# The role of macrophages in early pregnancy success

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# Abstract

Macrophages are abundant in the ovary and uterus, with their densities and distribution changing throughout the estrous cycle in response to the sex steroid hormones, estrogen ($E_2$) and progesterone ($P_4$). In the uterus, macrophages are present in the endometrium, where they are located in close spatial proximity with uterine epithelial cells, and the myometrium. In the endometrium, macrophages are thought to have key roles in tissue remodelling in preparation for embryo implantation and trophoblast invasion. In the ovary, macrophages are present in the thecal cell layer where they surround developing follicles, and are also present in functional corpora lutea. They are thought to have a role in ovulation and in the extensive remodelling that occurs during formation of the corpus luteum. Other studies have suggested that macrophages enhance the output of $P_4$ from luteal cells. Their accumulation in the corpus luteum during luteolysis, as well as in atretic follicles, is suggestive of a role in tissue remodelling and removal of cellular debris.

The specific role of macrophages during the pre- and peri-implantation period remains to be fully elucidated. Previous studies in mice lacking important macrophage-supporting cytokines suggest the role of these cells in endometrial receptivity and ovarian function may not be essential. However, interpretation of the importance of macrophages using these models is made difficult due to changes in macrophage phenotype, or incomplete macrophage depletion. The studies conducted herein make use of the *Cd11b-Dtr* transgenic mouse. The expression of the simian diphtheria toxin (DT) receptor (R) on CD11B-expressing macrophages enables transient and systemic depletion of these cells. The murine form of the DTR binds DT poorly and as such, injection of DT to wild-type mice has no effect. Immunohistochemistry and flow cytometry showed that using this model an 85% depletion of uterine macrophages and a 90% depletion of ovarian macrophages is achieved 24 h following DT injection to *Cd11b-Dtr* mice at estrus.

Macrophage depletion in *Cd11b-Dtr* mice during the pre- and peri-implantation period caused complete pregnancy failure with loss during the peri-implantation phase. A direct adverse impact of DT on developing embryos was excluded as a contributing factor. Macrophage depletion did not affect uterine epithelial cell proliferation that occurs in response to $E_2$. Stromal cell proliferation that occurs in response to $E_2$ and $P_4$ in early pregnancy, prior to differentiation into decidual cells, also occurs normally following DT-elicited macrophage depletion.

Macrophage depletion caused decreased expression of glycosylated structures involved in embryo attachment to uterine epithelial cells. There was a 25% reduction in the intensity of the lectin UEA-1 immunostaining (detects fucosylated structures on the uterine epithelium), and a 67% reduction in LewisX immunostaining. However, the process of artificially induced decidualisation was not impacted by macrophage depletion.

Investigation into the impact of macrophage depletion on ovarian function showed that corpus luteum function was compromised, as evidenced by a 77% reduction in circulating plasma $P_4$ levels on day 4.5 post coitum (pc). Importantly, pregnancy could be rescued by the exogenous injection of $P_4$ and could be sustained into late gestation when $P_4$ support was maintained. The impact of macrophage depletion of the expression of mRNAs encoding enzymes responsible for the synthesis of $P_4$ was investigated using qRT-PCR. There was no change in mRNA expression of *Star* or *Cyp11a1* following DT-elicited macrophage depletion, but there was a significant 29% decrease in *Hsd3b1* mRNA expression. *Hsd3b1* is the gene that encodes the protein HSD3B1, responsible for the conversion of pregnenolone to $P_4$.

Following macrophage depletion, ovaries from *Cd11b-Dtr* mice were hemorrhagic at autopsy, and histology of ovarian tissue revealed evidence of impaired structural integrity of the corpora lutea. Staining for endothelial cell specific markers showed that endothelial cells were also depleted from some corpora lutea; the extent of endothelial cell depletion varied between corpora lutea within the same ovary. The lymphatic vasculature surrounding corpora lutea appeared not to be affected by macrophage depletion.

In early pregnancy (day 1.5 and 2.5 pc), during the formation of the corpus luteum, the impact of macrophage depletion on genes involved in endothelial cell angiogenesis and survival was investigated. There was more than a 3-fold increase in the mRNA expression of *Vegfa*, but a significant decrease in the mRNA expression of *Vegfc, Vegfd*, *Flt-1* and *Kdr* at both of these time-points following macrophage depletion.

This study shows that macrophages are essential for the establishment of early pregnancy. In the endometrium, macrophages appear to influence uterine epithelial cell remodelling to increase the expression of markers of receptivity, and may facilitate the process of implantation. In the ovary, macrophages act to regulate the structure and function of the corpus luteum, potentially through

regulating the expression of VEGFs and their receptors. These studies reveal new functions for macrophages in reproductive events and indicate that macrophage involvement in ovarian angiogenesis and support of steroidogenesis warrants further investigation.

# Declaration

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# Publications arising from this thesis

1. Jasper MJ, Care AS, Sullivan B, Ingman WV, Aplin JD, Robertson SA. *Macrophage-derived LIF and IL1B regulate α(1,2)fucosyltransferase (Fut2) expression in mouse uterine epithelial cells during early pregnancy.* Biology of Reproduction, 2011 84(1); 179-88. Epub 2010 Sep 23.
2. Care AS, Jasper MJ, Ingman WV, Robertson SA. (In preparation). *Macrophages are essential for corpus luteum progesterone synthesis and establishing early pregnancy in mice.*
3. Care AS, Ingman WV, Jasper MJ, Robertson SA. (In preparation). *Macrophages are not required for the uterine proliferative response to steroid hormones or deciduoma formation in mice.*

# Abstracts arising from this thesis

**2011**

Care AS, Jasper MJ, Ingman WV, Robertson SA. *Macrophage regulation of corpus luteum angiogenesis in early pregnancy*. Oral presentation at the Society for Reproductive Biology, Cairns, Australia. October 2011.

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Care AS, Jasper MJ, Ingman WV, Robertson SA. *An essential role for macrophages in corpus luteum function and early pregnancy maintenance.* Poster presentation at the International Congress for Reproductive Immunology, Cairns, Australia. August 2010.

Care AS, Jasper MJ, Ingman WV, Robertson SA. *Macrophages are essential for maintenance of corpus luteum function in the mouse.* Oral presentation at the Society for the Study of Reproduction, Milwaukee, USA. August 2010

Care AS, Jasper MJ, Ingman WV, Robertson SA. *Macrophages regulate corpus luteum function and are essential for maintenance of early pregnancy.* Frontiers in Reproduction Symposium, Woods Hole, MA, USA. June 2010.

**2009**

Care AS, Jasper MJ, Ingman WV, Robertson SA. *Why the immune system counts during pregnancy.* Oral presentation at the Young Investigator Award finals, Royal Institution of Australia, Adelaide, Australia. October 2009.

Care AS, Jasper MJ, Ingman WV, Robertson SA. *Macrophages are essential for maintenance of early pregnancy through regulation of corpus luteum function.* Oral presentation at the Young Investigator Award semi-finals, Women's and Children's Hospital, Adelaide, Australia. September 2009.

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Care AS, Jasper MJ, Ingman WV, Robertson SA. *The role of macrophages in regulating uterine epithelial and stromal cell proliferation*. Poster presentation at the Society for Reproductive Biology, Melbourne, Australia. August 2008.

Robertson SA, Sharkey DJ, Bromfield JJ, Moldenhauer LM, Jasper MJ, LR Guerin, Care AS, JD Hayball. *Seminal plasma and male factor signalling for female tolerance of pregnancy*. Oral presentation at the Society for Reproductive Biology, Melbourne, Australia. August 2008.

Care AS, Ingman WV, Jasper MJ, Robertson SA. *Endometrial macrophage function: A novel mouse model.* Oral presentation at the University of Adelaide Postgraduate Research Expo. July 2008.

Care AS, Ingman WV, Jasper MJ, Robertson SA. *Assessment of endometrial macrophage function in early pregnancy: A novel mouse model.* Poster presentation at the Network in Genes and Environment in Development Forum, Palm Cove, Australia. June 2008.

Care AS, Ingman WV, Jasper MJ, Robertson SA. *A novel mouse model to assess endometrial macrophage function in early pregnancy*. Oral presentation at the Australian Society for Medical Research, Adelaide, Australia. June 2008.

**2007**

Care AS, Ingman WV, Jasper MJ, Robertson SA. *A novel mouse model for endometrial macrophage evaluation in early pregnancy.* Oral presentation at the Society for Reproductive Biology, New Zealand. September 2007.

Ingman WV, Care AS, Robertson SA, JW Pollard. *An acute macrophage depletion model reveals a role for macrophages in regulation of testicular steroidogenesis in vivo.* Oral presentation at the Society for Reproductive Biology, Christchurch, New Zealand. September 2007.

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# Abbreviations

| | |
|---|---|
| ADAMTS1 | a disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 1 |
| ATP | adenosine triphosphate |
| bFGF | basic fibroblastic growth factor |
| BM | bone marrow |
| bp | base pair |
| BrdU | bromodeoxyuridine |
| BSA | bovine serum albumin |
| CCL | chemokine (C-C) motif |
| *Cd11b-Dtr* | Tg(ITGAM-DTR/EGFP)34Lan, transgene insertion 34, Richard A Lang |
| CL | corpus luteum |
| COX-2 | prostaglandin-endoperoxide synthase-2 |
| CSF | colony-stimulating factor |
| Ct | cycle threshold |
| CXC | chemokine (C-X-C) motif |
| CYP11A1 | cytochrome P450, family 11, subfamily a, polypeptide 1 |
| CYP17A1 | cytochrome P450, family 17, subfamily a, polypeptide 1 |
| DAB | 3,3 diaminobenzadine |
| DAMPs | damage-associated molecular patterns |
| DAPI | 4',6-Diamidino-2-phenylindole dihydrochloride |
| DC | dendritic cell |
| DT | diphtheria toxin |
| DTR | diphtheria toxin receptor |
| $E_2$ | estrogen; estradiol; 17β-estradiol |
| eCG | equine chorionic gonadotropin |
| ECM | extracellular matrix |
| EGF | epidermal growth factor |
| EP | ectopic pregnancy |
| ER | estrogen receptor |
| FACS | fluorescence-activated cell sorting |

| | |
|---|---|
| FIGF | c-fos induced growth factor |
| FLT1 | FMS-like tyrosine kinase 1 |
| FSH | follicle-stimulating hormone |
| FUT | fucosyltransferase |
| GnRH | gonadotropin-releasing hormone |
| GROA | growth-related oncogene-alpha |
| hCG | human chorionic gonadotropin |
| HGF | hepatocyte growth factor |
| hMG | human menopausal gonadotropin |
| HMGB1 | high-mobility group box 1 |
| HPG | hypothalamic-pituitary-gonadal |
| HRP | horseradish peroxidase |
| HSD3B1 | hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1 |
| HSP | heat shock protein |
| i.p. | intraperitoneal |
| i.v. | intravenous |
| IFN | interferon |
| IGF | insulin-like growth factor |
| IL | interleukin |
| IL1R t1 | interleukin-1 receptor type-1 |
| IUGR | intrauterine growth restriction |
| IVF | *in vitro* fertilisation |
| KDR | kinase insert domain protein receptor |
| LH | luteinising hormone |
| LHR | luteinising hormone receptor |
| LIF | leukaemia inhibitory factor |
| LPS | lipopolysaccharide |
| LTP | *Lotus tetragonolobus purpureas* |
| LYVE1 | lymphatic vessel endothelial hyaluronan receptor-1 |
| mAb | monoclonal antibody |
| MCM | macrophage-conditioned media |
| MD | macrophage depleted |
| MFI | mean fluorescence intensity |

| | |
|---|---|
| MHC | major histocompatibility complex |
| MMP | matrix metalloproteinases |
| MPS | mononuclear phagocyte system |
| MT-MMP | membrane-type-1-MMP |
| MUC | mucin |
| NK | natural killer |
| NMS | normal mouse serum |
| O/N | overnight |
| op | osteopetrotic |
| $P_4$ | progesterone |
| PA | plasminogen activator |
| PAF | platelet activating factor |
| PAMPs | pathogen-associated molecular patterns |
| pc | post-coitum |
| PDGF | platelet-derived growth factor |
| PE | pre-eclampsia |
| PEC | peritoneal exudate cell |
| PECAM1 | platelet/endothelial cell adhesion molecule 1 |
| PG | prostaglandin |
| PIGF | progesterone-induced blocking factor |
| PMN | polymorphonuclear leukocyte |
| PR | progesterone receptor |
| PRL | prolactin |
| PRLR | prolactin receptor |
| qRT-RCR | quantitative real-time polymerase chain reaction |
| RBC | red blood cell |
| RT | reverse transcription |
| RT-PCR | real-time polymerase chain reaction |
| s.c. | subcutaneous |
| SEM | standard error of mean |
| SP | seminal plasma |
| STAR | steroidogenic acute regulatory protein |
| SV- | seminal vesicle deficient |

| | |
|---|---|
| TGF | transforming growth factor |
| TIMP | tissue inhibitors of matrix metalloproteinases |
| TLR | toll-like receptor |
| TNF | tumour necrosis factor |
| TUNEL | Terminal deoxynucleotidyl transferase dUTP nick end labeling |
| UEA-1 | *Ulex europaeus* |
| VEGF | vascular endothelial growth factor |

# Chapter 1

# Review of literature

## 1.1 INTRODUCTION

Macrophages are ubiquitous cells with roles in both innate and acquired immunity. However, these cells have roles extending far beyond host immunity. Macrophages play a homeostatic role within the body, achieved by their ability to secrete a diverse array of cytokines and growth factors with autocrine and paracrine actions. This enables them to participate in the resolution of inflammation, tissue remodelling and development, and the phagocytic removal of apoptotic cells and debris.

Macrophages are present in male and female reproductive tissues. This review will focus on macrophages within female reproductive tissues and their known or proposed contribution to reproductive function. Within the ovary, macrophage numbers fluctuate according to the estrous or menstrual cycle. They are also present in the cycling uterus, with their numbers increasing following insemination and during the early stages of pregnancy. In the ovary, there have been a number of studies implicating macrophages or their secretory products in the processes of follicular growth and atresia, ovulation, corpus luteum development/function and luteolysis. Macrophages in the oviduct are thought to regulate epithelial secretions which are important for embryo-maternal signalling that occurs prior to implantation. Macrophages are also proposed to assist with uterine cyclic remodelling, and in pregnancy they may participate in embryo attachment to endometrial luminal epithelium, decidualisation, and trophoblast invasion into the uterine stroma.

Many processes in ovarian development and blastocyst invasion require rapid and extensive angiogenesis. Ovarian lymphatic vasculature may also have important roles in follicular development and progesterone ($P_4$) transfer into the blood vasculature. These processes are discussed and the potential for macrophage involvement is reviewed.

## 1.2 THE IMMUNOLOGY OF PREGNANCY

The understanding of the immunology of pregnancy has come a long way since Medwar's hypothesis, which asserted the fetus was able to escape maternal immune surveillance due to a passive, suppressed, or indolent maternal immune system throughout the duration of pregnancy (Robertson, 2010, Mor and Abrahams, 2006). The presence of macrophages at the implantation site, and the high numbers of immune cells including T lymphocytes reactive with paternal MHC (major histocompatibility complex) and other antigens present at the fetal-maternal interface, is evidence that the maternal immune system is very aware of, and indeed responds to, the presence of the semi-allograft fetus. This

process begins at insemination in response to antigens and inflammatory mediators present in seminal fluid (Mor and Abrahams, 2006, Robertson, 2010).

Pregnancy has been viewed as a Th2-dominated condition, where anti-inflammatory Th2 cytokines predominate at the fetal-maternal interface, and pro-inflammatory Th1 cytokines such as interleukin 2 (IL2), tumour necrosis factor alpha (TNFA) and interferon gamma (IFNG) are suppressed; tight regulation of the balance between pro- and anti-inflammatory cytokines is considered a critical determinant to the success or failure of a pregnancy (Abrahams et al., 2004, Wegmann et al., 1993, Formby, 1995). Indeed, several lines of evidence support this phenomena, including the observation that Th1-dominated autoimmune disorders such as rheumatoid arthritis improve over the duration of pregnancy, while Th2-dominated conditions such as systemic lupus erythematosus become worse (Adams Waldorf and Nelson, 2008, Schulze-Koops and Kalden, 2001, Foster and Kelley, 1999). This theory is now being revised and considered an oversimplification of the complex interactions that occur in the immune environment of pregnancy (Mor and Cardenas, 2010, Mor et al., 2011). It is now recognised that particular events during pregnancy require a pro-inflammatory response from immune cells in order to be successful. This includes the post-mating inflammatory-like response - when suppressed, leads to reduced pregnancy success and a perturbed fetal growth trajectory (Bromfield, 2006), as well as implantation and trophoblast invasion (Fest et al., 2007). Evidence is accumulating to show that decidual macrophages are limited in their capacity to respond to infectious agents that would normally cause an inflammatory reaction and culminate in pregnancy loss. However, if the risk to maternal health becomes too great, then macrophages will respond in full force, albeit to the detriment of the pregnancy. Current research indicates that rather than pregnancy being a Th2 phenomena where Th1 immune responses are detrimental to conceptus survival, it appears to be a delicate balance between pro- and anti-inflammatory events. Macrophages are a major source of cytokines and growth factors, and are thought to be involved in the regulation of the delicate cytokine balance occurring at the implantation site (Abrahams et al., 2004).

### 1.3 MACROPHAGES

Cells of the mononuclear phagocyte system (MPS) are derived from the bone marrow. These progenitor cells differentiate to form monocytes, where they circulate in blood before entering the tissue to become tissue macrophages. In the 19$^{th}$ century, the work of Metchnikoff identified macrophages in a variety of different organs. He also recognised that the process of phagocytosis was not only a nutritional activity and a way of scavenging apoptotic cells, but it was a critical defence mechanism

against invading pathogens. It is now recognised that macrophages are ubiquitous immune cells with important roles in the innate and acquired immune responses, and further, these cells are involved in the maintenance of homeostasis within tissues due to their tissue remodelling capabilities and capacity to produce cytokines. Thus these remarkable cells do far more than scavenge debris after B and T lymphocytes have performed their duties (Hume et al., 2002, Chang, 2009, Cohen et al., 1999, Wu et al., 2004, Laskin et al., 2001).

Macrophages have been found in almost every tissue in the body. Resident tissue macrophages adapt to their local microenvironment, where their basal activity and their ability to respond to inflammatory mediators varies with the tissue in which they reside. Resident macrophages display local heterogeneity, but even within a given tissue, they are functionally heterogeneous. Thus, *in vivo*, macrophages have a diverse range of phenotypes (Hume et al., 2002, Laskin et al., 2001, Gordon, 2007).

Whilst residing in tissue, macrophages perform their sentinel and clearance functions, but are also capable of initiating acute inflammation and vascular changes, owing to their close association with vascular tissue (Gordon, 2007). Macrophages residing in tissues such as the kidney, pancreas and many endocrine organs, monitor physiological processes and contribute to homeostasis. They are rapidly recruited in response to any local disturbance (infection, cell turnover or wound healing, malignancy), and these recruited macrophages differ from local resident macrophages due to their activation state. Resident macrophages can also become activated, but whether they are able to respond to immunological stimuli to the same extent as recently recruited macrophages is questionable. It is suggested that they become less plastic with time in a specific location. In addition, the nature of an activated macrophage population is determined by the stimulus and their location (Hume et al., 2002). Dendritic cells (DCs) share many, though varied properties of macrophages, and could possibly be a further, likely irreversible stage of differentiation from a common lineage (Gordon, 2007).

Macrophages are often categorised as classically activated or “M1” macrophages, corresponding to Th1 driven responses. IFNG, colony stimulating factor 2 (CSF2), TNFA, and microbial products (lipopolysaccharide; LPS) elicit a classic M1 response, and these macrophages produce more Th1 cytokines, and participate in tissue destruction, killing of intracellular parasites, and tumour resistance. Alternatively activated, or “M2” macrophages, encompass all other macrophage phenotypes, but in

general correspond to Th2 responses. These M2 macrophages participate in tissue remodelling, angiogenesis, tumour promotion, immunoregulation and parasite encapsulation. Exposure to IL4, IL13 or IL10 can induce a M2 phenotype (Stout, 2010, Mantovani, 2006). However, it is suggested that the simplistic categorisation of macrophages into M1- or M2-type cells can lead to confusion and mistaken assumptions about the functions of these cells (Gordon, 2003). In general, however, categorisation of macrophages as either M1 or M2 – tissue destructive or tissue-reparative – is useful, but it must be accepted that not all macrophage phenotypes fit into this simplistic model (Stout, 2010). Mosser et al. (2008) suggest a new grouping of macrophage populations based on three different homeostatic activities. These groupings include wound-healing macrophages, classically activated macrophages involved in host defence, and regulatory macrophages that contribute to regulation of the host immune system. The authors emphasise that the behaviour of macrophages can be varied and overlapping, but the framework is useful in understanding and predicting macrophage functions in response to different environmental cues.

## 1.4 MACROPHAGES IN REPRODUCTIVE TISSUES

Macrophages are abundant in male and female reproductive tissue. Within the ovary and testis, these cells afford a close association with steroidogenic cells. The dynamics of uterine macrophage population are tightly controlled by the changing hormonal milieu (Cohen et al., 1999). The potential for macrophage involvement in female reproductive processes is detailed below.

### 1.4.1 Macrophages and ovarian function

The ovary is comprised of two principal parts; the central medulla, which is surrounded by the cortex. This highly organised structure contains germ cells (oocytes) and somatic cells (granulosa, theca and stromal cells), and interactions between these cell types control the formation of oocyte containing follicles, their growth and development, ovulation, and the formation of the corpus luteum (Richards and Pangas, 2010). The adult ovary contains growing and atretic follicles, and developing and regressing corpora lutea; the proportions of each depend on the stage of the ovarian cycle (Wu et al., 2004).

