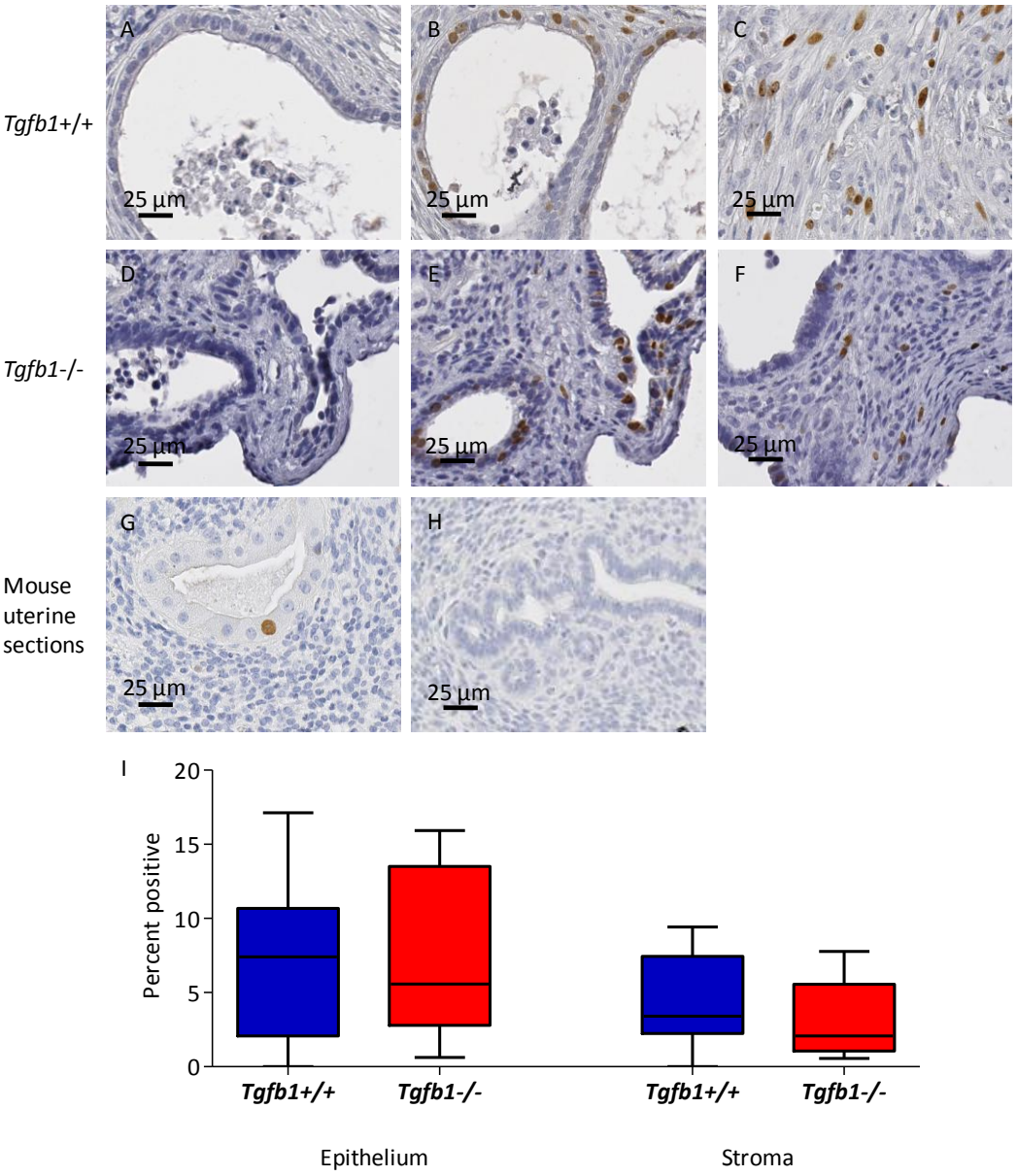


Figure 3. 7: BrdU immunostaining to localise proliferating cells in *Tgfb1*-wildtype (B and C) and –knockout (E and F) lesions in glandular epithelium (B and E) and stroma (C and F).

Omission of the primary antibody (PBS only) did not demonstrate specific staining (A and D). BrdU immunostaining was performed using a commercially available kit and the isotype of primary antibody is not disclosed by the manufacturer. Uterine sections from mice exposed (G) and unexposed (H) to BrdU were used as controls for specificity of the staining. The percent number for BrdU-positive cells was determined from five different fields. Data were analysed using the Mann Whitney U test ($p>0.05$). Boxes represent the 25^{th} to 75^{th} percentile, with median line; error bars represent minimum and maximum values (I).

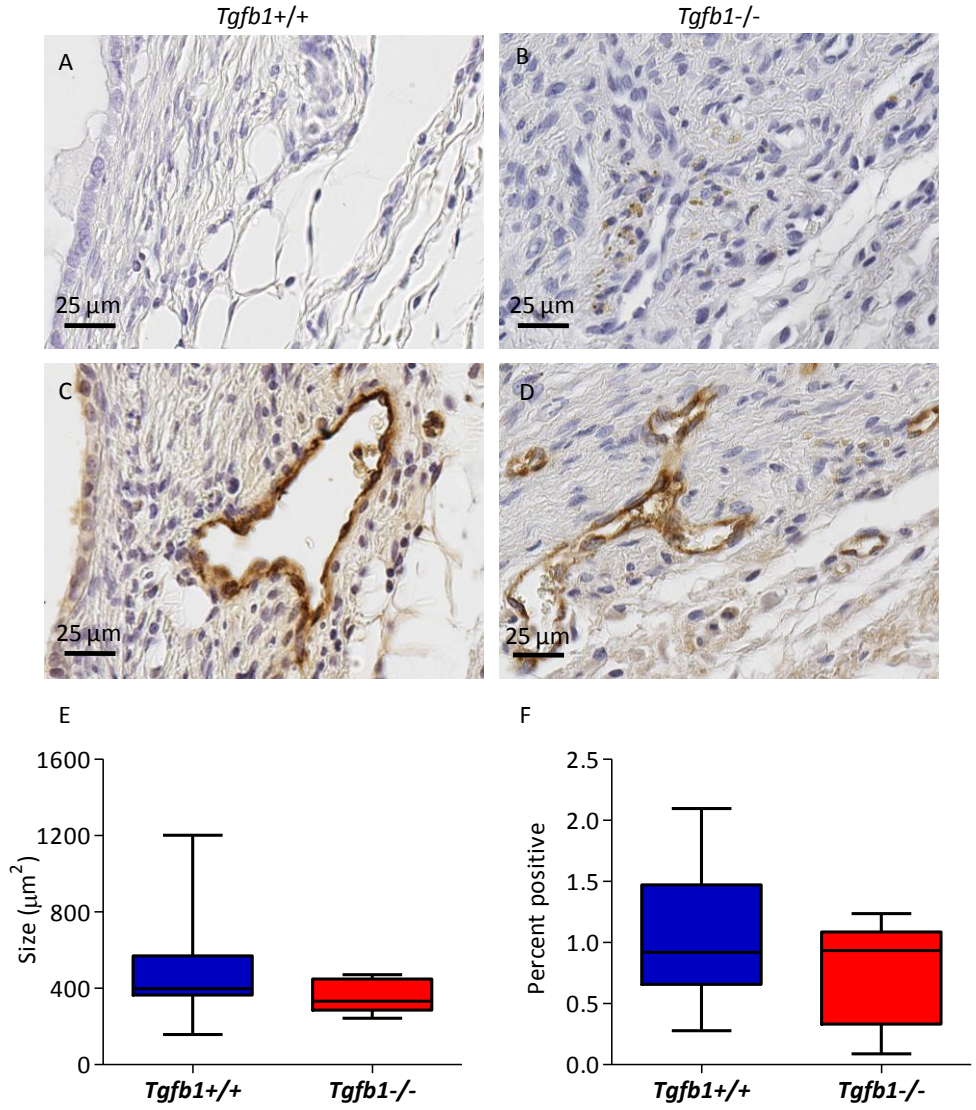


Figure 3. 8: von Willebrand Factor (vWF) immunostaining to localise blood vessels in *Tgfb1*-wildtype (C) and -knockout (D) lesions.

Omission of the primary antibody (PBS only) did not demonstrate specific staining (A and B). The size and percent area of the vWF-positive blood vessels were determined from five different fields using ImageJ software (E and F). Data were analysed using the Mann Whitney U test ($p>0.05$). Boxes represent the 25th to 75th percentile, with median line; error bars represent minimum and maximum values.

3.4 Discussion

3.4.1 Summary of findings

A similar proportion of both *Tgfb1*+/+ and *Tgfb1*-/- mice developed lesions after receiving human endometrial tissue implants. This implies that the early phases of ectopic endometrial lesion attachment and establishment were not impacted by a TGFB1 deficiency. This is consistent with the findings of Bruner-Tran *et al*. showing that TGFB1 did not influence the attachment of eutopic endometrial tissue in immunocompromised mice [260].

In contrast, Beliard *et al*. [261] suggested that pre-incubation of human endometrial cells with TGFB1 could promote their adhesion to the murine peritoneum *in vitro*. However, this was also seen when they incubated endometrial cells with IL-1β, IL-6 and TNFα. This suggests TGFB1 is not the only cytokine required for the attachment of human endometrial cells onto the murine peritoneal layer. Therefore, the absence of host TGFB1 did not impair the number of mice that developed lesions in our study.

The initial size of each endometrial piece was approximately 1 mm^3. Yet in the majority of mice, only one lesion was collected with dimensions larger than 1 mm^3 consistent with the endometrial pieces clumping together *in vivo* to form a single lesion.

The median weight of endometriosis-like lesions from *Tgfb1*-/- mice was significantly lower than the lesions from the *Tgfb1*+/+ mice. Although human derived TGFB1 from the endometrial tissue could have influenced the results, the absence of TGFB1 in host-derived cells effectively reduced the mass of lesions in the knockout mice, indicating its critical impact on normal lesion development.

We found host-derived TGFB1 was important in macrophage recruitment to the endometriotic lesions. Lesions from *Tgfb1*+/+ mice had a higher macrophage density than those from *Tgfb1*-/- mice, suggesting TGFB1 could be essential for macrophage signalling in the lesions. In the absence of TGFB1 in the host compartment, endometriotic lesions also contained a lower density of myofibroblasts compared to the TGFB1-replete host.

3.4.2 Validation of models

This is the first study to mimic endometriosis in *Tgfb1*-/- mice using human endometrial tissues. A number of studies have examined the importance of TGFB1 in endometriosis, but these were limited to *in vitro* experiments [260]. Due to the nature of *Tgfb1*-/- mice which have a shorter lifespan than other littermates and are prone to develop a wasting syndrome at the age of 12 weeks [197], the experiment was conducted before the mice reached 10 weeks of age.

It was not possible to explore lesion development at multiple time-points as only one *Tgfb1*-/- mouse was available for each experiment. A study by Hull *et al*. [146] using nude mice as an endometriosis model identified three key events: myofibroblast activity, macrophage infiltration and blood vessel formation that took place during endometriosis development. These events began at day 7 and reached their peak at day 14, as demonstrated by centrally localised myofibroblasts, massive influx of macrophages and fully formed blood vessels. As day 10 sits between these two time-points it represents the central phase of tissue remodelling and more importantly fits into the short experimental lifespan of the *Tgfb1*-/- mouse.

We also demonstrated that human endometrial tissues from different stages of the menstrual cycle have the ability to adhere and grow in the mouse models. Adherence of human endometrial tissues was not affected by host TGFB1 bioavailability as nearly all mice developed lesions after 10 days of tissue injections. The growth of the endometrial tissues in the ectopic environment was demonstrated by the presence of BrdU-positive cells in the glandular epithelium and stromal compartments of the lesions.

In the current study, endometrial samples from women with or without endometriosis and unknown disease status were used as similar number of lesions were seen in immunocompromised mice using these 3 types of endometriail tissues in studies by our group [146]. This published study shows that eutopic endometrial tissues from endometriosis-free patients readily implanted in mouse models, indicating that endometrial fragment attachment and growth is not highly dependent on the donors' disease status. In addition, all mouse models are not ideal and the use of human tissue in immunocompromised mouse models necessitates the availability of an endometrial sample in a small window of time. Because of this the parameters of disease status and stage of cycle need to be broad to successfully complete these experiments

Eutopic tissues (i.e. day 0) were histologically assessed using Noyes criteria as a reference for cycle stage and as a tissue control for staining. The weight and size of the eutopic endometrial tissue samples would be very arbitrary and the glandular and stromal volume fractions were completely different to xenografts in species composition, physiological status and cellular composition, so this comparison was not included. We wished to assess the difference in lesions from host deficient and replete environments, and the same T0 endometrium was used in both experimental arms. It

is demonstrated in **Figure 3.3** that although tissues were collected from women at secretory and proliferative stages of menstrual cycle, the absence of host TGFB1 effectively reduced the percent glandular area in all experiments independent of eutopic endometrial cycle stage. This evidence indicates that the cycle stage of tissue origin did not seem to impact the glandular morphology and size.

Damage afflicted by the 19G needle on endometrial tissues during injections was minimal and was not assessed. Although there was a possibility the tissues were damaged by the injections, this damage did not affect the attachment and growth of endometriosis-like lesions and any effects would be consistent across the experimental groups.

3.4.3 Morphology of the endometriosis-like lesions

In normal endometrium, TGFB1 is localised to both glandular and stromal cells [262], although TGFB receptors are more significantly expressed in glandular epithelial cells [145]. The presence of these receptors in epithelial cells demonstrates the importance of TGFB1 signalling in regulating epithelial cell activity. Therefore, the lack of TGFB1 ligand in the surrounding environment reduced the cellular activity of epithelial cells, which include the production of important cytokines and chemokines for ectopic tissue growth.

Glandular epithelial cells are also an important source of cytokines and chemokines, many of which are upregulated in endometriosis. In the literature, a few chemokines, including CCL16 and CCL21 were found to be upregulated in endometrial glands of patients with endometriosis [263]. As these chemokines are secreted by glandular cells,

they could be important for ectopic endometrial growth and chemotactic activity for immune cells, lymphocytes and monocytes.

An increase in glandular and luminal areas was noted in human endometrial xenografts in *Tgfb1* replete mice compared to *Tgfb1* null mice. The absence of host TGFB1 results in a decrease in the glandular epithelium activity as observed by reduced glandular and lumen area, hence affecting the growth of the endometriosis-like lesions as observed in the reduced weight of the lesions. This finding was in agreement with studies using immunodeficient mouse models (SCID and nude mice) with normal levels of TGFB1, as glands and lumen appeared to expand in xenograft over time [146, 264, 265]. The increase in glandular fraction was previously shown to be associated with the glandular modelling [146] and suggested to reflect enhanced secretion by glandular epithelium [213] under the influence of steroid hormones, including estradiol.

Thus, in lesions from *Tgfb1*-/- mice, the reduction in glandular and luminal areas could reflect diminished glandular secretion, which may disrupt the normal functions of ectopic endometrial glands. Given that *Tgfb1*-/- mice were reportedly having a normal uterine glandular structure [198], reduced glandular and luminal areas could be due to the xenograft-specific response to the host TGFB1 deficient environment. Although, the glandular area was significantly decreased in the absence of TGFB1, no difference was observed in the thickness of epithelial cell layer between both groups, suggesting that proliferative rate of epithelial cells was unaffected in the absence of host TGFB1 (as discussed in 3.4.6).

Many studies have implied that the endometrial gland development in animal models is mediated by estrogen (reviewed by [266]). In a neonatal mouse uterus, *Wnt* and *Hox*

gene families are important in uterine glandular development (reviewed by [266, 267]). Assuming the development of ectopic glands mimics its eutopic counterpart, the disruption in TGFB1 signalling may impair the normal remodelling of the glands, thus reducing the glandular area. Glandular growth is mediated through estradiol since estrogen deficient ovariectomised baboons had smaller endometrial glandular areas [268]. In an estrogen receptor α knockout mouse, the expression of estrogen receptor in the stromal compartment was critical for the normal growth of glandular epithelial cells in eutopic endometrium (reviewed by [269]), exemplifying the supporting role of stromal cells in glandular development. Although in our study, we observed a larger stromal area in the absence of host TGFB1, this did not translate into an increased glandular fraction. Thus, the quality of the stroma rather than the proportional quantity of stromal tissue appears to have a higher impact on glandular development.

3.4.4 Lower macrophage abundance in lesions from TGFB1 deficient mice

Post-mortem examinations in *Tgfb1*-null mice showed an excessive infiltration of macrophages in important organs, which eventually led to their premature death [199]. Therefore TGFB1 is a crucial growth factor in moderating macrophage functions associated with inflammation. In endometriosis-like lesions however, the number of macrophages (F4/80-positive cells) were significantly reduced in the periphery of the tissues (Figure 3. 5; p. 116). This observation suggests that TGFB1 is not only important for deactivation of macrophage inflammatory phenotype, but also for trafficking of these cells into transplanted tissues. In the absence of host TGFB1, fewer macrophages were guided into the tissues, thus resulting in a lower cell density.

Cytokines secreted by the glandular epithelium could also have a role in attracting macrophages into lesions. Therefore, the association between a lower glandular area per lesions and a lower macrophage density in the TGFB1 deficient lesions could be due to a causal correlation.

Conversely, a decrease in the glandular area fraction in lesions from *Tgfb1*-/- mice could be due to a deficiency in macrophage remodelling activity as a result of low abundance of macrophages around the lesions. Macrophage density in lesions from *Tgfb1*-/- mice was significantly reduced as compared to the lesions from *Tgfb1*+/+ mice. In this study, the lack of glandular area correlated well with the macrophage abundance in the periphery of endometriosis-like lesions. As macrophages could be important in glandular remodelling [270], the reduced number of macrophages was consistent with the lower glandular area fraction observed in the host-TGFB1 deficient lesions. A similar observation has been reported in a wound healing study showing that macrophage depletion reduced re-epithelisation, vascularisation and collagen deposition [112].

During the menstrual cycle, macrophage abundance has been suggested to be associated with the clearance of apoptotic cells and glandular regeneration [270]. Although mouse models do not have menstrual cycles, a reduction in macrophage number as the result of TGFB1 deficiency could have reduced the clearance of apoptotic cells and thus influenced glandular remodelling.

Many studies have shown that macrophages are important in the inflammatory response and also have a role in tissue remodelling and cellular proliferation [112]. Peritoneal macrophages, along with neutrophils secrete VEGF, demonstrating that

macrophages have an ability to remodel ectopic endometrial tissues [81]. Active macrophages stimulate epithelial cell proliferation *in vivo* directly via secretion of hepatocyte growth factor (HGF) [271]. However, in this current study, no difference was found in either stromal or epithelial proliferation in the absence of TGFB1 and in an environment of reduced macrophage abundance. This suggests that a deficiency in macrophage-derived growth factor that stimulates epithelial proliferation was not the predominant mechanism for reduced lesions size in TGFB1 deficient lesions.

The role of macrophages in tissue remodelling has been extensively studied in wound healing experiments. Gene expression studies show that wounding is one the key biological processes identified during the tissue remodelling processes of endometriosis [146]. Following wounding, activation of macrophages is demonstrated to be essential for the next healing step. Similar to endometriotic lesion development, macrophages play a central role in wound healing in phagocytosing cell debris and mediating recruitment of myofibroblasts and neovascularisation [113, 146].

3.4.5 Myofibroblast reduction in the absence of host TGFB1

Myofibroblasts are mesenchymal cells expressing α smooth muscle actin filament fibre and have increased contractile ability compared to fibroblasts. In this study, αSMA staining area was carefully determined in the stromal area of the lesions. Only the staining area localised to the cells with fibroblastic shapes was included in the analysis, consistent with the appearance of myofibroblasts. Although it is possible that vascular smooth muscle cells (VSMCs) could have been included in the analysis most would have been excluded by these precautions.

Myofibroblasts were present within the stromal areas of lesions from *Tgfb1*-/- and *Tgfb1*+/+ mice, although the expression of this protein was significantly lower in lesions from *Tgfb1*-/- mice where it was reduced by 47%. This number is very significant considering other cytokines, such as GM-CSF, are also able to induce the expression of myofibroblast marker, αSMA [132]. TGFB1 therefore appears to be the major cytokine for myofibroblast activation in endometriosis-like lesions.

Myofibroblasts are the source of collagen type I and fibronectin [126], which are usually associated with extracellular matrix remodelling. As myofibroblast density was reduced in the endometriosis-like lesions, it seems likely that the ECM phase in the lesions from *Tgfb1*-/- mice was impaired.

3.4.6 Cell proliferation and blood vessel were not affected in lesions from host deficient in TGFB1

As the weight of the endometriosis-like lesions was significantly affected, the mechanisms by which the endometriotic lesion growth could be affected are through lower blood supply and reduced cell proliferation in the absence of host derived TGFB1. Therefore, cellular proliferation and vascularisation were measured. However, these parameters were not significantly different in the lesions from the wildtype and null mutant mice.

Cellular proliferation is one of the key steps in the development of endometriosis. The presence of BrdU-positive cells in glandular epithelium and stroma proves that human endometrium has the ability to grow and proliferate even in the ectopic environment, thus supporting Sampson's implantation theory. Although human and mouse share

some homology in molecular structures, there are still some mismatches in molecule-ligand interactions. The ability of eutopic endometrium fragments to proliferate and form blood vessels shows this model is a suitable *in vivo* model to study endometriosis. Host TGFB1 did not appear to be critical in regulating proliferation of endometriotic cells in the mouse model. Similarly, TGFB1 also did not appear to be important in regulating proliferation of cells *in vitro* cultures of endometrial cells [188]. It may be that other cytokines, such as interleukin-4 (IL-4) can stimulate the proliferation of endometriotic lesions [272] while the absence of estrogen receptor appeared to affect the proliferative activity of endometriotic lesions [243].

Angiogenesis is important during lesion establishment and took place as early as day 7 in the nude mouse model of endometriosis [146]. Our results showed that at day 10, the abundance of von Willebrand Factor-positive blood vessels were not affected in the absence of host TGFB1. Therefore, bioavailability of TGFB1 at the site of implantation did not appear to be critical for the normal formation of blood vessels. The bioavailability of other vascular growth factors may compensate for the absence of TGFB1, e.g. macrophage inhibitory factor (MIF), TNFα and IL-6 [81, 273]. The inhibition of MIF in endometriosis-like lesions from the inhibitor-treated nude mice (n = 8) showed reduced expression of VEGF mRNA level compared to vehicle control (n = 7) [273]. Neutrophils and macrophages are the source of VEGF in a syngeneic endometriosis mouse model and both cells secreted VEGF in response to TNFα and IL-6 stimuli [81]. Undisrupted levels of TNFα and IL-6 in our knockout models therefore did not alter the availability of VEGF, which is likely to be critical for angiogenesis in endometriosis-like lesions.

Angiogenesis is an important event in endometriosis establishment. Recently, a group of macrophages were identified mediating this process [234]. A specific population of macrophages expressing Tie2, a tyrosine kinase receptor was identified to be pro-angiogenic and particularly important in establishing blood vessels in endometriosis-like lesions of mouse model [77]. This population of macrophages has been shown to infiltrate endometriotic lesions and to promote endothelial cell viability in a syngeneic mouse model of endometriosis. Another type of Tie2-expressing cell important for neovascularisation is endothelial cells. Our study however, did not observe any significant reduction in blood vessel formation in the TGFB1 deficient lesions. The development of blood vessels in these lesions may be independent on the level of TGFB1 and could also be mediated by other TGFB isoforms or other cytokines.

Endometrium is a vascular tissue and the transplanted eutopic tissues might have pre-existing blood vessels. Previous data [146] showed that eutopic tissues collected using pipelle suction curettage had vWF-positive blood vessels. Evidence in literature using animal models and human data suggests that blood vessel formation is one of the critical steps during lesion development [56, 146]. In these models, the majority of vessels were stained positively for murine specific endothelial cell markers demonstrating host derived neoangiogenesis, although a small number of human endothelial cells also made up the vasculature of lesions[56, 146].

Tgfb1-null mutant mice have unaffected levels of TGFB2 and TGFB3 isoforms. These isoforms have affinity towards the same receptors which activate TGFB1 signalling. Thus it seems likely that the compensatory actions of other TGFB isoforms increase the redundancy TGFB1 during critical events of endometriosis lesion development, such as in cell proliferation and angiogenesis.

Apoptosis factor was not examined in endometriosis-like lesions from *Tgfb1*-null mice. There was a possibility that the apoptosis was higher in the lesions from these null mutant mice compared to tissues from control mice. An increase in apoptosis rate could be caused by TGFB1-deficiency, hence resulted in smaller lesion.

3.4.7 Limitations of our model

This study showed the importance of TGFB1 in lesion development using a mouse model of endometriosis. The results presented are only possible with the use of animal models. Despite this, there are some limitations with the model which must be taken into account.

3.4.7.1 The absence of menstrual cycle

Endometriotic lesions are very responsive to the changes of estrogen and progesterone during menstrual cycles. For this reason, primates are the only *in vivo* model that truly fits this requirement. Our mouse model lacks the cyclical hormonal changes as the mice were ovariectomised and estrogen supplemented to maintain a constant hormone environment. This was done to minimise the estrous cycle-related estrogenic and progesteronic variations which could confound the effect of TGFB1 deficiency on lesion development. The endometrial graft tissues were allowed to grow for only 10 days essentially in an environment that simulates the follicular phase of the menstrual cycle. In a mouse model of endometriosis, progesterone has anti-proliferative effect on endometriosis-like lesion growth [274] and the absence of progesterone in the TGFB1 deficient model of endometriosis would therefore not reflect this aspect of human disease.

3.4.7.2 The use of non-menstrual tissues

Endometrial samples were collected from women at various stages of menstrual cycle at the time of surgery in an outpatient setting. It is not impossible to collect menstrual fluid for research with a menstrual cup, however the amount of tissue in this material is low and the mice rarely develop lesions. The functionalis layer of the endometrium was used and the endometrial tissues were sectioned into small pieces prior to injection, which approximates the likely state of endometrial tissue present in retrograde menstrual fluid. Previous work in our laboratory showed no influence of disease status and cycle stage on ectopic tissue growth [146]. In the current study, the number of mice receiving tissues from one patient was low (between 3 and 5 in each experiment). Given this low sample size, the stage of the menstrual cycle did not appear to affect the number of mice with lesions at the end of each experiment.

3.4.7.3 Short duration

The duration of this experiment is limited by the short lifespan of the *Tgfb1* null mutant mice as their health deteriorates as they get older. For this reason endometriosis-like lesions were only grown for 10 days. Despite the short timeframe when compared to human endometriotic lesion development, we were able to see the effect of TGFB1 deficiency within a 10 day period.

3.4.7.4 Species-specific cellular interactions

Endometriosis induction in the mouse models using human endometrial tissues could feasibly cause a species mismatch of ligand-receptor interactions in our *Tgfb1*-/- and *Tgfb1*+/+ mice. The TGFB1 protein however is conserved across mammalian species

with a 99% homology between human and murine protein sequences [275], so is likely to be functional in both the peritoneal and endometrial tissue in immunocompromised mouse models of endometriosis. Although there were interspecies differences, sufficient molecular communication from the murine host was clearly able to support the growth of human endometrial tissues enabling them to proliferate and form a blood supply.

3.4.8 Clinical importance

In this study, *Tgfb1*-/- mouse models were used to mimic the human condition of endometriosis. As genetic manipulation in primates is not possible, this study is the best and the most feasible way of representing human disease in a TGFB1 deficient host environment. *Tgfb1*-/- mice were bred onto SCID mice, which lack T and B lymphocytes to prevent the rejection of human endometrial fragments. These immunocompromised mice still possess innate immune cells, macrophages and neutrophils which are important for endometriosis initiation [81].

Subcutaneous injection of human eutopic endometrium onto TGFB1 deficient and replete mice demonstrated that stomal and epithelial remodelling is dependent on TGFB1. As this model only manipulates TGFB1 in the host, and not in the human endometrial fragments, the model affords the opportunity to manipulate the host response by targeting the TGFB1 pathway in the peritoneal environment. Immunohistochemistry results suggest that macrophages and myofibroblasts which support the growth of ectopic endometrial tissues were altered in the absence of TGFB1.

TGFB1 protein has been found at higher concentrations in the peritoneal fluid of women with endometriosis. The presence of this secreted cytokine in the peritoneal cavity seems to promote endometriosis development. In our study, TGFB1 acted directly or indirectly on macrophages and fibroblasts and these cells may mediate the functional influence of TGFB1 on ectopic endometrial lesion size.

In summary, host TGFB1 deficiency appears to reduce the weight of endometriosis-like lesions, associated with diminished macrophage infiltration and myofibroblasts activity. Targeting the host response rather than the actual lesion therefore might be a useful strategy to treat endometriosis in women. There are multiple pathways regulated by TGFB1 and further research is required to narrow down the pathways critical for endometriosis development.

3.5 Conclusions

Results from this study suggest that TGFB1 from the host is critical for the development of peritoneal endometriosis. TGFB1 may act through several pathways to mediate tissue remodelling in endometriotic lesions. Among the routes utilised by TGFB1 appeared to regulate lesion growth are glandular development, macrophage infiltration and myofibroblast activity. Thus, targeting molecules potentially involve in TGFB1 regulation could provide a novel treatment for endometriosis.