There is a dynamic relationship between the ovary and the pituitary gland, governed by positive and negative feedback regulation. Gonadotropin-releasing hormone (GnRH) is released in a pulsatile manner from the hypothalamus. This controls the release of follicle-stimulating hormone (FSH) and luteinising hormone (LH), also released in a pulsatile fashion from the anterior pituitary and responsible

for the control of many events in the adult ovary (Johnson and Everitt, 2007b, Richards and Pangas, 2010). These signals were originally believed to be sufficient for the control of ovarian function. A number of studies have revealed that ovarian control is far more complex and that growth factors produced locally are also important regulators of endocrine function (Brannstrom and Norman, 1993, Adashi et al., 1991).

Important studies implicating the immune system in the control of ovarian function show that thymectomy from neonatal mice causes developmental arrest of the ovary, with an absence of follicles and corpora lutea. Ovarian function can be restored by grafting of lymph node or thymic tissue (Nishizuka and Sakakura, 1969, Rebar et al., 1980). Rats treated with anti-thymocyte serum also suffer from impaired ovarian function (Bukovsky et al., 1977, Brannstrom et al., 1994a). The discovery of cytokines as a means by which cells communicate has enabled investigators to recognise links between the immune and endocrine systems. Evidence now suggests that the function of the ovary is influenced by leukocytes and cytokines (Tabibzadeh, 1994) in addition to gonadotropins and growth factors (Brannstrom and Norman, 1993).

Leukocytes traffic through the ovary, where their numbers fluctuate throughout the menstrual (Brannstrom et al., 1994b) or estrous cycle (Brannstrom et al., 1993, Petrovska et al., 1996). In the mouse, EMR1 (F4/80)-positive macrophages are found within the theca, stroma, and corpus luteum (Van der Hoek et al., 2000, Li et al., 1998). In the human, CD68 (macrosialin, FA11) positive macrophages have been detected in close association with vascular connective tissue and within the corpus luteum. CD68 is expressed by macrophages and some DCs (Gaytán et al., 1998, Wu et al., 2004).

#### *1.4.1.1 Macrophages and folliculogenesis*

Folliculogenesis is the process whereby a female germ cell develops within somatic cells and matures to become an oocyte capable of being fertilised (Knobil and Neill, 2006). Primordial follicles develop into primary, secondary, preantral, antral, and finally, preovulatory (mature) follicles. Secondary follicles are distinguished from primary follicles by the presence of more than one layer of granulosa cells, and the acquisition of an additional somatic cell layer, the theca. Thecal cells develop around the basement membrane and ultimately develop into the theca externa and the theca interna. Thecal development also coincides with the development of numerous blood vessels, while the granulosa cell layer is avascular (Knobil and Neill, 2006, Johnson and Everitt, 2007a). Proliferation of thecal and granulosa

cells, and the formation of an antrum, occur in response to FSH, estrogens, and locally produced factors. Only a small percentage of maturing follicles reach the preovulatory stage and ovulate, with the majority undergoing atresia, marked by granulosa cell apoptosis and follicle regression (Wu et al., 2004). As detailed below, macrophages have been implicated in the processes of folliclar growth and atresia.

Macrophages do not appear to be associated with primordial follicles, but they are present within the thecal layer surrounding growing follicles where they remain throughout follicle development (Wu et al., 2004). They are also a major cellular component in interstitial tissue between developing follicles. Macrophages are not present within ovarian follicles prior to ovulation (Simon et al., 1994a). It is proposed that thecal macrophages are a source of growth factors and cytokines that nurture developing follicles. It is possible that they promote cellular proliferation and follicle growth, as studies have shown that peritoneal macrophages are able to promote the proliferation of rat granulosa cells in an *in vitro* coculture system (Fukumatsu et al., 1992, Wu et al., 2004). In general, cytokines act in an inhibitory manner on immature ovarian follicles to prevent maturation of small follicles and instead, facilitate follicle growth (Brannstrom and Norman, 1993).

Vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), basic fibroblastic growth factor (bFGF), epidermal growth factor (EGF), transforming growth factor (TGF)A and B, and insulin-like growth factor (IGF) are some of the growth factors known to influence ovarian cell types and that impact follicle growth (Geva and Jaffe, 2000, Monget and Bondy, 2000, Ingman and Robertson, 2002, Richards et al., 2002, Wu et al., 2004). They are also macrophage-derived products, potentially implicating macrophages in the growth and development of follicles via paracrine actions of their secreted growth factors. Studies have investigated the effects of various cytokines that macrophages can produce and their impact on developing follicles, some of which are discussed in detail below. However, other leukocytes and somatic cells are present in the ovary and are also capable of producing these cytokines, making it difficult to decipher the contribution of individual cell types.

*1.4.1.1.1 Macrophage secretory products and folliculogenesis*

1.4.1.1.1.1 Interleukin 1

*Il1b* mRNA has been detected in the immature rat ovary (Hurwitz et al., 1991). IL1B has been reported to inhibit FSH-stimulated development of LH receptors, and to reduce secretion of $P_4$ and $E_2$ by undifferentiated rat granulosa cells (Gottschall et al., 1988, Gottschall et al., 1989, Kasson and Gorospe, 1989, Brannstrom and Norman, 1993). Brannstrom and Norman (1993) report that IL1B inhibits human chorionic gonadotropin (hCG)-stimulated androgen biosynthesis in thecal-interstitial cells of immature rats, and IL1 inhibits hCG-stimulated $P_4$ production by porcine granulosa cells obtained from medium sized follicles. However, IL1 stimulates proliferative growth of undifferentiated granulosa cells *in vitro* (reviewed, (Brannstrom and Norman, 1993). In general, it appears that in immature, developing follicles, IL1 stimulates growth but inhibits differentiation while the follicle and oocyte are maturing (Brannstrom and Norman, 1993).

1.4.1.1.1.2 CSF1

CSF1, also referred to as macrophage colony-stimulating factor, is a stimulator of macrophage proliferation and maturation, and a monocyte/macrophage chemoattractant (Stanley et al., 1983, Stanley et al., 1997, De et al., 1993). Mice that are homozygous for the recessive osteopetrotic (*op*) mutation (*Csf1*$^{op}$*/Csf1*$^{op}$ mice) suffer a number of pathologies, including deficiencies in macrophage and osteoclast cell populations. These deficiencies were found to be due to a complete absence of CSF1 (Wiktor-Jedrzejczak et al., 1990), but ostopertrosis, tooth eruption, and bone marrow cellularity can be corrected by daily injections of recombinant CSF1 (Wiktor-Jedrzejczak et al., 1991, Felix et al., 1990, Kodama et al., 1991, Pollard et al., 1991).

*Csf1*$^{op}$*/Csf1*$^{op}$ mice are severely depleted of macrophages in many tissues, including the ovary (Cohen et al., 1999). The *Csf1*-null mutation interferes with ovarian function, causing extended estrous cycles and a lower ovulation rate when compared to wild-type control mice, but this is due to dysfunction of the hypothalamic-pituitary-gonadal (HPG) axis rather than low macrophage numbers. The effects seen on the HPG axis can be overcome by the administration of recombinant CSF1 for during the first two weeks of life. These studies implicate macrophages in the development and function of the HPG axis prior to the onset of puberty (Cohen et al., 1997, Cohen et al., 2002).

EMR1-positive cells that surround developing follicles in wild-type mice are absent or significantly reduced in *Csf1$^{op}$/Csf1$^{op}$* ovaries (Cohen et al., 1997). The reduced macrophage numbers surrounding developing follicles may be a cause or an effect of the reduced follicle growth, marked by extended estrus cycles in *Csf1$^{op}$/Csf1$^{op}$* mice (Wu et al., 2004). Studies by Nishimura et al. (1995) showed that administration of human CSF1 to immature, gonadotropin-primed rats increased the number of ovarian macrophages associated with growing follicles, with the increase being statistically significant for mature follicles. Work conducted by Araki et al. (1996) in mature mice further implicates CSF1 in follicular growth, where untreated *Csf1$^{op}$/Csf1$^{op}$* mice were shown to have significantly less antral and mature follicles than control mice at proestrus. However, subcutaneous administration of recombinant human CSF1 daily from day 10 after birth, is able to significantly increase the number of antral follicles. The impaired proliferative capacity of granulosa cells in antral follicles in *Csf1$^{op}$/Csf1$^{op}$* mice is also restored by daily administration of CSF1. These experiments, whereby ovarian function and macrophage distribution are restored by the exogenous administration of CSF1, provide strong evidence that CSF1 and/or macrophages contribute to folliculogenesis. However, the situation is complicated because the CSF1 receptor is localised to oocytes in the developing follicle, implicating CSF1 in oocyte development, which may also be impaired in the *Csf1$^{op}$/Csf1$^{op}$* mouse (Arceci et al., 1992).

#### 1.4.1.1.1.3 CSF2

CSF2 (granulocyte-macrophage colony-stimulating-factor; GM-CSF) is a cytokine important for the survival, proliferation, differentiation and activation of myeloid leukocytes (granulocytes, macrophages and DCs (Inaba et al., 1992, Gasson, 1991)). CSF2 is expressed and secreted by cells from the human ovary, and is detected at higher levels in the follicular fluid and plasma of women undergoing ovarian hyperstimulation than from naturally cycling women (Jasper et al., 1996). This indicates that the production of CSF2 from ovarian cells may be regulated by gonadotropins or steroid hormones, as is the case in the uterus, with uterine epithelial cell synthesis of CSF2 being stimulated by $E_2$ (Robertson et al., 1996b, Jasper et al., 1996). In the mouse, the synthesis and release of CSF2 from ovarian macrophages increases with follicle development (Tamura et al., 1998, Wu et al., 2004).

#### 1.4.1.1.1.4 Other cytokines

Other cytokines potentially derived from macrophages that have been implicated in folliculogenesis include TNFA, IL6 and IFNG. Steroidogenesis in immature rat granulosa cells and thecal-interstitial cells is inhibited by TNFA. IL6 expression is linked with angiogenesis around developing mouse follicles. IFNG has been reported to inhibit FSH-stimulated $E_2$, $P_4$, inhibin and LH receptor formation in rat granulosa cells (Brannstrom and Norman, 1993). These studies, along with those reported for IL1, indicate that in the developing follicles cytokines act to suppress some of the actions of FSH that promote cell differentiation, and rather, promote granulosa and thecal cell proliferation to enable follicle growth before the cells reach a fully differentiated stage that occurs prior to ovulation (Brannstrom and Norman, 1993).

### *1.4.1.2 Macrophages and follicle atresia*

As previously highlighted, macrophages are normally restricted to the thecal layer of developing follicles, but in advanced stages of follicle atresia, they begin to infiltrate the granulosa cell layer in the rat (Gaytan et al., 1998) and human (Takaya et al., 1997, Best et al., 1996). Macrophage density in atretic follicles remains high throughout the estrous cycle in mice (Petrovska et al., 1996, Wu et al., 2004). Although granulosa cell apoptosis occurs in earlier stages of atresia, Gaytan et al. (1998) report that neighbouring granulosa cells appear to phagocytose apoptotic bodies in these early stages. One could postulate that as follicle atresia progresses, the capabilities of these non-professional phagocytes are overwhelmed, and macrophages invade to assist in the clearance and remodelling processes.

Macrophages may also promote follicle atresia. The macrophage-secreted product, TNFA, is able to induce granulosa cell apoptosis in early antral follicles *in vitro* (Kaipia et al., 1996, Wu et al., 2004). Thus it appears that macrophages, through their secretory products, are able to promote (via TNFA (Kaipia et al., 1996)) or inhibit (via epidermal growth factor (EGF) and TGFA; (Tilly et al., 1992)) granulosa cell apoptosis. The signals macrophages receive from their local microenvironment may determine which of these processes will occur.

### *1.4.1.3 Macrophages and ovulation*

There is a large body of work that implicates macrophages in ovulation. Macrophages are a known source of proteases and matrix metalloproteinases (MMPs) which may assist in follicle rupture at

ovulation. The process of ovulation has been likened to a classical inflammatory response because of the similarities in the extent of tissue remodelling that occurs and the common mediators liberated during these process (Espey, 1980). During inflammation, leukocytes quickly migrate to the site in response to chemotactic factors, where they become activated and produce enzymes and cytokines that enable them to orchestrate tissue breakdown and repair. Some of these substances, which can be produced by macrophages, include plasminogen activator (PA), collagenase, eicosanoids, platelet activating factor (PAF) and the cytokines IL1, TNFA and CSF2 have been implicated as key modulators of ovulation (Espey, 1980, Espey, 1994, Brannstrom et al., 1993). As discussed previously, the cytokines IL1 and TNFA are products secreted by activated macrophages, and have been shown to inhibit gonadotropin-stimulated events in steroidogenesis and receptor induction in undifferentiated granulosa and thecal-interstitial cells (Brannstrom and Norman, 1993, Brannstrom et al., 1993). However, these cytokines have very different actions in the mature, preovulatory follicle, where they stimulate $P_4$ production via their actions on theca cells.

Following the preovulatory LH surge, the follicle ruptures to allow extrusion of the oocyte. Macrophage populations in the rat ovary during the periovulatory period have been studied in detail (Brannstrom et al., 1993). Using the antibodies ED1 (reactive with blood monocytes and newly migrated macrophages) and ED2 (reacts with resident tissue macrophages), Brannstrom et al. (1993) reported an increase in macrophages in the periovulatory period. Specifically, there is a larger population of ED1-positive macrophages than ED2-positive macrophages at all stages of follicle development, implying that macrophages are being continuously recruited from the blood throughout follicle development. There is an increase in the density of ED1- and ED2-positive cells preceding ovulation, where they were detected beneath the surface epithelium at the follicle apex, implicating them in follicular rupture events (Brannstrom et al., 1993). The majority of macrophages present were positive for major histocompatibility complex (MHC)-II immunostaining, indicating an activated phenotype.

EMR1-positive macrophages have been detected surrounding the preovulatory follicle in the mouse (Hume et al., 1984). Macrophages have also been detected in the human follicle wall just prior to ovulation (Brannstrom et al., 1994b). There are reports detailing a significant increase in macrophage-like cells in the pig and the sheep at ovulation or immediately following follicle rupture (Standaert et al., 1991, Cavender and Murdoch, 1988, Brannstrom and Norman, 1993). After ovulation, CD11B-positive cells were detected within thecal and stromal cell layers in the mouse (Simon et al., 1994a).

Macrophages are present in human preovulatory follicular fluid obtained during follicle aspiration for *in vitro* fertilisation (IVF) procedures. Follicular fluid contains IL1, IL2, TNFA, CSF2, IFNA and IFNG, IL6, IL10 and CSF2. It is likely that macrophages are a major source of follicular fluid cytokines supporting a role for these cells in follicle growth and ovulation (reviewed, (Brannstrom and Norman, 1993, Wu et al., 2004).

A study by Hellberg et al. (1991) investigated the effect that leukocytes had on ovulation rate in the *in vitro* perfused rat ovary. While leukocytes alone did not increase the ovulation rate compared to control ovaries, leukocytes plus LH significantly increased the ovulation rate compared to ovaries perfused with LH only. However, the leukocyte mixture used in this study contained 15% granulocytes, 79% lymphocytes and 6% monocytes, making it difficult to determine whether an individual leukocyte population, or the cumulative actions of all were necessary to induce ovulation. Replicating this study and adding purified populations of granulocytes, lymphocytes or monocytes would help to determine whether an individual population is responsible for the increased ovulation rate, or whether these populations act in concert to achieve ovulation.

A study conducted by Van der Hoek et al. (2000) depleted ovarian macrophage populations by the intrabursal injection of clodronate liposomes; macrophage phagocytosis of clodronate liposomes causes them to undergo apoptosis. Injection of clodronate liposomes one day prior to gonadotropin-induced ovulation only achieved significant depletion of thecal CD68-positive cells. MHC-II- and EMR1-expressing populations in the stroma and theca appeared reduced following treatment, but this did not reach significance. Stromal CD68-positive cells were unaffected. However, when clodronate liposomes were injected three days prior to gonadotropin-induced ovulation, there was a significant decrease in thecal MHC-II- and CD68-positive cells, but not EMR1-positive cells; again, no stromal macrophage populations were affected by this treatment. Despite incomplete macrophage depletion, there was a significant decrease in the number of oocytes ovulated from ovaries treated with clodronate liposomes three days prior to gonadotropin-induced ovulation compared to control ovaries treated with saline liposomes. This model illustrates that partial macrophage depletion is sufficient to impede ovulation, providing further evidence for a supporting role of macrophages or their secretory products in ovulation.

#### *1.4.1.3.1 Macrophage secretory products and ovulation*

##### 1.4.1.3.1.1 CSF1

As mentioned previously, CSF1 administration increases the number of macrophages present in growing follicles (Nishimura et al., 1995, Araki et al., 1996), and this factor may influence ovarian macrophages in a way that promotes ovulation. Nishimura et al. (1995) conducted studies to determine the effect of exogenous CSF1 on the ovulation rate in immature, gonadotropin-treated rats. They revealed that CSF1 increases the ovulation rate in a dose-dependent manner. Intrabursal injection of an anti-CSF1 antibody significantly decreases the number of ova ovulated from the treated horn compared to the untreated contralateral horn.

Estrous cyclicity in the *Csf1$^{op}$/Csf1$^{op}$* mouse was shown to be perturbed due to impaired feedback regulation of the HPG axis, and can be restored by administration of recombinant CSF1 for the first two weeks of life (Cohen et al., 2002, Cohen et al., 1997). However, it is has not been determined whether this also improves the low ovulation rates in these mice. Other studies indicate that CSF1 can increase the ovulation rate. The study by Araki et al (1996) administered recombinant CSF1 from day 10 after birth. Although not sufficient to restore normal function of the HPG axis, as the critical period for HPG axis establishment is over the first two weeks of life (Cohen et al., 2002), this treatment was sufficient to markedly increase the number of oocytes ovulated when compared to *Csf1$^{op}$/Csf1$^{op}$* mice. However, the number ovulated was significantly less than achieved by wild-type animals. Thus CSF1 production in the ovary, and the resultant recruitment of macrophages, may be important in ovulation.

It has been shown in women treated with gonadotropins to retrieve oocytes for IVF that gonadotropins increase the ovarian CSF1 production (Nishimura et al., 1998). Serum concentrations of CSF1 increase throughout ovarian stimulation, but there are no significant changes in the CSF1 concentration of women who are poor responders to human menopausal gonadotropin (hMG). Follicular CSF1 concentrations were higher from follicles from which an oocyte could be retrieved compared to those where no oocyte could be retrieved. Such studies implicate CSF1 in follicular maturation processes, and in ovulation as an intraovarian regulator (Nishimura et al., 1998).

##### 1.4.1.3.1.2 CSF2 and IL1B

The ovulating ovary produces copious amounts of CSF2, with the amount being highest just prior to follicle rupture compared to preovulatory ovaries (Brännström et al., 1994). Studies by Jasper et al.

(2000) in *Csf2*$^{-/-}$ mice have shown that although there is no change in ovarian leukocyte numbers when compared to wild-type mice, there are alterations in phenotype. It has been reported that there is reduced expression of MHC-II on the cell surface of macrophages and DCs in *Csf2*$^{-/-}$ mice, indicating that these leukocytes are less activated than those in the ovaries of wild-type mice. *Csf2*$^{-/-}$ mice also display an increase in estrous cycle length, but there is no impact on the ovulation rate in an *in vitro* perfusion model (Jasper et al., 2000).

#### *1.4.1.4 Macrophages and luteinisation*

Luteinisation is a continuation of the ovulatory process. As discussed previously, just prior to ovulation there is an influx of leukocytes into the thecal layer of the follicle (Brannstrom et al., 1993). Following follicle rupture, the antrum becomes filled with blood and forms a central coagulum, which is later remodelled into connective tissue. The process of luteinisation is marked by rapid tissue remodelling and extensive proliferation of blood vessel capillaries that sprout towards the centre of the ruptured follicle and invade the previously avascular granulosa cells (Meyer and McGeachie, 1988, Reynolds and Redmer, 1999, Brannstrom and Norman, 1993). There is hypertrophy in the lutein cells as changes occur to enable them to become highly efficient steroid producing cells, including an increase in smooth endoplasmic reticulum and mitochondria (Leung and Adashi, 2004, Brannstrom and Norman, 1993). There are several lines of evidence that implicate macrophages and macrophage-derived products in the formation of the corpus luteum. For example, their proteolytic enzymes and cytokines may be important to enable the extensive angiogenesis and tissue remodelling that occurs, and they may enhance $P_4$ production from corpora lutea, as discussed below.

Within corpora lutea, there are a large number of EMR1-positive cells at estrus and day 1 post-coitum (pc), with the number on day 1 pc being slightly but significantly elevated above estrus levels (Cohen et al., 1997). There is a large increase in newly recruited macrophages (ED1-positive) in rat corpora lutea during pregnancy, with higher numbers in newly formed corpora lutea than those undergoing luteolysis. The number of ED1-positive cells in the pseudopregnant rat declines throughout the lifespan of the corpus luteum, prior to a second, but less substantial peak observed during luteolysis (Brannstrom et al., 1994a). ED2-positive tissue macrophages are present in lower numbers and do not change throughout the lifespan of the corpus luteum, while the number of activated MHC-II-positive macrophages increases during luteolysis (Brannstrom et al., 1994a, Brannstrom and Norman, 1993). CD11B-positive luteal cells are also found in the mouse (Tamura et al., 1998).

Several studies have detected macrophage-like cells in the human corpus luteum, where they predominate in the theca-lutein area compared to the granulosa-lutein area. Brannstrom and Norman (1993) report that macrophage numbers increase 2-3 fold from the early to mid-luteal phase. Other studies have shown that the proportion of macrophages do not change throughout the luteal phase (Castro et al., 1998).

Kirsch et al. (1981) showed that coculture of mouse peritoneal macrophages with granulosa cells or luteal cells causes increased $P_4$ secretion. Further, when luteal cells are co-cultured with corpora lutea-derived macrophages, greater levels of $P_4$ are produced than when cultured with peritoneal-derived macrophages, suggesting that the phenotype of ovarian macrophages is particularly suited to enhancing the function of luteal cells. Another study showed that coculture of human granulosa-lutea cells with peritoneal macrophages stimulates $P_4$ production (Halme et al., 1985). It has also been demonstrated that coculture of rat macrophages with granulosa cells enables enhanced granulosa cell proliferation (Fukumatsu et al., 1992). However, the work of Shakil et al. (1994) conflicts with this data and suggests that peritoneal macrophages have an inhibitory effect on $P_4$ production from granulosa cells in the rat. These studies differ in methodology because Shakil et al. (1994) isolated granulosa cells from proestrus rats and subsequently added gonadotropin to activate the cells and enable hormone production. The granulosa or luteal cells used in experiments by Kirsch et al (1981) were obtained from hormonally stimulated mice, and thus the cells already had the capability of producing hormone. Granulosa-luteal cells in the experiments by Halme et al. (1985) were obtained from patients undergoing IVF, and had also been exposed to gonadotropins.

Mating female mice with male mice that have had their seminal vesicle glands surgically excised ($SV^-$; sperm but no seminal fluid) results in a reduction in the number of infiltrating leukocytes, including macrophages, into the endometrium, when compared to matings with wild-type males (Robertson et al., 1996a). Studies extending from this observed fewer macrophages (measured by EMR1 and MHC-II immunohistochemistry) within corpora lutea of female mice the day after mating with $SV^-$ males, when compared to matings with intact or vasectomised (seminal fluid but no sperm) males, although this had no impact on serum $P_4$ levels (Gangnuss et al., 2004). Studies in pigs where gonadotropin-treated gilts received transcervically administered seminal plasma (SP) or PBS showed that treatment with SP resulted in a significant increase in macrophage recruitment to ovarian tissue, an increased weight of corpora lutea and increased plasma $P_4$ (O'Leary et al., 2006). From these studies, SP appears to increase macrophage number within the corpus luteum, and these macrophages may

potentially increase the $P_4$ output from the corpus luteum. Whether macrophage recruitment is a direct effect of signalling moieties within the seminal fluid, or mediated indirectly by causing the secretion of factors from the luminal epithelium, remains unresolved.

#### *1.4.1.4.1 Macrophage secretory products and luteinisation*

##### 1.4.1.4.1.1 CSF1

At estrus, EMR1-positive cells in corpora lutea are normally abundant, but are virtually absent in *Csf1*$^{op}$*/Csf1*$^{op}$ mice. However, during pregnancy, despite dysregulation of the HPG axis, the number of corpora lutea-associated macrophages is quickly restored to 35% of the density seen in wild-type mice. The corpora lutea from *Csf1*$^{op}$*/Csf1*$^{op}$ mice are able to produce sufficient $P_4$ to maintain pregnancy, although there seems to be a delay in their ability to do so when compared to wild-type animals. On day 5 pc, the level produced is 50% lower than in wild-type mice, but this difference disappears by day 8 pc (Cohen et al., 1997).

##### 1.4.1.4.1.2 CSF2

Ovaries from *Csf2*$^{-/-}$ mice treated with gonadotropin in an *in vitro* perfusion model produce significantly less $P_4$ than those from wild-type mice, and have a lower ovarian weight (Jasper et al., 2000). *Csf2* and its receptor were detected by real-time (RT)-PCR in human granulosa-lutein cell culture preparations, and in corpora lutea from the early, mid and late phase of the menstrual cycle. Culture supernatants obtained from granulosa-lutein cells and early and mid phase corpora lutea contain immunoactive CSF2 (Jasper et al., 1996). In the mouse, intense *Csf2* mRNA expression was detected by *in situ* hybridisation in luteal tissue (Tamura et al., 1998). As described earlier, the activation state of macrophages is perturbed in the *Csf2*$^{-/-}$ mouse, with less cell surface expression of MHC-II, a known activation marker for macrophages and DCs (Jasper et al., 1996). The activation state of corpora lutea associated macrophages may be important for them to carry out luteotrophic functions, and may offer an explanation for the reduced $P_4$ output from the ovaries of *Csf2*$^{-/-}$ mice. Further, studies report the presence of macrophages whose morphology suggests an activated state (spindle shaped or displaying dendritic features) within the human corpus luteum (Gaytán et al., 1998, Takaya et al., 1997, Wu et al., 2004).

##### 1.4.1.4.1.3 IL1 and other factors

IL1, potentially of macrophage origin, has been implicated in the processes occurring in the luteal phase. As reviewed by Brannstrom and Norman (1993), IL1 is elevated in the luteal phase in the

human. IL1 may be luteotrophic according to reports showing that recombinant IL1A increased basal and LH-stimulated $P_4$ secretion from luteinised granulosa cells, but IL1B appears not to affect steroidogenesis in human and bovine luteal cells. Other macrophage derived products that have been shown to increase $P_4$ production include EGF (Serta and Seibe, 1993, Kim et al., 1992) and TNFA (Yan et al., 1993).

#### 1.4.1.4.1.4 VEGFA

A critical step in the establishment of a functional corpus luteum is the neo-vascularisation that begins during the ovulatory process. This process seems to be dependent on VEGFA, with studies showing that inhibition of the actions of VEGFA prior to ovulation inhibits angiogenesis (Ferrara et al., 1998) by inhibiting endothelial cell proliferation, and thus the establishment of the dense vascular network, with an associated decrease in $P_4$ production (Fraser et al., 2000). Because macrophages are a source of angiogenic factors including VEGF, EGF and bFGF (Sunderkotter et al., 1994), they may facilitate these processes. This will be discussed in more detail later.

### *1.4.1.5 Macrophages and luteolysis*

Luteal regression, or luteolysis, involves the demise of luteal cells, with associated ischemia and apoptosis and a consequent fall in $P_4$ output (Johnson and Everitt, 2007a, Brannstrom and Norman, 1993). The luteolytic factor of uterine origin, prostaglandin-F2A (PGF2A), has been found to initiate this cascade of events in most mammals, with the exception of higher primates. In higher primates, in the absence of luteotrophic support in the form of chorionic gonadotropin secreted from the primate blastocyst, luteolysis will ensue (Johnson and Everitt, 2007a); a luteolytic signal appears not to be necessary.

Leukocytes are proposed to contribute to functional luteolysis via the secretion of cytokines that may inhibit steroidogenesis, and in structural luteolysis via initiating cell death and the phagocytic removal of dying cells, as well as the tissue remodelling that occurs as the corpus luteum becomes the corpus albicans (Brannstrom and Norman, 1993, Pate and Landis Keyes, 2001). Several studies in the rabbit (Bagavandoss et al., 1990), rat (Brannstrom et al., 1994a) and human (Lei et al., 1991, Brannstrom et al., 1994b) have reported an increase in macrophage density during luteolysis. There is an increase in activated MHC-II-expressing macrophages during luteolysis (Brannstrom et al., 1994a), indicating enhanced phagocytic capabilities. Studies have demonstrated that pro-inflammatory cytokines in the

corpus luteum can promote apoptosis as well as inhibit gonadotropin-stimulated steroidogenesis and stimulate luteal cell synthesis of PG (reviewed, (Pate et al., 2010).

*1.4.1.5.1 Macrophage recruitment signals/secretory products and luteolysis*

1.4.1.5.1.1 CCL2

CCL2 (chemokine (C-C motif) ligand 2; also known as macrophage chemoattractant protein (MCP)-1), a chemokine that controls macrophage migration, is implicated in macrophage recruitment during luteolysis. Senturk et al. (1999) report that CCL2 is expressed in the rat corpus luteum, with increased expression during luteolysis. The study found increasing macrophage numbers as luteolysis progressed, and increased CCL2 immunostaining and *Ccl2* mRNA expression in regressing corpora lutea compared to early stage corpora lutea. The source of CCL2 is unclear, with a report indicating that endothelial cells, fibroblasts, and macrophages can secrete this factor (Rollins et al., 1991). This study highlights that CCL2 production during luteal regression is likely to originate from the blood vessels, with increased CCL2 staining being detected in blood vessel walls surrounding the corpus luteum, than vessels distal to it. This implies that CCL2 may recruit macrophages from the blood (Senturk et al., 1999) and concurs with other reports showing increased CCL2 expression in regressing corpora lutea from the pig (Hosang et al., 1994), cow (Penny et al., 1998) and rat (Bowen et al., 1996, Townson et al., 1996).

1.4.1.5.1.2 TNFA

TNFA is a macrophage derived cytokine, and Benyo and Pate (1992) demonstrated that TNFA administered to bovine luteal cells causes a dose-dependent increase in the luteolytic PGF2A. TNFA also decreased LH-stimulated $P_4$ synthesis after 7 days in culture (Benyo and Pate, 1992, Pate et al., 2010). It has also been demonstrated in mouse granulosa cells that TNFA diminishes the gonadotropin-supported accumulation of $P_4$, largely by attenuating steps leading to $P_4$ production (Adashi et al., 1990, Andreani et al., 1991). TNFA also caused accumulation of PGE2 and PGF2A in human granulose-lutein cells (Wang et al., 1992). Resultant PGF2A causes increased activity of 20αHSD and thus increases $P_4$ catabolism (Leung and Adashi, 2004).

### 1.4.2 Macrophages and oviduct function

The oviduct is an often overlooked portion of the female reproductive tract. Its ciliated epithelial cells are important for transportation of the fertilised zygote into the uterine lumen for subsequent

implantation. However, oviductal epithelial cell secretions are likely to contribute to the health of the embryo and may play important roles in the embryo-maternal signalling that occurs. It has been demonstrated that the mRNA expression of *Lif* (Leukemia inhibitory factor; (Jasper et al., 2005, Bromfield, 2006)), *Csf2, Il6*, and *Igf2* (Insulin-like growth factor 2; (Bromfield, 2006)) are increased in the oviduct following exposure to SP. These cytokines and growth factors show embryotrophic properties within *in vitro* embryo culture systems (Schultz and Heyner, 1993, Sjöblom et al., 1999). In addition, LIF, CSF2 and IL6 are pro-inflammatory cytokines and may be involved in macrophage recruitment to the oviduct. *Lif*$^{-/-}$ mice have altered macrophage distribution patterns in the uterus, and *Csf2*$^{-/-}$ mice have altered activation phenotypes (Schofield and Kimber, 2005, Jasper et al., 2000).

Macrophages are present in the oviductal stroma, where they may regulate epithelial cell secretions. Following mating, SP induces an inflammatory-like response in the oviduct, similar to that characterised in the uterus. This response involves the recruitment of CD45-positive leukocytes, an effect extending as far as the infundibulum (Farr et al., 1995). In the case of ectopic pregnancy (EP), a condition unique to the human (Corpa, 2006), macrophage density is more than doubled at the tubal implantation site compared to tubal macrophage density in the absence of an EP. It is thought that aberrant macrophage behaviour may contribute to this life threatening condition (Tonello and Poli, 2007). Past infection or smoking are increased risk factors for EP, possibly due to alterations in the signals that macrophages receive, consequently directing their behaviour towards a trophic role in the oviduct in an attempt to repair tissue damage. This behaviour is normally restricted to the endometrium to assist with blastocyst implantation (Tonello and Poli, 2007). Tonello and Poli (2007) propose that macrophages may also impair tubal motility; tubal motility is influenced by $P_4$, $E_2$ and PG, with high levels of $P_4$ causing ciliary dysfunction (Paltieli et al., 2000). It is known that activated macrophages enhance $P_4$ secretion from granulosa and luteal cells (Halme et al., 1985), leading to increased macrophage production of PGE2 (Yagel et al., 1987). Tonello et al. (2007) speculate that this impairment of tubal motility may cause the embryo to be retained in the oviduct rather than being propelled to its rightful place in the uterine lumen. Also, enhanced LIF secretion from the tubal epithelium occurs following treatment of these cells with pro-inflammatory cytokines and this may alter the tubal epithelium into a receptive state. Increased LIF secretion may also be responsible for the increased macrophage density, which is decreased in *Lif*$^{-/-}$ mice (Stewart et al., 1992). These macrophages may perform tissue remodelling functions that aid the ectopic implantation of the blastocyst (Tonello and Poli, 2007).

### 1.4.3 Macrophages and uterine function

The uterus supports the transport, storage, and maturation of spermatozoa following copulation, provides an embryotrophic environment for the conceptus to implant and develop within, and delivery of the fetus at parturition. The uterus is not fully developed and differentiated at birth, instead this occurs postnatally in rodents, domestic animals, and humans. This establishes three classic histological elements of this tissue; the endometrium, myometrium, and the perimetrium (Spencer et al., 2005). The endometrium consists of three main tissues; the luminal epithelium, glandular epithelium and the stroma. Within the stroma are blood and lymph vessels and various other cell types, including fibroblasts and immune cells. Moving outward from the endometrium is the myometrium, which is made up of an inner layer of circular muscle, and an outer longitudinal layer (Finn, 1985). The myometrium is important during parturition but it is essential it remains quiescent during pregnancy, a task the hormone $P_4$ strictly regulates.

Macrophages are normal inhabitants of the cycling and pregnant uterus. In the cycling uterus, the density of macrophage populations changes in response to ovarian steroid hormones. Macrophage numbers are lowest at diestrus when $E_2$ levels are low, and increase at proestrus when $E_2$ secretion from maturing ovarian follicles is high (Cohen et al., 1999). Ovariectomy causes a significant decrease in the relative and absolute number of uterine macrophages. Numbers can be restored by exogenous hormone injections (De and Wood, 1990).

#### *1.4.3.1 Macrophages and uterine cyclic remodelling*

The cyclic events occurring in the uterus are governed by ovarian steroid hormones. Understanding the importance of the timing and concentration of hormones required to prepare the uterus for an implanting embryo has been largely achieved by detailed studies in ovariectomised mice. $E_2$ administered to ovariectomised mice causes the uterine luminal epithelial cells to undergo mitosis. Two days later, there is a wave of division in the glandular epithelium. If $P_4$ is administered two to three days following $E_2$ treatment, stromal cells that were unresponsive to $E_2$ undergo mitosis. It is presumed that this interval is necessary to enable changes to occur in the stromal cells that will enable their response to luteal $P_4$ and nidatory $E_2$. This schedule of hormone injections mimics natural fluctuations in early pregnancy in mice and women, where there are high levels of $E_2$ produced at proestrus, then there is little hormone exposure for a short period. The corpus luteum forms following ovulation, which then secretes $P_4$, responsible for epithelial cell differentiation in preparation for embryo implantation (Finn, 1985, Finn and Martin, 1969, Tong and Pollard, 2002).

There has been an ongoing debate questioning whether sex steroid hormones act directly on the uterus, and if so, whether their actions on one particular cell type might be mediated indirectly through another cell type. During the estrous cycle, uterine epithelial cells respond to ovarian steroid hormones by producing an array of cytokines and chemokines that cause macrophage recruitment to the uterus and regulate macrophage phenotype. In turn, growth factors and cytokines synthesised by macrophages potentially impact epithelial and stromal cell proliferation, secretory function and receptivity to embryo attachment (Tong and Pollard, 2002).

The development of the receptor null mutants, *Esr1*$^{-/-}$ (estrogen receptor 1 (alpha) null mutant) and *Pgr*$^{-/-}$ (progesterone receptor null mutant), provide a means to address these questions. Tissue recombinants between stroma and epithelium derived from immature mice that have steroid hormone receptors, or are devoid of them, have demonstrated that the proliferative actions of $E_2$ and the inhibitory actions of $P_4$ on epithelial cell proliferation are mediated through the stroma; cognate receptors in the uterine epithelium are not required (Cooke et al., 1997, Kurita et al., 1998a, Tong and Pollard, 2002). The studies addressing uterine epithelial cell proliferation suggest that the actions of $E_2$ on the uterine epithelium may be mediated in a paracrine manner by factors that are synthesised or released from the uterine stroma. This reveals an indirect mechanism responsible for epithelial cell proliferation (Cooke et al., 1997) and one in which macrophage secretory products could be involved. It should be noted that the uterine tissue utilised in the tissue recombinant experiments described is derived from neonatal mice. Because the embryological development of organs usually requires stromal-epithelial interactions, it is possible that the 'embryologic' phenotype in these grafts in maintained to some degree (Tong and Pollard, 2002). The manner by which stromal cell proliferation occurs in response to $E_2$ and $P_4$ is yet to be studied in detail, and the possibility for macrophage regulation of these events still remains.

There is ample research to support the idea that the local actions of sex steroid hormones are mediated in a paracrine manner by growth factors, and many of these growth factors are produced by the uterine epithelium (Pollard, 1990, Hunt, 1992, Robertson et al., 1992a). Research suggests that macrophages could also produce growth factors in response to sex steroid hormones (they contain ER and PR; (Huang et al., 2008), which could have paracrine actions on epithelial or stromal cells. Alternatively, stromal cells could induce the paracrine secretion of growth factors by stromal macrophages. Since stromal macrophages are in close juxtaposition to uterine epithelial cells at estrus,

these macrophages may also induce uterine epithelial cell proliferative response in a paracrine manner (Cooke et al., 1997, Kurita et al., 1998b). Studies in *Csf1$^{op}$/Csf1$^{op}$* mice do not support this possibility, since uterine luminal epithelial and stromal cell proliferation takes a normal course despite the low numbers of uterine macrophages in cycling animals (Cohen et al., 1997). However, since it has been indicated that the reproductive defects in the *Csf1$^{op}$/Csf1$^{op}$* mouse involve problems with feedback regulation of the HPG axis, they may not be an ideal model to address such a research question (Cohen et al., 1999). Additionally it is important to note that macrophage depletion in these mice is not complete (Robertson et al., 1998, Pollard et al., 1998).

#### *1.4.3.2 Macrophage recruitment following the post-mating inflammation-like response*

Following insemination, there is an inflammation-like response that occurs in the endometrium. This is characterised by recruitment of polymorphonuclear leukocytes (PMNs) from the blood and their transmigration into the endometrium and then through the luminal epithelium into the luminal cavity. This is closely followed by an influx of mononuclear cells, whose numbers are highest on day 1 pc, and begin to decline by day 3 pc in response to a $P_4$ induced shift in local milieu (De et al., 1991, Kachkache et al., 1991, Robertson et al., 1998).

Studies conducted by Robertson et al. (1996a) show that endometrial macrophage recruitment is inhibited by ablation of the seminal fluid stimulus, achieved by mating females with SV$^-$ males. Studies using this model have yielded interesting results, but interpretation of the direct role of macrophages as opposed to seminal factors is difficult. Mating with SV$^-$ males leads to compromised implantation, altered placental development, and adverse effects on fetal and postnatal growth, resulting in adult progeny with evidence of metabolic disorders (Bromfield, 2006). Further work is required to decipher whether these results can be attributed to reduced macrophage recruitment, the absence of seminal fluid (which itself contains many cytokines), adverse effects on embryo development, or a combination of these.

As eluded to above, investigation into the factors responsible for macrophage recruitment occurring in response to female sex steroid hormones, and following insemination, are on-going. There are a number of factors that are produced in response to $E_2$ or $P_4$ in the cycling and pregnant female, as well as factors in seminal fluid, that have been implicated. Some of these factors are discussed in more detail below.

*1.4.3.2.1 Macrophage secretory products and uterine function*

1.4.3.2.1.1 CSF1 and CSF2

CSF1 is produced by uterine epithelial cells in response to $E_2$ and $P_4$ (Bartocci et al., 1986, Pollard et al., 1987a). Additional studies reveal that ovariectomy causes complete loss of both uterine macrophages and CSF1, but systemic replacement of $E_2$ or $P_4$ is sufficient to restore macrophage and CSF1 levels to normal. Intrauterine administration of CSF1 is also able to restore uterine macrophage populations in ovariectomised mice, directly demonstrating the ability of CSF1 to recruit monocytes from the circulation into uterine tissue. Thus, $E_2$, $P_4$ and CSF1 increase macrophage density near uterine epithelial surfaces (De and Wood, 1990, Wood et al., 1992).

De et al. (1993) studied the relationship between macrophage numbers and CSF1 production in the pregnant mouse. They showed there is a high concentration of CSF1 on day 1 pc, and this correlates with a high density of macrophages mainly concentrated near the epithelium. As CSF1 bioactivity decreases from day 1 to day 3 pc, a concomitant decrease in uterine macrophages occurs and they are distributed evenly between the endometrium and myometrium. CSF1 bioactivity increases on day 4 and day 5 pc, when macrophages are again in close juxtaposition with the luminal epithelium. On days 6 and 7 pc, CSF1 bioactivity decreases (possibly due to epithelial cell destruction by the invading trophoblast cells); this causes macrophages to be displaced from the developing primary and secondary decidua and instead relocate in the deep endometrium and myometrium. CSF1 bioactivity then remains relatively high throughout the remainder of pregnancy. This study also notes that macrophages were not found within the uterine lumen.