Chapter 4

4.1 Introduction

4.1.1 Rationale for this study

In the previous chapter, host-derived TGFB1 was demonstrated to assist the lesion growth in a mouse model of endometriosis. The day-10 lesions from TGFB1 deficient hosts had lower macrophage numbers in the periphery and a lower percentage of myofibroblast positive areas. This adds to the body of literature that demonstrates an important role of macrophages in endometriosis-like lesion development. In this chapter, experiments to explore dynamic changes in macrophages during endometriosis-like lesion development were undertaken. A transgenic mouse strain that stably expresses enhanced green fluorescent protein (eGFP) only in macrophages was used as a model to track macrophages for endometriosis development.

Increased inflammatory cells and cytokine expressions have been reported in peritoneal fluid and lesions from women with endometriosis. The presence of iron and necrotic cells in the peritoneal cavity could contribute to initiation of this inflammatory reaction. Both iron and necrotic cells are present in retrograde menstrual fluid, which also contains endometrial tissue fragments. Activated macrophages have been identified to be higher in the peritoneal fluid of women with endometriosis than disease-free patients by 1.8-fold, as measured by acid phosphatase activity [34]. In a baboon model of endometriosis, intrapelvic injection of menstrual tissues increased the number of HLA-DR (human leukocyte antigen DR, an MHC class II molecule)-positive peritoneal cells [209]. Chemokines and cytokines such as RANTES [276], monocyte chemoattractant protein-1 (MCP-1) [277] and TGFB1 [166] were found to be at higher

concentrations in the peritoneal fluid and granulocyte-macrophage colony-stimulating factor (GM-CSF) [278] was highly expressed in the lesions from the women with endometriosis. These chemokines and cytokines have been demonstrated to increase macrophage recruitment [30, 279, 280] and activity which is increased in women with endometriosis. Macrophages are therefore likely to have a significant role in maintaining endometriotic lesions [82].

One of the most comprehensive studies showing the role of macrophages during endometriosis-like lesion development is reported by Bacci *et al*. [82]. In this study, female Balb/c mice were intraperitoneally grafted with syngeneic uterine tissues to create endometriosis-like lesions. Peritoneal macrophage depletion was achieved by injection of clodronate-containing liposomes at 0, 4 and 8 days post-tissue transplant to induce apoptosis in phagocytic cells which ingested these liposomes. Macrophage depletion before endometriosis initiation (day 0) in the host mouse resulted in a significant reduction in the weight of the lesions at day 12 [82]. The establishment of endometriosis-like lesion was shown to be dependent on F4/80-positive macrophages, as administration of anti-F4/80 intraperitoneally every 48 hours before and after the disease induction also resulted in a reduced lesion weight.

Liposome clodronate-mediated macrophage depletion at the mid stage of lesion growth (i.e. at day 4 and 8) was also associated with a significantly lower weight of lesions, although not to the same level as the depletion at the initial stage [82]. These results imply that although macrophages play significant roles throughout the process of endometriosis development in this mouse model, macrophage disruption during the initial establishment is more likely to reduce endometriosis development than disruption during progression. In the clinical setting, macrophages cannot be

functionally altered before lesion development, however this study showed the treatment can be effective in the mid stage of disease development with a similar therapeutic outcome. A limitation with Bacci's study however is the use of immunocompetent mice which requires implantation of syngeneic mouse uterine tissues. Myometrium is included in the transplanted tissue which differs from endometriotic lesions in women.

Macrophages are known to have a multiplicity of functions in addition to their role in inflammation. Unlike neutrophils, which are recruited at the onset of inflammation and then undergo apoptosis [281], macrophages are long-lived and several studies have shown that macrophages are able to change their phenotypes so suit their surrounding environment [87]. Chemokines such as RANTES (regulated on activation, normal T cells expressed and secreted) can also induce changes in macrophage phenotypes. For example, RANTES has been shown to induce macrophage recruitment and subsequently induced tolerance in macrophages by downregulating HLA-DR expression [30]. In a similar study, eutopic and ectopic tissues from women with endometriosis had higher level of RANTES expression than eutopic tissue from endometriosis-free women. Thus, RANTES could be important in assisting the growth of ectopic tissues.

4.1.2 Macrophage markers

In the current study, eGFP cells were immunohistochemically localised using markers associated with classically or alternatively activation macrophage. Markers for classically activated macrophages are MHC class II and iNOS, while markers for alternatively activated macrophages are arginase 1 and scavenger receptor A. These

markers were used to determine the macrophage phenotypes and hence their functions during endometriosis-like lesion development.

4.1.2.1 MHC class II

Major histocompatibility complex (MHC) class II molecules play an important role in presenting antigens to T cells to generate an adaptive immune response. This molecule is expressed by professional antigen presenting cells and lymphocytes. MHC class II molecule is comprised of A and E subregions [282]. TNFα and nitric oxide production by wound-derived MHC class II-deficient macrophages was significantly reduced when stimulated with LPS and IFNγ, compared to the wildtype controls [283]. This finding suggests that MHC class II molecules are important in inflammatory functions through production of cytokines.

Pro-inflammatory cytokines, IFNγ and the bacterial product, LPS participate in the regulation of MHC class II expression. IFNγ regulates the expression of MHC class II through the activation of MHC class II promoter transactivator (CIITA) *cis* elements [284]. LPS increases MHC class II expression transiently. In RAW264.7 cells, LPS induced the expression of MHC class II mRNA after 1-2 hours [285]. At 24 hours, LPS inhibited the expression of MHC class II mRNA [286]. The inhibition of MHC class II by LPS is mediated through suppression of the transcription factor, CIITA in the presence of LPS, which also involves ERK and p38 MAPK signalling pathways [286]. In reproductive tissues, GM-CSF was shown to mediate the expression of MHC class II by uterine macrophages during post-coital inflammation, suggesting an antigen presentation is regulated by this cytokine [287].

Besides primate studies, the association of macrophage MHC class II expression with endometriosis pathology has also been described in clinical and cell culture studies. Peritoneal macrophages from women with early (n = 38), mid (n = 12) and advanced stages (n = 26) of endometriosis had a lower expression of MHC class II molecule, HLA-DR than endometriosis-free women (n = 58) [288]. The suppression of HLA-DR molecules on the peritoneal macrophages of women with endometriosis appears to be crucial to provide immune tolerance, allowing the ectopic tissues to grow. This finding was also consistent in a later cohort studied by the same group [289]. The expression of HLA-DR on THP-1 cells, a macrophage cell line was downregulated when cultured with peritoneal fluid from 10 women with endometriosis in a dose dependent manner [290]. The inhibition of MHC class II on macrophages in this study was demonstrated to be dependent on the presence of high concentration of IL-10 in the peritoneal fluid of endometriosis patients. In this study, since no downregulation of mRNA transcript was observed, IL-10 in the peritoneal fluid may act post-transcriptionally on the MHC class II regulation. As MHC class II could provide a host protection against the lesion by presenting the antigens to T lymphocytes, the inhibition of this molecule by IL-10 in the peritoneal fluid may provide a way for the ectopic tissue to escape immune recognition.

In addition to IL-10, RANTES was also demonstrated to induce immune tolerance by downregulating the expression of MHC class II molecules on macrophages. The incubation of monocyte cells U937 with recombinant human RANTES showed suppression of MHC class II molecule, HLA-DR expression [30], in agreement with the aforementioned study by Lee *et al*. [290]. Lee *et al*. showed RANTES induced HLA-DR downregulation which appeared to be mediated by IL-10, as IL-10 level increased in

the culture media over time. Therefore, the expression of MHC class II in patients may be associated with increased surveillance and protection against ectopic endometrial tissues.

4.1.2.2 Inducible nitric oxide synthase (iNOS)

Nitric oxide synthase (NOS) is an enzyme that converts ι-arginine to ι-citrulline, releasing the free radical nitric oxide (NO) [291]. Inducible nitric oxide synthase (iNOS or NOS2) is predominantly expressed by monocytes and macrophages (reviewed by [291]). Nitric oxide (NO) production is downregulated by TGFB1 in the peritoneal macrophages [292].

The concentration of nitric oxide has been linked to endometriosis. NO levels were significantly higher in homogenised endometrial samples of peritoneal endometriosis patients (n = 17) than in similar sample from control women with myoma (n = 13), as measured using rapid-response chemiluminescence NO analyser in the follicular phase [293]. Peritoneal macrophages from women with endometriosis (n = 6) readily secreted higher level of NOS2 (iNOS) compared to those from control fertile women (n = 3) without any stimulation [294]. When the authors stimulated endometriotic peritoneal macrophages with IFNα, these cells significantly secreted NOS enzyme, measured 3 days later, than those from endometriosis-free women. The authors suggested that the higher expression of iNOS by macrophages and hence increased NO production in women with endometriosis may contribute to subfertility. Through production of NO, iNOS expression by endometriotic peritoneal macrophages could be a major contributor to inflammation in the peritoneal cavity of endometriotic patients.

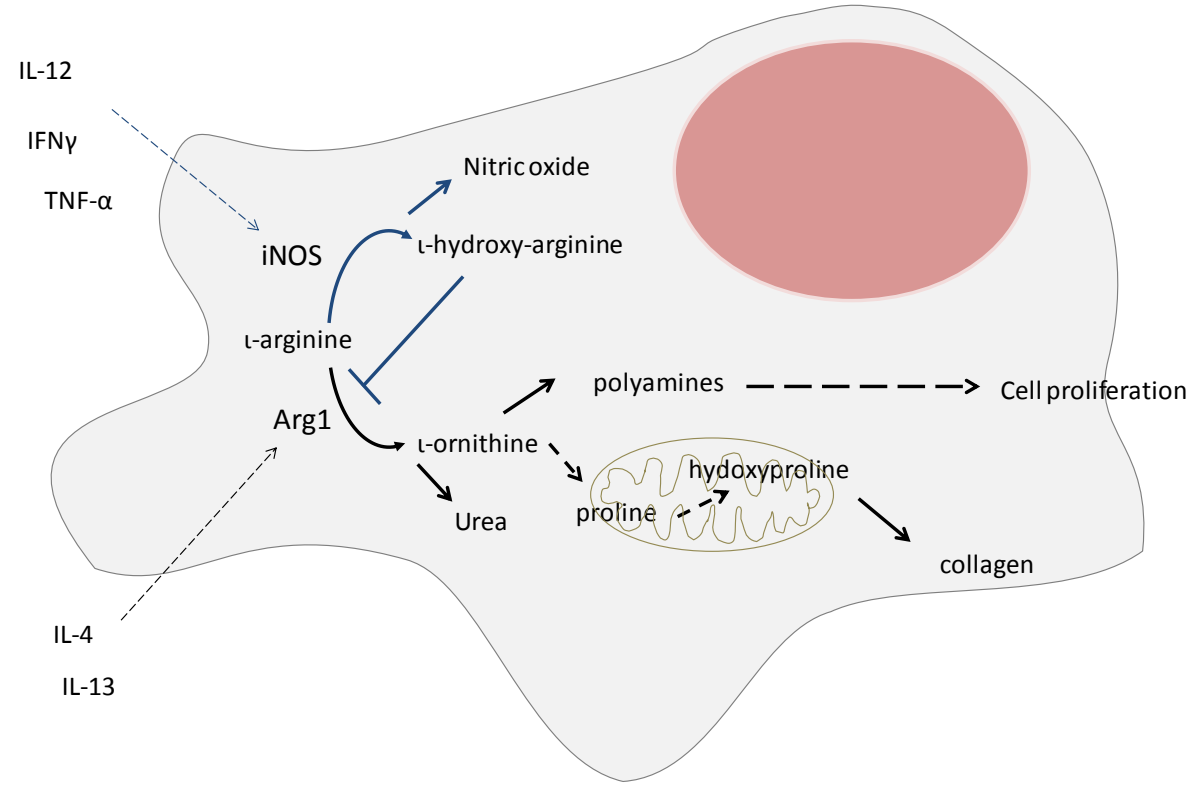


Figure 4 A: Nitric oxide synthase and arginase pathways in a macrophage. M1-induced cytokines such as IFNγ and TNFα drive the expression of inducible nitric oxide synthase (iNOS).

This enzyme synthesises the ι-arginine into ι-hydroxyl-arginine, releasing nitric oxide. Meanwhile, in M2 macrophages, IL-4 and IL-13 mediate the expression of arginase 1 (Arg1). Arginase 1 shares ι-arginine substrate with iNOS. iNOS enzymatic product, ι-hydoxy-arginine is an inhibitor of arginase 1. Arginase 1 converts ι-arginine to ι-ornithine, which is a precursor for polyamines, which is critical for cell proliferation and proline, a major component of collagen (reviewed by [295, 296]).

4.1.2.3 Arginase 1

Two isoforms of arginase exist in mammalian species, liver-specific arginase 1 and also arginase 2. Arginase is an enzyme that uses ι-arginine as a substrate component, like the NO synthases. Therefore, increased arginase 1 activity results in a decrease in NO production as reported by McLarren *et al*. [297], due to the competition with iNOS for their common substrate, ι –arginine. Arginase 1 converts ι-arginine to ι-ornithine, with urea as a by-product (reviewed by [298]). Arginase 1 protein expression was increased when murine peritoneal macrophages were exposed to LPS and also hypoxic

environment [299]. Arginase 1 is a marker for pro-tissue repair macrophages and its activation and suppression are dependent on cytokine stimulation. Cytokines IL-4 and IL-10 and prostaglandin E2 increased arginase activity in the bone marrow-derived mouse macrophages [300]. IFNγ in contrast decreased the level of arginase 1 in these cells and induced the level of nitric oxide synthase, even in the presence of IL-4 and IL-10. Parasite infection studies provide a lot of information regarding arginase 1 regulation in macrophages as arginase 1 expression was induced after exposure to Type 2 cytokines and schiotosoma eggs, and was suppressed by Type 1 cytokines, such as IFNγ [298]. Arginase 1 activity was proven to protect mice against adverse Type 2-driven inflammation in a schiotosoma infection model [101].

The importance of arginase 1-expressing macrophages in endometriosis remains unknown. So far no study has looked at the arginase 1 expression by macrophages during endometriosis development. One of the possible roles of arginase is to synthesise collagen fibres in the endometriotic lesions. The expression of arginase 1 as a marker for alternatively activated macrophages is only described in mouse. In contrast to murine monocytes, IL-4 and IL-13 cytokine stimulations did not induce *ARG1* mRNA expression in human monocytes, while the mannose receptor, another marker for alternatively activated macrophages was upregulated [301]. *ARG1* mRNA was detected in the eutopic endometrium from proliferative and secretory phases and its protein has been demonstrated to be localised in the epithelium [302]. In addition to cycling endometrium, this marker was identified in tissues from a syngeneic mouse model of endometriosis. *Arg1* transcript levels were increased by 25.8-fold in endometriosis-like lesions compared to uterine tissue at day 3 of tissue implantation, but did not differ significantly by day 29 [303]. The expression of arginase 1 therefore

could be critical for the early stage of tissue remodelling, but did not persist throughout lesion development.

4.1.2.4 Scavenger receptor

CD204 is a Class A scavenger receptor (SR-A) and was reported to be important during tumor development. In the literature, this class of scavenger receptor is also known as macrophage scavenger receptor 1 (Msr1) and it is expressed by human macrophages [304]. A tumor study showed that SR-A expression, in particular on macrophages mediates invasion and tumor progression which is critical in tumor development. The invasive properties of murine ovarian cancer cell line (ID8) were reduced by approximately 15-fold when cultured with SR-A-null macrophages compared to wildtype macrophages [305] in a 72-hour invasion assay in a modified Boyden chamber. Moreover, in a mouse model re-populated with SR-A-null macrophages, ID8 tumor growth was inhibited by approximately 2.3-fold. In concordance with these findings, macrophages expressing SR-A could be linked with endometriosis development which is dependent on tissue invasion and remodelling.

4.1.2.5 Macrophages in healing and repair process

The diverse roles of macrophages were demonstrated in a tissue wounding experiment when macrophages were transiently depleted. In a transgenic mouse expressing diphtheria toxin (DT) receptor exclusively on CD11b+ cells, macrophages were depleted by administration of this toxin at three phases of wound repair; early, mid and late stages [306]. In undepleted controls, alternative activation markers, F4/80+ cells positive for Fizz1 (resistin-like molecule α) and Ym-1 (an eosinophilic chemotactic

factor) were abundant at day 5 after injury and reduced at day 10. When macrophages were depleted by DT at the early phase of inflammation (day -2, -1, 2 and 4 of injury), the number of the F4/80+ cells expressing Fizz1 and Ym-1 was nearly undetectable at day 5. In addition, macrophage depletion at the early phase also suppressed vascularisation and contraction, as measured by the presence of CD31-postitive endothelial cells and αSMA-positive myofibroblasts, by approximately 4- and 9-fold respectively. Reduction in the levels of TGFB1 and VEGF-A- positive cells, by 1.5- and 3 fold respectively, was also seen. Macrophage reduction at mid-stage of repair (day 3, 4, 6 and 8) appeared to reduce the wound closure by decreasing the fibrinogen-positive area. Meanwhile, macrophage depletion at a late stage (day 8, 9, 11 and 13) did not affect the wound healing, showing a similar tissue architecture and morphology as the control. Hence, macrophages play significant roles at the early and mid-stage stage of repair process, but are less important at the latter. Stimuli for alternative macrophage activation were suggested to be only present in the early phase of repair, as reduced macrophage recruitment into the wounding site following DT administration consequently resulted in a lower number of Fizz1- and Ym-1-positive cells.

Classification of macrophages into M1 and M2 phenotypes is often viewed as being too simplistic. In a time course study, it is difficult to determine the phenotype of macrophages and often both types are present at once. For example, a flow cytometry analysis of infiltrating myeloid derived suppressor cells (MDSC) showed simultaneous expression of both M1 and M2 phenotypes. As discovered by Umemura *et al*., MDSCs in a murine tumor of colon adenocarcinoma and murine glioma cells at day 14 expressed both CXCL10 (M1) and CD206 (M2) [307]. A Western blot analysis also

demonstrated that MDSCs expressed M1 marker iNOS and M2 marker arginase 1. These cells therefore cannot be strictly classified into either M1 or M2. Over 90% of this MDSC population was made up of F4/80 positive cells [307]. Hence, *in vivo* M1 and M2 macrophages cannot be easily distinguished. As shown by Umemura *et al*., simultaneous expression of both markers by single cells means that macrophages could exist in intermediate phenotypes, or fluctuate between phenotypes, and possibly perform multiple tasks at a given time.

4.1.2.6 Macrophage polarisation in human tissues

Macrophages transitionally expressing different markers during inflammatory disease event are well described in mouse models. Although macrophage polarisation has been reported in the human tissues, the mechanisms and the markers could differ significantly. One of the examples is arginase 1 which has been reported as one of the markers for the M2 mouse macrophage population; however is not a marker for human M2 macrophages. Arginase 1 and Ym-1 are only specific M2 markers for mice but not human as IL-4 and IL-13 induction did not upregulate both markers in human monocytes [301, 308]. As depicted in Figure 1 B (page 40; Section 1.1.4.3 Macrophage Plasticity), undifferentiated M0 macrophages can be induced to M2 by IL-4/IL-13 stimulations *in vitro*. Markers used to identify this M2 macrophage population include scavenger receptors, CD163 and CD204, and mannose receptor CD206 [82, 309]. There are no available data however, demonstrating direct transformation from M1 to M2 either *in vitro* or *in vivo*.

4.1.2.7 Macrophage abnormalities in women with endometriosis

Women with endometriosis appear to have different distribution of immune cells compared to disease-free patients. The *Tgfb1*-/- mouse model suggests (Chapter 3) that macrophages are critical to endometriotic lesion development and this may be the reason behind the high number of macrophages in the women with endometriosis. Macrophage function may also be altered in endometriosis as in women with endometriosis (n = 10), peritoneal macrophages had lower CD36 mRNA and surface protein expression compared to endometriosis-free women (n = 11) [69]. Reduced CD36 expression in peritoneal macrophages of endometriotic women is associated with reduced phagocytic ability of these macrophages [69].

These abnormalities could be contributed by the presence of ectopic endometrial tissues, which consequently gives rise to a different immunological milieu. A study by Bacci *et al*. [82] showed that mannose and haemoglobin scavenger receptors, CD206 and CD163-positive macrophages were present in endometriotic lesions, but not in eutopic tissues, suggesting the abnormality observed is only specific to the endometriotic environment. Macrophage infiltration into the lesions is therefore likely to be emulated in xenografts from immunodeficient mouse models of endometriosis.

The importance of understanding macrophage behaviour in endometriosis is critical as this immune cell appears to have a significant role in the establishment of lesions. It may be that manipulation of macrophage behaviour could influence disease progression and modify symptoms such as subfertility and pain. The excess of inflammatory macrophages and cytokines in endometriotic women could be a contributing factor in the reduced fertility and successful pregnancy (reviewed by [310]). Furthermore, macrophages could contribute to and exacerbate the inflammatory process involved in endometriosis-related pain. The close proximity of nerve fibres to

CD68-positive macrophages in endometriotic lesions [311] suggests that macrophages may promote sprouting and re-generation of these fibres. We theorise that the macrophages are recruited to the endometriotic lesions and that these cells promote regeneration and growth of the ectopic endometrial endometrium.

4.1.3 MacGreen mice

To study macrophage distribution and phenotypes associated with these cells, a stable macrophage-specific marker is required. MacGreen mice are transgenic mice developed by Sasmono *et al*. [179] at the University of Queensland, Brisbane, Australia, which are characterised by the expression of enhanced green fluorescent protein (eGFP) under the *c-fms* promoter. The differentiation of macrophages from bone marrow progenitor cell is controlled by a lineage-specific growth factor, CSF-1. The *c-fms* gene encoding the CSF-1 receptor (CSF-1R) plays a critical role in macrophage precursor differentiation from progenitor cells. During murine embryo development, *c-fms* mRNA was detected in yolk sac phagocytes at 9.5 days post coitum, preceding many macrophage-specific markers, including PU.1 [312]. In embryonic and adult mice, the expression of CSF-1 is only restricted to the macrophages and trophoblasts (reviewed by [313]).

A plasmid containing the 3.5 kb *c-fms* promoter which drives the expression of the 10.5 kb eGFP gene was injected into eggs of C57/BL6/CBA donor mice and transferred into pseudo-pregnant mice [179]. The expression of eGFP is restricted to the bone marrow-derived myeloid cells as the majority of the eGFP cells are positive for CD11b. Alveolar, peritoneal, epidermal and retinal interstitial macrophages, Langerhans cells and Kupffer cells are among the macrophage populations detected to be positive for

eGFP in transgenic MacGreen mice [179]. All eGFP-positive cells in the macrophage-rich peritoneum express F4/80 and CD11b markers. One advantage of this model is the constitutive expression of eGFP although CSF-1R is not expressed constitutively by macrophages. Constitutive expression of this fluorescent protein allows visualisation, as CSF-1R is briefly expressed during development and the majority of $F4/80^{+}$ cells are dependent on CSF-1R in adult mice [314].

4.1.4 Summary

Macrophages are phenotypically altered in response to environmental stimuli during the course of inflammation and tissue repair. The dynamic changes in these phenotypes, characterised by different markers reflect diverse macrophage functions. There is limited evidence of macrophage phenotypic changes reported over the course of endometriosis development. Thus, in this chapter, eGFP-expressing macrophages (MacGreen) mice with an immunodeficient background were used to establish the specific macrophage activation markers during endometriosis-like lesion development.

4.2 Materials and methods

Materials and methods for this chapter are described in detail in Chapter 2. Briefly, eutopic endometrial fragments from pre-menopausal (n = 5) patients were subcutaneously injected into ovariectomised and estrogen supplemented MacGreen/SCID mice. At day 4, 7, 10 and 14 following tissue injection, the resulting lesions were collected and measured.

4.3 Results

4.3.1 Patient demographics

In total, endometrial tissues were obtained from five pre-menopausal patients, aged 32 to 39 years (median age = 35 years) at the time of surgery. All patients, except one (Patient #3) had a normal 28-day cycle, with no hormonal medications for 3 months prior to tissue collection. Four patients had laparoscopy surgery, while one patient did not have her peritoneal cavity visualised (**Table 4.1**). Of the four patients with a visualised pelvis, three did not have any visible endometriosis lesions and one had Stage 3 endometriosis.

4.3.2 Development of endometriosis-like lesions in MacGreen/SCID mice

Endometrial tissues from five patients were subcutaneously injected into 51 MacGreen-positive mice with SCID background to create endometriosis-like lesions. Each patient provided enough tissue for one MacGreen experiment. The mice were randomly divided into four groups, i.e. days 4, 7, 10 and 14. At four different time points, the mice were euthanized and the lesions were harvested. In these experiments (n = 5), 55-100% of mice developed one or more lesions (**Table 4.2**). In experiment 1, tissues from Patient 1 were injected into 10 mice in total. Two mice were randomly chosen and euthanised at day 4, 3 mice at day 7, 3 mice at day 10 and 2 mice at day 10. In total, 8 mice developed lesion(s) at the end of time points in Experiment 1 (percentage of lesion development for Patient 1 = 80%). Tissues from Patient 2 were transplanted into 10 mice in Experiment 2. At day 4 and day 7, xenografts were collected from 3 mice for each time point and from 2 mice, each for day 10 and day 14. Of 10 mice, 9 developed lesion(s) in Experiment 2 (percentage of

lesion development = 90%). Experiment 3 used the least number of mice, with 6. Two mice were allocated to day 4 and day 7 (n = 1/time point), while the rest (n = 4) were equally distributed for day 10 and day 14 (n = 2, each) and all mice developed lesion(s) (percentage of lesion development for Patient 3 = 100%). Meanwhile, tissues from Patient 4 were injected into 11 mice. In this experiment, 2 mice were euthanised and xenografts were collected at day 4. For day 7, 10 and 14, 3 mice were euthanised at each time point. In total, six of these mice developed lesion(s), (percentage of lesion development = 55%). Lastly, tissues from Patient 5 went into the highest number of mice with 14 in total. Three mice were randomly chosen and euthanised at day 4 after tissue injection. Xenografts were collected from 4 mice at day 7, 3 mice at day 10 and 4 mice at day 14. From 14 mice, 12 developed lesion(s) at the time points (percentage of lesion development = 86%).

In total, the number of mice which were found at autopsy to have developed lesions was 41 out of 51 (**Table 4.3**). From these 41 mice, 44 lesions collected had glandular structure, characteristic of endometrial glands, as determined by haematoxylin and eosin staining.

Endometrial tissues from one patient provided enough tissues for one experiment. In some experiments, when the donor tissues were in excess and/or insufficient recipient mice were available, one mouse received two endometrial injections. In the day 4 group, 11 mice were sacrificed and xenografts were collected. Of the 11 mice, 9 developed endometriosis-like lesions (percentage of lesion development at day 4 = 81.8%). After confirmation with haematoxylin and eosin staining, 11 lesions collected from this time point had endometrial glands, so all 11 were subsequently used for immunostaining.

Meanwhile, 14 mice were allocated into the day 7 group. At the end of the experiment, 10 mice had developed endometriosis-like lesions (percentage of lesion development = 71.4%). In total, 11 gland-positive lesions were collected at day 7.

In the day 10 group, 13 mice were allocated for xenograft collection. Of these 13 mice, 10 developed subcutaneous endometriosis-like lesions (percentage lesion development = 76.9%). The total number of gland-positive lesions collected from these mice was 10, which were later used for immunostaining. However, one lesion had to be excluded for the further immunofluorescent staining due to poor tissue morphology, leaving only 9.

In the day 14 group, endometrial tissues were grafted into 13 mice. At the end of the experiment, 12 mice developed visible lesions (percentage lesion development = 92.3%). In this group, each mouse had one lesion, thus 12 lesions were collected for day 14. Therefore, in total 44 endometriosis-like lesions were collected. The overall percentage lesion development was 80.4%.

4.3.3 Weight and size of endometriosis-like lesions

The median weight of lesions developed in MacGreen/SCID mice was not significantly different across the four time points [$p>0.05$, Kruskal-Wallis test; median (range) = 0.0064 g (0.0016-0.017 g) for day 4, 0.0081 g (0.0009-0.023 g) for day 7, 0.0071 g (0.0034-0.017 g) for day 10 and 0.0064 g (0.0031-0.014 g) for day 14] (**Figure 4.1B**). Similarly, no difference was observed in the size of the lesions (**Figure 4.1C**), between the four different days ($p>0.05$, Kruskal-Wallis test) [median (range) = 7.3 mm^3 (1-16 mm^3) for day 4, 2.8 mm^3 (2-18.8 mm^3) for day 7, 5.5 mm^3 (2-14 mm^3) for day 10 and 3.5 mm^3 (1-12 mm^3) for day 14].

4.3.4 Histology of endometriosis-like lesions

Similar to the endometriosis-like lesions examined in Chapter 3, endometrial tissues grafted into MacGreen mice were made up of stroma and glands (**Figure 4.2A**). The median of percent glandular area differed significantly across the four time-points ($p = 0.00090$, Kruskal-Wallis test; **Figure 4.2B**). At day 4, the median (range) percent of lesion area occupied by glandular epithelium was 6.7% (3-20.4%). This percent area was the lowest in comparison to the median values (ranges) at day 7 [11.2% (4.4-27%)], day 10 [24.1% (12.9-34.3%)] and day 14 [15.4% (10.5-45.5%)]. Compared to day 4 lesions, day 10 lesions recorded the highest area of glandular epithelium by 3.6-fold ($p<0.001$, Dunn's multiple comparison test). Meanwhile, the percent area of glandular epithelium at day 14 was significantly higher than at day 4 by 2.3-fold ($p<0.05$, Dunn's multiple comparison test). In contrast, the median value (range) for percentage stromal area was higher at day 4 [93.3% (79-95%)] compared to day 10 [75.9% (65.7-87.1%)] and day 14 [85.5% (54.6-89.6%)] ($p<0.01$ and $p<0.05$, respectively-Dunn's multiple comparison test) [median (range) = 87.3% (73-95.6%) for day 7; **Figure 4.2C**].