*Csf1*$^{op}$*Csf1*$^{op}$ are infertile and display severely reduced numbers of uterine macrophages (Pollard et al., 1991). However, following mating with *Csf1*$^{+}$*/Csf1*$^{op}$ males, pregnancy can be achieved, albeit at much lower rates than achieved by heterozygous females. It appears that the diminished pregnancy rate in these mice is due to their poor ovulation rates, as detailed above. EMR1-positive macrophages are not detected in uteri from cycling *Csf1*$^{op}$*/Csf1*$^{op}$ mice. They are detected in early gestation, but not beyond day 14 pc. The macrophages that are present in *Csf1*$^{op}$*/Csf1*$^{op}$ mice are uncharacteristically rounded and small, indicating an effect on macrophage phenotype resulting from the CSF1 deficiency (Pollard et al., 1991). Since macrophages are present in pregnant uteri of *Csf1*$^{op}$*/Csf1*$^{op}$ mice, it appears that *Csf1* is redundant and other factors contribute to macrophage recruitment during pregnancy. This

contrasts with the estrous cycle, when *Csf1* is not redundant but essential to maintain macrophage density.

CSF2 is another cytokine secreted by the uterine epithelium and it targets leukocytes of monocyte and granulocyte lineages. CSF2-expression is induced by $E_2$ in the cycling uterus, and is further increased in response to TGFB, a protein present in SP (Robertson et al., 1996b, Robertson and Seamark, 1990, Tremellen et al., 1998). Administration of CSF2 into the uterine lumen of ovariectomised mice causes the infiltration of macrophages, neutrophils and eosinophils at similar levels to those seen on day 1 pc. This observation, coupled with the known expression of the CSF2 receptor on leukocytes, positions this cytokine as a key mediator of the local recruitment and activation of leukocytes following mating (Robertson et al., 1994).

Thus, CSF2 was proposed as a cytokine that may compensate for the lack of CSF1 in $Csf1^{op}/Csf1^{op}$ mice during pregnancy. To explore this possibility, Robertson et al. (1998) generated mice deficient in both *Csf1* and *Csf2* ($Csf1^{op}/Csf1^{op}Csf2^{(-/-)}$). These studies demonstrated that neither cytokine is essential for macrophage recruitment, but they do appear to be involved in the regulation of macrophage distribution and activation status. The numbers and distribution of eosinophils, macrophages and neutrophils on day 1 and day 3 pc was not different in $Csf2^{-/-}$ mice compared to wild-type controls. There was, however, a reduction in the number of cells expressing MHC-II, with approximately 50% less expression seen on day 1 pc. This study and the study by Pollard et al. (1998) show that EMR1-positive macrophages are present in low numbers in estrus uteri of $Csf1^{op}/Csf1^{op}$ mice (periluminal macrophages are 35% of the mean number seen in heterozygous mice, and perimyometrial macrophages are 52.2%). Macrophages are seen to infiltrate the uterus on day 1 pc, although the numbers of these cells were consistently at the lower end of the range seen in heterozygous $Csf1^{+}/Csf1^{op}$ mice (periluminal macrophages on day 1 pc represent 53.2% of the mean number seen in heterozygous mice, while perimyometrial macrophages represent 49.6%)(Robertson et al., 1998, Pollard et al., 1998). Neutrophil and eosinophil populations are unperturbed in $Csf1^{op}/Csf1^{op}$ mice. In mice deficient in both cytokines, the number and distribution of leukocyte populations was similar to that seen in $Csf1^{op}/Csf1^{op}$ mice, and the major difference was less EMR1-positive macrophages in the day 1 pc uterus of double-knockout mice (Robertson et al., 1998).

Moldenhauer et al. (2010) showed in the $Csf2^{-/-}$ mouse that the gene deficiency does not alter the number of uterine $CD11C^{+}$ or $EMR1^{+}$ cells at estrus, or on day 0.5 or 3.5 pc. However, there were

fewer cells expressing the scavenger receptor (CD205) and MHC-II, and DCs had reduced levels of surface activation markers. This was associated with impaired activation of T cells, emphasising the importance for CSF2 in the ability of DCs and macrophages to activate adaptive immune responses.

The elegant studies described above clearly demonstrate that *Csf1* and *Csf2* are not essential for leukocyte recruitment during the post-mating response, and there are other factors involved in the regulation of uterine leukocyte populations. It is apparent that *Csf1* is involved in the regulation of macrophage numbers and distribution, as well as macrophage phenotype. *Csf2*-deficiency appears to mainly impact on macrophage phenotype, with their numbers and distribution being unchanged in its absence.

Further studies by Robertson et al. (1999) demonstrate a dramatic effect of *Csf2*-deficiency on offspring. While implantation rates are normal in the *Csf2*$^{-/-}$ mouse and viable pups are born, the mean litter size from *Csf2*$^{-/-}$ breeding pairs is 25% smaller at weaning compared to those from heterozygous breeding pairs, and this is attributed to fetal loss in late gestation. The rates of resorbing and malformed fetuses are twice as high, and there are reductions in fetal weight and the fetal:placental weight ratio, a measure of placental efficiency. Offspring from *Csf2*$^{-/-}$ mice suffer higher rates of mortality in the first three weeks of life, and the small size of the offspring persisted into adult hood. The growth retardation in offspring from *Csf2*$^{-/-}$ mice may be partially attributed to the alterations in placental structures. It is yet to be determined whether the fetal aberrations in the *Csf2*$^{-/-}$ mouse are due to altered macrophage or DC phenotypes and functions, but this seems likely given that macrophages are implicated in trophoblast invasion, as discussed later.

#### 1.4.3.2.1.2 Chemokines

Chemokines also contribute to regulation of uterine macrophage populations. Chemokines are a family of small, inducible, pro-inflammatory cytokines that are important in regulating leukocyte extravasion, chemotaxis, and activation during inflammation (Robertson et al., 1998). Robertson et al. (1998) investigated the expression of mRNAs encoding an array of C-C and C-X-C cytokines and identified some that could be candidates for the regulation of leukocyte recruitment in the 24 h following coitus. CCL5 (chemokine (C-C motif) ligand 5; also referred to as RANTES; Regulated upon Activation, Normal T-cell Expressed, and Secreted), CCL2 (MCP1; monocyte chemotactic protein-1), CCL3 (Mitogen-activated protein kinase associated protein 1; also known as macrophage inflammatory protein-1-alpha (MIP1A)) and CCL4 (MIP1B) are C-C chemokines that are chemoattractants and

activators of monocytes and macrophages. CCL5 and CCL11 (eotaxin; another C-C chemokine) also target eosinophils. RT-PCR showed that the mRNA expression of these chemokines was induced following coitus, with levels declining by day 3 or 4 pc to levels equivalent to or less than those seen at estrus. *Ccl11* mRNA expression was high at estrus and day 1 pc, before declining on day 3 pc (Robertson et al., 1998). *Cxc11* (Chemokine (C-X-C) motif; also known as KC) expression was moderately increased from estrus to day 1 pc, and not detected beyond this time point.

Wood et al. (1997) showed high levels of *Ccl5* and *Ccl2* expression on day 1 pc by northern blot analysis. To test whether CCL5 contributed to the macrophage recruitment seen following mating, recombinant human CCL5 was administered into the uterine lumen of normal cycling mice. This caused a marked increase in macrophage numbers within 1 h of treatment, with numbers still increasing 18 h following treatment.

Robertson et al. (1998) extended these studies by assessing the effect of steroid hormones on the expression of the chemokines investigated in ovariectomised mice that were untreated, or following treatment with $E_2$, $P_4$, and $E_2$ plus $P_4$. There was an increase in the synthesis of *Ccl3* mRNA following $E_2$ exposure, and an induction of *Ccl2* following exposure to $E_2$ plus $P_4$. *Ccl3* expression was inhibited in the presence of $P_4$ or $E_2$ plus $P_4$ (Robertson et al., 1998).

Similar studies were conducted by Wood et al. (1999) to determine the induction of various chemokines in response to $E_2$ or $P_4$ in ovariectomised mice using northern blot analysis. Similar results were obtained for *Ccl2,* where mRNA expression was induced in response to $E_2$ or $P_4$. There were discrepancies in some results, with Wood et al. (1999) showing an induction of *Cxcl11* by exposure to $E_2$ or $P_4$, while Robertson et al. (1998) showed that the levels were similar between untreated and $E_2$ treated mice, but $P_4$ or $E_2$ + $P_4$ treatment inhibited *Cxcl11* expression. These differences could be due to difference in mouse strains used in the studies, and different concentrations and preparations of hormones used.

The studies by Robertson et al. (1998), Wood et al. (1997, 1999) and Pollard et al. (1998) demonstrate an increase in *Ccl2*, *Ccl5* and *Ccl3* expression on day 1 pc. These studies highlight that ovarian steroid hormones, and active moieties in semen, are involved in the regulation of chemokine mRNA expression. The implicated chemokines may have key roles in regulating leukocyte populations by influencing extravasation and migration into the uterine stroma.

#### 1.4.3.2.1.3 LIF

LIF is a cytokine expressed by the uterine epithelium that is indispensible for uterine epithelial cell receptivity and has important roles in the process of decidualisation. LIF expression appears to be regulated by $E_2$, with transient LIF expression on day 1 pc by the uterine epithelium coinciding with the ovulatory $E_2$ surge, and on day 4 pc LIF is expressed specifically by the glandular epithelium in response to nidatory $E_2$ (Schofield and Kimber, 2005). Blastocyst implantation fails in mice lacking a functional *Lif* gene (*Lif*$^{-/-}$), an effect that can be rescued by exogenous administration of LIF on day 4 pc (Stewart et al., 1992).

Studies by Schofield and Kimber (2005) have shown that *Lif* is essential in the regulation of macrophage number and distribution in the early pregnant uterus. In wild-type mice, a high proportion of macrophages are situated near the uterine lumen on day 3 pc, and these cells relocate to the myometrium from day 4 pc onwards. There are significantly less macrophages in *Lif*$^{-/-}$ mice and the distribution does not follow the pattern seen in wild-type mice. It is speculated that these altered macrophage dynamics could prevent the cells from producing signals required to generate a receptive endometrium, and may be in part responsible for the infertility observed in the *Lif*$^{-/-}$ mouse.

LIF is also a macrophage secretory product that has been shown to enhance epithelial cell receptivity by enhancing epithelial cell expression of *Fut2* mRNA, a gene responsible for elevating epithelial cell fucosylated glycoprotein expression (Jasper et al., 2011). Neutrophil numbers in wild-type mice are high on day 1 pc, contributing to the inflammatory-like response detected following coitus, but in wild-type animals their numbers decrease between day 2 and day 3 pc. This reduction was not seen in *Lif*$^{-/-}$ and Schofield and Kimber (2005) suggest that this may be because LIF might promote apoptosis of neutrophils in the uterus, as it does so in other cell types such as mammary epithelial cells (Kritikou et al., 2003). Another possibility is that reduced macrophage numbers and their altered distribution means that macrophages may not be there to clear the neutrophils when the inflammation subsides. *Lif*$^{-/-}$ mice also have increased numbers of natural killer (NK) cells, with almost double the percentage seen in wild-type mice on day 4 pc (Schofield and Kimber, 2005).

### *1.4.3.3 Macrophage regulation of implantation*

The presence of leukocytes within the decidua and at the implantation site suggests that the innate immune system responds to antigens expressed by the semi-allogeneic fetus, and may have roles extending beyond host protection to infectious agents and participate in the establishment of a

microenvironment supportive of growth and resistant to harmful inflammatory immune reactions (Abrahams et al., 2004).

In rodents, macrophages are in close proximity with the implantation site, but as pregnancy progresses, they are excluded from the decidua and become distributed throughout the myometrium, endometrial stroma, and metrial gland (Tachi et al., 1981, Redline and Lu, 1988, Hunt, 1989). In contrast, a variety of leukocytes exist in the decidua during pregnancy in the human. Many of the leukocytes at the implantation site are decidual NK cells (60-80%), whose phenotype is distinct from peripheral NK cells. NK cell numbers decline throughout gestation and are absent at term. Macrophages constitute approximately 20-30% of decidual leukocytes and are present in high numbers throughout gestation (Lessin et al., 1988, Bulmer et al., 1988, Kabawat et al., 1985, Abrahams et al., 2004).

It has been suggested that macrophages are key antigen presenting cells (APC) in the decidua due to the abundance of $CD14^+$ decidual macrophages and the near absence of $CD14^-$ DCs (Houser et al., 2011, Gardner and Moffett, 2003). Houser et al. (2011) propose that MHC-II-expressing decidual macrophages are important in the establishment of fetal-maternal tolerance, as well as in tissue remodelling events required during pregnancy. In their study, they define two distinct subsets of $CD14^+$ decidual macrophages ($CD11C^{HI}$ and $CD11C^{LO}$) and through gene profile analysis they suggest that the former ($CD14^+CD11C^{HI}$) have roles in inflammation and antigen presentation, whereas the $CD14^+CD11C^{LO}$ cells express genes associated with extracellular matrix (ECM) formation and tissue growth. These decidual macrophage populations do not fit into the conventional M1/M2 classification as they are able to secrete both anti-inflammatory and pro-inflammatory cytokines, and the authors propose they may be essential to maintaining the cytokine balance required at the fetal-maternal interface (Houser et al., 2011).

Initially, the presence of macrophages at the maternal-fetal interface was thought to be a response to the immune recognition of paternal antigens. They have also been proposed to assist with tissue remodelling processes necessary to facilitate growth of extraembryonic tissue (Hunt, 1989). Abrahams et al. (2004) propose an essential role for macrophages in trophoblast invasion and spiral artery remodelling, and subsequent studies have given strong evidence to support the former (Fest et al., 2007). Indeed, studies have implicated macrophages in having the capability to impact all stages of the implantation cascade, and the discussion to follow will consider these studies in detail.

*1.4.3.3.1 Macrophage regulation of blastocyst apposition and adhesion*

Uterine epithelial cells express a variety of adhesion and anti-adhesion molecules on their surface, and this expression is regulated to provide a barrier to the developing embryo until the onset of uterine receptivity. At this time, embryo apposition with uterine epithelial cells precedes an attachment reaction, occurring between the luminal epithelium and the trophectoderm and causing apoptosis of the underlying epithelial cells in the mouse. In the human, it is suggested that the epithelium is displaced, but there is evidence that epithelial cell apoptosis may occur to enable the embryo to gain access to the underlying stroma. This attachment reaction occurs in the evening of day 4 pc in the mouse. Some molecules that provide a means of attachment are H type 1 and Lewis blood group antigens, and the integrin family of surface proteoglycans, for which ligands are expressed on the blastocyst. $E_2$ and $P_4$ are known to upregulate their expression on the luminal epithelium (Aplin, 1997, Lessey et al., 1996, Aplin and Kimber, 2004, Das et al., 1997b, Aplin, 2000).

*In vitro* studies conducted by Kosaka et al. (2003) suggest that endometrial epithelial cell receptivity in the human may be regulated by immune cells. Further, a study by Ideta et al. (2010) showed that uterine administration of autologous peripheral blood mononuclear cells in the bovine, significantly improved the pregnancy rate following subsequent embryo transfer. These studies support the hypothesis that macrophages, due to their intimate juxtaposition with luminal epithelial cells, could influence epithelial cell function and receptivity through a paracrine mechanism (Jasper et al., 2011).

There are a myriad of molecules implicated in promoting embryo adhesion in the receptive uterus, or preventing inappropriate attachment outside the window of implantation (Aplin, 1997, Aplin and Kimber, 2004). This review will focus on some whose expression may be regulated by macrophages.

1.4.3.3.1.1 Mucins

The uterine luminal epithelium contains a glycocalyx, of which the cell surface mucin MUC1 is a component. The purpose of the glycocalyx is to provide a barrier to inhibit cell adhesion by steric hindrance, but enable the diffusion of small molecules. Proteolytic cleavage of mucins is necessary to enable embryo apposition and attachment to the luminal epithelium. In mice and rats, MUC1 is downregulated at the time of implantation, under the control of maternal steroid hormones. MUC1 in the human appears to behave differently, with high expression throughout the receptive phase, although there are changes in its glycosylation. *In vitro* studies with human tissue have revealed that MUC1 disappears locally from cells surrounding the embryo following attachment. MUC4 also appears

to be down regulated in the rat (Aplin and Kimber, 2004). Macrophages may play a role in the regulation of anti-adhesion molecules, and this is supported by studies showing that coculture of human endometrial epithelial cell lines with human monocytes (U937 cells) caused the down regulation of *MUC4* mRNA expression, however, *MUC1* mRNA expression is upregulated (Nakamura et al., 2008, Robertson et al., 2008).

#### 1.4.3.3.1.2 Fucosyltransferases

As mentioned previously, matings in the absence of a seminal fluid stimulus results in reduced uterine macrophage recruitment. Studies by Jasper et al. (2011) utilised $SV^-$ males mated with untreated females to determine the impact of seminal fluid and reduced macrophage numbers on the expression of enzymes involved in the synthesis of adhesive glycoconjugates and anti-adhesion molecules. The study found a significant decrease in mRNA expression of the gene encoding fucosyltransferase-2 (*Fut2)*. To determine whether changes in fucosyltransferase expression may be due to the absence of the seminal fluid stimulus or reduced macrophage recruitment, an *in vitro* uterine epithelial cell-macrophage coculture system was assembled, where thioglycollate-elicited peritoneal macrophages were cultured with uterine epithelial cells. The expression of mRNAs encoding fucosyltransferases was evaluated. There was a significant increase in *Fut1* following 48 h coculture, and *Fut2* and *Fut4* mRNA expression was increased significantly following 24 h and 48 h coculture. Culture of epithelial cells with macrophage conditioned media (MCM) replicated the stimulatory effects of macrophage coculture on the mRNA expression of *Fut2* and *Fut4*. It was determined that these stimulatory effects of macrophages on *Fut2* mRNA expression could be elicited by the macrophage-derived cytokines LIF, IL1B and IL12. Neutralising antibodies to both LIF and IL1B used in combination substantially reduced the expression of *Fut2* mRNA, with expression being similar to that seen in control medium. However, this study did not find any changes in the expression of *Muc1* or *Muc4* mRNA in response to seminal fluid, or epithelial cell coculture with macrophages or MCM. It is possible that macrophage regulation of these factors is different in the mouse and human.

#### 1.4.3.3.1.3 IL1

IL1B can be produced by uterine macrophages (Pioli et al., 2006), has been immunolocalised to the endometrial stroma and endothelial cells (Simón et al., 1993), and is induced following mating and in response to $E_2$ and $P_4$ in mice (Wood et al., 1999). Another important mechanism in the embryo attachment and implantation may be the downstream events following activation of the IL1-receptor type I (IL1R tI) (Simon et al., 1994b). Signalling appears to occur exclusively via the type I receptor,

which binds both IL1A and IL1B. Blockade of the IL1R tI prevents many of the physiological responses associated with activation of this receptor by preventing signal transduction (Simon et al., 1998). Simon et al. (1998) have demonstrated that blocking the actions of IL1A and IL1B using the IL1R tI antagonist caused implantation failure in mice. *In vitro* culture experiments and embryo transfer experiments show that this implantation failure is not associated with any direct toxic effect of the receptor antagonist on developing embryos (Simon et al., 1994b, Simon et al., 1998). Indeed, the failure of implantation appears to be a result of down-regulation of the integrins α4, αv and β3 from the luminal epithelium of pregnant mice. In addition, plasma membrane microvilli do not transform as expected during the implantation window, retaining numerous long microvilli rather than a characteristically flattened surface with few low microvilli (Simon et al., 1998). These findings demonstrate another means by which macrophages and their secretory product may be important in implantation. However, since macrophages are not the only source of IL1B, these findings still need to be interpreted with care. In addition, transgenic mice lacking functional IL1R tI are reportedly fertile, even following the administration of the IL1R antagonist (Abbondanzo et al., 1996). This discrepancy is surprising and highlights the importance of other methods to validate the importance of individual factors, as there may be compensatory mechanisms in transgenic models to enable reproductive viability, that do not occur when pathways are blocked in wild-type animals.

#### *1.4.3.3.2 Macrophages and decidualisation*

The undifferentiated fibroblasts present within the endometrial stroma have the potential to differentiate into decidual cells (Finn, 1985). In order to provide the blastocyst with a vascular supply, stromal blood vessels undergo angiogenesis and increased permeability, while stromal fibroblasts differentiate into large decidual cells (Finn, 1985). In the human, decidualisation occurs during the luteal phase of every menstrual cycle, regardless of whether gamete fertilisation occurs. In the mouse decidualisation will not occur even in the right hormonal environment, without the presence of the correct stimulus at the surface of the luminal epithelium. This stimulus is the trophoblast but can be mimicked experimentally using oil droplets or by causing uterine trauma, enabling deciduomata formation (Finn, 1985).

The extensive remodelling occurring within the endometrial stroma during decidualisation is a process in which macrophages may be involved. This process requires the breakdown of existing matrix and deposition of new components. MMPs and their inhibitors, tissue inhibitors of metalloproteinases (TIMPs), are involved in the regulation of this process. Because macrophages are a source of MMPs (Goetzl et al., 1996), it is possible that they play an important role in the endometrial remodelling

associated with decidualisation (Curry and Osteen, 2003, Das et al., 1997b). DCs are important for the formation of the decidua, which fails following DC depletion in pregnant or artificially induced uteri (Plaks et al., 2008). Given the overlapping functions of DCs and macrophages, the role of macrophages in this process warrants further investigation.

*1.4.3.3.3 Macrophages and trophoblast invasion*

Following attachment to receptive endometrial epithelial cells, the embryo breaches the epithelium and the trophoblast cells gain access to the underlying stroma. The ability of macrophages to alter transport properties and barrier integrity of epithelial cells *in vitro* provides a means by which they may facilitate trophoblast breaching of the epithelial cell surface during implantation (Zareie et al., 1998).