Macrophages identified on the basis of eGFP expression were seen to infiltrate into the endometriosis-like lesions as early as day 4 and were localised in the stromal compartment of the lesions. On day 4, eGFP-macrophages appeared to be restricted around the edge of the lesions. By day 7 to day 14, the infiltration of eGFP-positive macrophages into the lesions was more pronounced than at day 4 (**Figure 4.3A**). The number of eGFP-expressing macrophages per viewing field however was not significantly different across the four time points (**Figure 4.3B;** $p>0.05$, Kruskal-Wallis test) [median (range) = 78.6 cells/field (52.2-98.1 cells/field) for day 4, 86.9 cells/field

(56.6-141.5 cells/field) for day 7, 73.2 cells/field (60.2-113.4 cells/field) for day 10 and 74.8 cells/field (56.2-114.4 cells/field) for day 14].

4.3.5 Immunofluorescent staining to localise macrophage activation markers, collagen and TGFB1

4.3.5.1 Antibody binding specificity

To determine if the primary antibody binding was specific to the antigens they were raised against, normal isotype-matched immunoglobulins (Ig) were applied to the tissue sections at the same Ig concentration as the antibodies (**Figure 4.4**). Anti-MHC class II isotype matched IgG2aκ, did not develop any positive signals when applied to the endometriosis-like tissue. Similarly, when the lesion section was incubated with the rabbit IgG in replacement of the anti-iNOS antibody, only some weak staining was observed and it was not consistent with the positive signal produced by the anti-iNOS antibody. Unimmunised goat IgG was used to determine the specificity of the arginase 1 antibody antigen binding, and did not develop any positive staining on tissue section. An IgG2b isotype-matched control for anti-CD204 antibody was applied to tissue sections and no staining was observed. Lastly, the specificity of collagen type 1 antibody was tested with an unimmunised rabbit IgG. This polyclonal IgG did develop weak staining but it was not consistent with the positive signal.

Each primary antibody was tested for its binding specificity. Human eutopic endometrial and mouse uterine sections were independently incubated with primary antibodies to evaluate the species specificity (**Figure 4.5**). The antibody used to recognise MHC class II was raised specifically against murine MHC class II 1a antigen. Upon incubation with this antibody, only murine tissue section developed signal, while

human sections did not. An iNOS antibody was raised against human NOS2 antigens. In both human and mouse endometrial sections, iNOS was mainly localised in the glandular epithelium and a small amount of staining was present in the stromal compartments. Anti-arginase 1 antibody was developed against liver-specific arginase. This antibody only specifically recognised arginase 1 on murine cells, and produced a weak staining when applied onto human tissue. Macrophage scavenger receptor was identified using an antibody which was developed against CD204. The binding of this antibody was limited only to murine cells, and no fluorescent staining was detected in the human endometrial sections. Type 1 collagen fibres were localised using an antibody which was raised against murine antigen. Although the manufacturer claims less than 0.01% cross-reactivity to human collagen fibres, with the protocol used for mouse tissue a positive signal in human endometrial section was also seen, suggesting this antibody was cross reactive to human-derived collagen.

4.3.5.2 Macrophage activation markers

CSF-1R-eGFP macrophages were evaluated for expression of their activation markers by staining endometriosis-like lesion sections for different macrophage-specific surface markers. These macrophages were evaluated for their expression of the inflammatory markers, MHC class II and iNOS, and the pro-tissue remodelling markers, arginase 1 and scavenger receptor A. Macrophages positive for each marker were counted to determine the absolute cell number per field and the percentage of macrophages expressing each marker. In each analysis, non-parametric Kruskal-Wallis test was performed to analyse the median difference across four time points, followed by

Dunn's multiple comparisons test to determine differences between medians at two time points.

4.3.5.2.1 MHC class II

MHC class II macrophages (MHC+eGFP+) were present in the periphery and stroma of endometriosis-like lesions across the four time points. At day 4, the majority of eGFP macrophages were also MHC class II-positive (**Figure 4.6A**). The number of eGFP-macrophages expressing this antigen presenting molecule (Total MHC+/eGFP+ cells) significantly differed in association with time point ($p = 0.0080$, Kruskal-Wallis test). The median (range) number of cells expressing both MHC class II and eGFP was significantly higher at day 4 [26.4 cells/field (19.5-40.2 cells/field)] than day 7 [16.4 cells/field (5.7-28.5 cells/field)] by 2.5-fold ($p<0.01$, Dunn's multiple comparison test) [median (range) = 21 cells/field (14.7-25.3 cells/field) for day 10 and 21 cells/field (11.5-30.3 cells/field) for day 14] (**Figure 4.6B**). Supporting this finding, the analysis of medians using Kruskal-Wallis test showed a significant difference in the percentage of macrophages expressing MHC class II molecule (% MHC+/eGFP+) across four time points ($p = 0.027$) (**Figure 4.6C**). Dunn's multiple comparison test demonstrated that 35.9% (range = 24.8-57.2%) of macrophages expressed MHC class II molecule at day 4, which was 1.6-fold higher than day 7 [median (range) = 22.7% (11.5-39.9%)] ($p<0.05$) [median (range) = 36.2% (22-38.4%) for day 10 and 34.3% (23.6-38.3%) for day 14].

4.3.5.2.2 Inducible nitric oxide synthase (iNOS)

iNOS-positive macrophages (iNOS+/eGFP+) appeared to be more abundant at the earlier time points, day 4 and day 7, than later stages of lesion development (**Figure**

4.7A). Analysis of differences between groups using the Kruskal-Wallis test demonstrated that the number of eGFP-macrophages expressing iNOS (Total iNOS+/eGFP+) differed among the four time points ($p<0.0001$) (**Figure 4.7B**). The number of iNOS-positive macrophages in day 4 [median (range) = 35.1 cells/field (29-68 cells/field)] was higher than day 10 [19.2 cells/field (10.8-28.7 cells/field)] and 14 [15.2 cells/field (2.8-27.3 cells/field)] ($p<0.5$ and $p<0.01$, respectively). This marker was also highly expressed at the early stage of endometriosis development, as the median number of iNOS-expressing macrophages was higher at day 7 [58.8 cells/field (21.7-86.5 cells/field)] compared to day 10 and 14 ($p<0.01$ and 0.001, respectively). Similarly, the median of percentage of eGFP macrophages positive for iNOS (% iNOS+/eGFP+) significantly differed across the four time points ($p<0.0001$, Kruskal-Wallis) (**Figure 4.7C**). Dunn's multiple comparison test revealed that the percentage of iNOS-positive macrophages at day 4 [median (range) = 41.1% (33.5-72.3%)] was higher than day 10 [24.8% (8.6-42.1%)] and day 14 [20.4% (3.9-35.2%)] ($p<0.05$ and $p<0.01$, respectively). Likewise, macrophages expressing iNOS at day 7 [47.7% (29.7-68.2%)] were more abundant than those from day 10 and 14 ($p<0.01$ and $p<0.001$, respectively, Dunn's multiple comparison test).

4.3.5.2.3 Arginase 1

Cells positive for macrophage marker (eGFP) and arginase 1 (Arg1+eGFP+) were highly abundant at day 7 compared to the other stages of lesion development (**Figure 4.8A**). Analysis of differences between the groups using Kruskal-Wallis test showed that the number of macrophages positive for arginase 1 (Total Arg1+/eGFP+ cells) differed among the four time points ($p = 0.0011$). The number of macrophages expressing

arginase 1 peaked at day 7 [median (range) = 41.7 cells/field (25.2-60.5 cells/field)] in the endometriosis-like lesions. This median was significantly higher than medians (ranges) for day 4 [26.4 cells/field (9-39 cells/field)], day 10 [21.7 cells/field (14.7-41.5 cells/field)] and day 14 [24.6 cells/field (10.7-36.3 cells/field)] ($p<0.05$ for day 7 vs. day 4, $p<0.01$ for day 7 vs. day 10 and day 14, Dunn's multiple comparison test) (**Figure 4.8B**). Furthermore, there was a significant difference in the percentage of macrophages positive for arginase 1 (% Arg1+/eGFP+) ($p = 0.0058$, Kruskal-Wallis test) (**Figure 4.8C**). The population of macrophage positive for arginase 1 at day 7 [median (range) = 57% (45.3-85.6%)] was 1.3-fold higher at day 4 [43.3% (20.5-60%)] and 1.5-fold than day 14 [37.8% (18.3-53.9%)] ($p<0.05$, Dunn's multiple comparison test) [median (range) = 47% (24.4-56.4%) for day 10].

4.3.5.2.4 Scavenger receptor A, CD204

Scavenger receptor A, CD204-positive macrophages (CD204+eGFP+) were present at four different stages of endometriosis-like lesion development. Macrophages expressing this marker appeared to be more abundant at day 14 than the earlier time points (**Figure 4.9A**). The non-parametric Kruskal-Wallis test analysis of medians showed that the number of cells expressing CD204 and eGFP (Total CD204+/eGFP+) was significantly different across four time points ($p = 0.028$). The number of these macrophages peaked at the last time point, day 14, suggesting these cells accumulated over time. Further analysis comparing the medians (ranges) between two time points demonstrated that the median number of CD204-positive macrophages at day 10 [23 cells/field (15.8-51.3 cells/field)] was lower than day 14 [47.3 cells/field (25.5-74.7 cells/field)] by 51% ($p<0.05$) (**Figure 4.9B**) [median (range) = 32 cells/field (14.7-47.8

cells/field) for day 4 and 32.3 cells/field (14.7-39.8 cells/field) for day 7]. Further analysis on the macrophage population using Kruskal-Wallis test showed the median for the percentage of CD204-positive cells (% CD204+/eGFP+) significantly differed across four time points ($p = 0.017$). Analysis of medians between two time points revealed that significantly more macrophages express CD204 at day 14 [65.5% (40.2-74.7%)] than day 7 [51.1% (35.5-58.9%)] by 28% ($p<0.05$, Dunn's multiple comparison test; **Figure 4.9C**) [median (range) = 53% (40.2-59.2%) for day 4 and 51.4% (27-66.7%) for day 10].

4.3.5.3 Type 1 collagen

Collagen infiltration of endometriosis-like lesions was noted at day 4 and the later time points. Stromal areas positive for this collagen were mostly in the periphery and in some lesions, the collagen was present in the areas proximate to epithelium. At day 4, macrophages (eGFP+) were only restricted to the periphery and the presence of collagen type 1 was less pronounced. At day 7 to day 14 of endometriosis-like lesion development, macrophages were localised inside the collagen-positive stroma (**Figure 4.10A**). Analysis of medians of percent positive area using Kruskal-Wallis test showed significant difference between four groups ($p = 0.020$). There was a trend in increased collagen intensity throughout endometriosis-like lesion development. The percent positive area of collagen was significantly increased by 1.7-fold in day 14 [36.7% (20.7-49.6%)] compared to the earliest time point, day 4 [21.9% (14.8-33.2%)] ($p<0.05$, Dunn's multiple comparison test; **Figure 4.10B**) [median (range) = 27.7% (15.8-57.3%) for day 7 and 34.8% (25.1-59.3%)] for day 10].

4.3.5.4 Active TGFB1 staining

Active TGFB1 protein was present in the epithelial lining and stroma of day 10 endometriosis-like lesions (**Figure 4.11**). Majority of eGFP-positive macrophages in examined sections did not appear to co-localise with TGFB1 staining, although some cells seemed to be in close proximity with this cytokine.

Table 4. 1: Clinical data of the recruited patients for eutopic endometrial tissue collection

Experiment/Patient	Age at the time of surgery	Stage of cycle	Revised AFS score
1	32	Proliferative	No (S)
2	35	Proliferative	Asymptomatic (Pelvis was not visualised)
3	32	Late secretory	No (S)
4	35	Menstrual	Stage III
5	39	Late secretory	No (S)

No (S) = surgically proven no endometriosis

Table 4. 2: The number of CSF-1R-eGFP (MacGreen) mice receiving tissues from each patient and the percentage of mice developed lesions at the end of time points

Experiment / Patient	Number of mice/time point				Mice with lesion(s) (%)
	Day 4	Day 7	Day 10	Day 14	
1	2	3 (2)	3 (2)	2	80
2	3	3	2 (1)	2	90
3	1	1	2	2	100
4	2 (0)	3 (2)	3 (2)	3 (2)	55
5	3	4 (2)	3	4	86

( ) = The number of mice developed lesions at each time point

Table 4. 3: The number of MacGreen mice allocated for each time point.

Day	Total number of mice	Number of mice with lesion(s)	Number of lesions
4	11	9	11
7	14	10	11
10	13	10	10
14	13	12	12
Total	51	41	44

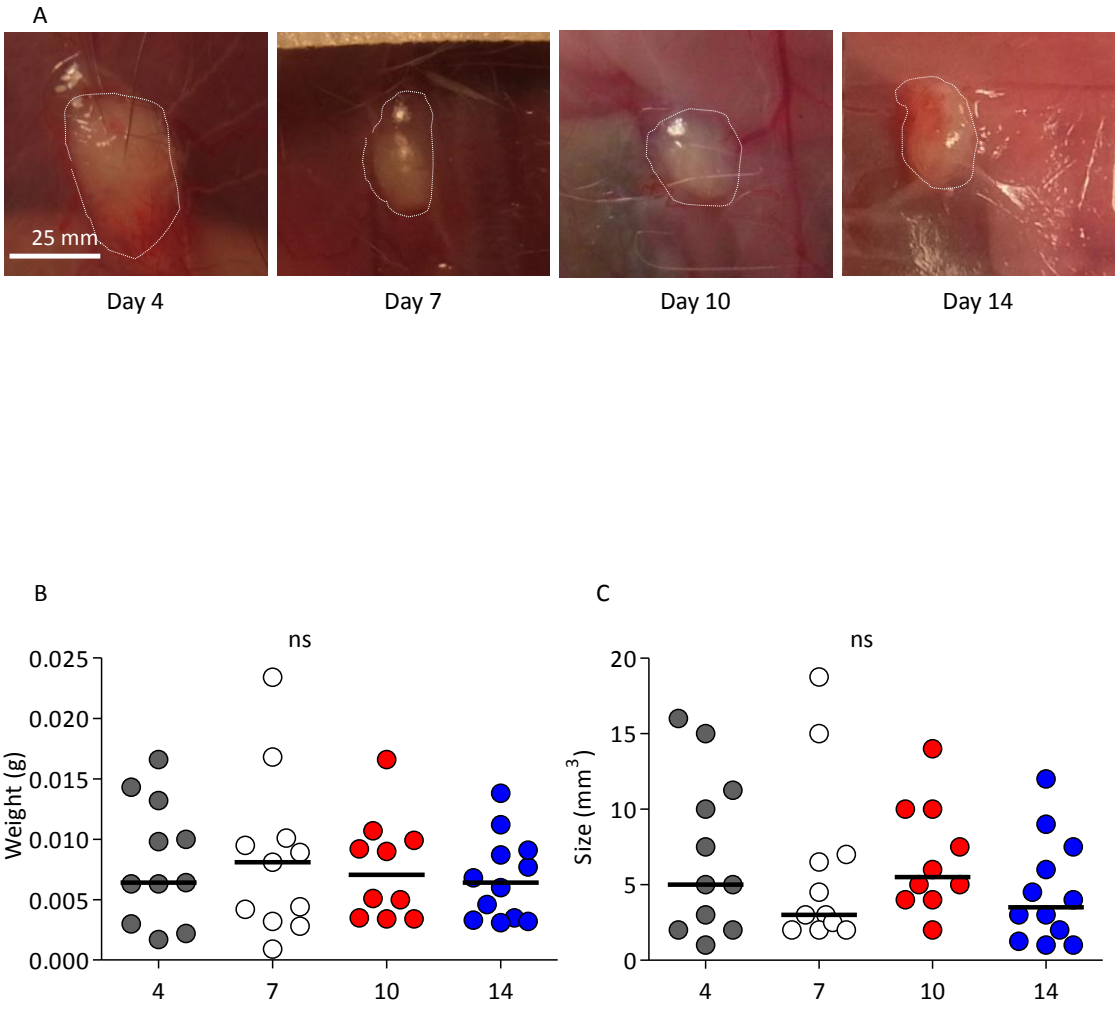


Figure 4. 1: Endometriosis-like lesions development in MacGreen/SCID mice.

(A) Xenografted human endometrial tissues formed subcutaneous lesions in these immunodeficient transgenic mice. (B) The weight and (C) size of lesions were compared between four groups using non-parametric Kruskal-Wallis test (ns, $p>0.05$). Each symbol represents data from one lesion, with median for each data set shown as a bar.

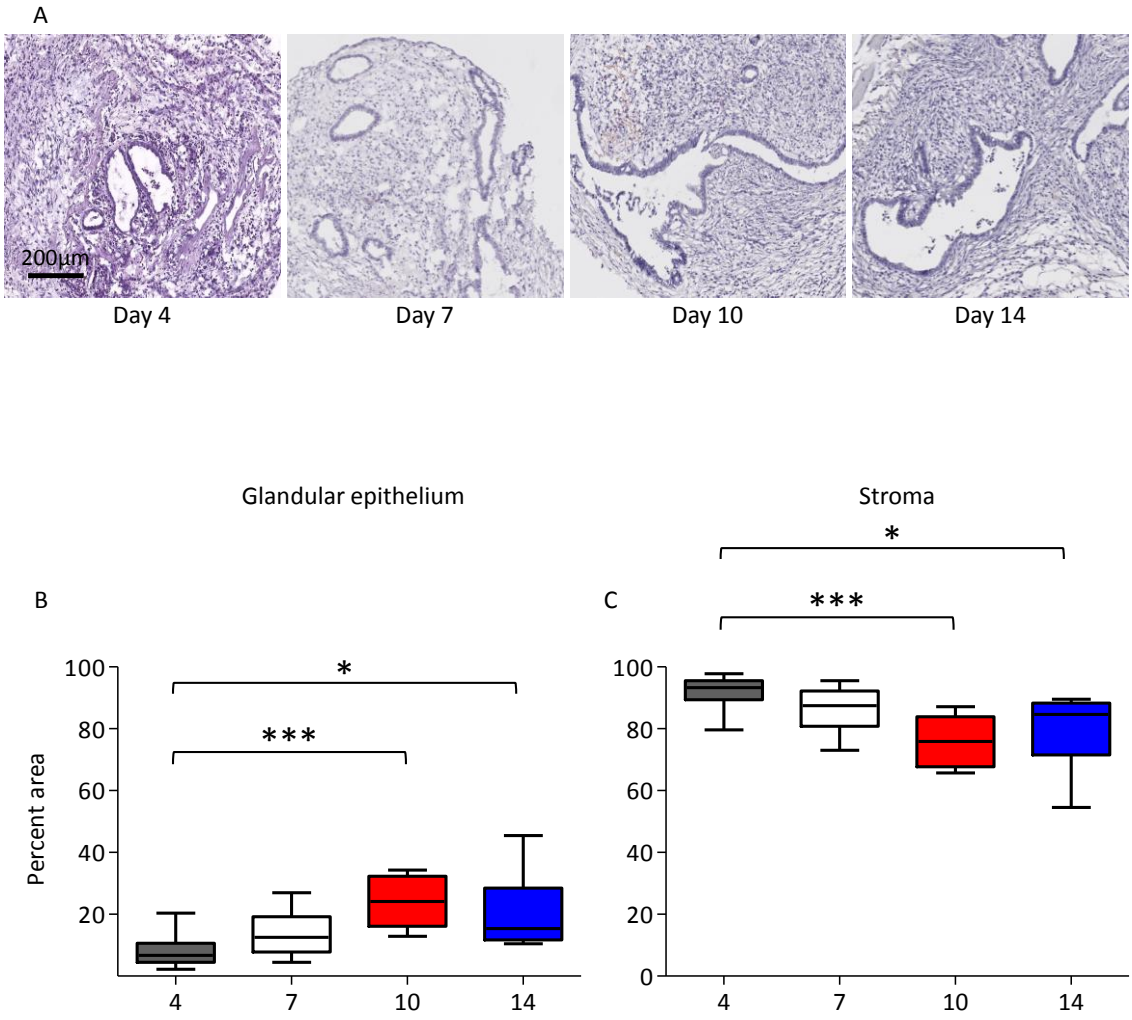


Figure 4. 2: Haematoxylin and eosin staining of endometriosis-like lesions.

For each time point, one representative section was chosen and measured. (B) The percent glandular and stromal areas of lesions at four different time points. The medians were analysed using non-parametric Kruskal-Wallis test, with significance inferred at $p<0.05$, followed by Dunn's multiple comparisons test (* $p<0.05$; *** $p<0.001$). Boxes represent the 25th and 75th percentile, with median line; error bars represent minimum and maximum values.

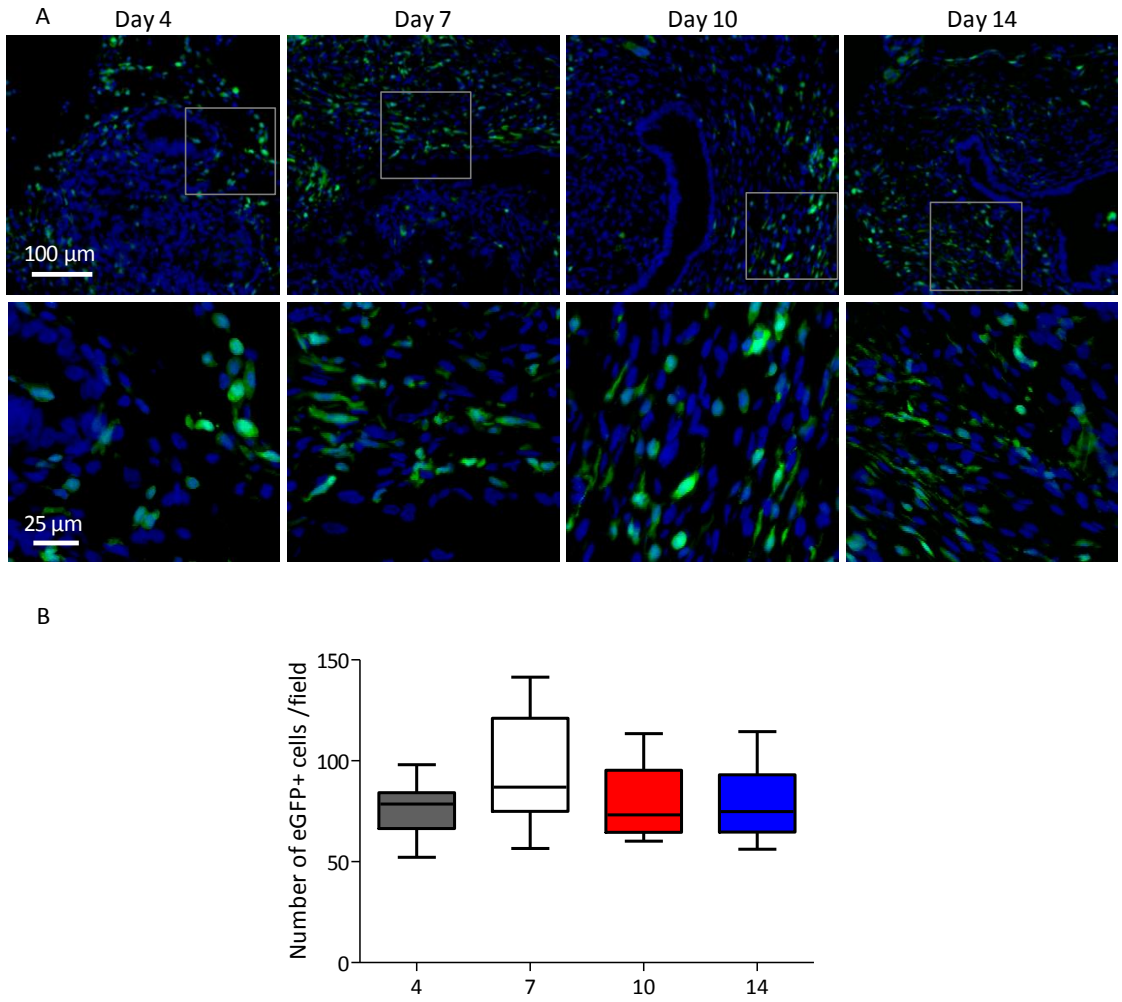


Figure 4. 3: Infiltration of macrophages (eGFP-positive cells) into endometriosis-like lesions at four time points.

(A) Host macrophages expressing eGFP under *c-fms* promoter were present in the periphery and centre of the lesion stroma. The eGFP images were merged with nuclear staining, DAPI (blue) to reveal individual cells. The number of macrophages was enumerated in the stromal compartment and averaged. (B) The abundance of macrophages was not statistically different across time points of lesion development ($p>0.05$, Kruskal-Wallis test). Boxes represent the 25^{th} and 75^{th} percentile, with median line; error bars represent minimum and maximum values.

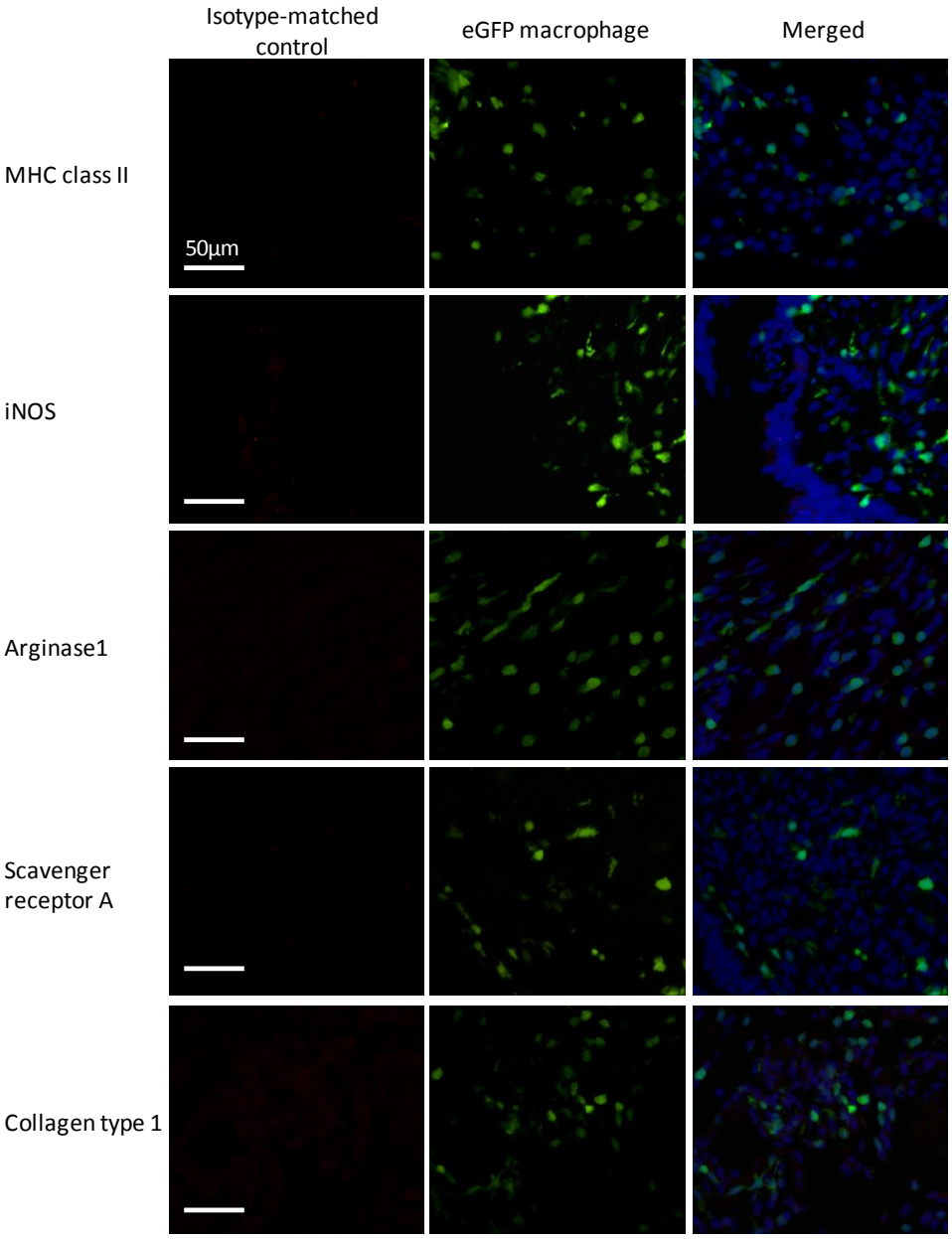


Figure 4. 4: Isotype-matched control for each immunostaining condition.

The specificity of each primary antibody was confirmed by substituting the primary antibody with an isotype-matched immunoglobulin (IgG). Endometriosis-like lesion sections were incubated with non-immunised rat IgG2aκ for MHC Class II immunofluorescent staining, rabbit IgG for iNOS and collagen type 1, rat IgG2b for scavenger receptor A and goat IgG for arginase 1. The immunofluorescent signals (red) developed were merged with the macrophage marker, eGFP (green) and nuclear staining, DAPI (blue). Scale bar = 50 µm.