Trophoblasts are invasive cells, bearing a striking resemblance to tumour cells with respect to their rapid proliferation, tissue invasion, and abilities to avoid destruction by immune cells. However, in a healthy pregnancy, the extent of invasion is limited, and macrophages may be involved in this process by secretion of growth inhibiting cytokines such as TNFA and TGFB. The latter has growth promoting effects on cells of mesenchymal origin, but inhibitory effects of cells of epithelial origin and lymphocytes (Hunt, 1989). This cytokine is also able inhibit trophoblast DNA synthesis, further emphasising a role in limiting the extent of trophoblast invasion, which if excessive, could be detrimental to maternal health. The inhibitory effects of TGFB on lymphocyte proliferation may also be important in protection of the semi-allograft conceptus by inhibiting anti-fetal lymphocyte activities (Hunt, 1989).

1.4.3.3.3.1 MMPs and trophoblast invasion

The invasion of trophoblast cells into the stroma and decidua are highly regulated processes occurring via remodelling of the ECM. Trophoblast cells are equipped with a plethora of proteases to enable them to invade the vessel walls. Included in their armoury of proteases are the MMPs, which have been shown to degrade basement membrane matrix components. Isolated first trimester trophoblasts or extravillus trophoblasts *in situ* have been shown to produce MMP1, 2, 9, 12 and 14 (also known as membrane-type-1-MMP (MT1MMP)) (Harris and Aplin, 2007). As mentioned above, macrophages are also sources of MMPs, including MMP1, 2, 3, 9, 7 (matrilysin) and 12 under the influence of cytokines, ECM and PGE2 (Goetzl et al., 1996). Macrophage derived MMPs degrade connective tissue in the course of remodelling (Goetzl et al., 1996), and may assist trophoblasts with invasion and remodelling that they perform in maternal spiral arteries (in the human) and stroma.

The expression of MMPs and their inhibitors are under strict spatial and temporal regulation. In the mouse, there is a marked upregulation of *Mmp2* mRNA in the subepithelial stroma between day 3 and 5 pc, coinciding with the pre- and peri-implantation period (Das et al., 1997b). In the rat, *Mmp2* is also induced in the pre-implantation period, and expression of *Mmp7* was shown to be highest just after mating (Zhao et al., 2002). A role for inflammatory cell recruitment following mating is supported by the finding that MMP2 expression is reduced in the implantation site following matings in golden hamsters that lacked their male accessory gland fluids (Chow et al., 2003).

#### 1.4.3.3.3.2 Macrophage inflammatory mediators and trophoblast invasion

As discussed previously, pregnancy is a Th2 dominated condition. However, the process of implantation and trophoblast invasion appears to require the release of pro-inflammatory stimuli (Fest et al., 2007, Wira et al., 2005). Suppression of this inflammatory response can result in implantation defects leading to placental insufficiency and altered fetal growth trajectories (Tremellen et al., 1998, Bromfield, 2006). This concurs with reports showing decreased expression of the pro-inflammatory cytokines IL1A and IL6 in secretory phase endometrium from women suffering from recurrent pregnancy loss (Jasper et al., 2007).

Bacteria and viruses are able to ascend from the lower female reproductive tract to the uterine lumen, and this can cause inflammation. It has been suggested that this ascending microflora may actually support implantation by triggering inflammation (Fest et al., 2007, Palmarini et al., 2001, Crane-Godreau and Wira, 2004). Trophoblast cells express receptors that recognise pathogen associated molecular patterns (PAMPs), namely toll-like receptors (TLRs), and are key players in the activation of the innate immune system. Activation of some of these trophoblast TLRs causes production of pro-inflammatory chemokines including CCL5, CCL2, IL8 and growth-related oncogene-alpha (GROA) which have strong chemoattractant properties and may recruit monocytes to implantation site.

Studies by Fest et al. (2007) have demonstrated that first trimester trophoblast cells induce monocyte migration and secretion of pro-inflammatory cytokines and chemokines. There was a significant increase in the secretion of the cytokines IL6, IL8, TNFA, and the chemokines GROA, CCL2, CCL4 and CCL5. This was demonstrated using an immortalised monocyte cell line (THP-1 cells) and with primary $CD14^+$ monocytes isolated from peripheral blood of healthy female volunteers.

#### *1.4.3.3.4 Macrophage removal of apoptotic cells*

Another proposed role for macrophages during implantation is in the removal of apoptotic trophoblasts (Abrahams et al., 2004). Apoptosis during implantation enables tissue remodelling of the maternal decidua and invasion of the developing embryo. It is a process that occurs in the trophoblast layer throughout gestation in uncomplicated pregnancies, suggesting that constant cell turnover at the implantation site is necessary for placental growth and function (Piacentini and Autuori, 1994, Smith et al., 1997a, Abrahams et al., 2004). The ability of macrophages to recognise and swiftly remove apoptotic cells is critical to the maintenance of homeostasis in the body. Macrophages have several receptors to enable this process (Savill et al., 2002, Henson et al., 2001). The removal of apoptotic cells occurs 'silently' in the absence of inflammation. In fact, the engulfment of apoptotic cells by phagocytosis elicits an active process with powerful anti-inflammatory and immunosuppressive effects, marked by the release of anti-inflammatory cytokines such as TGFB, PGE2 and IL10, and suppression of TNFA secretion, inducing them into a tolerogenic state (Savill et al., 2002, Voll et al., 1997, Abrahams et al., 2004).

If the removal of apoptotic cells is impaired due to failure of normal clearance mechanisms, or if the system is overwhelmed by excessive apoptosis (the local capacity for phagocyte-mediated clearance is exceeded), cells progress to a state of post-apoptotic (or secondary) necrosis, releasing pro-inflammatory stimuli that could spark a damaging immune response (Henson et al., 2001, Savill et al., 2002, Silva et al., 2008). In fact, pregnancies complicated by pre-eclampsia or intrauterine growth restriction (IUGR) have been show to involve higher levels of first trimester placental apoptosis, accompanied by insufficient trophoblast invasion (Allaire et al., 2000, DiFederico et al., 1999, Smith et al., 1997b). Failure to phagocytose apoptotic trophoblast could cause the cells to undergo secondary necrosis, which considering the semi-allogeneic nature of the placenta, is particularly concerning. Trophoblast cells contain proteins that are antigenically foreign to the maternal immune system. If these cells were to undergo secondary necrosis, mediating the release pro-inflammatory and pro-immunogenic intracellular contents, this could initiate an immunological response detrimental to fetal survival (Abrahams et al., 2004).

#### *1.4.3.3.5 Macrophages and spiral artery remodelling*

Uterine NK cells are thought to be the major leukocyte with roles in trophoblast invasion and human spiral artery remodelling (Hanna et al., 2006), although evidence suggests that macrophages may also

participate in this process (Smith et al., 2009). In the human, macrophages have been located near spiral arteries where they may assist the process of trophoblast invasion. Importantly, there are marked differences in the distribution of macrophages under pathological conditions such as pre-eclampsia (PE) (Kim et al., 2002, Reister et al., 2001). In healthy pregnancies, trophoblasts transform and infiltrate the wall of spiral arteries, while macrophages remain in the surrounding stroma. In PE pregnancy, macrophages surround the spiral arteries that trophoblasts fail to transform; the distribution of macrophages resembles a barrier between the trophoblast and the spiral artery (Abrahams et al., 2004). Abrahams et al. (2004) suggest that in healthy pregnancies, macrophages assume the role of support cells, assisting the invasive trophoblast cells. However, in pathological conditions, macrophages become a barrier to trophoblast cells, inhibiting their invasion and differentiation, and preventing spiral artery remodelling by initiating trophoblast cell apoptosis. This proposal, although speculative, highlights the importance of the microenvironment macrophages are exposed to in determining their function within tissues. The signals they receive and the environment they reside within will determine whether they are supportive of or detrimental to pregnancy.

## 1.5 Angiogenesis in the female reproductive tract

Angiogenesis is the formation of new blood vessels by endothelial cell proliferation and outgrowth of pre-existing vessels. The adult vascular endothelium is generally quiescent, except in cases of tissue injury or tumour growth. The female reproductive tract is a notable exception, with the ovary, uterus and placenta undergoing dynamic, periodic growth and regression, with marked changes in blood flow to enable the supply of nutrients and hormone precursors required in the establishment of pregnancy. Based on tissue weight, the ovary, placenta and uterus receive some of the greatest blood flow rates, when functioning at full capacity, than any other tissue in the body (Reynolds et al., 1992, Fraser and Lunn, 2000).

### 1.5.1 Ovary

In the ovary, intense angiogenesis occurs during follicular development, ovulation, and during corpus luteum formation. Primordial and primary follicles receive nutrients by passive diffusion, but at the antral stage when the follicle increases in size, follicular angiogenesis begins, coinciding with the development of the thecal layer (Fraser and Lunn, 2000, Fraser, 2006). This capillary network expands with the rapidly growing follicle, and remains confined to the thecal layer until breakdown of the basement membrane occurs at ovulation (Fraser, 2006). It is interesting to note the parallels, with macrophages also being localised within the thecal layer surrounding developing follicles, and they

also are not associated with primary follicles (Wu et al., 2004). It is thought that follicular angiogenesis may control dominant follicle selection, as well as follicle atresia. The pro-angiogenic vascular endothelial growth factor (VEGF) family consists of VEGFA (often referred to as VEGF), B, C, FIGF (VEGFD) and platelet-derived growth factor (PDGF). VEGFA is the predominant pro-angiogenic factor, acting through the tyrosine kinase receptors KDR (VEGFR2) and FLT1 (VEGFR1). Typically, *VEGF* mRNA is expressed in the granulosa and thecal cells in secondary follicles, but is not expressed prior to this stage. Studies in the marmoset, where the actions of VEGFA are blocked by various inhibitors (reviewed, (Fraser, 2006, Fraser and Lunn, 2000) have revealed that this factor is essential for follicular growth beyond the small antral stage. Studies in the rat (Ferrara et al., 1998) showed that blocking the actions of VEGFA prior to gonadotropin treatment caused the ovaries to have only small antral follicles and few corpora lutea; the corpora lutea appeared avascular with central ischemic necrosis (Fraser and Lunn, 2000).

As described previously, ovulation is followed by the rapid development of the corpus luteum. A mature corpus luteum is comprised of 50% endothelial cells, and it receives the greatest supply of blood compared to other compartments of the ovary. Blood flow within the ovary is highly correlated with $P_4$ secretion (Reynolds et al., 1992). Numerous studies have shown that the actions of VEGFA are also essential for function of the corpus luteum in the rat (Ferrara et al., 1998) and in the primate (reviewed, (Fraser and Lunn, 2000).

#### *1.5.1.1 Macrophages and ovarian angiogenesis*

Evidence supports a role for macrophages in the supply of trophic factors that support blood vessel growth, including VEGF, EGF and bFGF (Sunderkotter et al., 1994). Thus, as the follicle grows and its demand for nutrients and substrate escalates to enable enhanced steroidogenic activity, macrophages may support the angiogenic processes that accompany this follicular growth. Following ovulation, macrophages are spread throughout the corpus luteum amongst the endothelial cells. The intimate juxtaposition of macrophages with endothelial cells in the thecal layer of developing follicles, and within the corpus luteum, coupled with their known ability to secrete pro-angiogenic factors, makes it tempting to speculate that these cells may play important roles in ovarian angiogenesis and vascular support.

### 1.5.2 Uterus

The growth and regression of blood vessels that occur in the endometrium of primates is regulated by $E_2$ and $P_4$. Growth of the endometrial vasculature begins in the follicular phase, and continues throughout the luteal phase of the menstrual cycle (Rogers et al., 2009, Reynolds et al., 1992).

Increased uterine vascular permeability and angiogenesis are hallmark events in implantation and decidualisation (Matsumoto and Sato, 2006). The primary purpose of these events is the development of new blood vessels to establish a placental blood supply. Placental vascular growth begins early in pregnancy, and continues throughout gestation to keep up with the increase in blood flow required to satisfy the metabolic demands of the developing fetus. Inadequate development of placental vasculature may contribute to clinical conditions such as embryonic wastage and low birth weight (Reynolds et al., 1992).

In the rodent, $E_2$ is responsible for this increase in vascular permeability, leading to stromal oedema and a marked increase in uterine wet weight. This transpires at sites where the initial attachment reaction between the uterine epithelium and the blastocyst occurs, followed by stromal cell decidualisation and angiogenesis. Expression of VEGF and its cognate receptors is spatially and temporally regulated in the rabbit, rat and mouse uterus (Das et al., 1997a, Rabbani and Rogers, 2001, Chakraborty et al., 1995). The actions of VEGF are critical for early pregnancy success; blocking the actions of VEGF during the peri-implantation period using VEGF antiserum caused implantation failure in mice (Rockwell et al., 2002)

It is suggested that the blocking of endometrial angiogenesis in the mouse may be partially mediated by neutrophils. VEGF-containing neutrophils infiltrate the endometrium following a single $E_2$ injection. Preventing this neutrophil influx using anti-neutrophil serum or the GR1 (LY6G; lymphocyte antigen 6 complex, locus G) monoclonal antibody, partially blocks the endometrial endothelial cell proliferation normally seen in response to $E_2$ (Heryanto et al., 2003, Heryanto et al., 2004, Rogers et al., 2009). Heryenato et al. (2004) propose that this reduced proliferation is due to decreased VEGF, caused by inhibiting neutrophil migration, but they acknowledge that there are still VEGF-positive cells present after the induction of neutropenia.

$P_4$ is reported to stimulate endometrial endothelial cell proliferation, but this is only partially mediated by VEGF. Interestingly, priming with $E_2$ prior to injection of $P_4$ caused inhibition of $P_4$-mediated angiogenesis (Rogers et al., 2009).

#### *1.5.2.1 Macrophages and uterine angiogenesis*

As described previously, macrophages are a known source of VEGF (Sunderkotter et al., 1994) and VEGF is expressed by unidentified stromal cells throughout the endometrium and adjacent to blood vessels, with a similar distribution to that reported for macrophages (Shweiki et al., 1993, Halder et al., 2000). In addition macrophages produce other angiogenic factors and agents that influence vascular permeability, including IL1, IL6, TNFA, bFGF, and EGF (Yoshida et al., 1997).

## 1.6 Lymphangiogenesis in the female reproductive tract

The primary function of the lymphatic system is the return of protein-rich fluid back to the circulating blood, following exudation out of capillaries and into interstitial space due to blood pressure. Importantly, this system is also responsible for the trafficking of immune cells to lymph nodes from the periphery. Lymphangiogenesis is the formation of new blood vessels. Lymphatic development requires the actions of VEGFC and FIGF, acting through the FLT4 (VEGFR3)(Alitalo et al., 2005, Oliver, 2004)).

### 1.6.1 Ovary

In the macaque, lymphatic vessels have been identified within the ovarian stroma and thecal layer of preovulatory follicles, and in the corpus luteum (Xu and Stouffer, 2009). Staining intensity of LYVE1 (lymphatic vessel endothelial hyaluronan receptor 1; a lymphatic vessel specific marker) increased from the early to mid stage within the corpus luteum, where it remained elevated thereafter. Expression of *VEGFC*, *FIGF* and *FLT4* mRNA were detected by RT- PCR, with the levels expression of *VEGFC* and *FLT4* correlating with the immunohistochemistry results and increasing from the early to mid-luteal phase, but then decreased in the late-luteal phase. *FIGF* mRNA expression was elevated in the early-luteal phase, declined during the mid-luteal phase, before increasing again in the late-luteal phase (Xu and Stouffer, 2009).

Lymphatic supply to the ovary appears to be important for fertility. ADAMTS1 (a disintegrin-like and metallopeptidase (reprosylin type 1) with thrombospondin type 1, motif 1) is an extracellular protease that has been detected in the rodent ovary, and *Adamts1* null mutant mice are subfertile. These mice display impaired follicular development, ovulatory disruption, and the prepubertal mouse ovary lacks

normal lymphatic development. At postnatal day 10 and 21, there is a complete absence of lymphatic vessels in *Adamts1* null mutant mice, as determined by LYVE1 immunohistochemistry, compared to a complex lymphatic structure in heterozygous mice (Brown et al., 2006). The postnatal development of the ovarian lymphatic vasculature was found to require the presence and hormonal regulation of the lymphangiogenic growth factors *Vegfc* and *Figf*, and their receptor *Flt4* (Brown et al., 2010).

The lymphatic system appears to be important in the transport of hormones, with $P_4$, $E_2$ and inhibin being measured in ovarian lymph. It is proposed that these hormones are transferred back into circulation in the blood by retrograde transfer (Lindner et al., 1964, Stefanczyk-Krzymowska and Krzymowski, 2002, Stefanczyk-Krzymowska et al., 2002, Findlay et al., 1986). Studies in the ewe have revealed a large increase in lymph flow from the ovary when a functional corpus luteum is present (Morris and Sass, 1966, Hein et al., 1988). The lymphatics in the ewe are small during the follicular phase, but after ovulation and coincident with the development of the corpus luteum, there is a large volume of lymph that forms in the ovary and this flow remains high throughout the luteal phase (Lindner et al., 1964), indicating cyclic remodelling of lymphatic vasculature. It has been documented that the mean concentration of $P_4$ in ovarian lymph is 1000-fold higher than that detected in jugular vein plasma (Lindner et al., 1964, Staples et al., 1982, Hein et al., 1988).

The finding that higher $P_4$ concentrations in ovarian lymph compared to ovarian venous blood suggests that $P_4$ transport by ovarian lymph is quantitatively more important than transport via ovarian blood, and that ovarian lymph is an important source of $P_4$ in peripheral plasma (Hein et al., 1988). Another reason for the high concentration of $P_4$ in ovarian lymphatic is likely to be due to the high permeability of blood capillaries within corpora lutea, especially during their early formation, with the leakage of protein from blood to the lymph being their highest in the per unit weight of the luteal ovary of the ewe than in any other organ examined (Morris and Sass, 1966). This research suggests that ovarian lymphatics are important for ovarian function, and are important carriers of $P_4$ during the luteal phase.

### 1.6.2 Uterus

The myometrium contains a network of lymphatic vessels (Ueki, 1991, Uchino et al., 1987), but reports on the distribution of these vessels in the endometrium are conflicting. Some studies report the presence of lymphatic vessels in the functional zone (Blackwell and Fraser, 1981), while others report that they were only detected in the endometrial basalis (Uchino et al., 1987). Donoghue et al. (2007) report that lymphatic vessels in the functional zone were sparsely distributed, but in the basalis the

vessels were larger and closely associated with spiral arterioles (Rogers et al., 2009). As described, lymphatic vessels play important roles in maintaining fluid balance and immunosurveillance, and evidence indicates a role for macrophages in lymphangiogenesis.

### 1.6.3 Macrophages and ovarian and uterine lymphangiogenesis

CD11B-positive macrophages have been implicated in inflammatory lymphangiogenesis in the mouse cornea (Maruyama et al., 2005), and thus may play a role in the development of uterine and ovarian lymphatics, in particular after ovulation and coitus, which are inflammatory event marked by a large influx of macrophages and other leukocytes (Espey, 1980, Robertson et al., 1998). It is proposed that macrophages may assist in the process of lymphangiogenesis by transdifferentiating and incorporating into the endothelial layer, or by stimulating the growth of pre-existing lymphatic endothelial cells through their ability to express pro-lymphangiogenic growth factors, including VEGFC and FIGF. In addition, macrophages have been shown to express FLT4, and *in vitro* studies show that VEGFC induces macrophage chemotaxis (Kerjaschki, 2005, Maruyama et al., 2005, Granata et al., 2010, Cursiefen et al., 2004, Schoppmann et al., 2002).

## 1.7 ANIMAL MODELS TO ASSESS MACROPHAGE FUNCTION

A number of models have given a valuable insight into the potential role of macrophages in reproductive processes, as discussed previously. However, these models are insufficient to determine the functional role played by the macrophage, due to either incomplete macrophage depletion (Clodronate liposomes (Van der Hoek et al., 2000), matings with $SV^{-}$ males (Robertson et al., 1996a) and $Csf1^{op}Csf1^{op}$ mice (Pollard et al., 1991, Robertson et al., 1998)), alterations in macrophage phenotype ($Csf2^{-/-}$ mice (Jasper et al., 2000)), or problems with feedback regulation of the HPG axis ($Csf1^{op}Csf1^{op}$ mouse (Cohen et al., 2002)). The fact that macrophages are still present in the aforementioned models makes it extremely difficult to differentiate the precise role that macrophages are playing amongst the intricate network of cell interactions and secretory products.

More recently, the *Cd11b-Dtr* mouse was created (Duffield et al., 2005a), providing a new means of assessing the importance of macrophages in early pregnancy. This model exploits the fact that murine diphtheria toxin receptor (DTR) binds diphtheria toxin (DT) poorly, but expression of the simian *DTR* under the macrophage specific promoter, *Cd11b*, renders CD11B-expressing macrophages sensitive to DT (Saito et al., 2001, Glenny and Allen, 1922). Studies utilising these mice may provide a more conclusive insight into the important role that macrophages might play in reproductive processes.

## 1.8 SUMMARY, HYPOTHESES AND AIMS

There is substantial evidence showing macrophages are in close proximity to target cells during various reproductive events, including folliculogenesis, ovulation, luteinisation, insemination, implantation and trophoblast invasion. However, there is little understanding of the mechanism by which they exhibit their proposed roles. This is largely due to the absence of models than enable sufficient macrophage depletion without other confounding effects. The known and proposed roles of macrophages are summarised in Figure 1.1.