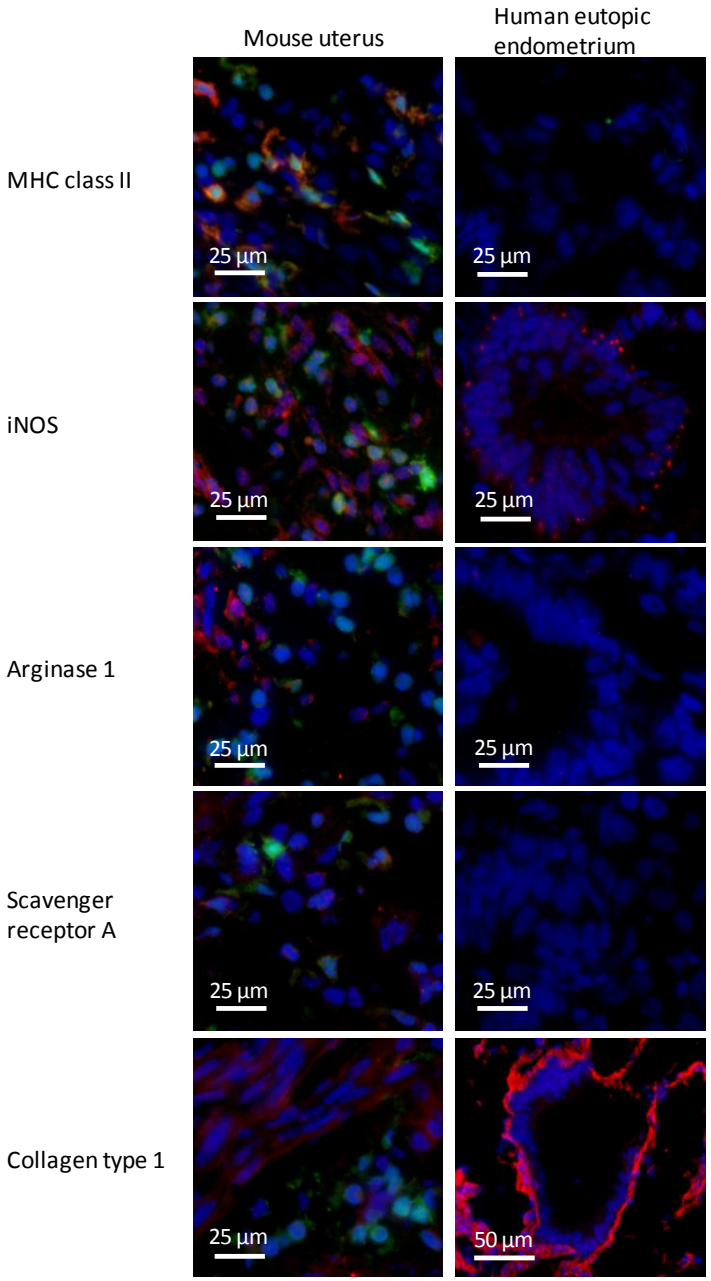


Figure 4. 5: Species specificity for each primary antibody tested on murine and human tissues.

Sections of murine uterine (left panel) and human eutopic endometrial (right panel) were independently incubated with anti-MHC Class II, anti-iNOS, anti-arginase 1, anti-scavenger receptor A (anti-CD204) and anti-collagen type 1. The immunofluorescent signals (red) developed were merged with the macrophage marker, eGFP (green) and nuclear staining, DAPI (blue).

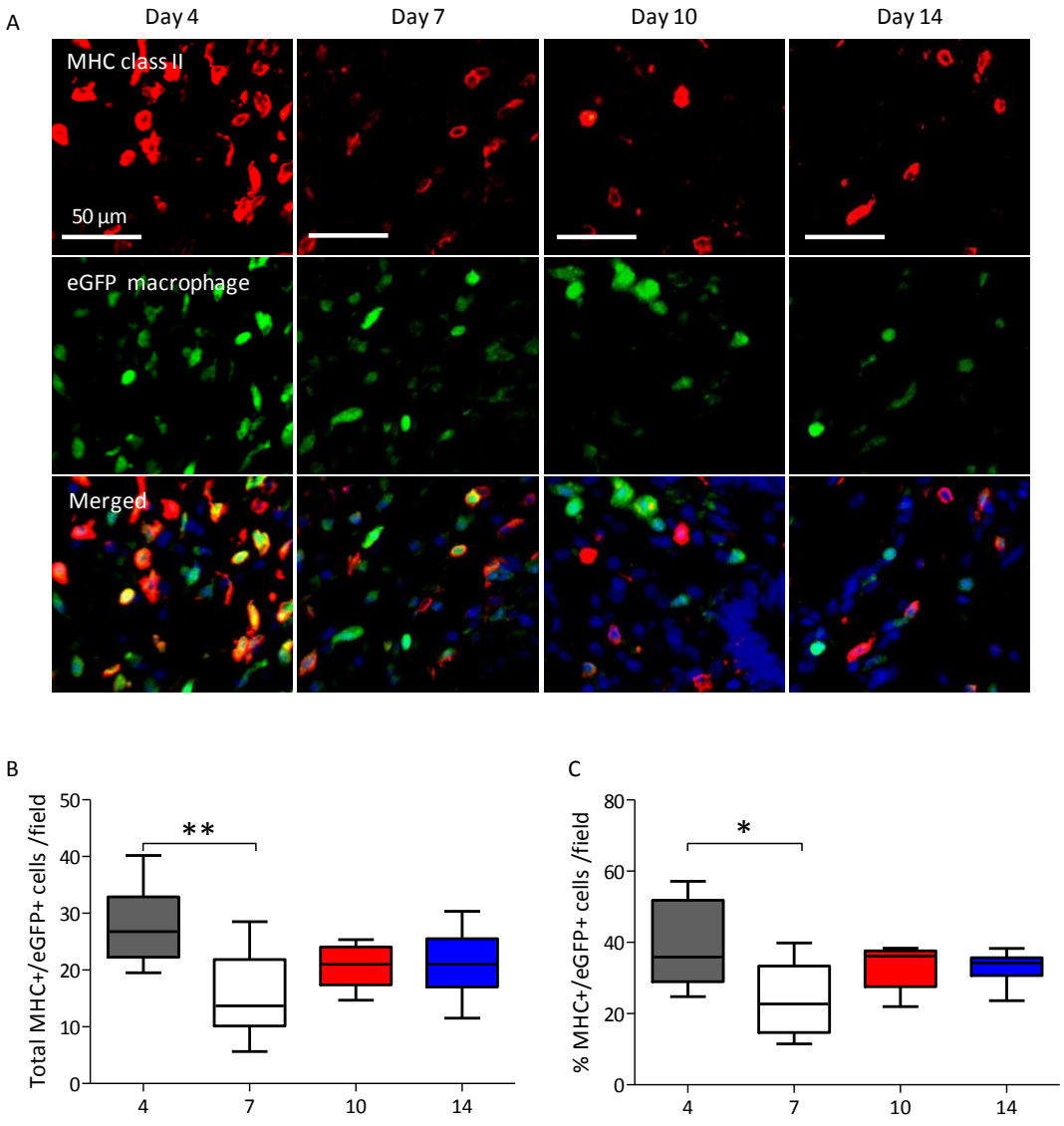


Figure 4. 6: MHC class II was localised in association with eGFP-positive macrophages.

(A) MHC class II-positive cells (red) were present in the stroma of the endometriosis-like lesions at four time points, day 4, 7 10 and 14 (top panel). The expression of endogenous enhanced green fluorescent protein (eGFP) which labelled CSF-1R-positive macrophage population in these lesions (middle panel). The immunofluorescent images were merged with nuclear staining, DAPI (blue) to reveal MHC class II-positive macrophages (bottom panel). (B) The number of MHC class II-positive cells was averaged from six representative fields of the lesions. (C) The percentage of eGFP-macrophages which also expressed MHC class II at each time point. Data were analysed using Kruskal-Wallis test, with significance inferred at $p<0.05$, followed by Dunn's multiple comparison test (*denotes $p<0.05$, ** $p<0.01$). Boxes represent the 25^{th} to 75^{th} percentile, with median line; error bars represent minimum and maximum values. Scale bar = 50 µm.

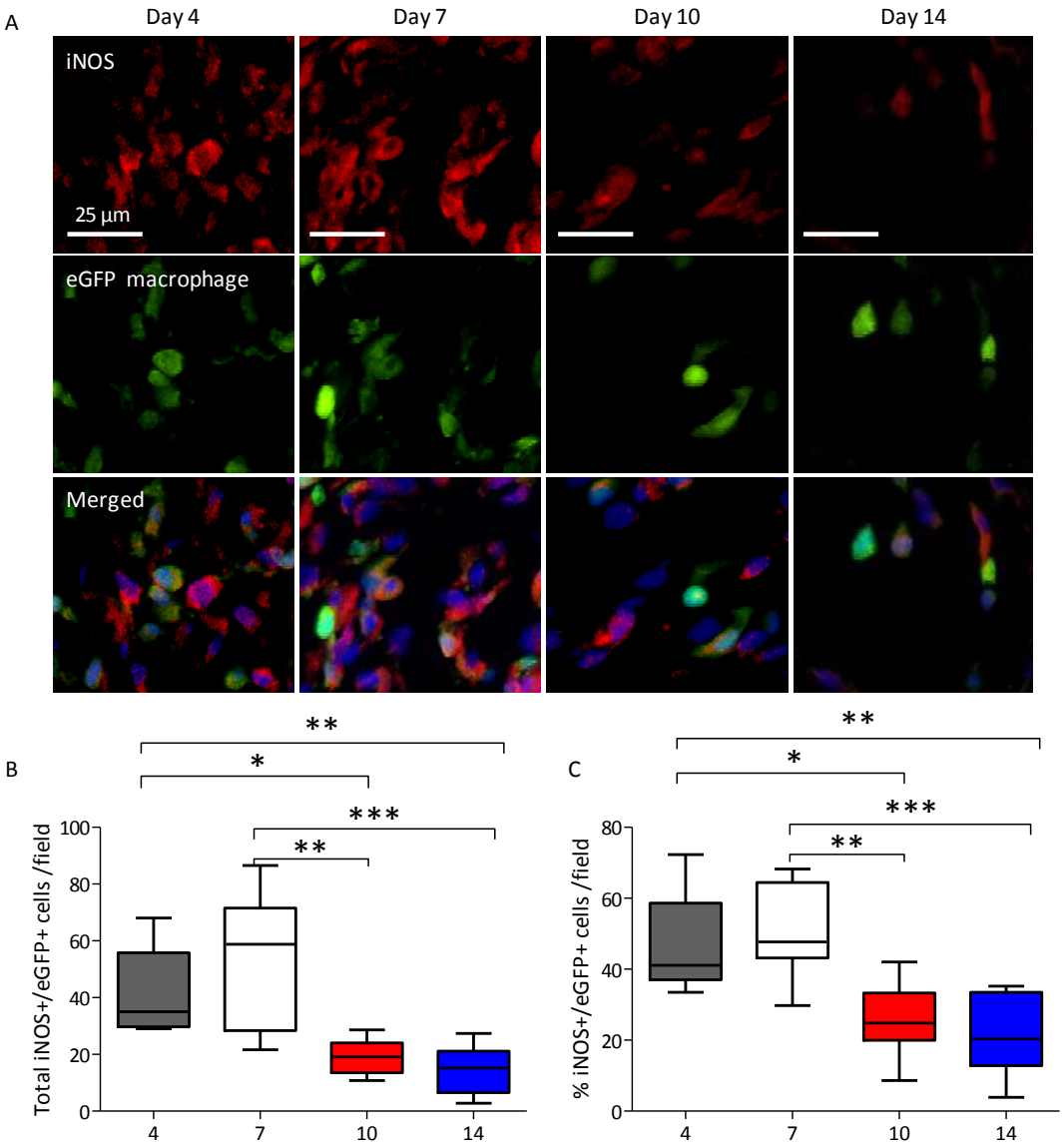


Figure 4. 7: Inducible nitric oxide synthase (iNOS) expression was localised with eGFP-positive cells.

(A) iNOS-positive cells (red) were present in the stroma and epithelium (not shown) of the endometriosis-like lesions from four time points, day 4, 7 10 and 14 (top panel). The expression of endogenous enhanced green fluorescent protein (eGFP) which labelled CSF-1R-positive macrophage population in these lesions (middle panel). The immunofluorescent images were merged with nuclear staining, DAPI (blue) to reveal iNOS-positive macrophages. (B) The number of iNOS-positive cells was averaged from six representative fields of the lesions. (C) The percentage of eGFP macrophages positive for iNOS at each time point. Data were analysed using Kruskal-Wallis test, with significance inferred at $p<0.05$, followed by Dunn's multiple comparison test (*denotes $p<0.05$, ** $p<0.01$, *** $p<0.001$). Boxes represent the 25th to 75th percentile, with median line; error bars represent minimum and maximum values. Scale bar = 25 µm.

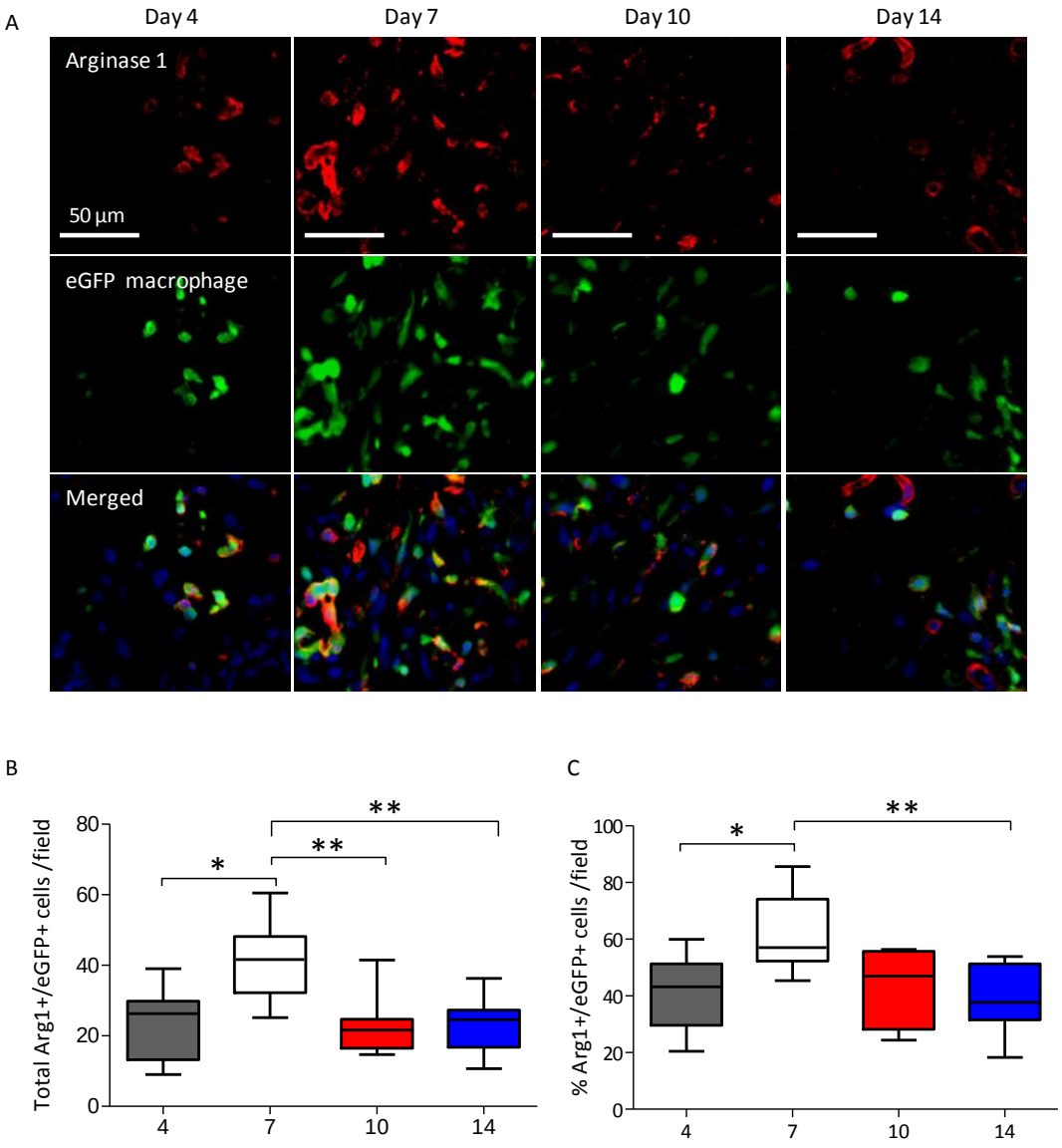


Figure 4. 8: Arginase 1 was identified in association with eGFP-positive macrophages.

(A) Arginase 1-positive cells (red) were present in the stroma of the endometriosis-like lesions from four time points, day 4, 7 10 and 14 (top panel). The expression of endogenous enhanced green fluorescent protein (eGFP) which labelled CSF-1R-positive macrophage population in these lesions (middle panel). The immunofluorescent images were merged with nuclear staining, DAPI (blue) to reveal arginase 1-positive macrophages (bottom panel). (B) The number of arginase 1-positive cells was averaged from six representative fields of the lesions. (C) The percentage of eGFP-macrophages which also expressed arginase 1 at each time point. Data were analysed using Kruskal-Wallis test, with significance inferred at $p<0.05$, followed by Dunn's multiple comparison test (*denotes $p<0.05$, ** $p<0.01$). Boxes represent the 25^{th} to 75^{th} percentile, with median line; error bars represent minimum and maximum values. Scale bar = 50 µm.

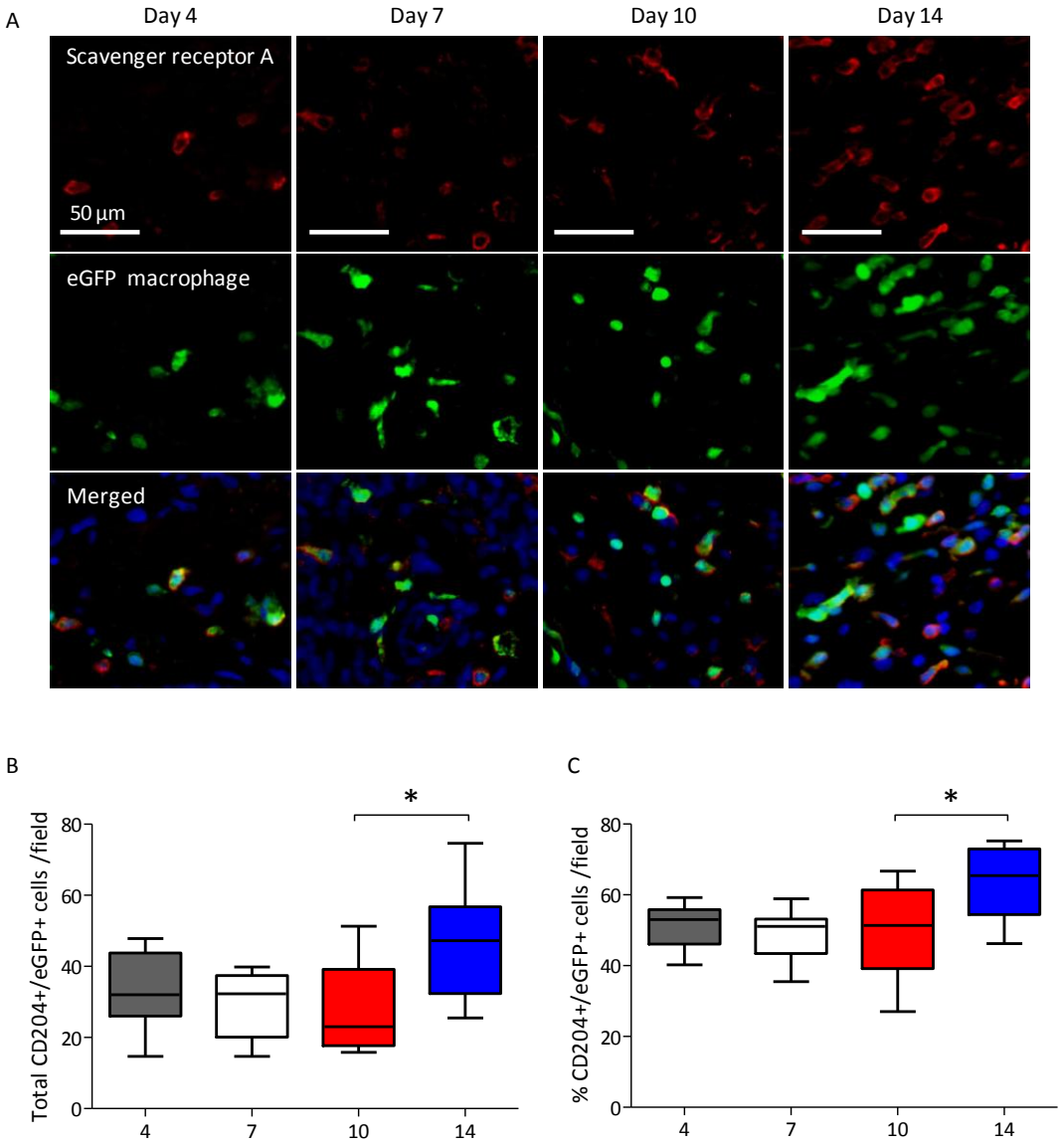


Figure 4. 9: Scavenger receptor A, CD204 localised to eGFP-positive cells.

(A) CD204-positive cells (red) were present in the stroma of the endometriosis-like lesions from four time points, day 4, 7 10 and 14 (top panel). The expression of endogenous enhanced green fluorescent protein (eGFP) which labelled CSF-1R-positive macrophage population in these lesions (middle panel). The immunofluorescent images were merged with nuclear staining, DAPI (blue) to reveal CD204-positive macrophages. (B) The number of CD204-positive cells was averaged from six representative fields of the lesions. (C) The percentage of eGFP-macrophages which also expressed CD204 at each time point. Data were analysed using Kruskal-Wallis test, with significance inferred at $p<0.05$, followed by Dunn's multiple comparison test (*denotes $p<0.05$). Boxes represent the 25^{th} to 75^{th} percentile, with median line; error bars represent minimum and maximum values. Scale bar = 50 µm.

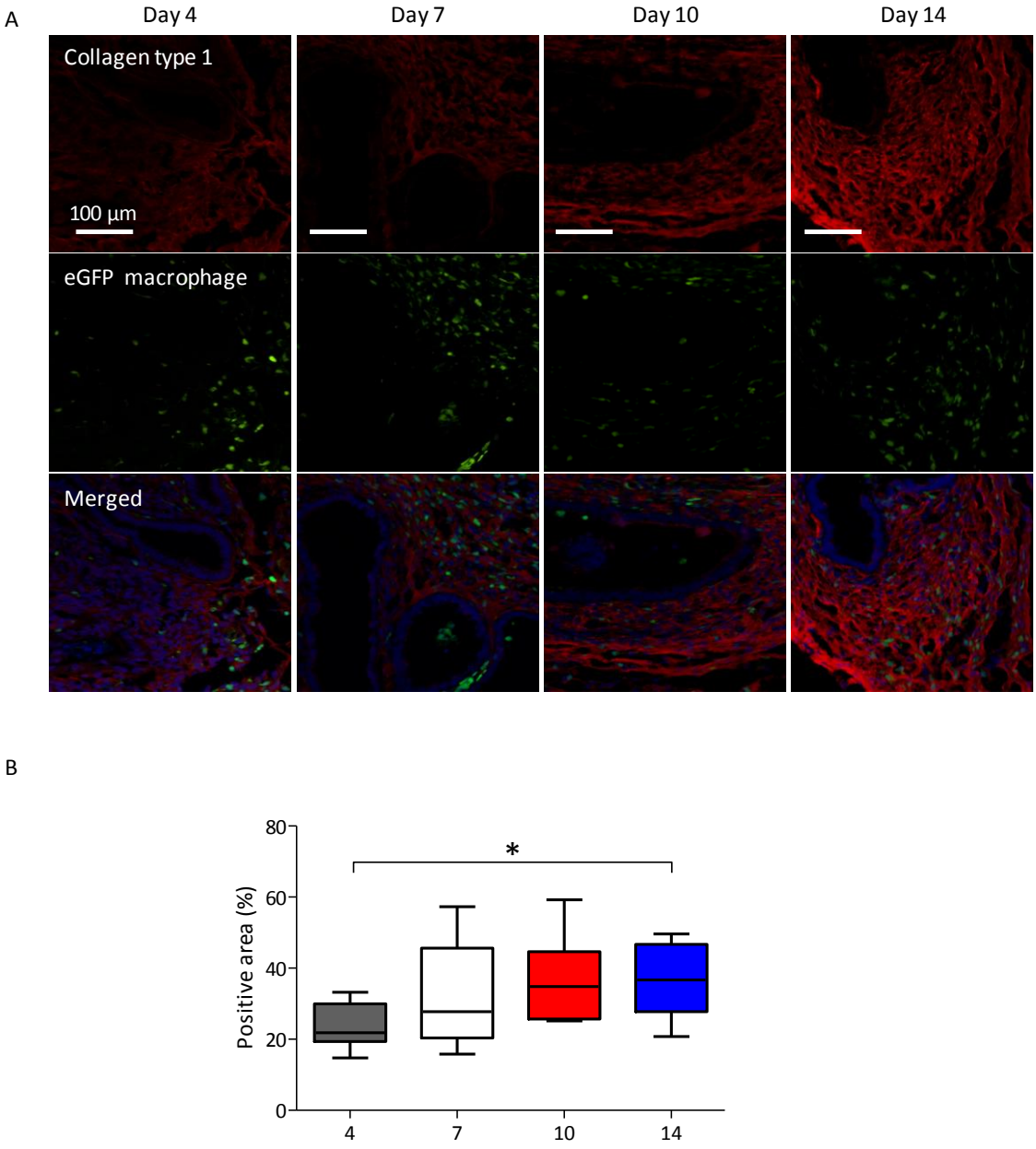


Figure 4. 10: Collagen type 1 deposition in endometriosis-like lesions.

(A) Collagen type 1 fibres (red) were present in the stroma of the lesions at four time points, day 4, 7 10 and 14 (top panel). The immunofluorescent images were merged with the endogenous eGFP (green), which expressed by CSF-1R-positive macrophage population in these tissues (middle panel) and nuclear staining, DAPI (blue) to reveal collagen and macrophage close proximity (bottom panel). (B) The area positive for collagen was averaged from six representative fields of the lesions. Data were analysed using Kruskal-Wallis test, with significance inferred at $p<0.05$, followed by Dunn's multiple comparison test (*denotes $p<0.05$). Boxes represent the 25^{th} to 75^{th} percentile, with median line; error bars represent minimum and maximum values. Scale bar = 100 µm.

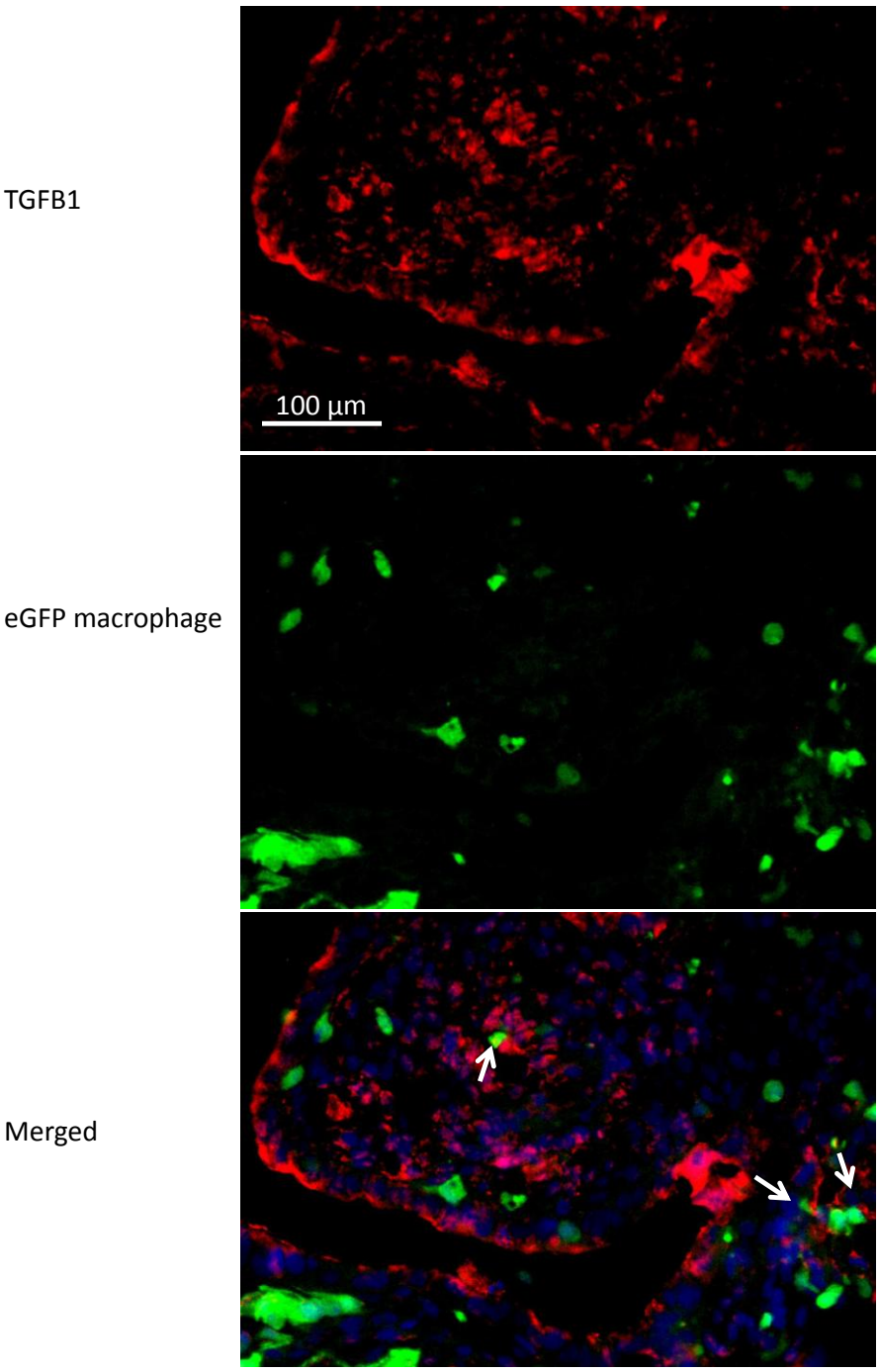


Figure 4. 11: TGFB1 localisation in association with eGFP macrophages in endometriosis-like lesions at day 10.

The active TGFB1 protein (red) was localised in the stroma and the lining of glandular epithelium of the lesions. The immunofluorescent images were merged with nuclear staining, DAPI (blue) to reveal the location of the staining. Some macrophages (eGFP) were in the close proximity with the TGFB1 (white arrows, bottom panel), although much of the TGFB1 staining appears to be associated with the extracellular matrix.

4.4 Discussion

This study examined the role of macrophages in endometriosis-like lesion development in immunocompromised mice. At the earlier stages of endometriosis development, inflammatory macrophages appeared to be more predominant, as demonstrated by the higher total number and percentage of MHC class II- and iNOS-positive macrophages. Both markers were abundant in day 4 to day 7 lesions compared to the later stages of endometriosis development. The expression of macrophage markers associated with the tissue repair arginase 1 appeared to overlap with inflammatory markers indicating a subtle shift in function over time. The marker of alternative activation, scavenger receptor A CD204 expression was significantly higher at day 14 than day 10 suggesting its upregulation over time. Unlike other macrophage markers studied in this chapter, which fluctuated over the time points, the number of CD204-positive macrophages seemed to be progressively increased during lesion development. This gradual increase in CD204-positive macrophages from day 7 of lesion development, suggests that the macrophages expressing this marker played important role in ectopic endometrial remodelling, particularly at day 14.

In MacGreen mice, eGFP is expressed by mainly macrophages, although other type cells have also been reported to express this marker, depending on the tissue locations. The promoter for the *c-fms* gene was used to drive macrophage-specific expression of eGFP since the gene product CSF-1R is crucial for development, proliferation and survival of macrophage population. CSF-1R is involved in macrophage generation, with *Csf1r* gene deletion resulted in a reduction of the number of F4/80-positive macrophages by 30.5% [124]. However, eGFP was also shown to be expressed by

some CSF-1R-negative macrophages, presumably because eGFP was retained from CSF-1R-positive precursor cells [315]. Thus, all myeloid cells with a brief expression of surface marker CSF-1R would appear as eGFP-positive. Macrophages typically have been identified using the classical F4/80, CD11b and CD68 markers. These markers can however be shared with some other immune cells, such as dendritic cells (CD11b) and neutrophils (CD11b and CD68). Thus, in immunohistochemistry studies, more than one marker is needed for definitive macrophage identification.

The number of eGFP macrophages did not change significantly across time points. This finding is in agreement with the observations by Bacci *et al*. [82] in a syngeneic mouse model of endometriosis. In this study, Bacci *et al*. showed that the percentage of murine peritoneal cells expressing F4/80 and CD11b, sorted by flow cytometry, was not significantly different throughout the 12-day experiment. The percentage F4/80-positive cells showed a small increase from day 0 to 4, followed by a trend to reduction towards the end of the experiment. The percentage of CD11b+ cells was also not affected during the 12-day endometriosis-like lesion growth in Bacci's experiment. Therefore, the steady level of macrophages in endometriosis-like lesions suggests that these cells are involved in all the critical steps of disease progression.

4.4.1 Glandular fraction was increased during endometriosis development

The lesion composition in the two groups of wildtype mice, without any growth factor deficiency, was expected to contain the same amount of glandular areas. However, the median for glandular area recorded from *Tgfb1*+/+ mice was 50% (Chapter 3), while MacGreen (in this chapter) median was 24% at 10 days post-injection. The different

genetic background of the host mice (Balb/c of SCID/MacGreen versus mixed C3H/Sve of *Tgfb1*+/+) as well as variability between patient tissues could contribute to the structural differences of the lesions, and consequently the glandular fraction of the tissues. Although standard haematoxylin and eosin staining was used for both experiments, lesions were processed in different ways (paraffin embedded for *Tgfb1*+/+ and frozen for SCID/MacGreen) which may also contribute to this disparity.

An increase in the glandular fraction at day 10 and day 14 when compared to day 4 could be due to three reasons. Firstly, the number of glands might increase over the time course of endometriosis-like lesion development as the result of newly generated glands. Thus, the total lesion area was higher in the later time points. The presence of endometrial stem cells in these tissues may explain why the cells have the capacity to form new glands in the presence of cytokines. Secondly, the increased size of the glandular lumen over time may explain why the percent glandular area was higher in day 14 and 10 than day 4. Thirdly, continuous estrogen supplementation over the course of the experiment may also have contributed to the increase in the size of the glandular epithelium. In two studies by Guo *et al*. and Alvarez Gonzalez *et al*., the experimental SCID mice were supplemented with 17β-estradiol. Guo *et al*. [316], described that the morphology of human endometrial graft as having pseudostratified nuclei and noted an increase in lumen size at day 4 compared to the pre-grafted endometrium. This is consistent with our findings which showed that endometrial explants were able to grow and the content of the tissues was dynamically altered. Similarly, Alvarez-Gonzalez *et al*. [317] also observed an increased lumen size at day 14 post-tissue implantation and sparsely dense stroma, compared to day 7. In Guo's study however, at 31 days post-tissue implantation, the xenografted endometrial tissue

started to disintegrate. Therefore, the growth of glandular area is continuous but is not indefinite, especially for xenografts.

Two-way communication is likely to occur between epithelial cells and macrophages in endometriotic lesions and it is possible that macrophages regulate the epithelial remodelling in ectopic endometrial tissues. It is known that the presence of ectopic endometrial tissues attracts macrophages into the peritoneal cavity [67]. Among the important chemoattractant and cytokines responsible for attracting macrophages/monocytes and also released by infiltrated macrophages are RANTES [276], IL-1 [318] and TNFα [319]. The secretion of these cytokines is regulated by estrogen and progesterone (reviewed by [320]), suggesting a role for these steroid hormones in macrophage recruitment into tissues.

The increase in the glandular area over time could also be contributed to by the macrophages. In a co-culture study using primary human macrophages and endometrial epithelial cells, peritoneal macrophages from women with endometriosis induced a higher relative proliferative capacity of endometrial epithelial and stromal cells when compared to macrophages from endometriosis-free women [321]. Thus, peritoneal macrophages alone, particularly from endometriosis women, have the ability to support the growth of endometrial epithelial cells *in vitro*.

As previously stated, macrophage cytokines are also likely to affect stromal cells. There was an increase in collagen 1 positive area over time as macrophage phenotype subtly shifted into a healing function (M2). This suggests that the later subtype of macrophages could be important for this collagen deposition by fibroblasts. Another type of macrophage Tie2 (as described in Chapter 1) was demonstrated to be

important for the formation of blood vessels [77]. Therefore, in endometriosis-like lesions, macrophage phenotypes played significant roles during the growth of the tissues. It is unknown if macrophage signalling to stromal cells can affect stroma-epithelium interactions or if macrophages can directly alter epithelial cell activity. *In vitro* models need to be established to further elucidate this concept.

Macrophages are also the source of enzymes that induce tissue breakdown and repair during the menstrual cycle. Macrophages were noted throughout menstrual cycle, most notably during secretory phase, expressing extracellular matrix proteases, such as MMP-9 [322]. An increase in MMPs, in particular MMP-1, MMP-2 and MMP-9 molecules was noted after progesterone withdrawal at day 28 and day 31 in a xenograft model of menstrual tissue repair exposed to steroid hormone treatment, mimicking a menstrual cycle [316]. Since the model used in this chapter was not exposed to progesterone, matrix degradation was not observed, explaining the ongoing increase in glandular area in the endometriosis-like lesions until day 14. In a mouse model of menstrual tissue repair, MMP-9 appeared to be critical for re-epithelialisation of the endometrial and was expressed mainly by neutrophils and some macrophages [241]. In addition to having a role in tissue breakdown, MMP-9 also reportedly has a role in tissue repair. Therefore, the epithelial repair as part of tissue remodelling could be mediated by MMP-9 from macrophages and neutrophils in this MacGreen mouse model.

4.4.2 Macrophage phenotypes dynamically changed during endometriosis development

In a mouse study, the absence of macrophages did not impair the implantation of ectopic endometrium, however the tissues failed to grow and vascularise appropriately [82], suggesting a role of macrophages in the remodelling of endometriotic lesions. In our study, the phenotype of macrophages in endometriosis-like lesions changed at four different time points.

There are two possible explanations to clarify the macrophage phenotype change in endometriosis-like lesions. The original immature cells could become activated in the presence of inflammatory stimuli. As the concentrations of these stimuli receded, the damaged tissues may produce other cues for the macrophages to transdifferentiate into a healing subtype. This was demonstrated *in vivo* by Mooney *et al*. [87] using a non-SCID MacGreen mouse model. Mooney and colleagues discovered that some eGFP-positive macrophages ended up as myofibroblasts, co-expressing αSMA. This observation suggests that macrophages have the dynamic ability to exert different functions depending on the stage of tissue repair. Further evidence by Lucas *et al*. [306], also favours the theory that the original (pre-existing) macrophages are important for the subsequent M2 phenotype. Lucas *et al*. reported that DT-injection resulting in the loss of systemic macrophages during mid-phase of tissue repair did not affect the healing of skin wounds.

On the other hand, using tumor mouse models, Movahedi *et al*. [323] showed that circulating monocytes expressing high level of Ly6C were precursors to M1 and M2 phenotypes of macrophages and Ly6C-positive cells were present throughout the

experiment. Therefore, there was a constant influx of circulated immature monocytes and these cells could be activated by the microenvironmental cues present in the tissues. There was a possibility that transdifferentiation and an ongoing influx of less differentiated that then mature depending on the environment in MacGreen/SCID mouse model. These theories can only be proven using macrophage depletion or macrophage labelling techniques in the current endometriosis model.

4.4.2.1 The role of iNOS during early stage of endometriosis development

Immunohistochemical staining of iNOS demonstrated that it was mainly localised in the glandular epithelium of pre-menopausal endometrium [324], particularly from the secretory phase [325]. This confirms our observation that the immunofluorescent signal against iNOS was localised to the epithelium of eutopic endometrial sections. Besides glandular epithelium, iNOS can also be expressed by peritoneal macrophages after inflammatory activation [326] and occasionally the stromal cells and smooth muscle cells [325]. The presence of iNOS in menstrual endometrial tissues is thought to be involved in tissue breakdown and cell apoptosis (reviewed by [327]). Nitric oxide (NO) synthesised by iNOS could regulate a range of macrophage activities, in addition to their main function to kill extracellular pathogens. iNOS regulation was demonstrated to be controlled by the pro-inflammatory transcription factor NFκB (nuclear factor kappa B) in a murine macrophage cell line, RAW 264.7 [328]. An increased activation of NFκB was observed in the peritoneal red lesions (n = 3) [329] and also in the peritoneal macrophages of 22 women with endometriosis [330]. Estrogen receptor β (ERβ) also plays a role in regulating iNOS expression in macrophages. LPS-induced iNOS activity

was reduced in the presence of ERβ agonist in peritoneal macrophages from endometriotic women [326]. Therefore, NFκB and estrogen receptor β could together play an important role in regulating iNOS expression by macrophages during the early inflammatory phase of endometriosis-like lesion development.

The specific role of iNOS-positive macrophages in mediating the growth of endometriotic lesion is still unknown. The presence of iNOS at higher concentrations in peritoneal macrophages of endometriosis patients [294] could be associated with the increased activation of these macrophages [331]. This macrophage activation could then be necessary for iNOS activity in assisting endometriosis-like lesion growth. One of the possible roles of iNOS activity in endometriosis is by mediating angiogenesis via NO synthesis. Inhibition of NO by a NO synthase inhibitor (L-NAME) reduced the VEGF-induced proliferation and organisation of human endothelial cells in a 3-dimensional culture [332]. Although, the endothelial nitric oxide synthase (eNOS) rather than iNOS could be responsible for this effect, there is a possibility that iNOS-positive macrophages are also important for the formation of blood vessels through the production of NO. Another important role of iNOS is in tissue re-epithelisation during tissue repair. The inhibition of iNOS (by an inhibitor L-NIL) markedly reduced the number of proliferating Ki-67-positive keratinocytes [by 68% (d3) and 54% (d5)] and epithelial cells in wounded skin during the tissue repair phase in a mouse model at day 3 and 5 [333]. Thus, the role of iNOS is not simply limited to inflammation, but this enzyme is also important for angiogenesis and tissue regeneration. Importantly, early iNOS expression during time course experiment could suggest this enzyme was necessary for endometriosis-like lesion development.

4.4.2.2 MHC class II in endometriosis-like lesion inflammation

MHC class II expression at day 4 was associated with inflammation in the endometriotic lesions which is consistent with its role as an antigen presenting molecule in inflammation. High expression in day 4 xenografts is consistent with the histology findings of necrosis and inflammation at this time and chemoattractants released in this environment are likely to recruit neutrophils and macrophages [81, 146] and promote their activation.

In patients with endometriosis, MHC class II molecule (HLA-DR) is present and is associated with macrophage activation. For example, the activity of acid phosphatase, a measure of macrophage activation, was more than 50% higher in peritoneal fluid from 15 women with endometriosis than in 10 disease-free patients [34]. The expression of this molecule was upregulated following endometriosis induction in baboons [67], suggesting ectopic endometrial tissues stimulated the expression of MHC class II.

In addition to modulating inflammation, MHC class II could also play a role in tissue remodelling. In a wound healing study, fibroblast proliferation and collagen synthesis was reduced by 4.8- and 2.3-fold, respectively in MHC class II-deficient mice at day 10 post-wounding compared to wildtype control [283]. These findings could be explained by the existence of two subsets of MHC class II during tissue inflammation and in the subsequent tissue remodelling. These two subsets of MHC class II-expressing macrophages were demonstrated by Wang *et al*. [334] and Movahedi *et al*. [323] in tumors developed from injections of murine hepatocellular carcinoma and mammary carcinoma cells in mouse models. Two subsets of macrophage-positive MHC class II were assessed by flow cytometry, and characterised by the low- (MHC class II^{hi}) and

high- (MHC class II^{lo}) MHC class II expressions. The infiltrating macrophages into tumor tissues were preferentially MHC class II^{hi} and this subset of macrophages downregulated their MHC class II expression to become MHC class II^{lo} [334]. MHC class II^{lo} was shown to express high level of M2-associated mRNA transcripts such as *Arg1* (arginase 1), *Ym-1*, *Tgfb1* (TGFB1) and scavenger receptor *Cd163*, scavenger receptor A surface marker and also MMP-9 and VEGF proteins compared to MHC class II^{hi}, thus contributing to tumor development. In contrast, both studies showed that M1-associated mRNA transcripts were upregulated in MHC class II^{hi}, such as *Nos*2 (iNOS) and *Il1b* (IL-1β). Therefore, MHC class II expression appears to be dynamically regulated in the tumor depending on the stage of disease, which could also apply for endometriosis-like lesions.

The immunofluorescent staining used in our study was less sensitive in allowing the distinction between low and high expression of MHC class II on macrophages; therefore the MHC class II^{lo} population might appear either negative or faintly positive in the endometriosis-like lesions. In our study, MHC class II macrophages were higher at day 4 than day 7 and persisted, albeit at low levels at the later time points of lesion development, day 10 and 14. This was also demonstrated in Wang's study, in which macrophages recruited into expressed high level of MHC class II before being progressively replaced by a MHC class II^{lo} phenotype.

4.4.2.3 Arginase 1 expression by macrophages

Arginase 1-postive macrophages were more abundant at day 7 of endometriosis-like lesion development compared to other three time points. The expression of this protein is associated with tissue remodelling step of tissue healing [335]. It was suggested

that arginase 1 mediates the inflammation elicited by iNOS. Stimulations of mouse macrophages by LPS induced the expression of *Nos2* (iNOS) mRNA as early as 3 hours in culture, while *Arg1* mRNA only peaked at 9 hours [336]. Consistent with the mRNA expression, arginase 1 protein was only present after 12 hours of LPS-stimulation. This delayed expression of arginase 1 was suggested to be important for controlling the production of NO [336] as arginase 1 competes with iNOS for the common substrate, L-arginine [297].

The *Arg1* transcript has previously been identified to be upregulated in endometriosis-like lesions at day 3 in the early stages of tissue remodelling [303]. In our study, arginase 1-positive macrophages were present in a notable number as early as day 4 and were abundant by day 7 of endometriosis-like lesion development. Macrophages expressing this marker resolve rather than participate in tissue inflammation.

Expression of arginase 1 by tumor-associated macrophages is linked to an increased ability to mediate tissue remodelling *in vivo*. For instance, in a murine tumor model, the *Arg1* mRNA transcript was identified in a subset of macrophages with low MHC class II expression [323]. In addition, MHC class II^{lo} macrophages co-cultured with chicken chorioallantoic membranes (CAM) exhibited increased pro-angiogenic capacity, as demonstrated by a higher number of vessels, than did the MHC class II^{hi} macrophages, which had lower *Arg1* gene expression [323]. This subset of the macrophage population appears to assist tissue remodelling via altered arginase 1 production.

4.4.2.4 Scavenger receptor A CD204 in endometriosis-like lesion development

In women with endometriosis, the peritoneal macrophages expressing scavenger receptor were proposed to be less efficient in clearing red blood cells and apoptotic endometrial cells [337], thus resulting in the lesion growth. In another study, co-culture of M2 (scavenger receptor, CD163+) macrophages with endometrial stromal cells resulted in an increase in CD204 expression [338]. This could be as the result of upregulation by macrophages upon encounter with endometrial stromal cells. The authors however incorrectly used CD204 as a marker to stain stromal cells, where vimentin would be more appropriate. In human macrophages, CD204 was demonstrated to be critical for the uptake of acetyl low-density lipoproteins (AcLDL) [339]. Besides class A scavenger receptor, class B receptor has also been described in endometriosis and its expression was higher in the endometriosis lesions than in the paired eutopic tissues [340]. This receptor is primarily involved in steroidogenesis [341], which is important in maintenance of ectopic endometrial tissues.

Scavenger receptor plays an important role in scavenging dead cells, demonstrated by a redundant function of scavenger receptor class A (SR-A), CD204 and class B, CD36. For example, CD204-null mutant mice exhibited normal phenotypes and their clearance of apoptotic cells was not impaired [342] due to compensation by a class B scavenger receptor, CD36 in the absence of CD204. Additionally, Neyen *et al*. [305] demonstrated that SR-A-deficient mouse macrophages had reduced ability to mediate the invasion and progression of tumor tissues. Therefore, SR-A-positive macrophages are important for normal tumor development. In our study, CD204-positive

macrophages were identified in the endometriosis-like lesions at all four time points and were significantly more abundant at day 14. Although our initial expectation was to have negligible SR-A macrophages early on, this was not the case. Our data and Neyen's study showed that SR-A-expressing macrophages are important in the early stages of lesion development and this expression may be needed to enhance cell growth. The expression of scavenger receptor A was also increased following endometriosis induction in a mouse model of endometriosis. An injection of syngeneic epithelial or stromal cells, into the peritoneal cavity of mouse increased the SR-A activity as measured by the internalisation of fluorescent-labelled AcLDL [89]. In this study, compared to stromal cells, ectopic endometrial epithelial cells had a higher ability to recruit macrophages owing to their increased secretion of stimuli such as MCP-1 as early as 4 hours post-injection.

SR-A also participates in the clearance of apoptotic cells, following inflammation. In the absence of SR-A (in CD204-null mice), inflammation failed to be resolved [343] primarily via reduction in the uptake of apoptotic cells [344]. Similarly, SR-A is also important in suppressing LPS-induced TLR4-mediated inflammatory as demonstrated by an excessive infiltration of macrophages following LPS injection in CD204-null mice (SRA-/-) [345]. Therefore scavenger receptor plays an important role in regulating inflammation, presumably to prevent excessive cell damage and mediate tissue remodelling.

4.4.3 Possible factors mediating macrophage polarisation in endometriosis

In vitro, non-polarised macrophages can be induced by IFNγ into inflammatory/M1 phenotypes and further stimulated using IL-10 or IL-4 to develop M2 phenotypes [82, 346].

Iron, apoptotic cells and hypoxia may induce a similar change in *in vivo* macrophage polarisation.

4.4.3.1 Iron homeostasis

Peritoneal iron metabolism has been linked to the pathogenesis of endometriosis. Haemosiderin (an iron storage complex in cells) is commonly seen in macrophages laden in surgically-confirmed endometriotic women and rarely in the control patients [347]. Furthermore, peritoneal fluid from women with endometriosis at secretory phase had higher iron concentration (n = 19) than the disease-free women at similar stage cycle (n = 16) [348]. This finding is in agreement with Lousse *et al*. [349] who showed peritoneal fluid of women with endometriosis to have a higher iron concentration than controls at secretory phase of menstrual cycle. Both studies also reported higher ferritin (a protein structure that stores iron) concentration in peritoneal fluid and a higher ferritin optical density in peritoneal macrophages [349] from women with endometriosis.

In human, clearance of circulating haemoglobin by macrophages via scavenger receptor CD163 is the only known way to remove free circulating iron [350]. CD163 is also one of the markers of M2 macrophages in human and mouse. Macrophages expressing scavenger receptor, CD163 were found to be significantly increased in the endometriotic peritoneum and lesions compared to endometriosis-free peritoneum [82]. Increased level of these receptor-expressing macrophages could be as the result of peritoneal iron overload observed in endometriosis women.

4.4.3.2 Apoptosis cells

Apoptotic leukocytes may induce polarisation of macrophages, shifting their phenotype from M1 to M2 phenotype [351]. Little is known if phagocytosis can influence the macrophage to change its phenotype. Macrophages were involved in the clearance of apoptotic thymocytes via expression of scavenger receptor A (CD204) through the Mer receptor tyrosine kinase (Mertk) pathway [344]. CD204-mediated uptake of apoptotic cells was shown *in vitro* and *in vivo* which was affected in CD204-null mice [344], and this is likely to be translatable to endometriosis conditions. Some necrotic cells are present in the displaced retrograde endometrial tissues inside the peritoneal cavity. These necrotic cells may attract macrophages and induce a macrophage phenotype shift.

4.4.3.3 Hypoxia

Low oxygen tension (hypoxia) can also trigger macrophage polarisation in damaged tissues via hypoxia inducible factor (HIF) pathways. In endometriotic lesions, hypoxia-associated proteins have been identified early in lesion development. The stromal compartment of lesions demonstrated positive staining for the hypoxia marker, hypoxyprobe-1 an hour after endometriosis induction which remained for 48 hours in a C57/Bl6 endometriosis mouse model [181]. Seven days after tissue transplantation, the endometrial transplant was negative for hyproxyprobe-1. It is likely that neovascularisation improved the oxygen supply to lesions during this time. The presence of hypoxia in endometriosis was also demonstrated in a clinical study. Ectopic stromal cells (n = 5) showed more than a two-fold increase in protein expression of the transcription factor, HIF-1α isoform compared to the paired eutopic

cultures [352]. The inhibition of HIF-1α by 2-methoxyestradiol was found to reduce the lesion size of endometriosis-like lesions in a mouse model, through down-regulation of angiogenic factors, in particular VEGF [181]. Hypoxia contributes to endometriosis establishment and HIF-1α is the key molecule regulating this low oxygen condition.

Data in tumor studies suggested that monocytes and macrophages are recruited to hypoxic sites along a hypoxic-induced chemoattractant gradient (reviewed by [353]). The findings in an *in vitro* study using HIF-1α-null mice also showed that this transcription factor is important for macrophage invasion and motility. In a modified Boyden chamber, HIF-1α-null peritoneal macrophages had a 60% reduced invasive capacity and cell motility was inhibited by 50% and 75% in normoxia and hypoxia, respectively [354]. It is likely that an increase in HIF-1α reflects the hypoxic environment encountered by ectopic tissues in the peritoneal environment. This environment is likely to trigger macrophage activation. Our study provides new data on macrophage polarisation in ectopic endometrial tissues.

Tumor-associated macrophages (TAMs) are commonly identified in hypoxic conditions in cancers. In endometrial cancer tissues, TAMs were abundant in the hypoxic/necrotic areas [355], although TAMs were only localised with a single marker, CD68 which does not accurately identify this sub-population of macrophages. There is evidence that chemokine receptor CXCR4 and its ligand CXCL12 might be responsible for TAM recruitment into the hypoxic sites (reviewed by [356]). An M2-associated macrophage population was identified in the hypoxic regions. Movahedi *et al*. [323] demonstrated in a mammary adenocarcinoma mouse model, that the hypoxic regions contained cells positive for CD11b but expressed low levels of MHC class II, suggesting an M2 phenotype. The presence of low oxygen area in tissues could stimulate the recruitment

of macrophages with M2 phenotypes to encourage blood vessel formation, thus improving oxygen supply to this site.

Although hypoxia could be one of the key mechanisms that triggers macrophage polarisation, these low oxygen conditions can also reduce the expression of scavenger receptors, particularly CD204. When the murine macrophage cell line, RAW264 was exposed to 1% oxygen environment, *Msr1* mRNA [a gene which encodes for scavenger receptor A (CD204)] expression was reduced in a time- and HIF1α-dependent manner [357]. Additionally, LPS stimulation and hypoxia reduced the expression of *Msr1* mRNA. Macrophage expression of CD204 is associated with an anti-inflammatory phenotype [357]. This scavenger receptor reduces the production of the pro-inflammatory cytokine, TNFα and moderates inflammation by clearing pathogens and inflammatory stimuli. Suppression of scavenger receptor CD204 in hypoxic conditions was suggested to be essential in allowing an inflammatory response and killing of intracellular pathogens in a low oxygen environment. Hypoxia could therefore regulate the expression of M2-associated phenotypes depending on the stage of infection and inflammation.

4.4.3.4 Other factors that could contribute to macrophage polarisation

Dioxin (TCDD) and estradiol have also been described as inducers of macrophage polarisation. The combination of dioxin and estradiol was shown to increase protein expression of chemokine receptor type 1 (CCR1) ligand in U937 monocyte cell line [30]. This receptor serves as a binding ligand for RANTES, among other molecules. The expression of RANTES has been shown to induce tolerance in immune U937 cells by suppressing MHC class II molecules, HLA-DR [30]. The expression of CCR1 was even

higher when a combination of dioxin and estradiol or estradiol alone was added to the co-culture of U937 cells and primary endometrial stromal cells and peritoneal mesothelial cells. Thus, this finding suggested that physical contact of macrophages with both endometrial and mesothelial cells increased macrophage sensitivity to RANTES, hence rendering them more tolerant.

Mice with systemic depletion of estrogen and progesterone showed impaired tissue remodelling. Ovariectomised female mice in a wound healing study showed a delayed in tissue healing compared to an intact control [358]. Exogenous estrogen supplementation into the ovariectomised mice decreased the infiltration of macrophages and inflammatory cytokine TNFα mRNA. Macrophages expressing M2 marker mRNA, Ym-1 were also more abundant in the estrogen-supplemented mice compared to estrogen-depleted ovariectomised mice. Therefore, in the absence of estrogen and progesterone, inflammatory M1 macrophages were more abundant and tissue repair was delayed, while estrogen supplementation partially restored this deficiency by suppressing the infiltration of inflammatory macrophages and enhancing M2 macrophages. Estrogen regulation of specific macrophage phenotypes was demonstrated to be mediated through macrophage inhibitory factor (MIF) [359]. This mechanism could also be applicable in the endometriosis lesion development in which estrogen is associated with the endometriosis pathogenesis. Estrogen could contribute to lesion growth by modulating the functions of macrophages in this tissue.

4.4.4 The role of macrophages in collagen deposition

Macrophage depletion was shown to delay the tissue remodelling process in endometriosis. One of the steps demonstrated to be affected when macrophages were

depleted is collagen deposition. Disruption of diphtheria toxin (DT)-expressing CD11b cells reduced the macrophage population at day 1, 3, 5 and 7 post tissue wounding, compared to the non-treated control [112]. In the macrophage-depleted mice, collagen intensity as detected by Masson's Trichrome staining was significantly reduced at day 7 and 10 by approximately 2.5- and 1.3-fold, respectively. Given that DT was only administered at the early stage of tissue wounding (immediately before and 48 hours after), this study demonstrated that the recruited macrophages to the wounded sites were important in collagen synthesis at the later stage. This finding was in agreement with another study which showed that DT- depleted macrophages at the initial stage (48 and 24 hours prior and 48 hours after) of a wound healing experiment reduced collagen deposition at day 14, while depletion at later stage did not significantly affect tissue repair process [306]. In the current study, the increased expression of collagen type 1 coincided with the changes in the phenotype of macrophages from inflammation to tissue remodelling.

Macrophages could play a pivotal role in the increase of this protein in endometriosis-like lesions. Macrophage depletion was shown to delay the tissue remodelling process in endometriosis. Therefore, in the endometriosis-like lesions, macrophage recruitment could be important for the increased collagen fibres seen at day 14. Macrophage depletion prior and during the inflammatory process would affect the collagen synthesis and hence tissue development.