The experiments described in this thesis aim to address the following hypotheses:

- Macrophages play an essential role in the female reproductive tract during early pregnancy
- Macrophages have a key role in endometrial tissue remodelling in the estrous cycle and during early pregnancy to promote implantation success and optimal pregnancy outcome
- Macrophages have a key role in the development and function of the corpus luteum in early pregnancy

The experiments described in this thesis will address the following aims:

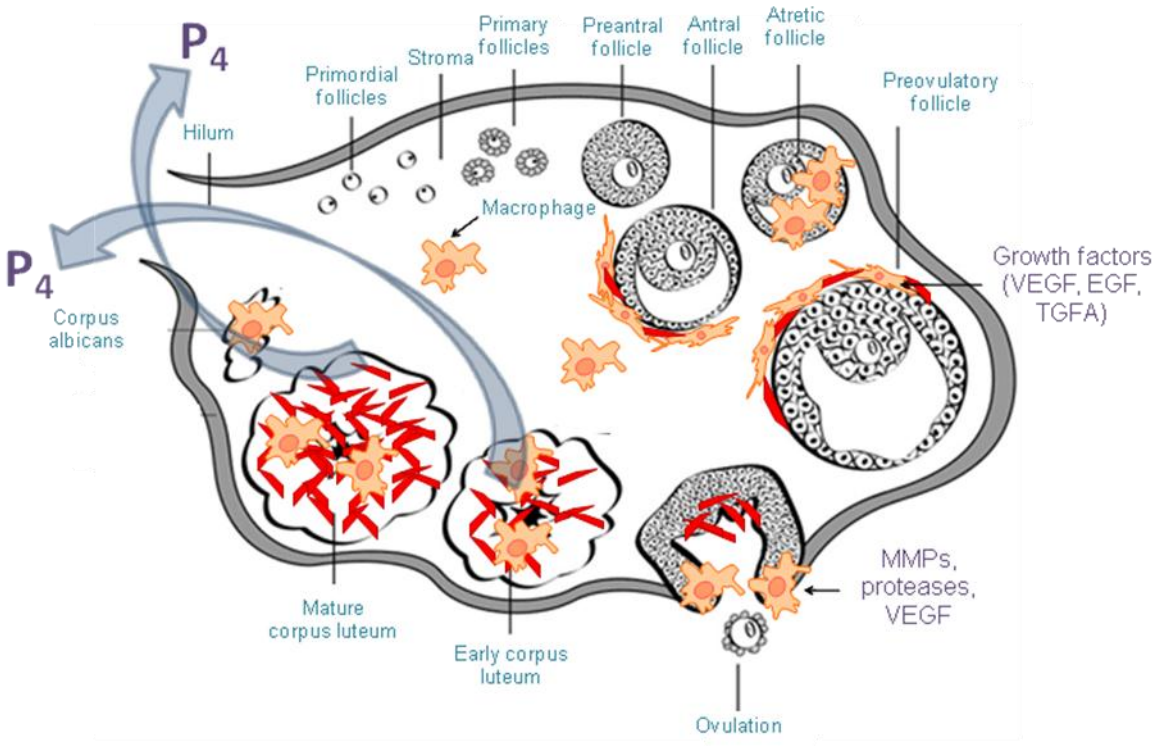
- To characterise the extent of macrophage depletion from female reproductive tissues using the *Cd11b-Dtr* mouse model
- To examine the effect of endometrial macrophage depletion on cyclic endometrial tissue remodelling processes associated with receptivity for embryo implantation
- To examine the impact of macrophage depletion in ovarian function in early pregnancy

**Figure 1.1 Current understanding of macrophage involvement in ovarian function, endometrial remodelling and embryo implantation**

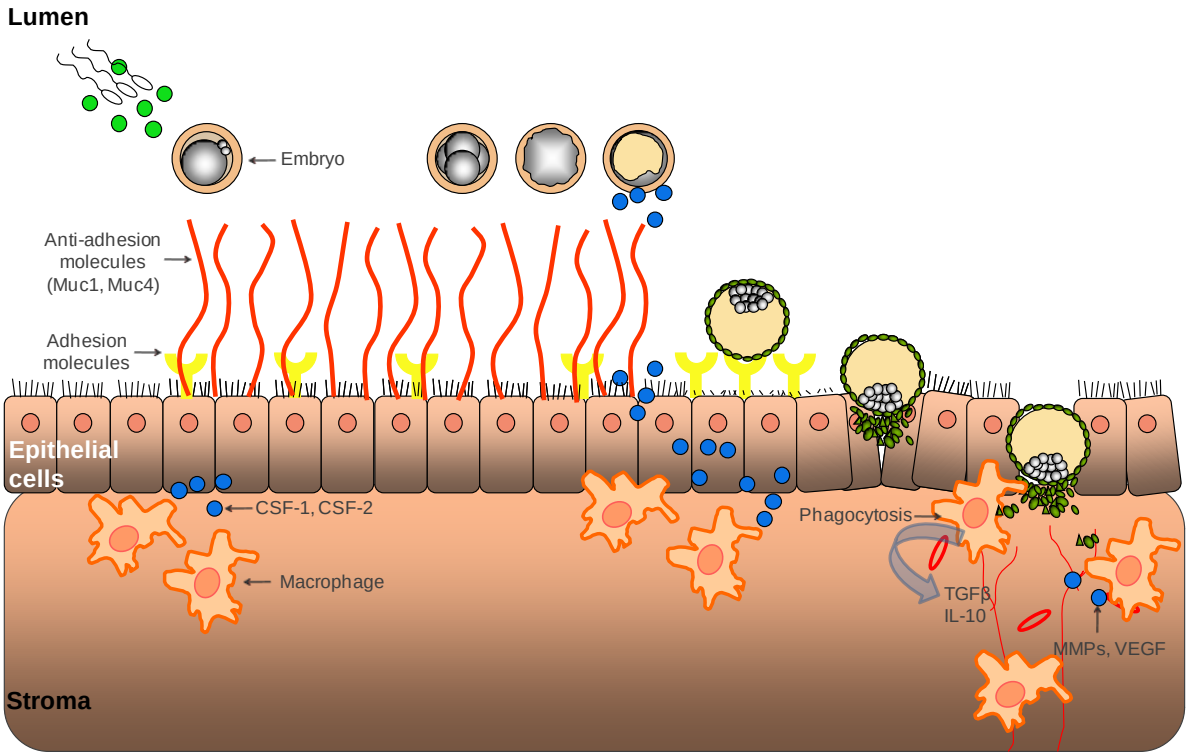
A. Macrophages are abundant in the ovary, where they surround developing follicles and may enhance follicular growth by their secretion of growth factors and cytokines, and their remodelling capabilities. Macrophages are also a source of VEGF and may provide trophic support to enhance the development of the essential blood supply required to support the advanced stages follicular development. Macrophages localise at the apex of the preovulatory follicle, and through their secretion of MMPs, proteases and pro-inflammatory cytokines, are thought to contribute to the process of follicle rupture and ovulation. Following ovulation, macrophages are abundant in the blood filled antrum as endothelial cell angiogenesis begins; macrophages may be essential to the extensive remodelling that occurs during corpus luteum formation, and may provide trophic support to enhance angiogenesis. Macrophages may also enhance the steroidogenic output of the functional corpus luteum. Macrophage numbers increase in luteolytic corpora lutea and atretic follicles, where again, they may be involved in tissue remodelling associated with these processes, and may scavenge apoptotic cells.
Image used with permission and adapted from (Buse et al., 2008)

B. Macrophages are abundant in the cycling and pregnant uterus. Their numbers fluctuate in response to hormonal milieu during the estrous or menstrual cycle. In concert with the actions of $E_2$ and $P_4$, macrophages act to facilitate endometrial remodelling and embryo implantation. $E_2$, produced by the ovary at estrus causes endometrial epithelial cells to secrete CSF1, CSF2 and chemokines, causing macrophage recruitment into the endometrial stroma. Factors in the seminal plasma cause further macrophage recruitment following coitus. Macrophage cytokines including LIF and IL1B, may cause the upregulation of adhesion molecules on the epithelial cell surface via their actions of *Fut2* expression. Unknown macrophage secretory products could possibly act to down regulate MUC1 and MUC4 from the glycocalyx. In combination with signals secreted from the embryo and epithelial cells, macrophages may act to facilitate the attachment reaction. Macrophage derived MMPs and VEGF are thought to facilitate endometrial stromal remodelling to enable the formation of the decidua. Trophoblast cells secrete factors to recruit macrophages to the implantation site. Macrophages may assist the trophoblast cells to invade and remodel uterine arteries by secreting VEGF and MMPs. The swift removal of apoptotic trophoblasts by macrophages could prevent activation of conceptus-reactive T cells. The process of phagocytosis causes macrophages to secrete anti-inflammatory Th2 cytokines such as TGFB and IL10. Macrophages may work to sustain the delicate balance between pro-inflammatory processes to enable implantation, and anti-inflammatory processes to prevent a detrimental immune response.

A


P4
Primordial follicles
Stroma
Primary follicles
Preantral follicle
Antral follicle
Atretic follicle
Preovulatory follicle
Hilum
Macrophage
P4
Corpus albicans
Growth factors (VEGF, EGF, TGFA)
MMPs, proteases, VEGF
Mature corpus luteum
Early corpus luteum
Ovulation

B


Lumen
Embryo
Anti-adhesion molecules (Muc1, Muc4)
Adhesion molecules
Epithelial cells
CSF-1, CSF-2
Macrophage
Phagocytosis
TGFβ
IL-10
MMPs, VEGF
Stroma

# Chapter 2

# Materials and Methods

## 2.1 ANIMALS AND SURGERIES

### 2.1.1 Mice

All mice used in these studies were bred and housed in the University of Adelaide Laboratory Animal Services at the Medical School Animal House in a specific pathogen-free facility. Food and water were provided *ad libitum* and a 12:12 h light-dark cycle was maintained within the enclosure. Animals were maintained and used in experiments under the NHMRC Australian Code of Practice for the care and use of animals for scientific purposes.

All female mice were virgin prior to the onset of any experiment. All experiments involving cycling or pregnant mice utilised females between 6 and 12 weeks of age at the time of treatment. For all experiments involving bone-marrow transplanted mice, females were approximately 5 weeks of age when irradiation and bone-marrow transplant procedures were performed.

*Cd11b*-diphtheria toxin receptor [*Dtr*; *Hbegf* (heparin-binding EGF-like growth factor)] mice [Tg(ITGAM-DTR/EGFP)34Lan] (Duffield et al., 2005a) carry a transgene containing a fusion product of the simian DTR and green fluorescent protein under the control of the CD11B (ITGAM; integrin alpha M) promoter. Mice are on the FVB/NJ background and were kindly provided by Professor Richard Lang University of Cincinnati, Cincinnati, OH, USA). Administration of DT to *Cd11b-Dtr* mice causes acute and systemic ablation of CD11B-expressing macrophages while administration of DT to FVB/NJ control animals has no effect (Saito et al., 2001, Glenny and Allen, 1922). This is because murine DTR binds DT with low affinity, but mice can be rendered sensitive to DT by the transgenic expression of the simian DTR, which binds DT with high affinity.

### 2.1.2 General Procedures

All surgical instruments were sterilised by autoclaving, or were submerged in 70% ethanol (ANALAR, Melbourne, Australia) prior to use. For anaesthesia, mice received an intra-peritoneal (i.p.) injection of 15 µl/g body weight of 2% Avertin (tribromoethanol; Sigma-Aldrich, St. Louis, USA), or alternatively, anaesthesia was induced using fluorothane (Veterinary Companies of Australia, Sydney, Australia). Surgical incisions were closed using 9mm MikRon® wound clips (Becton Dickinson, Sparks, USA), which were removed 2 weeks following surgery. Following anaesthesia, mice were kept on a 37$^{o}$C heating pad until the following morning. When mice regained consciousness, they were injected

subcutaneously with buprenorphine analgesia (Temgesic; Intervet, Schering-Plough, Victoria, Australia). Buprenorphine was administered at 0.8 µg/10 g of body weight.

### 2.1.3 Bone Marrow Chimera protocol

Donor bone marrow was harvested from the tibia and femur of *Cd11b-Dtr* male or female mice and kept on ice in RPMI-1640 Medium (JRH Biosciences, Lenexa, USA). Cells were centrifuged (300xg, 10 min, 4$^{o}$C) and red blood cells (RBC) were removed by flash-lysis; cells were resuspended in 900 µl of Milli-Q water (Millipore, MA, USA) before immediately adding 100 µl of 10X phosphate buffered saline (PBS, Dulbecco's $Mg^{2+}$ $Ca^{2+}$ free). Cells were resuspended in RPMI at a concentration of 5 x $10^6$ cells/200 µl medium (25 x $10^6$ cells/ml). Wild-type FVB/NJ female mice at 5 weeks of age were irradiated with 900 cGy using a linear accelerator at the Royal Adelaide Hospital, Adelaide. Irradiated mice received 5 x $10^6$ bone marrow cells from *Cd11b-Dtr* mice by an i.v. injection in the lateral tail vein on the day of irradiation (to subsequently be referred to as *Cd11b-Dtr*-BM mice). *Cd11b-Dtr*-BM mice were left to recover for 6 weeks before ovariectomy, and two weeks following surgery, experimental protocols began. This allowed for at least 8 weeks for complete bone marrow reconstitution. Peritoneal cells from all *Cd11b-Dtr*-BM mice were analysed by flow cytometry at autopsy to ensure $EMR1^+$ and $CD11B^+$ cells were present in control mice, and depleted in DT-treated mice (section 2.2.7), to show that the BM transplant procedure had been successful.

### 2.1.4 Ovariectomy

*Cd11b-Dtr*-BM mice underwent a bilateral ovariectomy as described previously (Hogan et al., 1986, Olson and Bruce, 1986). Briefly, mice were anaesthetised under Fluorothane anaesthesia, shaved in the dorsal region, and swabbed with 70% ethanol. A small longitudinal dorsal incision was made, followed by a retroperitoneal incision above the ovarian fat pad. The ovary and associated fat were exteriorised and the oviduct was severed close to the uterotubal junction. The remaining reproductive tract tissue was returned to the peritoneal cavity and the procedure was repeated on the other side. The skin was closed using wound clips.

### 2.1.5 Induction of deciduoma

This procedure was conducted while mice were being treated with estradiol and progesterone to enable deciduoma formation (see section 2.5.4). Mice that underwent this procedure had previously undergone ovariectomy under Fluorothane anaesthesia. This procedure was performed under Avertin anaesthesia. This was to avoid the repeated use of Avertin which can be an irritant. Briefly, 2 to 3 weeks following the ovariectomy procedure, *Cd11b-Dtr*-BM were anaesthetised, and uteri were

accessed as described for ovariectomy (Section 2.1.4). The fat pad was clamped with tweezers, externalised, and held with a small surgical clamp the enable the left horn of the uterus to remain externalised. 10 µl of sesame oil (Sigma-Aldrich, St. Louis, USA) was injected at the proximal end of the uterine horn, at the uterotubal junction, using a 50 µl glass syringe (SGE Analytical Science, Victoria, Australia) and a 26 gauge needle (BD Biosciences, Franklin Lakes, USA), and care was taken to only handle the uterotubual junction with tweezers and not to traumatise the uterus. Following the injection of oil, the horn was replaced inside the peritoneal cavity. The same procedure was performed on the right horn, with insertion of a sterile needle only as a sham control. This procedure was performed with the aid of a dissection microscope. The skin was closed using wound clips.

### 2.1.6 Macrophage Depletion

*Cd11b-Dtr* or *Cd11b-Dtr*-BM mice received DT from *Corynebacterium diphtheria* (Sigma, St Louis, MO) to elicit macrophage depletion. Doses of 25 ng/g or 10 ng/g of body weight were utilised, and DT was diluted in PBS to obtain the appropriate dose, which was given as an i.p. injection. DT was made up fresh each day from 1 mg/ml aliquots and excess was discarded.

### 2.1.7 Estrous cycle tracking

Estrous cycles were tracked by cytological analysis of daily vaginal smears, as described previously (Allen, 1922, Bronson et al., 1966). Vaginal spears were conducted between 8:00 – 11:00 h. Briefly the vagina was flushed with the aid of a pipette tip containing 20 µl of PBS and the recovered fluid was viewed as a wet, unstained preparation on a microscope slide with a coverslip. Proestrus was indicated by the presence of bright, rounded epithelial cells, with few other cells being present and minimal leukocytes. Estrus was identified when epithelial cells had begun to cornify, but before there was a large number of clumped, cornified epithelial cells (Metestrus-1). Mice were tracked through one full cycle before being utilised for experiments.

### 2.1.8 Matings

Female mice were housed with a proven-fertile stud male (> 14 weeks of age) and checked each morning between 9:00 and 11:00 h for the presence of a vaginal copulatory plug (2:1 female to male ratio). The morning a plug was found was regarded as day 0.5 pc. If an experimental treatment was given to mice at or before 12:00 h, it was regarded as day 0.5 pc. If an experimental treatment was given at or after 12:00 h, it was regarded as day 1.0 pc, and this methodology was applicable for all subsequent days of pregnancy.

### 2.1.9 Identification of implantation sites

To identify implantation sites on the afternoon of day 4.5 pc or day 5.5 pc, before they are clearly visible to the naked eye, mice were anaesthetised with Avertin (Sigma-Aldrich) and then injected i.v. via the lateral tail vein with 0.4% trypan blue solution (Sigma-Aldrich) diluted 1:1 with PBS. After 10 min later, mice were sacrificed and the uteri were dissected and assessed for clearly delineated blue bands as evidence of early implantation sites.

### 2.1.10 Assessment of day 17.5 pc pregnancy outcomes

On day 17.5 pc (or earlier if mice were moribund) mice were killed by cervical dislocation between 10:00-12:00 h. The uterus from each female was removed, and the number of viable, dead and resorbing implants was counted. An implantation site included both viable and non-viable implantations. A pregnant mother was defined as a mother carrying implantation sites (either viable or non-viable). Each viable fetus was dissected from the amniotic sac and umbilical cord; fetal and placental weights were recorded.

### 2.1.11 Plasma collection

Approximately 1 ml of blood was collected by cardiac puncture from anaesthetised mice (Avertin; Sigma-Aldrich) using a 20G needle (BD Biosciences) containing approximately 50 µl (3 USP units) of heparin (300 USP units per vial of heparin sodium salt diluted in 5 ml PBS; Sigma-Aldrich). Blood was left at 4$^{o}$C for 1-2 h before being centrifuged at 1500xg for 10 min to recover serum. Plasma samples were stored at -80$^{o}$C for later analysis of estradiol and progesterone concentration (Section 2.7.1).

### 2.1.12 Estradiol for injection

17β-estradiol ($E_2$; Sigma-Aldrich) was dissolved in absolute ethanol and diluted in sesame oil (Sigma-Aldrich) to obtain the required concentration (500 ng/ml, 100 ng/ml or 1 µg/ml). Vehicle control was made where absolute ethanol only was diluted in sesame oil.

### 2.1.13 Progesterone for injection

To dissolve $P_4$ (Sigma-Aldrich) in absolute ethanol, it was made at a concentration of 100 mg/ml and heated to 56$^{o}$C for approximately 10 min. It was then diluted in sesame oil (Sigma-Aldrich) to obtain the required concentration. Vehicle control was made where absolute ethanol only was diluted in sesame oil at the same concentration.

## 2.2 FLOW CYTOMETRIC ANALYSIS FOR IDENTIFICATOIN AND QUANTIFICATION OF IMMUNE CELL POPULATIONS

Peritoneal exudate cells (PEC), uteri, ovaries and spleens were collected following cervical dislocation.

### 2.2.1 PEC collection

Peritoneal exudate cells (PEC) were collected by peritoneal lavage using 5 ml of ice cold PBS containing 0.1% BSA (Albumin from bovine serum, Sigma) and 0.025% sodium azide [Sigma-Aldrich); fluorescence-activated cell sorting (FACS) buffer]. For quantification of total cell numbers, PEC were collected in 4 ml of FACS buffer, and 100 µl (2.5%) of this suspension was transferred into a separate tube (see section 2.2.6).

### 2.2.2 Uteri collection

Uteri were trimmed of excess fat and slit longitudinally using spring-loaded scissors and chopped into small pieces prior to being enzymatically digested into a single cell suspension for 1 h at 37$^{o}$C [RPMI containing 10% FBS, 0.015M HEPES (JRH Biosciences), and 2000 U/ml Type1A Collagenase from *Clostridium histolyticum* (Sigma-Aldrich) and 25 ug/ml DNase 1 (Sigma-Aldrich)]. The cell suspension was mixed every 15 min during the digestion. Cells were passed through a 70 µm nylon strainer (BD Falcon, Bedford, USA) before being washed in FACS buffer, centrifuged (300xg, 5 min, 4$^{o}$C) and prepared for flow cytometric analysis.

### 2.2.3 Ovary collection

Both ovaries were collected and the oviduct was removed with the aid of a dissection microscope. Ovaries were chopped into small pieces and enzymatically digested as described previously (see section 2.2.2).

### 2.2.4 Spleen collection

Each spleen was harvested and made into single cell suspensions by homogenising the tissue; this was achieved by gently grinding the tissue between the frosted ends of two glass slides. Liberated cells were suspended in 1 ml of 1 x RBC lysis buffer (0.155 M $NH_4Cl$, 10 mM $KHCO_3$, 99.2 µM EDTA disodium salt in RO water). For quantification of total cell numbers, 100 µl (10%) of this cell suspension was transferred into a separate tube (see section 2.2.6).