4.4.5 The role of collagen in macrophage functions

In addition to providing structural support for the newly developed lesions, collagen type 1 could also carry a role for macrophage functions. Collagen type 1 however did

not play a significant role in inducing polarisation in the macrophage population [360]. Stimulation by cytokines, LPS/IFNγ and IL-4/IL-13, were more effective at inducing human macrophages into M1 and M2 phenotypes, respectively *in vitro*. M1 macrophages exhibited suppressive actions on collagen type 1. There is also a preference in the phenotype of macrophages that stimulate the expression of collagen fibres. Culture media from M1 macrophages suppressed the expression of type 1-collagen mRNA, *Col1A*, in human dermal fibroblasts compared to unstimulated and M2 macrophages after 6 days [361]. This finding showed that cytokines released by the macrophages with M1 phenotype are more likely to inhibit collagen type 1 synthesis while M2 cytokines could favour this process. This explains our observation of an increase in collagen type 1 deposition, which might have been accelerated as the abundance of M2 macrophages increased as the lesion developed.

The presence of collagen type 1 may also assist in macrophage motility. Macrophage scavenger receptor A has a specific ligand, called collagenous domain, which was shown *in vitro* to adhere to the denatured, non-fibrillar form of collagen type 1 [362]. Binding of macrophages to collagen type 1 is suggested by Gowen *et al*. [363] to be important for the retention of these cells at the wounding sites, enabling subsequent tissue remodelling. During tissue breakdown, collagen is digested by proteases, hence assistance is required from macrophages to regenerate. This also explains macrophage inability to bind to the native, undenatured form of collagen type 1 [363]. The presence of this ligand for scavenger receptor is consistent with an important role for M2 macrophages in mediating tissue repair process.

4.4.6 Rationale for using immunofluorescence

Immunofluorescence was employed to study the populations of macrophages over time because it allowed an *in situ* localisation of these cells in endometriosis-like lesions. In addition, since host TGFB1 deficiency reduced the number of macrophages (Chapter 3), as unravelled using an immunolocalisation technique, it was appropriate to use the same approach to analyse different phenotypes of macrophages in this chapter. Besides immunofluorescence and immunohistochemistry, another approach that could be used to analyse the dynamic macrophage population during endometriosis development is flow cytometry.

Fluorescence Activated Cell Sorting (FACS) or flow cytometry would allow co-expression of macrophage activation markers to be studied. This method is more quantitative than immunofluorescent morphometric analysis which permits co-localisation of macrophage markers and eGFP. The main disadvantage of FACS is that the whole lesions had to be digested and processed (as single-cell suspensions) before they could be examined for macrophage markers. This process would reduce the information regarding the location of the cells and may inadvertently include murine cells not present in lesions. Lesion digestion in this technique would prevent the assessment of morphology. For these reasons, immunofluorescence was employed to localise eGFP expression and macrophage-specific markers.

4.5 Conclusions

In this series of experiments, the markers of macrophage activation were found to be differentially expressed throughout the course of endometriosis development. Classically activated macrophage markers iNOS and MHC class II were preferentially

expressed at the earlier time points. This suggests that inflammatory macrophages are more dominant during the early phase of development of the lesion. Meanwhile, the expression of markers indicative of alternatively activated macrophages was more evident at the mid through late stages of endometriosis-like lesion development. Therefore, these alternatively activated macrophages appear to be critical for lesion development during the later stage of the disease process. As the various markers are likely to indicate different functions of these cells, macrophages could play multiple roles in endometriosis development at different stages.

Additionally, collagen type 1 fibres were found to be present in the endometriosis-like lesions. Their abundance was increased as the lesion developed. Macrophages could assist in collagen deposition potentially through the expression of scavenger receptor. Similarly, collagen could facilitate in regulating macrophage migratory properties.

Chapter 5

5.1 Introduction

5.1.1 TGFB1 and macrophages

The studies described in Chapter 3 show that host-derived TGFB1 is critically important in supporting the growth of ectopic endometrium in a mouse model of endometriosis. Through analysis of specific markers of activation throughout lesion development, we demonstrate that macrophages are a key cell type altered in this model, exhibiting dynamic alterations in phenotype during lesion development (Chapter 4). TGFB1 is the key regulator of macrophage phenotype and function, and we wished to further explore the role of TGFB1 in endometriotic lesion development and specifically its action in modulating macrophage activity.

TGFB1 has stimulatory and inhibitory effects on macrophages and their migratory behaviour. Bansoni *et al*. [364] showed that TGFB1 inhibited CD11b expression in the human monocyte cell line (U937) and in primary human monocytes *in vitro* and decreased cell migration by 65% [364]. However, TGFB1 promoted migration of murine macrophages (RAW 264 cells) after short-term culture (1 hour) [177], with long term exposure (24 hours) to TGFB1 inhibiting migration. Therefore, TGFB1 appears to exert differential effects on macrophage migration in time- and species-dependent manner.

5.1.2 Macrophage phenotypes during cellular inflammation and repair

Emerging evidence suggests that macrophage populations can be classified based on their roles during inflammation. Activation markers are used to identify different populations of macrophages, known as “M1” and “M2” macrophages. The M1

macrophage population is typically present at the onset of inflammation and is capable of killing and phagocytosing microbes. M2 macrophages are potent inhibitors of inflammation and mediate tissue repair following the inflammatory response (see Figure 1 B; page 40).

In our study, pro-inflammatory M1 macrophages were identified using markers that reflect their function. MHC class II and inducible nitric oxide synthase (iNOS or NOS2) are two markers that are associated with the pro-inflammatory functions of murine macrophages [97, 200] and are negatively regulated by TGFB1. Increased expression of MHC class II was observed in the pulmonary venous endothelium and in the lymph nodes of *Tgfb1* null mutant mice [199]. Similarly, MHC class II expressing cells were reduced in the mammary glands of mice transplanted with TGFB1 deficient mammary epithelium [365]. Inflammation in *Tgfb1* null mice can be alleviated by disabling MHC class II expression, increasing the lifespan of these mice [200]. Monocytes and macrophages are the main immune cells expressing iNOS (reviewed in [291]) which catalyses the production of free radical nitric oxide (NO) [291]. Increased expression of iNOS occurs in peritoneal macrophages, mammary gland macrophages and in the glomerulus of kidney of *Tgfb1* null mutant compared to TGFB1 replete mice [292, 365, 366], and corresponds with increased serum levels of NO [366]. Combined, this suggests that iNOS and MHC class II expression, and the pro-inflammatory phenotype of macrophage is suppressed by TGFB1.

Macrophage-specific pro-tissue remodelling markers, arginase 1 and scavenger receptor A were used to localise M2 macrophage phenotypes with remodelling and tissue repair functions [97, 323]. TGFB1 inhibited expression of scavenger receptor A (*Msr1*) mRNA in a human macrophage cell line (THP-1) and in primary human

monocytes [367]. This suppression was mediated by downstream signalling SMAD2 and SMAD3 pathways [368]. Reduced expression of scavenger receptor is associated with the loss of the phagocytic ability of peritoneal macrophages which is required for tissue remodelling. However, TGFB1, SMAD2 and SMAD3 also promote tissue remodelling activities of macrophages by promoting arginase activity [369, 370]. Increased arginase 1 production in macrophages enhanced proliferation of human breast carcinoma cells (cell line ZR-75-1) in a co-culture system [371]. Arginase 1 also competes with iNOS for a common substrate, L-arginine, and thus suppresses inflammation though inhibition of nitric oxide. Although TGFB1 has been shown to regulate arginase 1 *in vitro*, little is known whether this action is translated to *in vivo* models.

TGFB1 may also promote tissue remodelling through the deposition of collagen. TGFB1 stimulated transcription of pro-collagen type 1 genes (*COL1A1* and *Col1a2*) in human and murine lung fibroblasts [372, 373], through SMAD3 signalling [374]. Collagen has been suggested to provide a framework for alternatively activated M2 macrophages in both normal development and in cancer [375].

5.1.3 Summary

Evidence in the literature suggests that TGFB1 favours the presence of M2-expressing macrophages *in vivo* and *in vitro*. Using the *Tgfb1*-knockout mouse model of endometriosis, we sought to delineate the effects of TGFB1 on regulation of specific macrophage phenotypes in day 10 of endometriosis-like lesions. This time point was chosen because the majority of important cellular functions, such as MHC class II-positive cell infiltration, collagen deposition and neoangiogenesis have been shown to take place between day 7 and day 14 post-tissue implantation in the nude mouse

model of endometriosis [146]. The following experiments extend our findings on macrophage involvement in TGFB1-mediated endometriotic lesion development.

5.2 Materials and methods

Materials and methods for this chapter are described in detail in Chapter 2. Briefly, eutopic endometrial fragments from pre-menopausal (n = 5) patients were injected subcutaneously into ovariectomised and estrogen supplemented *Tgfb1*-null mutant (*Tgfb1*-/-) mice (n = 5), wildtype littermates (*Tgfb1*+/+) (n = 9) and heterozygous littermates (*Tgfb1*+/-) (n = 7). Ten days following tissue injection, the lesions were dissected, weighed and frozen in OCT compound for histological analysis. To determine the effects of host TGFB1 deficiency on the macrophage functions of mouse model of endometriosis, only lesions from *Tgfb1*-/- mice and *Tgfb1*+/+ were further examined.

5.3 Results

5.3.1 Patient demographics

Endometrial tissues were obtained from five pre-menopausal patients, aged between 27 and 37 years (median age = 29 years) at the time of surgery. All patients had a normal 28-day cycle, with no hormonal medications for 3 months prior to tissue collection. Four patients had laparoscopy surgery, while one patient did not have her peritoneal cavity visualised (**Table 5.1**). Of the patients with a visualised pelvis, two did not have any visible endometriosis lesions and two had Stage 1 endometriosis.

5.3.2 Weight of endometriosis like lesions

At day 10 following subcutaneous injection of human endometrial fragments, endometriosis-like lesions were observed in 4 out 5 *Tgfb1*-/- mice, and in all 9 *Tgfb1*+/+ mice (p = 0.36, Fisher's exact test). Thus 80% of *Tgfb1*-/- mice and 100% of the *Tgfb1*+/+ control mice developed lesions. Each mouse received a single injection except for one *Tgfb1*-/- mouse that received two injections, thus 5 lesions were collected from *Tgfb1*-/- mice, and 9 lesions from *Tgfb1*+/+ mice.

The weight of the lesions harvested from *Tgfb1*-/- mice [median (range) = 0.0035 g (0.0013-0.0068g)] was 2.2-fold lighter than the lesions collected from *Tgfb1*+/+ mice [0.0078 g (0.0033-0.022 g)] (p = 0.0145, Mann Whitney U test; **Figure 5.1A**). This finding supports the observations described in Chapter 3. Furthermore, the combined median weight of lesions from *Tgfb1*-/- mice [median (range) = 0.0028 g (0.0013-0.016 g)] of the two experiments was significantly lower by 10-fold compared to those from *Tgfb1*+/+ mice [0.014 g (0.0013-0.12 g)] (**Figure 5.1B**). Lesions from *Tgfb1*-/- mice were also significantly lighter than lesions from heterozygous (*Tgfb1*+/-) mice [0.011 g (0.007-0.020 g)] by 73.8%.

5.3.3 Histology of the lesions

Routine haematoxylin and eosin staining of sections was performed to examine the morphology of the tissues. Endometriosis-like lesions were seen to be composed of glandular and stromal compartments, consistent with the origin of these tissues (**Figure 5.1C**).

5.3.4 Immunofluorescent staining of endometriosis-like lesions

5.3.4.1 Assessment of non-specific antibody binding

Although we have previously demonstrated antibody and species binding specificity (Chapter 4), tissues were tested from mice from different background mouse strain (*cfms-eGFP* C57Bl/6 *Prkdc*^{scid}) to *Tgfb1* null mice (mixed background of CF1/129/C3H/BalbC *Prkdc*^{scid}). Therefore we wished to confirm specificity of these antibodies for use in the *Tgfb1* null mouse background strain. Endometriosis-like sections were incubated with matched isotype control immunoglobulins (IgGs) at the same concentrations as the primary antibodies (**Figure 5.2**). Anti-MHC class II isotype matched IgG2aκ, did not show positive staining when applied to the endometriosis-like lesions (**Figure 5.2A, B, C**). Similarly, when the lesion section was incubated with the rabbit IgG in replacement of the anti-iNOS antibody, limited non-specific background was observed (**Figure 5.2D, E, F**) which was clearly less intense and easily distinguished from the positive signal observed with the anti-iNOS antibody. Unimmunised goat IgG was used as a negative control for arginase 1 antibody antigen binding, and non-specific binding was not observed in tissue sections (**Figure 5.2G, H, I**). No staining was observed when an isotype-matched control for anti-CD204 antibody, IgG2b, was applied to sections (**Figure 5.2J, K, L**). Lastly, the specificity of collagen type 1 antibody was tested with an unimmunised polyclonal rabbit IgG, weak background staining was detected which was not consistent with positive staining for collagen (**Figure 5.2M, N, O**).

5.3.4.2 Species specificity of antibodies

The specificity of each primary antibody was determined by incubation with human endometrial and mouse uterine sections. Anti-MHC class II antibody reacted with mouse (**Figure 5.3A**) but not human cells (**Figure 5.3B**). Although iNOS antibody was raised against antigen of human origin, it recognised both human and mouse cells (**Figure 5.3C** and **D**). Arginase 1 antibody bound strongly to mouse cells with limited reactivity to human cells (**Figure 5.3E** and **F**). An antibody against scavenger receptor CD204 only localised cells of murine origin and no signal was detected when applied onto human tissues (**Figure 5.3G** and **H**). Collagen fibres were identified using an antibody reactive to type 1 collagen and identified collagen fibres in human as well as murine tissues (**Figure 5.3I** and **J**).

5.3.4.3 F4/80+ macrophage population

Similar to previous observations, F4/80-positive cells were localised mainly in the periphery of lesions and were less abundant centrally. In this chapter, the total number of F4/80-positive macrophages was determined in both central and peripheral locations (**Figure 5.4**). The median number of F4/80-positive cells in the periphery and centre of lesions from *Tgfb1*-/- mice was reduced by 50% compared to lesions from *Tgfb1*+/+ mice [median (range) = 41.2 cells/field (33.8-47.2 cells/field) and 62 cells/field (47-85.5 cells/field), respectively] ($p = 0.001$, Mann Whitney U test; **Figure 5.4B**). When centrally located macrophages were analysed, F4/80-positive macrophages in lesions from *Tgfb1*-/- mice [median (range) = 34.6% (13.3-41.6%)] were 1.2-fold lower than macrophages in lesions from *Tgfb1*+/+ mice [median (range) = 41.9% (24.5-63.5%)] ($p = 0.021$, Mann Whitney U test; **Figure 5.4C**).

5.3.4.4 Inflammatory macrophage markers

Cells expressing MHC class II were more abundant in the periphery of lesions and were sparse in the centre of the endometriosis-like lesions (**Figure 5.5A**). The median number of MHC class II-positive cells in lesions from *Tgfb1*-/- mice was 4.1-fold lower than lesions from *Tgfb1*+/+ mice [median (range) = 7.8 cells/field (5.8-16 cells/field) and 32.3 cells/field (22.3-53.2 cells/field), respectively] ($p = 0.0005$, Mann Whitney U test) (**Figure 5.5B**).

Cells expressing iNOS were identified in the epithelium and stroma of endometriosis-like lesions from the *Tgfb1*+/+ and to a lesser extent in the *Tgfb1*-/- mice (**Figure 5.6A**). A significant 1.8-fold reduction in the abundance of iNOS-positive stromal cells was observed in lesions from *Tgfb1*-/- mice compared to those in *Tgfb1*+/+ mice [median (range) = 11.2 cells/field (8.5-19.5 cells/field) and 20.2 cells/field (14-25.7 cells/field), respectively] ($p = 0.068$, Mann Whitney U test) (**Figure 5.6B**).

5.3.4.5 Tissue repair macrophage markers

Macrophage markers which are normally associated with tissue repair were also analysed. No difference was observed between the median number of arginase 1-positive cells in lesions from *Tgfb1*-/- and *Tgfb1*+/+ mice [median (range) = 22.8 cells/field (19.2-28 cells/field) and 23.5 cells/field (13.8-34.2 cells/field), respectively] ($p>0.05$) (**Figure 5.7B**).

The majority of CD204-positive cells were localised in the periphery of the lesion with relatively lesser number centrally (**Figure 5.8A**). CD204-positive scavenging cells were significantly decreased by 52.7% in lesions from *Tgfb1*-/- mice compared to those from

Tgfb1+/+ mice [median (range) =24.2 cells/field (22.7-31 cells/field) and 51.2 cells/field (30.2-64.5 cells/field), respectively] (p = 0.02) (**Figure 5.8B**).

Collagen type 1 immunostaining was restricted to the periphery of lesions from *Tgfb1*-/- mice, while it was more abundant and centrally localised in lesions from *Tgfb1*+/+ mice (**Figure 5.9A**). The percent positive area for collagen type 1 was significantly decreased by 1.5-fold in lesions from *Tgfb1*-/- mice compared to lesions from *Tgfb1*+/+ mice [median (range) = 30.1% (28.5-43.3%) and 45.4% (41.8-56.3%), respectively] (p = 0.0034, Mann Whitney U test; **Figure 5.9B**).

Table 5. 1: Clinical data of the recruited patients for eutopic endometrial tissue collection

Experiment/Patient	Age at the time of surgery	Stage of cycle	Revised AFS score
1	37	Late Secretory	Pelvis not visualised
2	28	Early Secretory	No (S)
3	27	Proliferative	No (S)
4	29	Late secretory	Stage 1
5	29	Proliferative	Stage 1

No (S) = surgically proven no endometriosis

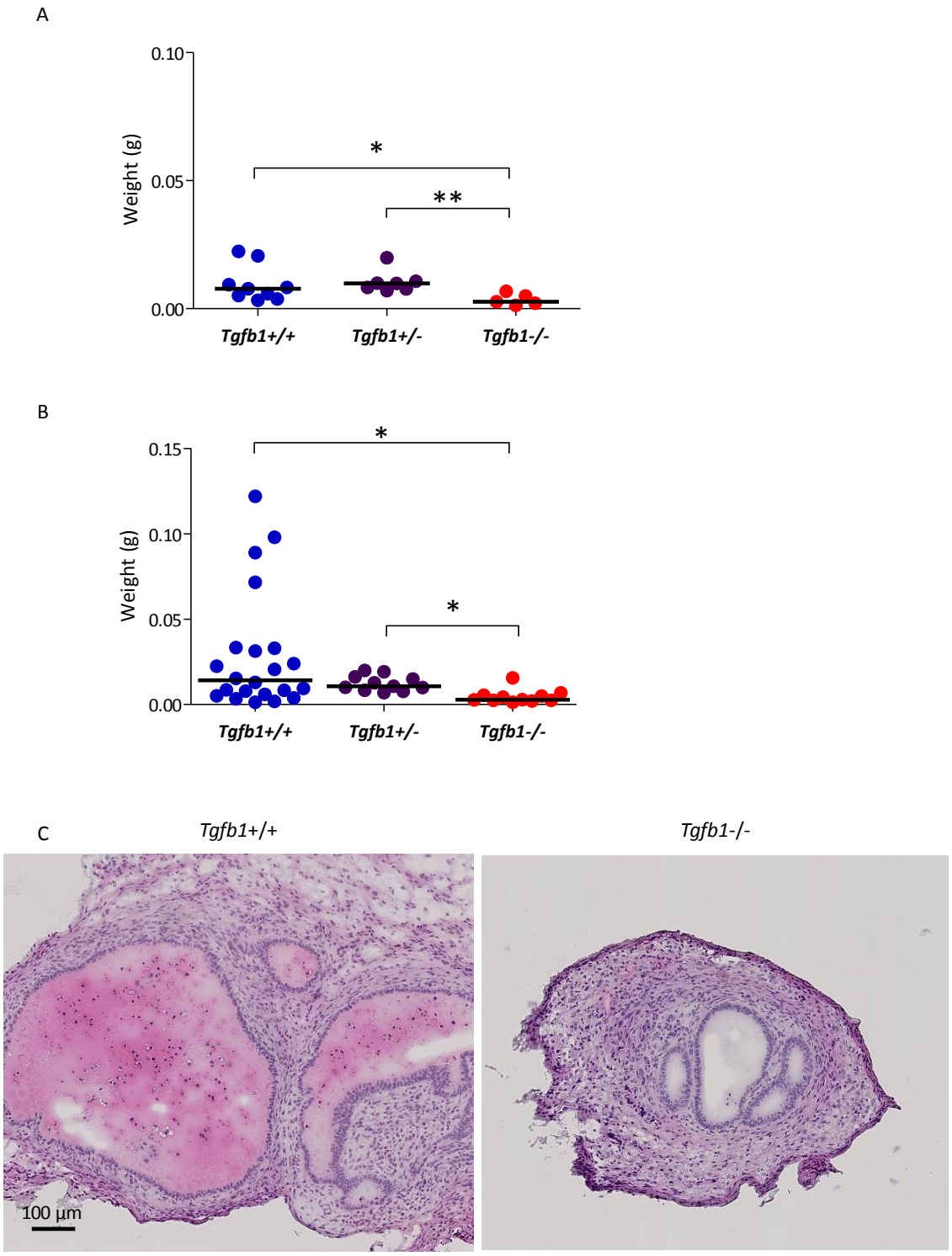


Figure 5. 1: Endometriosis-like lesions at day 10 from *Tgfb1*-null mutant mice (*Tgfb1*-/-) and wildtype control (*Tgfb1*+/+).

The combined weight of endometriosis-like lesion from two experiments (Chapter 3) (B). Morphology of the lesions stained using haematoxylin and eosin (C). Each symbol represents the weight of endometriosis like lesions per injection with horizontal bar denotes the median for each group. Data were analysed using Mann Whitney U test, with significance inferred at $p<0.05$ (denoted by *, ** $p<0.01$).

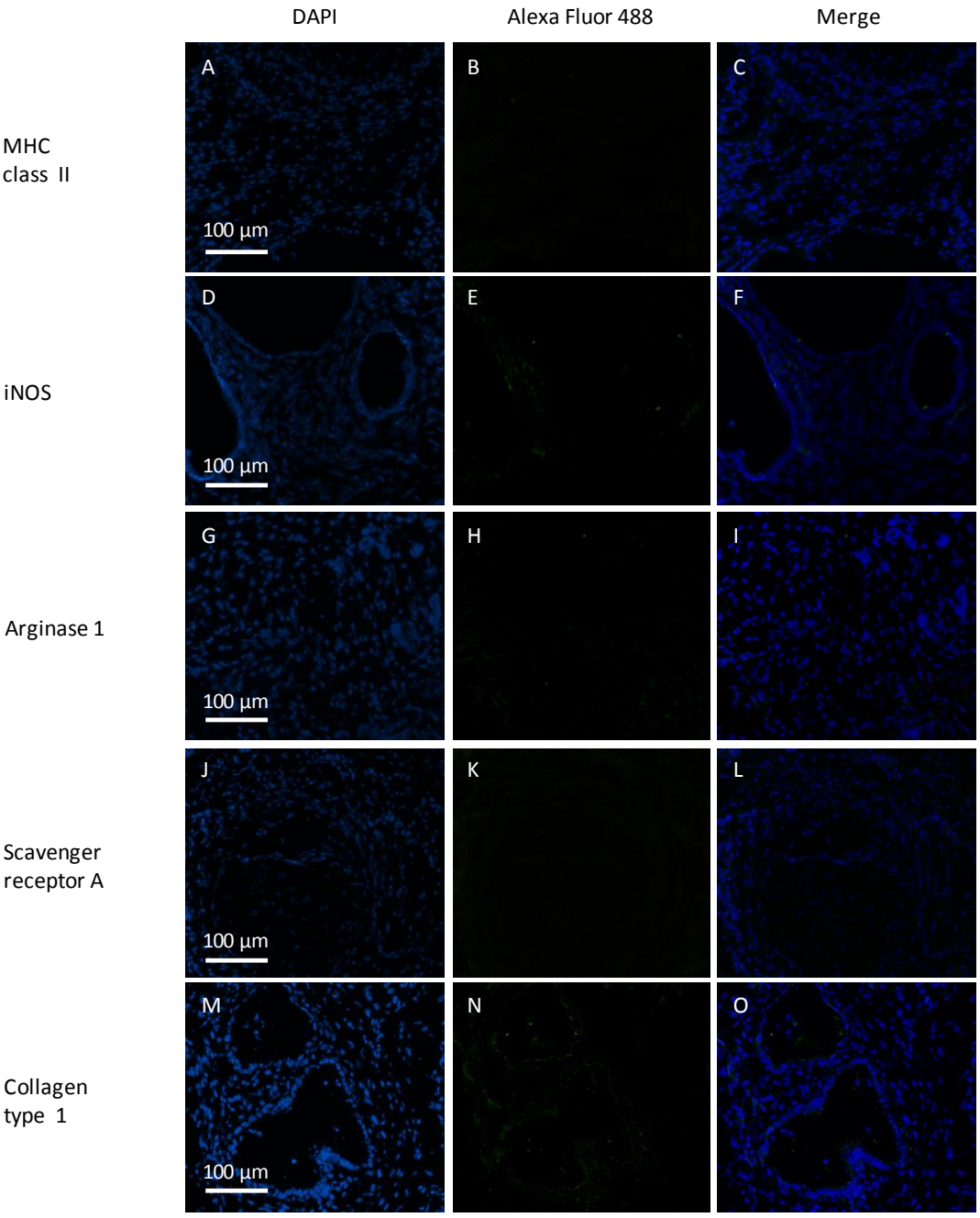


Figure 5. 2: Isotype-matched control for each immunostaining condition.

The specificity of each primary antibody was confirmed by substituting the primary antibody with an isotype-matched IgG. Endometriosis-like lesion sections were incubated with non-immunised rat IgG2aκ for MHC class II (A, B and C), rabbit IgG for iNOS (D, E and F) and collagen type 1 immunofluorescent staining (M, N and O), rat IgG2b for scavenger receptor (J, K and L) and goat IgG for arginase 1 (G, H and I). The immunofluorescent signals (green) developed were merged with the nuclear staining, DAPI (blue).

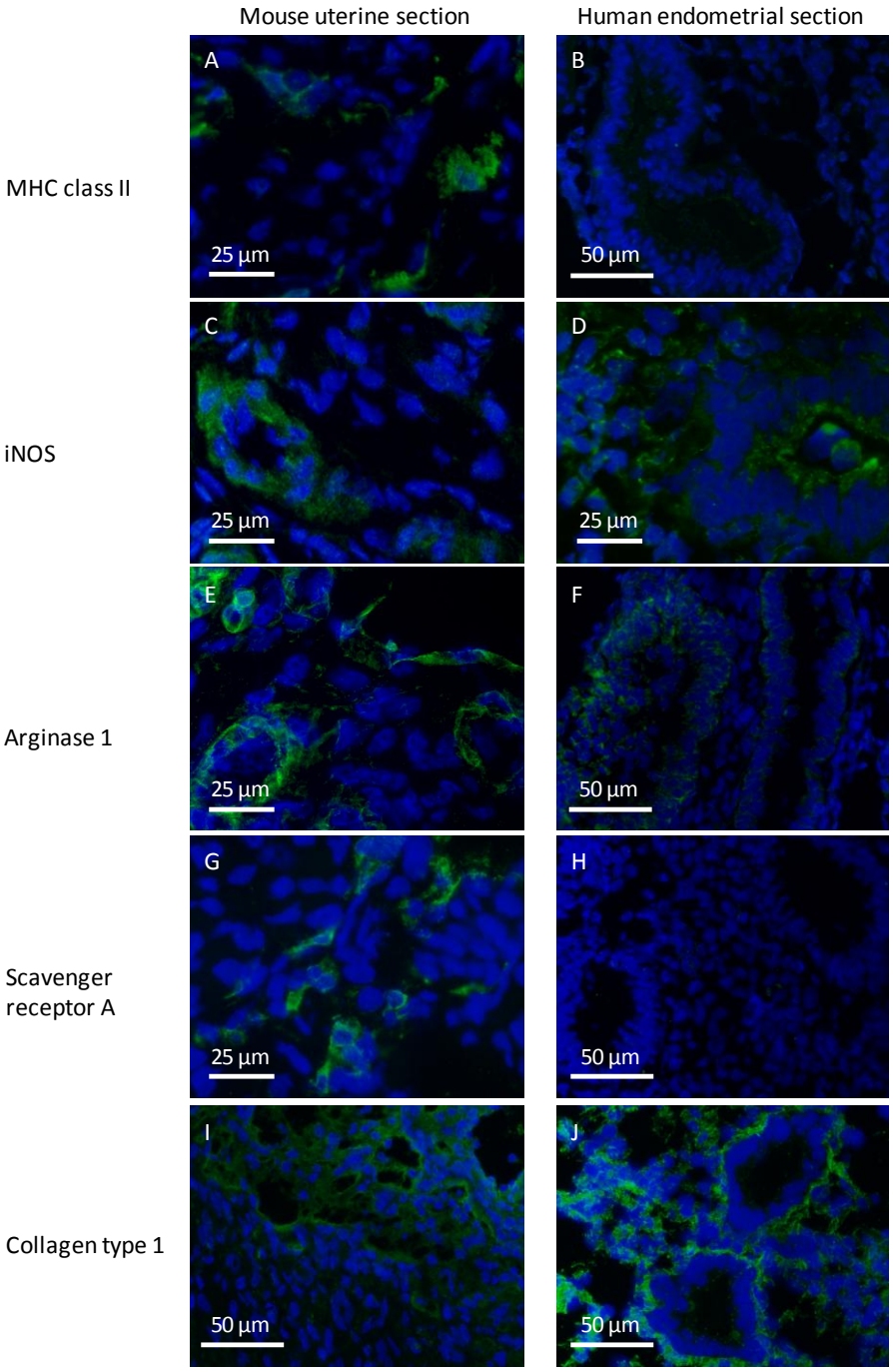


Figure 5. 3: Species specificity for each primary antibody as tested on murine and human tissues.

Sections of murine uterine (A, C, E, G and I) and human endometrial (B, D, F, H and J) were independently incubated with anti-MHC class II (A and B), anti-iNOS (C and D), anti-arginase 1 (E and F), anti-CD204 (scavenger receptor) (G and H) and anti-collagen type 1 (I and J).

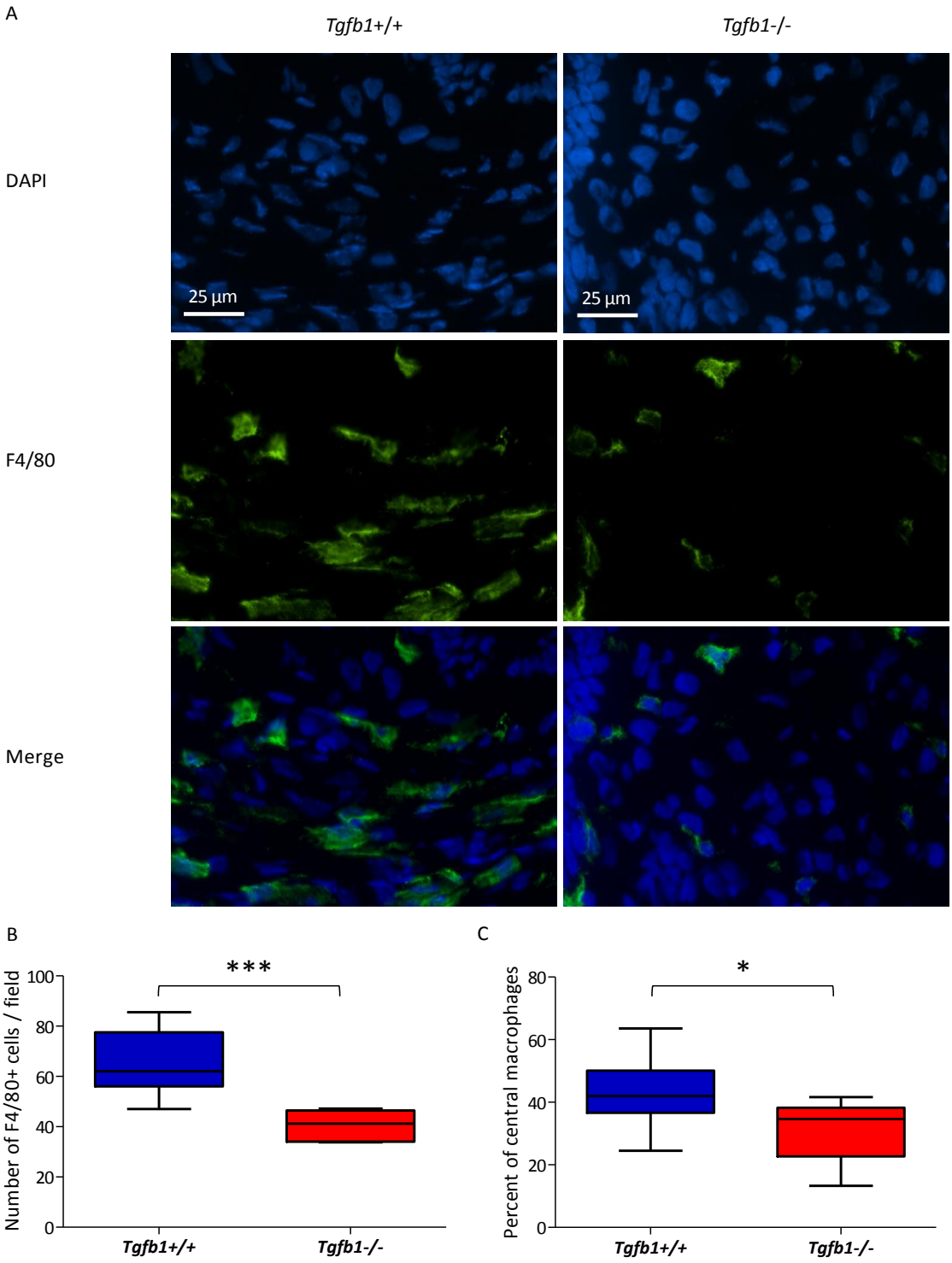


Figure 5. 4: Infiltration of macrophages (F4/80+ cells) into endometriosis-like lesions at day 10 from *Tgfb1*+/+ and *Tgfb*1-/- mice.

(A) Host macrophages (green) were present in the periphery and centre of the lesion stroma. F4/80 immunofluorescent images were merged with nuclear staining, DAPI (blue) to reveal individual cells. (B) Macrophage abundance was reduced in *Tgfb1*-/- lesions globally (*** $p<0.001$) and centrally (* $p<0.05$, Mann Whitney U test; C). Boxes represent the 25th to 75th percentile, with median line; error bars represent minimum and maximum values.

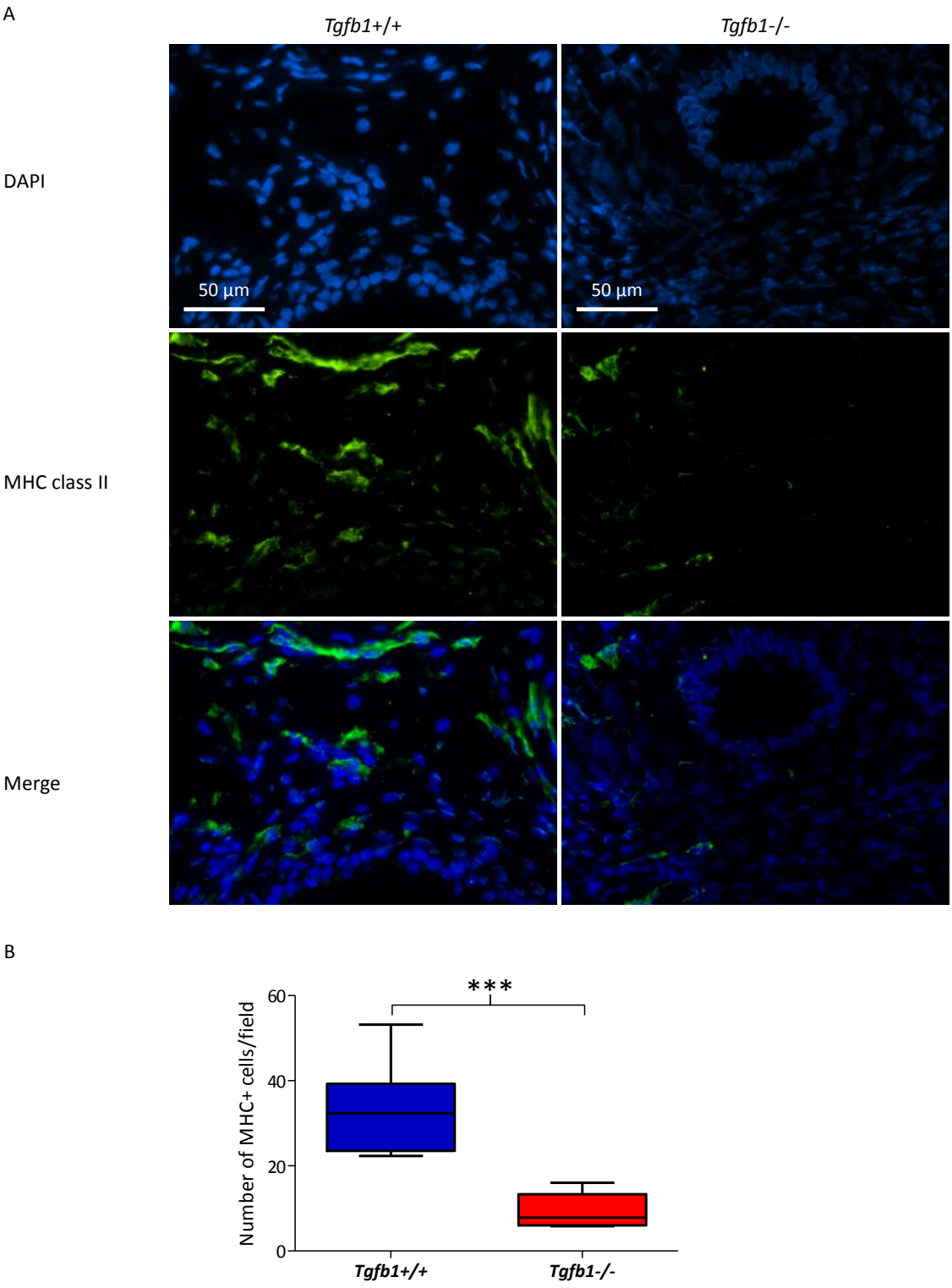


Figure 5. 5: Expression of MHC class II macrophages in endometriosis-like lesions at day 10 from *Tgfb1*+/+ and *Tgfb1*-/- mice.

(A) MHC class II-positive cells (green) were present in the periphery and centre of the lesion stroma. The nuclear staining, DAPI (blue) reveals tissue morphology. (B) The abundance of MHC class II-positive cells was reduced in lesions from *Tgfb1*-/- mice (*** $p<0.001$, Mann Whitney U test). Boxes represent the 25^{th} to 75^{th} percentile, with median line; error bars represent minimum and maximum values.

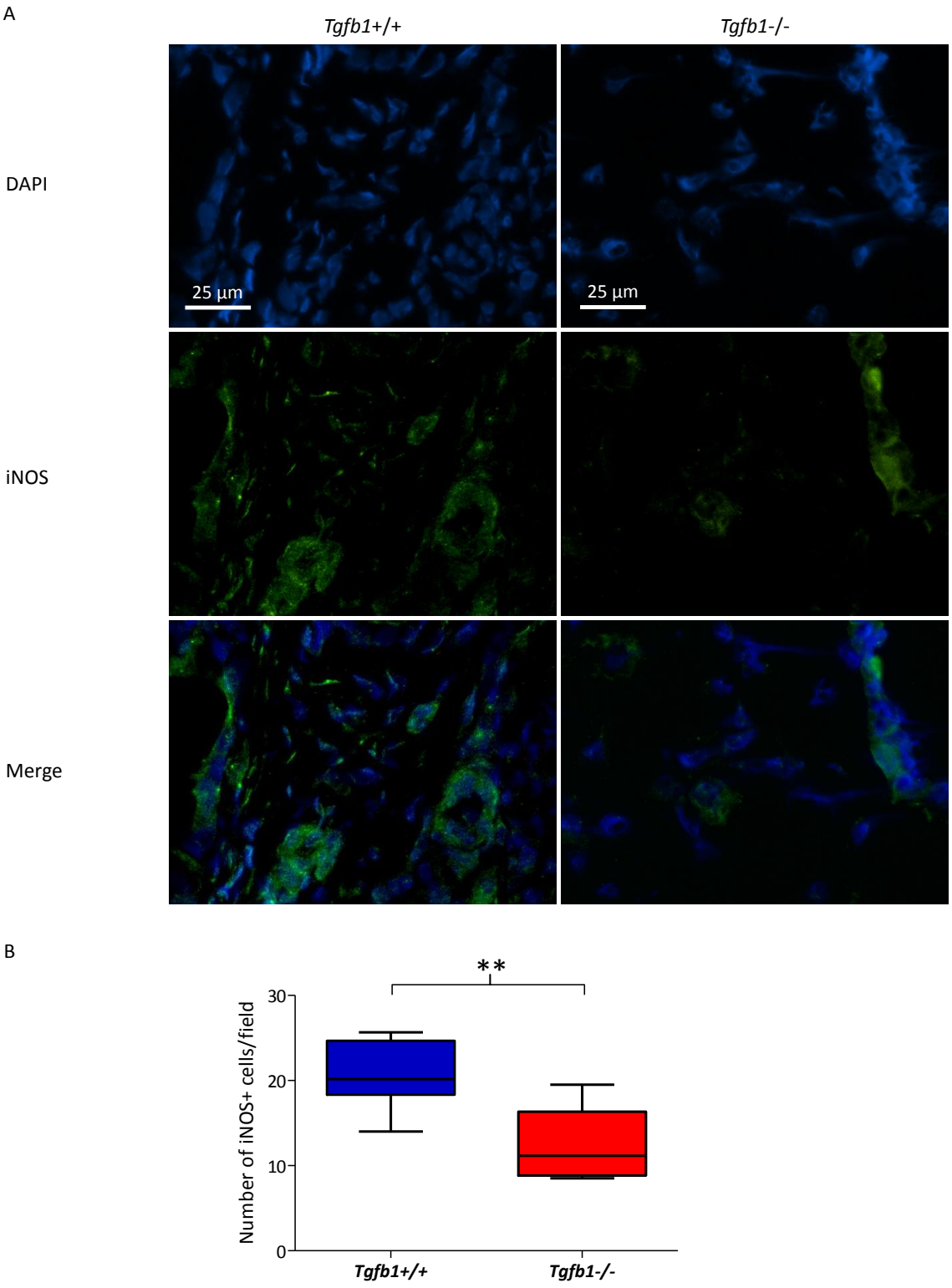


Figure 5. 6: Inducible nitric oxide synthase (iNOS)-positive cells in endometriosis-like lesions at day 10 from *Tgfb1*+/+ and *Tgfb1*-/- mice.

(A) iNOS-positive cells (green) were expressed by the epithelial and stromal cells. iNOS fluorescent images were merged with DAPI staining (blue) to reveal individual cells. (B) The number of iNOS-positive stromal cells was reduced in lesions from *Tgfb1*-/- mice (** $p<0.01$; Mann Whitney U test). Boxes represent the 25th to 75th percentile, with median line; error bars represent minimum and maximum values.

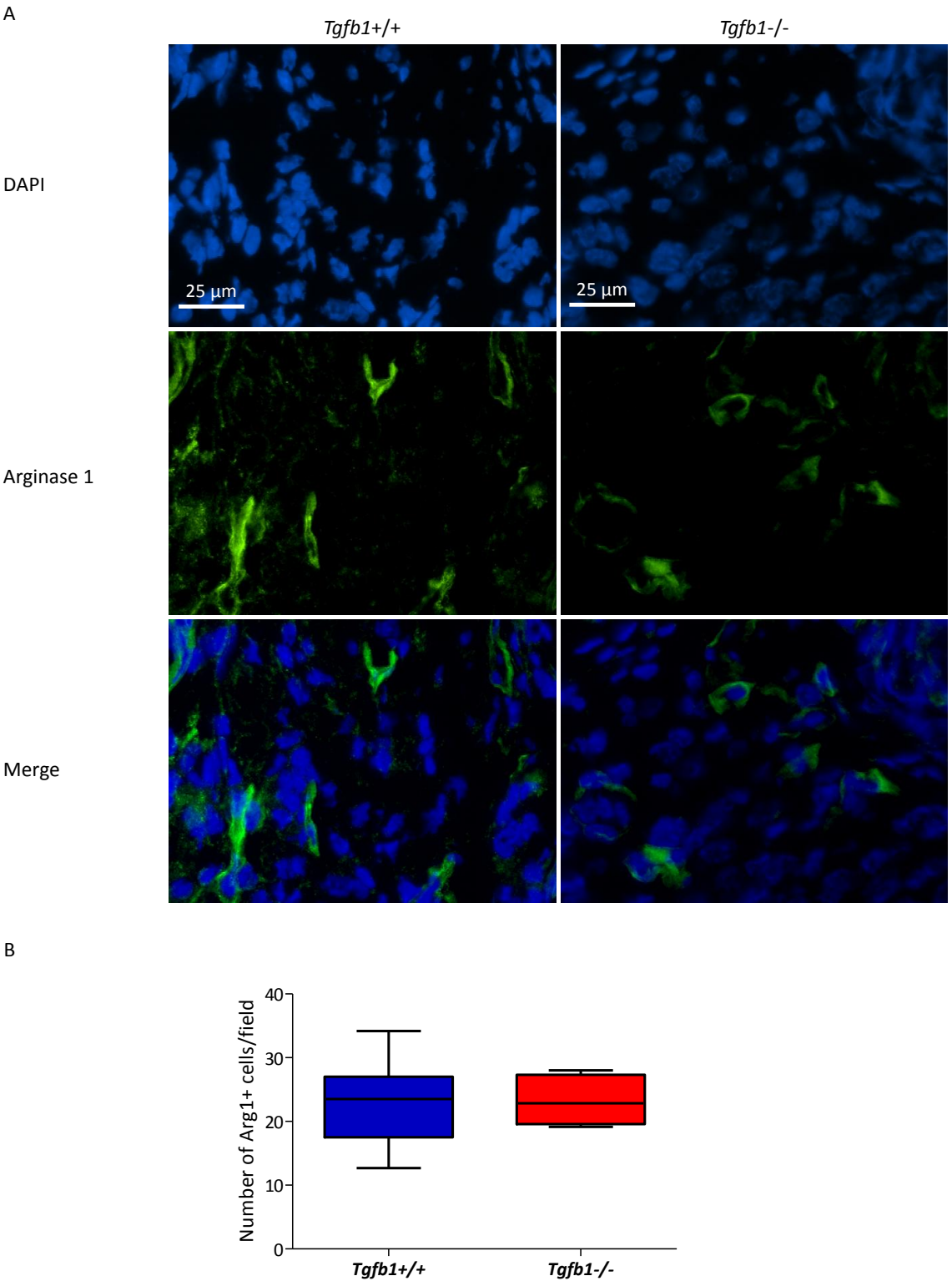


Figure 5. 7: Infiltration of arginase 1 (Arg1)-positive cells into endometriosis-like lesions at day 10 from *Tgfb1*+/+ and *Tgfb1*-/- mice.

(A) Arg1-positive cells (green) were present in the stroma of the lesion. Immunofluorescent images were merged with nuclear staining, DAPI (blue) to reveal individual cells. (B) The number of Arg1-positive cells in lesions was comparable between two groups. Boxes represent the 25th to 75th percentile, with median line; error bars represent minimum and maximum values.

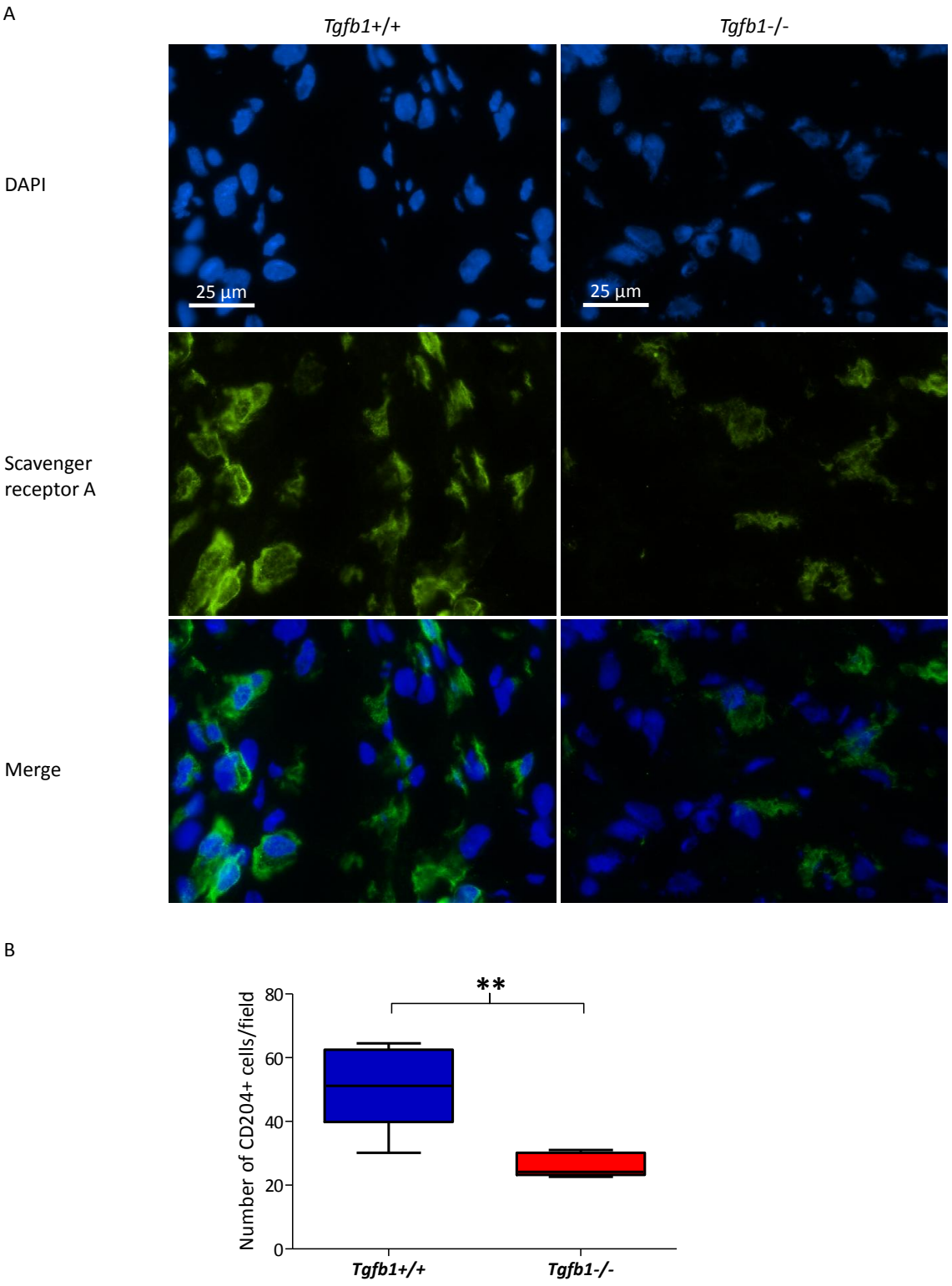


Figure 5. 8: Scavenger receptor A (CD204) expression in endometriosis-like lesions at day 10 from *Tgfb1*+/+ and *Tgfb1*-/- mice.

(A) CD204-positive cells (green) were present in the periphery and centre of the lesion stroma. Immunofluorescent images were merged with nuclear staining, DAPI (blue) to reveal individual cells. (B) The number of CD204-positive cells was lower in *Tgfb1*-/- lesions (** $p<0.01$, Mann Whitney U test) (C). Boxes represent the 25th to 75th percentile, with median line; error bars represent minimum and maximum values.

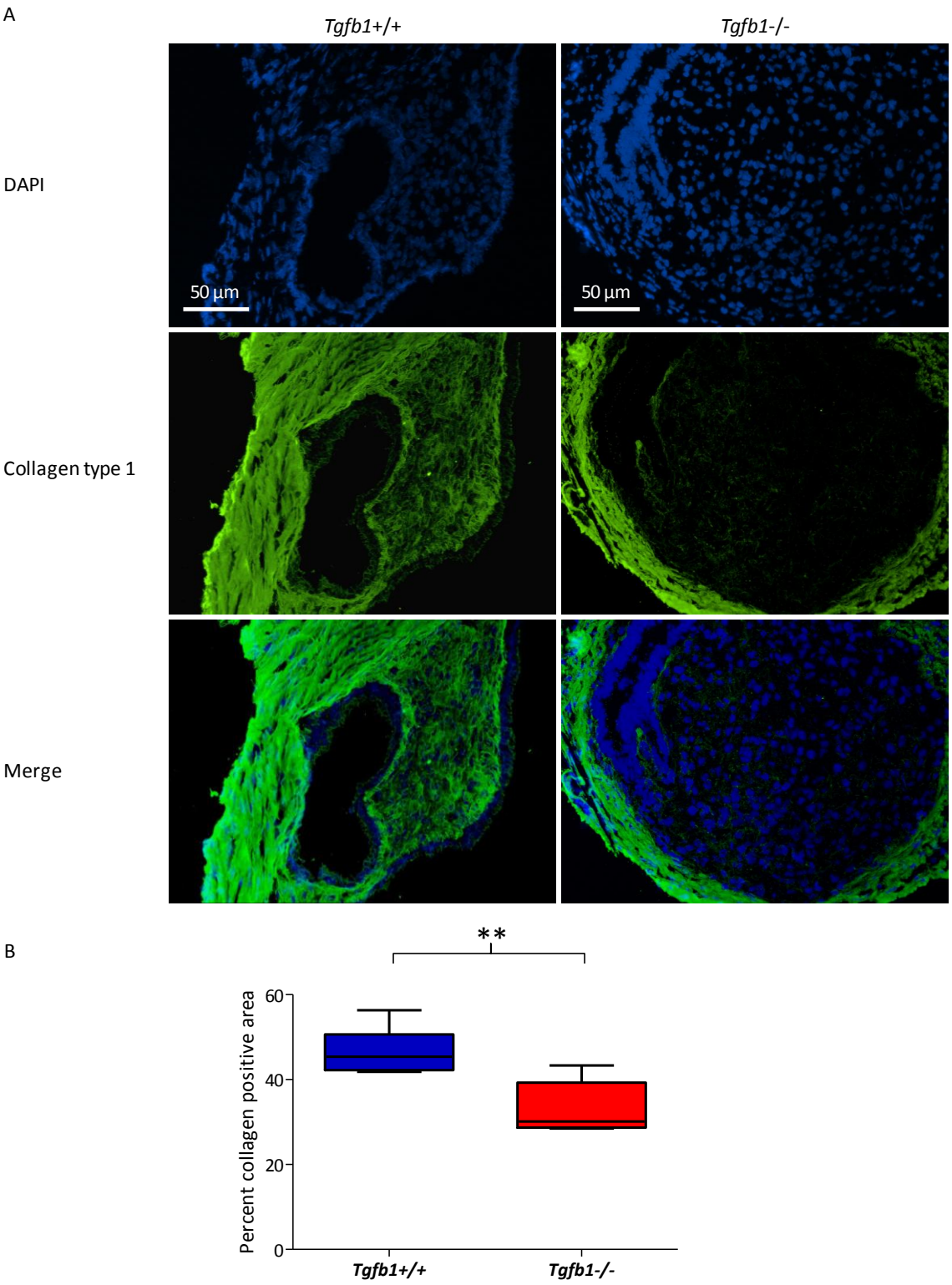


Figure 5. 9: Collagen type 1 deposition in endometriosis-like lesions.

(A) Collagen type 1 fibres (green) were present in the stroma of the lesions from *Tgfb1*+/+ and *Tgfb1*-/- mice. The immunofluorescent images were merged with nuclei staining, DAPI (blue) to reveal collagen-positive area. (B) Collagen positive area was reduced in lesions from *Tgb1*-/- mice (** $p<0.01$, Mann Whitney U test). Boxes represent the 25th to 75th percentile, with median line; error bars represent minimum and maximum values.

5.4 Discussion

In this Chapter, we have extended initial observations on the effect of host-derived TGFB1 on endometriotic lesion development to explore dynamic changes in macrophage phenotype affected by TGFB1. As the model of transplantation of human endometrium into TGFB1 deficient or replete host mouse was the same as that described in Chapter 3, the results on lesion development and weights can be combined for the two experiments. We have observed that comparable numbers of endometriosis-like lesions developed in *Tgfb1* null and replete mice, and the weight of the lesions was significantly lower when host mice were deficient in TGFB1. Therefore, our initial conclusions that tissue attachment is unaffected by TGFB1 deficiency, and that host derived TGFB1-deficiency suppresses lesion development, are confirmed in this larger combined cohort.

The combined data (Figure 5. 1B, p. 206) of experiments in Chapter 3 and the current study show that lesions from *Tgfb1*-null mice are consistently lighter than those from wildtype controls. There were some variations in the weight of the wildtype lesions, with four lesions in Chapter 3 were noticeably heavier than the rest. There are several reasons to account for the variations in lesion weight harvested from wildtype mice. The heterogeneity of endometrial tissues and mouse models could be a major source of difference. Since endometrial tissues were collected from different women at various stages of cycle and varying disease status, this factor might have a significant impact on the growth of these tissues ectopically. Pre-existing blood vessels and resistance towards induced apoptosis and immune cell clearance could also be the major influences on the growth of endometrial tissues ectopically.

Wildtype mice used in this study all had intact *Tgfb1* gene, however they differed individually. Some experimental mice could be physically larger than the others, which could have been influenced by their litter size and the maternal care they received. They could also be exposed to different elements, for example immunological challenges (prior to and/or during experiments) which were beyond the experimenter's control.

5.4.1 Macrophage infiltration was impaired in lesions from *Tgfb1*-null mutant mice

The total number of F4/80 positive macrophage was significantly reduced in lesions from *Tgfb1* null mice, which is consistent with our previous observation (Chapter 3). In Chapter 3, macrophages were counted only in tissue periphery. This result showed that TGFB1-deficiency reduced the trafficking of these cells into the lesions. In the current chapter (Chapter 5), the locations of macrophages were analysed peripherally and centrally to add a body of information on total macrophage number in lesions. Using the same immunofluorescent images and counting method, the percentage of central macrophage numbers were determined. As the percentage of macrophages located centrally was lower in lesions from TGFB1-deficient mice, it is now understood that TGFB1 did in fact affect the penetration of these cells into the tissues.