### 2.2.5 Labelling of single cell suspensions

Single cell suspensions of PEC, from uteri, from ovaries, and splenocytes were washed with 2 ml of FACS buffer and centrifuged (300xg, 5 min, 4$^{o}$C). Fc Receptors were blocked by resuspending cells in 50 µl of FACS buffer containing 0.5 µg anti-Fc-γIIR antibody (FcBlock, BD Pharmingen, BD Biosciences, San Diego, USA) for 10 min at 4$^{o}$C. Surface markers were labelled by adding 50 µl of FACS buffer containing 0.5 ug each of Alexa Fluor® 488 conjugated anti-EMR1 antibody (F4/80; BioLegend, San Diego, USA), allophycocyanin (APC) conjugated CD11B, Alexa Fluor® 488 anti-CD11C, fluorescein isothiocyanate (FITC) conjugated anti-CD3, phycoethrin (PE)-Cy5.5 conjugated anti-RB6 (which detects LY6G/C), and PE-Cy7 conjugated anti-CD45 (all BD Pharmingen) for 30 min at 4$^{o}$C (Table 2.1). Cells were then washed twice with 2 ml of FACS buffer, followed by centrifugation. Data were analysed using FACS Canto (BD Biosciences, San Jose, USA) using FACS Diva software (version 6.0, BD Biosciences). Gates were applied to the forward scatter/side scatter dotplots to exclude cell debris. Propidium iodide (5 µl at 50 µg/ml to a 300 µl cell suspension; Sigma-Aldrich) was added to labelled cell suspensions prior to flow cytometry to allow exclusion of non-viable cells from analysis.

**Table 2.1 Monoclonal antibodies used in flow cytometric analyses**

| mAb | Conjugate | Clone | Isotype | Alternative names | Reactive lineages | Source |
|---|---|---|---|---|---|---|
| CD45 | PE-Cy7 | 30-F-11 | rat (IgG2b) | CD45 (LCA, Ly-5, T200) | pan leukocytes | BD Pharmingen |
| EMR1 | PE | BM8 | rat (IgG2a) | F4/80 | macrophages, eosinophils | eBioscience |
| | Alexa Fluor 488 | BM8 | rat (IgG2a) | | | BioLegend |
| CD11B | APC | M1/70 | rat (IgG2b) | Mac-1, ITGAM, Integrin αM | monocytes, granulocytes, NK cells | eBioscience |
| CD11C | Alexa Fluor 488 | N418 | rat (IgG) | ITGAX, Integrin αX | DCs, subset IEL, some activated T cells | eBioscience |
| CD3 | FITC | 17A2 | rat (IgG2b) | CD3 Molecular Complex | thymocytes, mature T lymphocytes | BD Pharmingen |
| RB6-8C5 | PerCP-Cy5.5 | RB6-8C5 | rat(IgG2b) | Gr-1 | granulocytes, monocytes | BD Pharmingen |

IEL; intestinal intraepithelial leukocytes

### 2.2.6 Total cell number quantification

The total cell number of RB6-expressing cells was quantified as described previously (Guerin et al., 2011, Guerin, 2010). 100 µl (1/40$^{th}$) of the original PEC population, and 100 µl (1/10$^{th}$) of the original splenocyte population was incubated in 10 µl of FACS buffer containing FcBlock for 10 min at 4$^{o}$C. Cells were then incubated with 10 µl of FACS buffer containing FITC anti-CD3 for 30 min at 4$^{o}$C. The final volume was made up to 1 ml by the addition of 860 µl of FACS buffer, and 20 µl of CountBright Absolute Counting Beads (Molecular Probes, Invitrogen, Carlsbad, USA). Flow cytometry was performed, and 1000 CountBright Absolute Counting Beads were counted. The population of CD3$^{+}$ cells was determined based on FITC expression, and the total number of these cells was determined using the following equation:

PEC:

Total number of CD3$^{+}$ cells = Number of CD3$^{+}$ cells/1000 * total number of beads added * 40

Spleen:

Total number of CD3$^{+}$ cells = Number of CD3$^{+}$ cells/1000 * total number of beads added * 10

From this, the total number of neutrophils (RB6$^{+}$ cells) was determined based on their relative ratio to CD3$^{+}$ cells. CD3$^{+}$ cells were chosen as this population has been shown not to be altered following DT-elicited macrophage depletion (see Table 3.1; (Duffield et al., 2005a, Cailhier et al., 2005)).

### 2.2.7 Flow cytometry of PEC cells to ensure macrophage depletion

At autopsy, PEC were collected as described in section 2.2.1, Fc receptors were blocked as described in section 2.2.5, and cells were labelled with PE anti-EMR1 (eBioscience, San Diego, USA) and APC anti-CD11B (BD Pharmingen) to identify macrophage populations. This protocol was carried out following any experiment with wild-type and *Cd11b-Dtr* or *Cd11b-Dtr*-BM mice to confirm that DT treatment had resulted in macrophage depletion.

## 2.3 IMMUNOHISTOCHEMISTRY

### 2.3.1 Tissue collection, embedding and sectioning

#### *2.3.1.1 Fresh-Frozen Tissue*

Uteri or ovaries were collected from mice at autopsy and trimmed of excess fat and connective tissue. Tissue was then embedded in Tissue-Tek OCT compound (Sakura Fintek, Torrance, USA) and frozen

in nitrogen-cooled isopentane (Unilab, Ajax Finechem, Tarren Point, Australia), and stored at -80°C. Tissue was sectioned at 6 µm using a Leica CM 1850 cryostat (Leica Microsystems, NSW, Australia) and disposable microtome blades (Feather, Osaka, Japan) and SuperFrost Plus microscope slides (Menzel-Gläser, Braunschweig, Germany). Slides were allowed to dry for approximately 1 h before being stained or were stored desiccant (silica gel; Sigma Aldrich) at -80°C.

#### *2.3.1.2 Paraffin*

Uteri were collected and trimmed of excess fat and connective tissue, and fixed in 4% paraformaldehyde (Sigma-Aldrich) in PBS (pH 7.4) for 24 h at 4°C. Tissue was then washed three times in 1 x PBS over the following two days, and late on the second day, tissue was transferred to a 70% ethanol solution where it remained until tissue processing. Tissue was processed and embedded using the Leica TP1020 Tissue Processor (Leica Microsystems) involving the following dehydration and embedding protocol; 1 h 75% ethanol, 1 h 85% ethanol, 1 h 90% ethanol, 1 h 96% ethanol, 2 x 1 h absolute ethanol (ANALAR), 2 x 1 h 100% Xylene (Ajax Finechem), 2 x 1 h paraffin wax (Ajax Finechem) under vacuum conditions. Tissue was moulded immediately into wax blocks and stored for sectioning on a Leica Rotary Microtome (Leica Microsystems). Sections were cut into 6 µm sections and fixed onto SuperFrost Plus slides (Menzel-Gläser) using a 45°C water bath. Slides were dried overnight (O/N) at 37°C. Blocks and sections were stored at room temperature prior to staining.

##### *2.3.1.2.1 BrdU Incorporation*

Two hours prior to sacrifice, mice received an i.p. injection of 100 µl of 10 mg/ml BrdU (Sigma, St Louis, USA). To identify proliferating cells in paraffin embedded uterine tissue, the bromodeoxyuridine (BrdU) *In-Situ* Detection Kit (BD Pharmingen), containing a biotinylated anti-BrdU antibody, was used according to the manufacturer's instructions. Tissue was counterstained with haematoxylin, as described in section 2.3.2.3.

### 2.3.2 Immunohistochemistry Protocol

#### *2.3.2.1 Fresh frozen tissue*

##### *2.3.2.1.1 DAB detection*

Primary antibodies used with this protocol included supernatants from the EMR1 hybridoma (American Type Culture Collection) and the MTS-12 hybridoma, a kind gift from Richard Boyd (Monash University, Melbourne, Australia). Fresh frozen tissue sections were fixed in cold 96% ethanol (ANALAR) for 10

min at 4°C. Slides were then washed in PBS (3x5 min) and then blocked in PBS containing 1% BSA (PBS-BSA; Sigma; 1 x 5 min). All incubations were performed in a humidified chamber. Primary antibody was applied to sections in the afternoon either as neat supernatant containing 10% normal mouse serum (NMS, Sigma-Aldrich) or diluted in PBS-BSA with 10% NMS, and the slides incubated at 4°C O/N. The following morning, a biotinylated secondary antibody (Rabbit anti-rat IgG, biotinylated; Dako, Glostrup, Denmark) was diluted 1:400 in PBS-BSA containing 10% NMS and incubated for 2 h at RT. Slides were then incubated with avertin-horseradish peroxidase (HRP; Dako) and detection of bound antibody was performed using 3,3 diaminobenzadine (DAB; SigmaFast tablets, Sigma) according to the manufacturer's instructions. DAB was neutralised using a 4% sodium hypochlorite solution (Unilab, Ajax Finechem) prior to disposal. Negative controls were incubated as described above but with the primary antibody omitted. Tissue sections were counterstained with haematoxylin as described in section 2.3.3.3, before being dehydrated in 95% (5 min) and absolute ethanol (5 min), and cleared in 2 changes of Safsolv (Ajax Finechem) for 5 min. Slides where left to dry O/N before coverslips were mounted using DPX mountant (Merck, Darmstadt, Germany). Some tissue required endogenous peroxidase activity to be neutralised. To do this, tissue was incubated with 0.3% hydrogen peroxide (Sigma-Aldrich) in PBS for 10 min before the primary antibody was applied.

#### *2.3.2.1.2 Immunofluorescence*

Primary antibodies used with this protocol include the Alexa Fluor® 488 conjugated anti-EMR1 (diluted 1:50), Alexa Fluor® 647 conjugated anti-PECAM1 (diluted 1:100; both Biolegend, San Diego, CA), anti-LYVE1 rabbit polyclonal (diluted 1:100; Millipore Corporation, MA, USA), *Ulex europaeus agglutinin 1* (UEA-1; specific for Fucα1-2Gal) and *Lotus tetragonolobus purpureas* (LTP; specific for Fucα1-2Gal, Fucα1-3GlcNAc, and Fucα1-4GlcNaC; both Sigma and both used at 10 µg/ml), and rat anti-mouse LewisX [3-fucosyl-N-acetyl-lactosamine, CD15 (1:50 dilution; Santa Cruz Biotechnology, Santa Cruz, USA] . The protocol was performed as described in section 2.3.3.1.1, except primary antibodies were diluted in PBS-BSA containing 10% NMS and 10% normal rabbit serum (Sigma-Aldrich). All primary antibodies were incubated O/N at 4°C in a humidified chamber. LYVE1 was detected using Alexa Fluor® 594 goat-anti rabbit IgG (Invitrogen, Carlsbad, CA). Biotinylated lectins were detected by incubating slides with streptavidin-FITC (Dako, Glostrup, Denmark) diluted 1:40 in PBS-BSA plus 10% NMS for 40 min at 4°C. LewisX was detected by incubation with FITC conjugated anti-rat IgG (Dako) diluted 1:40 in PBS-BSA plus 10% NMS, for 30 min at 4°C. If a counterstain was required, slides were incubated with 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI dihydrochloride, Sigma, St Louis, USA) for 20 min. Slides were mounted with coverslips using

fluorescent mounting medium (Dako, Glostrup, Denmark) and were stored at 4°C in the dark until image capture.

#### *2.3.2.1.3 Alkaline phosphatase staining*

Fresh frozen tissue sections were fixed and washed as described in Section 2.3.2.1.1. A SigmaFast BCIP/NBT (5-Bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium; Sigma Aldrich) tablet was dissolved in Milli Q water and the tissue sections were covered in the solution. After 5-10 min, sections were washed with PBS and counterstained using Eosin (Sigma-Aldrich) that was diluted 1:2 in 70% ethanol. Tissue was dehydrated and coverslips applied as described in Section 2.3.2.1.1. BCIP/NBT acts as a precipitating substrate for the detection of alkaline phosphatase activity, whereby the development of a purple reaction product is indicative of alkaline phosphatase activity.

### *2.3.2.2 Paraffin embedded tissue*

The antibody used in this protocol was anti-EMR1 rat monoclonal antibody (1:50 dilution; Caltag Laboratories, Burlingame, USA). Tissue sections were dewaxed in Safsolv (Ajax Finechem) and rehydrated through graduated dilutions of ethanol for 5 min each (2 x 100%, 1 x 90% and 2 x 70% ethanol) followed by 1 x 5 min in MQ water, and 1 x 5 min in PBS. Endogenous peroxidase activity was quenched by incubating slides with freshly prepared 30% hydrogen peroxide (Sigma-Aldrich) solution in methanol (Sigma-Aldrich; solution contained 10 ml hydrogen peroxide, 100 ml methanol, 90 ml water) for 15 min. Slides were blocked with 15% NMS and 15% normal rabbit serum in PBS for 30 min at 37°C, before incubation with anti-EMR1 antibody (Caltag Laboratories) overnight at 4°C in a humidified chamber. Sections were then incubated with biotinylated secondary antibody at 1:100 for 40 min at RT (Biotinylated rabbit anti-rat IgG; Vector Laboratories) and ABC Elite kit (Vector Laboratories, Burlingame, USA). Sections were treated with streptavidin-conjugated HRP (Vectastain ABC kit; Vector Laboratories, Burlingame, USA), and detection was performed using DAB (SigmaFast tablets, Sigma) according to the manufacturer's instructions. Tissue sections were counterstained, as described in section 2.3.3.3, before being rehydrated through a graduated increase in ethanol concentration (the reverse of the dehydration protocol) and cleared in two changes of Safsolv (Ajax Finechem) for 5 min. The slides were dried O/N and then mounted with coverslips using DPX mountant (Merck, Darmstadt, Germany).

### *2.3.2.3 Haematoxylin counterstain*

Tissue sections were counterstained with haematoxylin (Sigma-Aldrich) for approximately 30 s, before rinsing in MQ water. Tissue sections were then dipped in 0.5% HCl for 5 s, and again rinsed in MQ

water. To enhance the blue counterstain, tissue was incubated in tap water (high $Ca^{2+}$ solution) for 2 min, and rinsed in MQ water.

#### *2.3.2.4 Haematoxylin and eosin stain*

Tissue sections were counterstained with haematoxylin and then stained in eosin (Sigma-Aldrich) diluted 1:2 in 70% ethanol for 30 s prior to dehydration, clearing and mounting, as described above.

#### *2.3.2.5 Image capture and cell quantification*

Stained tissue sections were captured as a digital image using a Nanozoomer 1.0 (Hamamatsu, Shizouka, Japan) at a zoom equivalent to either a 20x or 40x objective lens. Endometrial area and lumen perimeter were calculated via the manual tracing of these regions on the section image using NDP View software (Hamamatsu). Staining was quantified using the video image analysis software, Video Pro (Leading Edge Software, Adelaide, Australia). The area of positive staining was calculated as the percent positive staining (DAB) of the total stained (haematoxylin + DAB) area, expressed as a percentage. A minimum of two non-serial sections were assessed for each sample, and 2-6 fields were analysed per section.

To determine the percentage of endogenous peroxidase-positive eosinophils, slides were stained with DAB and haematoxylin only. The percent of DAB-positivity from these slides was subtracted from the total positivity detected for EMR1 staining.

To determine the percent of proliferating cells, individual nuclei in uterine luminal epithelium were counted using the Image J (NIH, Maryland, USA) cell counter function, where BrdU incorporated proliferating cells were counted as were the non-proliferating cells (haematoxylin stained). To determine the percent of proliferating stromal cells, online ImmunoRatio software (Institute of Biomedical Technology, University of Tampere, Finland; http://imtmicroscope.uta.fi/immunoratio/) was used, where images were uploaded and the size of the nuclei determined, and the number of DAB-positive (BrdU incorporated) nuclei and the number of haematoxylin-positive nuclei were counted, and the percentage of proliferating nuclei was calculated.

Image capture of LewisX stained luminal epithelium was collected using the Radiance 2100 laser scanning confocal microscope (Olympus and Biorad). Image capture of uterine luminal epithelium stained with the lectins UEA-1 or LTP, were collected using the Nikon C1 laser scanning confocal microscope (Olympus and Nikon). Using ImageJ software, the mean staining intensity (mean grey-scale value) was determined for the apical one-third of the luminal epithelial layer was defined for each section, and a mean value of 4-6 sections per uterus was calculated. All of the luminal epithelium for a

section was usually visible within the field. If this was not the case, the average staining intensity for the field was calculated as follows:
The mean fluorescent intensity (MFI) for each field was multiplied by the area per field. This was then divided by the total area of all fields, to obtain an average MFI for the section. Images of various uterine immune cells in uteri of *Cd11b-Dtr* mice were collected using the Nikon C1 laser scanning confocal microscope. Images of PECAM1 and EMR1 positive cells were collected using the Radiance 2100 laser scanning confocal microscope. The mean staining intensity of PECAM1 in a defined endometrial area (excluding the uterine stroma and luminal epithelial cells) was determined, and a mean value of 4-6 tissue sections per uterus was calculated (ImageJ).
To score gland positivity, within a section the number of glands with proliferating glandular epithelium were counted and assigned a score based on the percentage of proliferating (BrdU-positive) cells. For example, if there were 11 glands that were each 5-20% BrdU-positive, glands were assigned an average score of 12.5%. Four uterine tissue sections per animal were scored in this manner, and the average percentage of BrdU-positive glandular epithelial cells calculated per mouse.

#### *2.3.2.6 Ovarian follicle and corpora lutea classification*

One haematoxylin-stained ovary tissue section per mouse was analysed. Follicles were categorised as preantral (primordial, primary and secondary follicles), antral (early antral, antral and pre-ovulatory follicles) or atretic follicles. Only follicles with a clearly defined developmental stage and visible oocyte were included in this analysis. All corpora lutea and the those displaying an abnormal/disorganised structure were recorded. Corpora albicantia were also counted.

## 2.4 EMBRYO CULTURE

### 2.4.1 *In vitro* embryo culture

#### *2.4.1.1 Ovarian hyperstimulation*

5 IU of equine chorionic gonadotropin (eCG; Folligon; Intervet, Bendigo, Australia) in 0.9% saline (Baxter Healthcare, Sydney, Australia) was injected (i.p.) to pre-pubertal *Cd11b-Dtr* mice at 3-4 weeks of age (12-14 g body weight) at 13:00 h. 47 h later mice received 5 IU of human chorionic gonadotropin (Chorulon; Intervet). Females were then housed with proven fertile stud *Cd11b-Dtr* males and checked the following day for the presence of a copulatory plug.

#### *2.4.1.2 Embryo collection and culture*

Embryos were collected as described below. Prior to culture, medium G1 and G2 sequential culture medium ± DT (Sigma-Aldrich) was equilibrated at 37°C in 5% oxygen and 6% $CO_2$. See Table 2.2 and 2.4 for G1 and G2 medium formulation. Embryos were cultured in 20 μl droplets of culture media under mineral oil (Sigma-Aldrich). Pulled glass Pasteur pipettes (Chase Instruments, New York, USA) were used to place 12-15 embryos into each droplet of equilibrated culture media. Embryos were scored once daily to determine their developmental stage.

##### *2.4.1.2.1 One-cell embryos*

On the day of the plug (15:00 h; day 1.0 pc) mice were sacrificed and the ovaries, oviducts and proximal end of the uterus were excised and placed in warm MOPS- buffered medium (Table 2.3 and 2.4). Zygotes were recovered from the ampullary region of each oviduct and were exposed to 0.5 mg/ml of hyaluronidase (Sigma-Aldrich) for up to 1 min to remove cumulus cells before being washed in MOPS buffer. Embryos recovered from each mouse were allocated one of two treatment groups: (1) Culture in G1 medium, (2) Culture in G1 medium + DT (25 ng/ml). At the 8-cell stage (day 3 of culture) embryos were transferred to pre-equilibrated G2 culture medium where they remained until the blastocyst stage.

##### *2.4.1.2.2 Eight-cell embryos*

On day 2.5 pc, mice were sacrificed and their oviducts and uteri were excised as one piece and placed in warm MOPS buffer. Uteri were flushed using a 30G needle, and the ampullary region of the oviduct was torn open to collect compacted morulla and early blastocysts. Embryos recovered from each mouse were cultured in either G2 medium or G2 medium + DT (25 ng/ml). Embryos remained in this culture medium until blastocyst stage.

### 2.4.2 *Ex vivo* blastocyst culture

Wild-type and *Cd11b-Dtr* female mice were naturally mated with stud males of the same genotype, and checked daily for the presence of a copulatory plug, where upon mated female mice were then separated from males. On day 2.5 pc, mice received an injection of DT (Sigma-Aldrich) and 24 h later on day 3.5 pc (between 9:00 and 11:00 h) mice were sacrificed and their oviducts and uteri excised. Embryos were recovered as described in Section 2.4.1.2.2 except that the embryo culture medium was low glucose DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sigma-Aldrich), 2 mM L-glutamine (BDH laboratory supplies, Poole, UK) and antibiotics ((Penicillin/Streptomycin;

Sigma-Aldrich)(DMEM-FBS)). The handling medium was also DMEM-FBS with the addition of 20 mM HEPES (Sigma-Aldrich). Embryos were scored and photographed (Olympus IX71 microscope and camera) for their developmental stage upon recovery from the reproductive tract, and then once daily until blastocysts were hatched and had attached to the culture dish.