Moreover, the percentage of macrophages located centrally was also lower. Given that the lesions from *Tgfb1* null mice were smaller in mass, the recruitment and migration of macrophages is clearly critically impaired by TGFB1 deficiency. A similar role for TGFB1 has been found both *in vitro* and *in vivo*, TGFB1 induced migration of human peripheral monocytes in culture [376], and mediated monocyte migration in a rat model

of synovial inflammation [377]. However, TGFB1 exerts inhibitory actions on differentiated macrophages (reviewed by [378]), and we sought to explore macrophage phenotypes in endometriosis-like lesions affected by TGFB1 deficiency through the analysis of macrophage activation markers.

5.4.2 Inflammatory markers were altered in the lesions with host deficient TGFB1

Profound effects of TGFB1 deficiency on the abundance of MHC class II- and iNOS-positive cells in endometriosis-like lesions were observed. The significant decrease in M1 activation markers in lesions from *Tgfb1* null mice was surprising, previous studies have suggested TGFB1 acts to dampen pro-inflammatory responses as MHC class II-positive cells [199] and iNOS-positive cells are generally more abundant in *Tgfb1* null mice [202, 366]. Although other TGFB isoforms, TGFB2 and TGFB3 are present and active in these mice [199], TGFB1 deficiency alone resulted in reduced abundance of MHC class II and iNOS and thus the effect on M1 macrophage markers is restricted to the TGFB1 isoform.

The reduced abundance of MHC class II- and iNOS-positive cells in lesions from *Tgfb1* null mice could be the result of impaired recruitment of macrophages into the lesions in the absence of host TGFB1. TGFB1 and its downstream pathway molecules, such as SMAD3, have been previously linked to local immune cell infiltration. In a wound healing model, *Smad3*-null mutant mice healed quicker than heterozygous and wildtype controls, with wound closure occurring by day 2, rather than by day 3 [379]. This was associated with reduced monocyte infiltration and local inflammation in the *Smad3*-/- mice. Similarly in our model, TGFB1 deficiency resulted in reduced

macrophage abundance in the lesions which could account for reduced M1 macrophage abundance.

The abundance of MHC class II, a marker that mediates antigen presentation by macrophages, increases during the early stages of development of endometriosis-like lesions (Chapter 4). A similar finding was made using a baboon model of endometriosis where MHC class II protein HLA-DR was increased in the peritoneal fluid following intrapelvic injection of endometrial tissues [67]. The presence of ectopic endometrium appears to trigger recruitment of immune cells expressing MHC class II. These cells are likely to be highly pro-inflammatory, as demonstrated in a study of murine hepatoma xenograft tumors, where macrophages with high expression of MHC class II also expressed high levels of iNOS mRNA [334]. As the lesions from the wildtype TGFB1 were heavier and the cells expressing MHC class II and iNOS were more abundant, the inflammatory milieu at the early stage of endometriosis development may be critical for lesion development, and could account for reduced lesion development in the absence of host-derived TGFB1.

In addition to inflammation, iNOS production of nitric oxide (NO) has also been reported to be involved in ECM remodelling. Examples in trophoblast cell culture and *in vivo* cutaneous repair demonstrated that NO increased proteases, MMP-2 and MMP-9 which are important in ECM breakdown [380] and also re-epithelisation and keratinocyte proliferation [333]. Thus, the reduced number of iNOS-positive cells in the lesions from *Tgfb1* null host mice may explain the propensity for reduced lesion development.

5.4.3 Alternatively activated markers were differently regulated in lesions from *Tgfb1*-null mutant mice

Arginase 1 and scavenger receptor A are markers used to identify M2 alternatively activated macrophages. Arginase 1-positive cells have been described as promoting tissue remodelling in a wound healing study [381]. The absence of TGFB1 in host tissues did not alter the total number of cells expressing arginase 1. This finding suggests that there may be compensatory mechanisms to promote optimal arginase activity within the lesions, or that lesion development is delayed in *Tgfb1* null mice.

From our findings described in Chapter 4, day 7 was the peak time for arginase expression in the normalised TGFB1 environment. Thus, the tissue remodelling phase involving arginase 1-positive cells is expected to be resolved by day 10 in *Tgfb1* replete mice. A delay in tissue remodelling in *Tgfb1* null mice could account for the increased presence of arginase 1-positive macrophages at day 10. TGFB2 and 3 isoforms may also regulate arginase 1 in a compensatory manner; therefore the absence of type 1 TGFB may not significantly alter the expression of this activation marker. For instance, TGFB2 was shown to upregulate arginase 1 protein and mRNA expression in the primary human lung (small airway) epithelial cells [382]. Alternatively, increased arginase 1 expression in the absence of TGFB1 could be due to compensation by other cytokines. *In vitro* stimulation of primary murine (bone marrow-derived) macrophages using IL-4, IL-13 and IL-10 increased the arginase 1 surface marker [383]. Thus, there is a possibility that overexpression of these cytokines in the TGFB1-deficient environment promotes recruitment of arginase 1-positive cells.

However, the marked decrease in M1 macrophages, together with the clear decrease in absolute abundance of F4/80-positive macrophages, suggests that the ratio of M2 arginase 1-positive macrophages to M1 pro-inflammatory macrophages is increased, resulting in a microenvironment with reduced capacity for inflammatory activity but little impairment to tissue remodelling. Regardless of the mechanistic pathway that results in normal arginase 1-positive cells in lesions from *Tgfb1* null mice; this again suggests that pro-inflammatory pathways are a critical component of the host response for robust lesion development.

The number of scavenger receptor A-positive (CD204) macrophages was reduced in xenografts from *Tgfb1* null mice. This suggests that a TGFB1 deficiency either regulates the expression of CD204 on macrophages or reduces the CD204-positive macrophage infiltration into the lesions. In lung adenorcarcinoma [384] and pancreatic cancer [385] tissues, macrophage expression of CD204 was associated with increased tumor growth and this receptor is commonly expressed by tumor-associated macrophages [386, 387]. Scavenger receptor A-expressing macrophages phagocytose apoptotic cells, scavenge debris and moderate inflammation during tissue remodelling events (reviewed by [388]). When supernatants from co-culture of the ovarian cancer cell line, SKOV3 and CD204-expressing macrophages, were introduced to an endothelial cell line *in vitro*, the endothelial cell migration was increased by 4.1-fold compared to the control [386]. This demonstrates a pro-angiogenic property of scavenger receptor-positive macrophages, which might be important for tissue remodelling. *In vivo*, similar cellular behaviours may occur, as 66% of macrophages from ascites from 10 patients with ovarian cancer expressed CD204 [389]. The abundance of scavenger receptor-expressing macrophages was suggested by the authors to be involved in maintaining a microenvironment that

favours cancer tissue growth. Thus, in our study, reduced abundance of CD204-positive cells in lesions from *Tgfb1* null mice may suppress the development of lesions *in vivo*, presumably by impairing tissue regeneration.

Although our data suggest that host TGFB1 is required to promote abundant CD204-expressing cells in endometriosis-like lesions, others have reported that TGFB1 inhibits macrophage expression of CD204. Culture of both human peripheral monocytes and the monocytic cell line THP-1 with TGFB1 reduced *CD204* mRNA expression in a time-dependent manner [367]. However, THP-1 cells readily expressed CD204 after activation by phorbol 12-myristate 13-acetate (PMA, an agent to activate monocytes), and this was associated with increased secretion of TGFB1 [386]. Therefore a long term exposure to TGFB1 may downregulate the expression of CD204 *in vitro* as we have observed *in vivo*.

Chuang *et al*. [304] demonstrated that scavenger receptor class B, CD36-positive macrophages were reduced in the peritoneal cavity of women with endometriosis and the reduced expression of this receptor was linked to impaired phagocytic ability. CD36-positive cells have a similar function as scavenger receptor A, CD204-positive cells in the mouse model [342]. Therefore, in endometriosis pathogenesis, CD204 may share a similar function, which is phagocytosis of apoptotic cells during tissue remodelling.

5.4.4 Collagen type 1 expression was reduced in the lesions from *Tgfb1*-null mice

Different types of collagen have been identified in human endometrial tissues. Type I, III and IV collagen fibres were localised in both eutopic and ectopic tissues [390]. In the normal human endometrium, collagen type 1 is localised in the stroma and the intensity is dependent on menstrual cycle stage. The high estrogen environment of the proliferative phase is thought to induce proliferation of stromal cells resulting in increased collagen type 1 during the secretory phase [391]. *COL1A1* gene, which encodes the collagen type 1 monomer, was one the predicted targets of 22 differentially expressed miRNA between eutopic and ectopic endometrial biopsies of 8 patients [392] and was the most abundant transcript in endometriosis-like xenografts in a nude mouse model of endometriosis [146]. Therefore, this collagen type appears to be integral to the pathogenesis of endometriosis.

The xenograft area in *Tgfb1* null mice that stained positive for collagen 1 was reduced by 1.5-fold compared to xenografts from TGFB1 replete mice and limited collagen was observed in the central regions of these tissues (**Figure 5.9A**). Therefore, host TGFB1 appears to play a central role in promoting collagen type 1 deposition in the endometriosis-like lesions. Macrophages could also regulate stromal cell synthesis of collagen either via TGFB1 or production of other factors that promote collagen deposition. Selective CD11b-positive macrophage depletion via diphtheria toxin in a transgenic mouse model reduced Masson's Trichrome stained collagen area by approximately 50% and 31% at day 7 and day 10, respectively following an induced wound [112]. Similarly, in a myocardial injury study in mice by van Amerongen *et al*. [113],

macrophage depletion by the liposome clodronate reduced collagen type 1 by 3.9-fold in injured cardiac tissues at day 4. In the van Amerongen's study, the decrease in collagen type 1 infiltration was associated with a decrease in TGFB1-secreting macrophages as shown by the co-localisation of immunofluorescent signals. Therefore collagen deposition could be mediated by macrophages via secretion of TGFB1.

TGFB1 of macrophage origin may also have a key role in collagen secretion from stromal fibroblasts in endometriosis-like lesions. Although there is lack of evidence in endometriotic lesions to prove this observation, data obtained from other tissues nevertheless showed macrophages are partly responsible for collagen deposition. Mice with a deletion in the colony stimulating factor-1 gene ($Csf1^{op}/Csf1^{op}$) exhibit a severe reduction in tissue macrophages [124], associated with reduced fibrillar type 1 collagen in pubertal mammary tissue [375]. In this study, fibrillar collagen was detected by the resonant emission of polarised light (second harmonic generation signal). Monomeric collagen, detected by immunofluorescence was not altered in *Csf1* null mice, suggesting that macrophages did not promote collagen synthesis but rather the organisation and deposition of the fibrillar collagen structure. LPS-activated macrophages were also able to induce type 1 collagen expression by pancreatic stellate cells at both the mRNA and protein levels *in vitro* [393].

In addition to a reduction in TGFB1-driven macrophage infiltration, the decrease of collagen type 1 immunostaining could be associated with the lower numbers of myofibroblasts in the endometriosis-like lesions. Results from Chapter 3 demonstrated that α smooth muscle actin (αSMA)-expressing myofibroblasts were reduced in the absence of host TGFB1 by 47%. One of the reported extracellular matrix components produced by myofibroblasts to assist the tissue remodelling process is collagen type 1

[394]. Therefore, a reduced abundance of myofibroblasts is likely to lead to reduced type 1 collagen production.

Compensatory mechanisms that act to maintain extracellular matrix homeostasis also exist, and can become perturbed in the absence of TGFB1 or normal myofibroblasts activity. *αSMA* null mice exhibited excessive collagen secretion [395], with the level of pro-collagen type 1 mRNA being 2.2-fold higher in *αSMA* null kidney cells grown *in vitro* compared to kidney cells from wildtype control mice. Penile tissue from *Tgfb1* null mice also exhibited excessive type 1 collagen, could also be due to other pathology such as erectile dysfunction [396]. In our study, human endometrial grafts are likely to secrete TGFB1, perhaps preventing potential compensatory mechanisms from being switched on in the TGFB1-deficient environment.

5.4.5 Limitations of this study

There are some limitations of this study in addition to those described in Chapter 3. Firstly not all cells expressing the macrophage markers investigated here are macrophages, as was demonstrated by dual labelling studies in Chapter 4. In addition to macrophages, MHC class II is also expressed by dendritic cells, iNOS expression is shared with epithelial cells and arginase 1 antigen is also expressed by fibroblasts, among other cells. However, there were clear differences in the abundance of cells expressing these markers, which is likely to have a functional consequence for the development of endometriosis-like lesions.

Secondly, F4/80 antibody used to identify macrophages is not expressed by all macrophages. Macrophages express different markers throughout their development and activation in tissues [397]. F4/80 is not always expressed by myeloid phagocytic cells

most notably blood monocytes, progenitor cells and some macrophage populations in lymphoid tissues, lung and skin (reviewed by [179])[398]. Although a substantial volume of published literature has utilised F4/80 as a single marker for macrophages, it should be recognised that in endometriosis-like lesions some of the macrophages may be positive for the examined marker but negative for F4/80.

Thirdly, the composition of the lesions was markedly different in *Tgfb1* null mice, with reduced glandular fraction and reduced αSMA-positive myofibroblast abundance compared to lesions grafted to TGFB1 replete mice. In this study, we are unable to distinguish the cause from the effect, as the altered composition could differentially affect macrophage abundance and phenotype.

5.5 Conclusions

Macrophages appear to play critical roles during the tissue remodelling stage of endometriosis-like lesion development and their abundance within the lesion during development is regulated by TGFB1. A null mutation in the *Tgfb1* gene in host mice resulted in reduced abundance of inflammatory cells expressing MHC class II and iNOS, and could be the result of reduced macrophage infiltration. Although the abundance of arginase 1-positive cells were not affected in lesions from *Tgfb1* null mice, reduced lesion weight may suggest that arginase 1 expression was delayed in the absence of host TGFB1. Reduced abundance of CD204-positive cells in lesions from *Tgfb1* null mice may indicate that these cells are important in moderating inflammation by scavenging debri and apoptotic cells for a normal lesion development at day 10. Reduced collagen type 1 in the absence of host-derived TGFB1 may partially account

for the reduced lesion and may be a consequence of reduced macrophages or myofibroblasts within the lesion.

The specific phenotypes of macrophages active in endometriosis-like lesions, and the reduction of these cells in the absence of TGFB1, suggest the inflammatory process during the establishment phase of endometriotic lesions is critical for the development of the pathology. Suppression of TGFB1 and the inflammatory process may offer new targets for therapeutic strategies to reduce the development of endometriosis.

Chapter 6

6.1 Introduction

Despite advances in technology and improved knowledge about endometriosis development, this debilitating disease remains a problem for women of reproductive age. Lack of suitable models and the heterogeneity of the disease hinder research and development of new therapeutic interventions. Data from murine models suggest that endometriosis development, which involves the interaction between the endometrial tissues and the host peritoneum, occurs via a series of events involving initially inflammation and tissue breakdown then healing and repair.

Immune cell infiltration into endometriotic lesions is critical to normal ectopic endometrial tissue establishment. Macrophages and neutrophils are elevated in the peritoneal cavity of women with endometriosis [399, 400]. Higher concentrations of extracellular matrix (ECM)-related proteins (indicative of tissue remodelling), such as MMP-2, MMP-3 and urokinase-type plasminogen activator (uPA) are identified in the peritoneal fluid and lesions of women with endometriosis [65, 401]. These deviations from the normal peritoneal environmental suggests that inflammation and tissue repair events are important in human disease establishment. If these cellular events could be disrupted, the growth of endometriosis lesion could be presumably suppressed, reducing the disease severity. Thus, it is important to define the key cellular pathways and molecular events that are involved in the initial establishment of the endometriosis lesions.

6.1.1 TGFB1 is critical for a normal tissue development

TGFB1 is emerging as a critical modulator of the peritoneal-endometriotic lesion interaction [146]. This pleiotropic cytokine was elevated in the peritoneal fluid of women with endometriosis [166]. The experiments reported in this thesis provide evidence that host-derived TGFB1 mediates important events during endometriosis development, and that TGFB1 acts through regulation of macrophages, myofibroblasts and collagen. As the likelihood of lesion development from transplanted fragments was not influenced by TGFB1 availability but instead lesions size was diminished, this cytokine is likely to exert its main effects after the early phases of lesion attachment and establishment.

6.1.2 TGFB1-regulated modelling of endometriosis-like lesions

We observed a reduction in the weight of lesions which developed in hosts deficient in TGFB1 compared to the lesions from hosts with normal TGFB1 level. Further analysis found that parameters including the glandular area, macrophage density and myofibroblast staining were significantly affected in the TGFB1-deficient lesions. Therefore, by suppressing TGFB1 in the ectopic lesion vicinity, glandular development and macrophage and myofibroblasts activity could be interrupted. As these events were identifiably important for lesion growth, their disruption could suppress lesion growth.

6.1.2.1 Glandular area

In the literature, there are several studies which show that endometrial xenografts in immunodeficient mice had a gradual increase glandular area [146, 316] over time. Similarly, we observed an incremental increase in the proportion of lesion area

occupied by the glands in the MacGreen/SCID mouse model throughout lesion growth. The absence of host TGFB1 diminished the glandular volume, as demonstrated by the smaller glands and lumen, and a 32% reduction in the total glandular fraction in lesions from *Tgfb1*-/- mice. The decrease in glandular area is physiologically significant since the epithelium is the source of important cytokines and chemokines implicated in normal development of endometrial tissues at ectopic sites. In addition, TGFB1 immunostaining is localised to the epithelium of endometriosis-like lesions (Figure 4.11).

6.1.2.2 Macrophage recruitment

In addition to the structural morphology of the tissues, TGFB1 is likely to regulate immune cell activity in the endometriosis-like lesions. Macrophage density within 100 µm from the periphery of a lesion was reduced by 66% in the absence of host TGFB1. Furthermore, in a TGFB1-deficient environment there was a1.2-fold reduction in the percentage of macrophages in the central part of the lesions (Chapter 5). As reported in Chapter 4, macrophages expressed different activation markers throughout disease progression in a mouse model, despite the number of macrophages (CSF-1R-eGFP) being comparable between the four time points of endometriosis-like lesion development. It appears that macrophages are important to the development of ectopic endometrial tissues in the mouse model and the function of macrophages changes over the time course of lesion establishment and progression.

This observation is important as human ectopic endometrial tissue is generally collected from patients at only one time point. The variability of transcript and protein expression seen in most human endometriotic tissues may represent lesions at

differing stages of tissue breakdown and repair. It is not possible to study macrophage progression in human disease for ethical reasons since multiple surgeries to obtain lesions at different time points are not feasible. Our observations in the mouse model imply that macrophages are likely to be constantly present during development of the human endometriotic lesions, and they may have multiple and fluctuating functions at different stages of disease progression.

6.1.2.3 Myofibroblast activity

αSMA staining in lesions from *Tgfb1*-/- mice was reduced by 47% compared to lesions from *Tgfb1*+/+ mice. TGFB1 is known to upregulate the expression of αSMA *in vitro* [131], and this is consistent with the observations in our *in vivo* mouse model. As myofibroblasts secrete higher levels of collagens and other molecules, they are likely to have a significant role in laying down ECM during the tissue remodelling events required for normal lesion growth. van Kaam *et al*. [145] demonstrated that myofibroblasts were present in 17 deeply infiltrating endometriotic lesions. We observed a 1.5-fold fold reduction in the collagen type 1-positive area when endometrial xenografts were grown in the TGFB1-deficient environment (Chapter 5). TGFB1 induced the secretion of collagen type 1 by fibroblasts, which could be one of the important events in TGFB1-mediated lesion growth. Blocking collagen deposition in the lesions may decrease lesion burden, thus reducing the disease severity.

6.2 Macrophages have different phenotypes

6.2.1 Inflammatory macrophage markers

Macrophages expressing MHC class II and iNOS were preferentially abundant in the early stages of endometriosis-like lesion development. The expression of inflammatory

markers early in the development of endometriosis suggests that macrophages are induced in the initial inflammatory process in lesions and that this inflammatory phenotype resolves by day 7 to 10. The downregulation of these macrophage inflammatory markers by day 7 to 10 may facilitate tissue remodelling and reduce oxidative stress-mediated cell death due to nitric oxide release from inflammatory cells in the lesions. In addition, the initial recruitment of inflammatory macrophages into the tissues might be important in the normal progression of macrophage function, since the number of alternatively activated macrophages were reduced when inflammatory macrophages were depleted [306]. The presence of inflammatory cells was thought to have provided the stimulus for the alternative activation of macrophages. In the absence of TGFB1, the proportion of inflammatory cells expressing MHC class II was reduced, and this could cause a delayed expression of arginase 1 (Chapter 5).

6.2.2 Tissue repair

As the lesion developed MHC class II- and iNOS-expressing macrophages was superseded by arginase 1- and scavenger receptor-expressing macrophages later in lesion development. This population of macrophages favours tissue remodelling [305, 335], and it our mouse model took place from day 7 until day 14 of lesion development. Arginase 1 is a specific marker for alternative activation in murine macrophages [301], whereas human macrophage surface markers, such as mannose receptor and scavenger receptor would need to be utilised to assess alternative activated macrophages in human tissues.

In human disease pathology, the peritoneal environment may play an important role in stimulating alternative activation of macrophages. Bacci *et al*. [82] demonstrated a

greater infiltration of M2 macrophages, expressing haemoglobin scavenger receptor CD163 and mannose receptor CD206, into endometriotic lesions than into peritoneal tissues from disease-free women. The abundance of tissue repair macrophages in endometriosis suggests an important supporting role in lesion growth and remodelling. The stimuli that initiate the transformation of inflammatory macrophages to tissue repair phenotypes in these lesions remain unknown. On the basis of studies in other tissue systems, apoptotic cells, iron and hypoxia contribute to the phenotype changes seen in macrophages [323, 350, 351]. Further studies will be required to investigate the precise role of these factors in regulating macrophage phenotype in endometriosis lesion.

6.3 TGFB1-deficiency alters macrophage phenotypes

Compared to the lesions from *Tgfb1* replete mice, a lower percentage of MHC class II and scavenger receptor A (CD204)-positive cells were present in endometriosis-like tissues from *Tgfb1-/-* mice (Chapter 5). In Chapter 4, we identified arginase 1-positive macrophages as more abundant at day 7, thus the expression of arginase 1 at day 10 in lesions from *Tgfb1-/-* mice may represent delayed lesion development associated with a delayed transition of macrophage phenotype, or it may be that a TGFB1 deficiency suppresses inflammation and promotes a remodelling phenotype at all time points.

6.4 Collagen type 1

In addition to being an extracellular matrix component, collagen type 1 also has an important role in modulating macrophage functions. In inflammatory tissues, the interaction between collagen type 1 and macrophages is important for phagocytosis [402]. Human monocytes that encounter collagen type 1 *in vitro* had increased Fc

receptor expression, which mediated the engulfment of opsonised bacteria (*Escherichia coli* and *Staphylococcus aureus*). The adhesion of human blood monocytes to collagen type 1 was shown to be specifically mediated by CD11c and CD18 receptors (also known as $\alpha_X\beta_2$ integrins, gp150-95) [403]. Additionally, as described in Chapter 4, collagen type 1 may provide a binding site for macrophage scavenger receptor to bind and remain at the sites of tissue healing [362]. Collagen type 1 and macrophages may have reciprocal regulatory roles as macrophages can also promote collagen fibre reorganisation in a developing mammary gland [375]. Excessive collagen in endometriosis is associated with the fibrotic changes which are commonly described in this disease.

Macrophages also regulate collagen deposition and infiltration. In a peritoneal fibrosis model, macrophage depletion with clodronate-containing liposome reduced collagen type 1 protein and mRNA (*Col1a1*) levels when compared to controls with intact macrophage function [404]. Similarly, CD11b-positive macrophage depletion using the diphtheria toxin reduced collagen type 1 mRNA by approximately 50% in a renal fibrosis mouse model [405]. It thus appears that macrophages are directly involved in the production of collagen type 1 and this may also occur in endometriosis.

In our study, collagen type 1 at day 14 was increased by 1.7-fold compared to day 4. The increased secreted collagen type 1 was associated with the abundance of an alternatively activated macrophage population associated with tissue healing (at day 7 and day 14). The collagen type 1 deposition was reduced when xenografts were grown in a TGFB1 deficient environment, suggesting the involvement of TGFB1-mediated signalling in collagen production.

Since TGFB1 is identified as a key determinant of collagen type 1 deposition in endometriosis-like lesions, this raises the possibility of manipulating the collagen content by targeting this cytokine. If proven effective, this action could eventually suppress the normal development of the lesion. Since there is lack of understanding of the role of collagen type 1 in the pathophysiology of endometriotic lesions, more studies need to be done to determine if this protein is crucial for normal growth of these tissues. Similarly, further investigations would be needed to assess how to specifically target endometriosis tissue or whether there would be off-target effects of suppressing collagen type 1 since it is the most abundant collagen fibre in the human body.

6.5 Future directions

The reduced weight of endometriosis-like lesions in the absence of host TGFB1 suggests that targeting host TGFB1 may be an effective way of interrupting lesion growth. Since TGFB1 plays a wide range of important roles such as providing protection against auto-immunity and tissue healing, TGFB1 needs to be specifically targeted in endometriosis tissue and perhaps only moderately suppressed, in order to prevent adverse effects in other tissues. Pharmaceutical agents directed towards molecules in downstream TGFB1 regulatory pathways, such as SMAD proteins may have fewer undesirable side effects in comparison to directly targeting TGFB1 proteins. In addition, microRNA (miRNA) that specifically suppresses TGFB1 mRNA could be an effective intervention to reduce TGFB1 bioavailability and to reduce lesion size. *Tgfb1* mRNA contains 5'- and 3'- untranslated regions, in which regulatory proteins can bind to repress translation (reviewed by [406]). miR663 and miR744 target 3'UTRs of Tgfb1 mRNA with miR744 has higher ability to suppress TGFB1 synthesis than miR663 [407].

Targeting TGFB1 signalling could also be achieved through SMAD7, TGFB Type II receptor (TGFBR2) and latency-associated peptide (LAP). SMAD7 inhibits the phosphorylation of SMAD2 and SMAD3, which is critical for TGFB signalling [155]. Blocking TGFBR2, a receptor binding for TGFB1 could also antagonise TGFB1 activity [154, 252] in endometriosis tissues. An overexpression of LAP in a transgenic mouse model was shown to suppress the bioactive TGFB1 by blocking this protein and neutralises its effects *in vivo* [408]. Multiple opportunities are available to block the action of TGFB1 in endometriotic tissues, however further research is required to confirm their efficacy.

We observed different macrophage phenotypes over the progression of endometriosis-like lesion development in a mouse model. Previously, only a few studies have examined the different phenotypes of macrophages in peritoneal fluid, serum or lesions from patients with endometriotic disease. To extrapolate the observations in the current mouse study to human tissues, the phenotype of macrophages in different activation states needs to first be established. Comparisons of macrophage markers in ectopic versus paired eutopic and healthy controls, using flow cytometry and microarray will provide more information about the regulation of immune cells in the pathogenesis of endometriosis.

6.5.1 Potential treatments for endometriosis

Given that inflammatory macrophages expressing MHC class II and iNOS were more abundant at the early time points of lesion development, it seems reasonable that suppression of this macrophage population may interrupt the subsequent events which lead to the growth of endometriosis-like lesions. A member of the PPARγ agonist family of inflammatory inhibitors, rosiglitazone, was shown to reduce the size

of ectopic endometrium from baboon and rat models [409, 410]. This agonist suppressed the progression of ectopic endometrial tissues by reducing inflammation, proving inflammation is one of the important events in endometriotic lesion formation. Since PPARγ agonists are known to enhance differentiation of human monocytes to M2 macrophages *in vitro* [95], the use of this inhibitor to treat endometriosis may warrant further research.

Thus, by using information on how macrophages and TGFB1 may regulate endometriosis-like lesion development, we have identified two possible strategies to suppress the lesion growth. The first possibility is to target TGFB1 in the host tissues, since the absence of host TGFB1 in mice clearly can reduce the lesion burden. As xenograft-derived TGFB1 was not altered, this reduction was not dependent on the TGFB1 levels of endometrial xenograft, but only the host-derived TGFB1. By suppressing this cytokine, collagen content and macrophage number might be subsequently reduced. Secondly, since the alternatively activated macrophage population mediates the tissue remodelling events, targeting this population could be an effective way to reduce development of the lesion. These therapeutic targets may be beneficial for women, and are likely to have less undesirable side effects than treatments targeting steroid hormone production which can create hypoestrogenism.

6.6 Summary

This thesis has led to development of new mouse models to study endometriosis *in vivo*. Additionally, macrophages and myofibroblasts have been shown to be an integral component of endometriosis-like lesion development. We also identified TGFB1 as a critical growth factor for lesion growth and a potential therapeutic target. In the

future, this information could improve the health of women with endometriosis disease.

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Appendix

Statement of Authorship

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Author Contributions

By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

Name of Principal Author (Candidate)	Mohammad Zahied Johan
Contribution to the Paper	Performed all experiments, collected and analysed data, interpreted data and contributed to manuscript.
Signature	Date 14/11/13

Name of Co-Author	M. Louise Hull (co-first author)
Contribution to the Paper	Organised tissue collection, supervised development of work, helped in data interpretation, wrote manuscript, manuscript evaluation and acted as corresponding author.
Signature	Date 13/11/12.

Name of Co-Author	Sarah A. Robertson
Contribution to the Paper	Supervised development of work and helped to evaluate and edit the manuscript.
Signature	Date 15/11/13

Name of Co-Author	Wendy L. Hodge
Contribution to the Paper	Provided assistance in human endometrial tissue collection
Signature	Date 13/11/13

Statement of Authorship

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Contribution to the Paper	Supervised in data interpretation and helped to evaluate and edit the manuscript.		
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