**Table 2.2 Composition of G1 and G2 embryo culture medium**

| Component (mM) | G1 | G2 |
|---|---|---|
| NaCl | 85.16 | 85.16 |
| KCl | 5.5 | 5.5 |
| NaH2PO4.2H2O | 0.5 | 0.5 |
| $CaCl_2.2H_2O$ | 1.8 | 1.8 |
| $MgSO_4.7H_2O$ | 1.0 | 1.0 |
| $NaHCO_3$ | 25.0 | 25.0 |
| Na pyruvate | 0.32 | 0.1 |
| Na lactate (L-isoform) | 10.5 | 5.87 |
| Glucose | 0.5 | 3.15 |
| Glutamine | 1.0 | 1.0 |
| Taurine | 0.1 | 0.0 |
| Non-essential amino acids | All | All |
| Essential amino acids | None | All |
| EDTA | 0.01 | 0.0 |
| Human serum albumin | 5.0 g/l | 5.0 g/l |

All reagents from Sigma-Aldrich. For essential and non-essential amino acids, see Table 2.4. Adapted from (Gardner and Lane, 1997)

**Table 2.3 Formulation of MOPS buffer for embryo handling**

| Component (g/L) | MOPS |
|---|---|
| NaCl | 5.552 |
| KCl | 0.41 |
| $NaH_2PO_4$-$H_2O$ | 0.035 |
| $MgSO_4$-$7H_2O$ | 0.246 |
| Na Lactate (L) | 1.17 |
| Glucose | 0.09 |
| Penicillin (optional) | 0.06 |
| $NaHCO_3$ | 0.21 |
| Phenol Red | 0.001 |
| Pyruvic Acid | 0.035 |
| $CaCl_2$-$H_2O$ | 0.246 |
| Glutamax solution | 10 ml |
| Taurine | 0.0125 |
| Non-Essential amino acids | 10 ml |
| MOPS | 4.197 |
| BSA | 0.00000 4 |

Glutamax solution (Invitrogen). All other reagents from Sigma-Aldrich. Non-Essential amino acids solution, see Table 2.4. Adapted from (Gardner and Lane, 2004).

**Table 2.4 Concentration of amino acids used in G1, G2 and MOPS medium**

| Non-essential amino acids | Concentration (mM) | Essential amino acids | Concentration (mM) |
|---|---|---|---|
| Alanine | 0.1 | Arginine | 0.6 |
| Asparagine | 0.1 | Cystine | 0.1 |
| Aspartate | 0.1 | Histidine | 0.2 |
| Glutamate | 0.1 | Isoleucine | 0.4 |
| Glycine | 0.1 | Leucine | 0.4 |
| Proline | 0.1 | Lysine | 0.4 |
| Serine | 0.1 | Methionine | 0.1 |
| | | Phenylalanine | 0.2 |
| | | Threonine | 0.4 |
| | | Tryptophan | 0.05 |
| | | Tyrosine | 0.2 |
| | | Valine | 0.4 |
| | | Glutamine | 1.0 |

All amino acids purchased from Sigma-Aldrich. Adapted from (Gardner and Lane, 1997).

## 2.5 EXOGENOUSE HORMONE TREATMENT PROTOCOLS

In these experiments, all mice were ovariectomised *Cd11b-Dtr*-BM and experiments began approximately 2-3 weeks following surgery. In all experiments, mice were primed with two s.c. injections of 100 ng of estradiol (Sigma-Aldrich) on subsequent days. Experimental protocols following priming and the times of DT-elicited macrophage depletion (or PBS control) are illustrated in the figures below. Hormones were prepared as described in 2.1.12 and 2.1.13. 2 h prior to sacrifice, mice received an injection of BrdU (Sigma-Aldrich; Section 2.3.1.2.1); these injections were staggered across the group so that all mice were killed exactly 2 h following the BrdU injection. In all experiments, PEC were collected as described in Section 2.2.7 and uteri were collected as described in Sections 2.3.1.1 and 2.3.1.2.

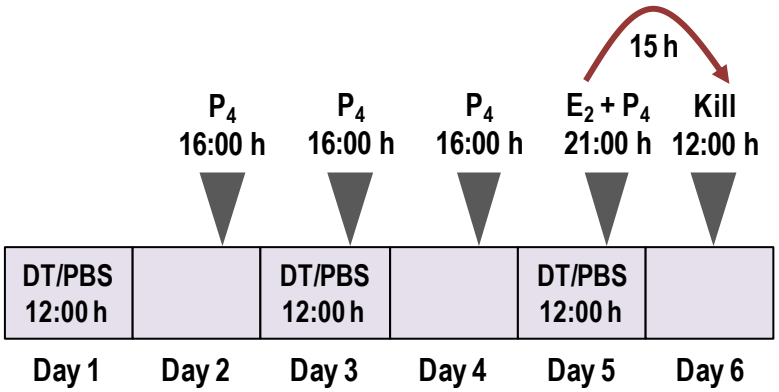
### 2.5.1 Induction of uterine epithelial cell proliferation

This experimental protocol began 6 days following the final $E_2$ priming injection (section 2.5). Maximal epithelial cell proliferation was induced by a single injection of 50 ng of $E_2$ (Sigma-Aldrich) at 21:00 h and mice were sacrificed 15 h later (Tong and Pollard, 1999). Mice were treated with PBS (control group) or DT (macrophage-depleted group) at 12:00 h.

### 2.5.2 Induction of stromal cell proliferation

This experimental protocol began 6 days following the final $E_2$ priming injection (section 2.5). Maximal stromal cell proliferation was induced by 4 consecutive days of $P_4$ injections (1 mg; Sigma-Aldrich) with one single injection of $E_2$ (50 ng; Sigma-Aldrich) at the same time as the final $P_4$ injection; mice were sacrificed 15 h after the final hormone injection (Tong and Pollard, 1999). Mice were treated with PBS (control group) or DT (macrophage-depleted group) as illustrated in Figure 2.1.

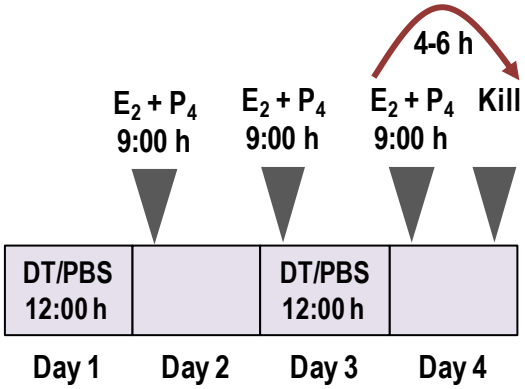
**Figure 2.1 Protocol to elicit uterine stromal cell proliferation**




### 2.5.3 Induction of uterine receptivity

This experimental protocol began 2 days following the final $E_2$ priming injection (section 2.5). Mice were treated for three consecutive days with $E_2$ (10 ng; Sigma-Aldrich) and $P_4$ (500 µg; Sigma-Aldrich), this hormone injection regime is designed to mimic the hormonal environment in early pregnancy. Mice were killed 4-6 h following the final hormone injection, when there is maximum sensitivity to a decidual stimulus (Finn and Martin, 1972, Finn et al., 1989, Robertson et al., 1996b). Mice were treated with PBS (control group) or DT (macrophage-depleted group) as illustrated in Figure 2.2.

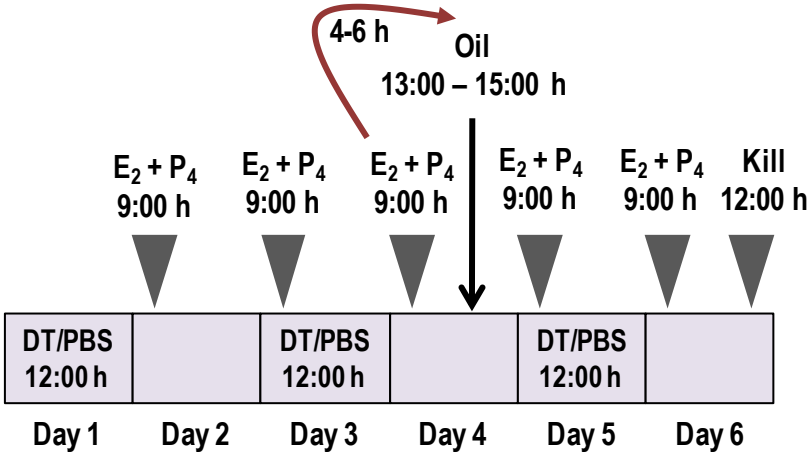
**Figure 2.2 Protocol to elicit uterine receptivity to a blastocyst**




### 2.5.4 Induction of deciduoma

This experiment was conducted as described in Section 2.5.3, except oil was instilled into the uterine lumen (Section 2.1.5) at the time of maximal sensitivity to a blastocyst (4-6 h following the hormone injection on day 4). Mice were treated with PBS (control group) or DT (macrophage-depleted group) as illustrated in Figure 2.3. To assess differentiation of decidual cells, alkaline phosphatase staining was performed as described in Section 2.3.2.1.3.

**Figure 2.3 Protocol to elicit deciduoma formation**



## 2.6 QUANTITATIVE REAL-TIME PCR

### 2.6.1 RNA extraction

Ovaries were dissected under RNase free conditions, oviducts and excess fat were removed, and the tissue was snap frozen in liquid nitrogen and stored at -80$^{o}$C until processing (one ovary per mouse for quantitative real-time (qRT)-PCR, the other was collected for immunohistochemistry, Section 2.3.1.1). Total ovarian RNA extraction was performed by adding 300 µl of Trizol (Invitrogen, Carlsbad, USA) to tubes containing 1.4 mm ceramic beads (Geneworks, Adelaide, Australia). Ovaries were added and the Precellys (Bertin Technologies, Montigny le Bretonneux, France) was used to homogenise tissue (2 x 15 s). 60 µl of chloroform (Unilab, Ajax Finechem, Tarenpoint, Australia) was then added and samples were gently mixed by hand. Samples were then centrifuged at 12000xg for 15 min at 4$^{o}$C. Approximately 150 µl of the aqueous RNA phase was removed and placed into a fresh tube. An equal volume of isopropanol (Sigma-Aldrich) was added and 0.5 µl of glycogen (Invitrogen), and the sample was precipitated overnight at -20$^{o}$C. The following morning, samples were pelleted at 16000xg for 30 min at 4$^{o}$C. The pellet was then washed twice with ice-cold 75% ethanol and centrifuged at 12000xg for 15 min at 4$^{o}$C. Excess ethanol was removed and the pellet was air dried for 30-45 min before being resuspended in 20 µl of RNase free Milli-Q water. Samples were then treated with DNase to remove contaminating DNA; using reagents supplied in a TURBO DNA-free kit (Life Technologies, Applied Biosystems, Carlsbad, USA) according to the manufacturer's instructions. 2 µl of 10x DNase I buffer and 1 µl of DNase I were added to each sample and incubated for 30 min at 37$^{o}$C. Following this, 5 µl of DNase Inactivating Reagent was incubated with each sample for 2 min. Samples were centrifuged at 12000xg for 2 min at 4$^{o}$C, and the RNA-containing supernatant was transferred to a clean tube. Extracted RNA was quantified using a NanoDrop Spectrophotometer (Thermo Scientific, Wilmington, USA).

### 2.6.2 Reverse Transcription and cDNA generation

Samples were resuspended at 3 µg/10 µl in RNase free Milli-Q water prior to incubation with 1 µl of 500 µg/ml random hexamers (Geneworks) at 70$^{o}$C for 10 min. Samples were then chilled on ice for 5 min. Reverse transcription (RT) master mix [4 µl of RT buffer, 1 µl of RNase inhibitor (Invitrogen), 1 µl of DTT, 2 µl 10 mM dNTPs (AMRAD Pharmacia Biotech, Melbourne, Australia) and 1 µl Superscript III enzyme (Invitrogen)] was added to each sample and incubated at 25$^{o}$C for 5 min, and then 43$^{o}$C for 90 min. The RT reaction was stopped by heat inactivation by incubating samples at 94$^{o}$C for 5 min. For quantitative real-time PCR (qRT-PCR), cDNA was diluted 1:1 with milli-Q water and stored at -20$^{o}$C.

For each experiment, all RNA samples were reverse transcribed in a single batch and all were assayed with a given individual primer set in the same PCR run.

### 2.6.3 Oligonucleotide primer design

Oligonucleotide primer pairs were designed using Primer Express version 2 software (Life Technologies, Applied Biosystems). Messenger RNA sequences were downloaded from the Entrez nucleotide database, accessible from the National Centre for Biotechnology Information website (http://www.ncbi.nlm.nih.gov). Primers were designed to meet previously optimised criteria to suit the ABI Prism 7000 Sequence Detection System (Applied Biosystems), and where possible, were designed to span an intron-exon boundary, to ensure absence of amplification of contaminating genomic DNA. All primers were purchased from Geneworks (Adelaide, Australia). A list of all sequences for target genes analysed, including product size and Genebank accession number are provided in Table 2.5. Primer specificity was determined by gel electrophoresis (2% agarose gel; Promega, Madison, USA) to confirm the correct product size and analysis of the dissociation curve and gel electrophoresis was used to exclude the formation of primer dimers or non-specific products. PCR products were sequenced to confirm the identity of the amplified sequences (Flinders Medical Centre Sequencing Service, Adelaide, Australia) except for those from the *Cyp11a1* and *Pecam1* primers.

### 2.6.4 Quantitative Real-Time PCR

Using the primers shown in Table 2.4, qRT-PCR was performed on cDNA samples using an ABI Prism 7000 Sequence Detection System (Applied Biosystems) and a 2 x SYBR Green PCR master mix (Applied Biosystems) according to the manufacturer's instructions. The optimal primer concentration was determined by conducting serial dilutions of primer sets with a positive control sample cDNA. Each reaction (20 µl total) contained 3 µl of cDNA and 0.5 µM 5' and 3' primers. The efficiency of each primer set was determined by regression analysis across serial dilution of cDNA and only primers consistent with the housekeeper (*Actb*) primer set were accepted. 18S primers (Ambion, Applied Biosystems, Austin, USA) were used according to the manufacturer's instructions; the optimal primer:competimer ratio was determined to be 2:8. The negative control included in each reaction contained Milli-Q water substituted for cDNA (non-template control) and showed that primers did not produce primer dimers as evidenced by no amplification.

Optimal PCR conditions were: 2 min at 50°C, 5 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Amplification plots for each sample were obtained and cycle threshold (Ct) limits determined using the ABI 7700 software package (Applied Biosystems).

All samples were amplified in duplicate for each primer set. The quantity of cDNA for each sample was normalised to the housekeeper genes *Actb* and 18S using the $2^{-\Delta\Delta Ct}$ method; this determined the Ct difference between the housekeeper gene and the gene of interest (ΔCt). This difference was then subtracted from the average difference for the designated control group (ΔΔCt). Relative change in cDNA quantity was calculated by multiplying the cDNA quantity by a constant so that the level of expression for control tissues equalled an arbitrary number of 100. This allowed the data for all samples for each primer set to be expressed relative to the mRNA content of control tissue.

**Table 2.5 PCR primers for qRT-PCR**

| Target mRNA | 5'-3' Primer sequence | Product size (bp) | Genebank |
|---|---|---|---|
| *Actb* | 5' TGTGATGGTGGGTATGGGTC | 162 | M10277 |
| | 3' ACACGCAGCTCATTGTA | | |
| *Star* | 5' CCGGAGCAGAGTGGTGTCAT | 158 | L36062 |
| | 3' TGCGATAGGACCTGGTTGATG | | |
| *Cyp11a1* | 5' CACTCCTCAAAGCCAGCATCA | 92 | NM_019779 |
| | 3' ACGAAGCACCAGGTCATTCAC | | |
| *Hsd3b1* | 5' GGACAAAGTATTCCGACCAGAAAC | 103 | NM_008293 |
| | 3' CAGGCACTGGGCATCCA | | |
| *Cyp17a1* | 5' TGGCTTTCCTGGTGCACAA | 81 | M64863 |
| | 3' GTGTTCGACTGAAGCCTACATACTG | | |
| *Emr1* | 5' CACATCCAGCCAAAGCAGAA | 111 | NM_010103.2 |
| | 3' CTCGGATGCTTCCACAATCTC | | |
| *Pecam1* | 5' ACTCACGCTGGTGCTCTATGC | 116 | NM_008816 |
| | 3' GGGTCAGTTGCTGCCCATT | | |
| *Vegfa* | 5' GCAGGCTGCTGTAACGATGA | 197 | NM_009505 |
| | 3' CGCTCTGAACAAGGCTCACA | | |
| *Vegfb* | 5' CCCCTGTGTCCCAGTTTGAT | 126 | NM_011697 |
| | 3' TGCCCATGAGTTCCATGCT | | |
| *Vegfc* | 5' AGACGTTCTCTGCCAGCAACA | 96 | NM_009506 |
| | 3' CAGGCATCGGCACATGTAGTT | | |
| *Figf* | 5' GTGGACCGCACATGACGTT | 223 | NM_010216 |
| | 3' CAGGCTGGCTTTCTACTTGCA | | |
| *Flt1* | 5' GTGGCTGCGACCCTCTTTT | 246 | NM_010228 |
| | 3' GCCAAATGCAGAGGCTTGA | | |
| *Kdr* | 5' GCCTTGATCTCGCCTATGGAT | 142 | NM_010612 |
| | 3' TTGTTTGGCCGGGTCTGTAG | | |

## 2.7 Assays

### 2.7.1 Estradiol and Progesterone

Radioimmunoassay kits were used to measure the concentration of estradiol and progesterone (DSL4800 and DSL-3400, respectively; Diagnostic Systems Laboratories; Webster, USA) in plasma (Section 2.1.10). Assays were carried out by Dr. D. Kennaway and Ms A. Voultsios according to the manufacturer's instructions. For each experiment, samples from DT-treated wild-type and DT-treated *Cd11b-Dtr* mice were measured in a single assay. The lower limits of detection were 5 pg/ml estradiol and 0.3 ng/ml progesterone. The within-assay coefficients of variation were 10.0% (estradiol) and 7.0% (progesterone). The lower limits of detection were 5 pg/ml estradiol and 0.3 ng/ml progesterone.

## 2.8 Statistical Analysis

Data are presented as the mean ± SEM (standard error of mean). Data were assessed for normal distribution with a Shapiro-Wilk normality test using GraphPad Prism 5 (GraphPad software Inc, San Diego, USA) or SPSS Statistics Version 17.0 (IBM Corporation, Armonk, NY, USA). If data was normally distributed, data were analysed using an Unpaired t test. If data were not normally distributed, or normal distribution could not be determined due to small sample size, data were analysed using a Mann-Whitney U test. Kruskal-Wallis test and post-hoc Dunn's test were used when comparing more than two treatment groups where data were not normally distributed. Categorical data were compared using Fisher's exact test or Chi-Square test; actual data were used, not percentages. To determine *P* value between two or more groups, a Fisher's exact test was performed on all possible combinations to determine the difference between each group. Within analyses, individual datum points were excluded as outliers if they were greater than 2 standard deviations from the mean. The difference between groups was considered statistically significant if $P < 0.05$. Statistical significance was assessed as follows: *$P < 0.05$, **$P < 0.01$, *** $P < 0.001$ and ****$P < 0.0001$. Where more than two groups were compared, different superscripts were used to indicate statistical significance between groups.

# Chapter 3

# Effect of macrophage depletion on leukocyte parameters in the female reproductive tract and on embryo implantation

## 3.1 INTRODUCTION

There have been a number of studies that recognise the potential importance of macrophages in the reproductive tract. Despite a lot of speculation about an important role, studies have failed to determine whether these cells are essential for reproductive function, or rather, supportive of reproductive processes (Hunt and Robertson, 1996).

Studies carried out in our laboratory show that endometrial macrophage recruitment is reduced by ablation of the seminal fluid stimulus, by mating females with seminal vesicle deficient males (SV-) (Robertson et al., 1996a). This leads to compromised implantation, altered placental development, and adverse effects on fetal and postnatal growth, resulting in adult progeny with evidence of metabolic disorders (Bromfield, 2006). However, it is difficult to decipher whether these results can be attributed to reduced macrophage recruitment, the absence of seminal fluid (which itself contains many cytokines), or a combination of both.

To date, the most well studied model of macrophage deficiency is the *Csf1*$^{op}$*/Csf1*$^{op}$ mouse. However, both male and female *Csf1*$^{op}$*/Csf1*$^{op}$ mice exhibit fertility defects due to dysregulation of the HPG axis resulting from the *Csf1*-deficiency. Further, this model is not particularly useful for studying the contribution of macrophages to reproductive events as they are recruited into the ovary and uterus following coitus (Cohen et al., 1997, Pollard et al., 1991, Robertson et al., 1998). This suggests that cytokines other than CSF1 are responsible for macrophage recruitment during pregnancy. CSF2 was one candidate cytokine suspected of such a role, but studies involving *Csf1*$^{op}$*/Csf1*$^{op}$*Csf2*$^{(-/-)}$ double-knockout mice refute this possibility as there is a similar increase in macrophage recruitment following syngeneic matings with cytokine replete males (Robertson et al., 1998). This research indicates that other molecules contribute to macrophage recruitment following coitus, and are sufficient to compensate in the absence of CSF2 and CSF1. Several macrophage chemokines have since been identified that may fulfil this role (Robertson et al., 1998, Pollard et al., 1998, Wood et al., 1997, Wood et al., 1999).

To further the understanding of macrophage contributions to reproductive events, the *Cd11b-Dtr* mouse is valuable, enabling transient, systemic depletion of CD11B-expressing macrophage populations (Duffield et al., 2005a). This model is advantageous as the mice are essentially no different from wild-type FVB/NJ mice until DT is administered, and therefore their growth and

development prior to the initiation of experiments is unperturbed. This model has been used in the following experiments to elucidate the role of macrophages in early pregnancy.

## 3.2 EFFECT OF MACROPHAGE DEPLETION ON REPRODUCTIVE TRACT IMMUNE CELL POPULATIONS

There are no studies that thoroughly characterise the impact that macrophage depletion by DT administration in the *Cd11b-Dtr* model might have on the various immune cell populations in the uterus and ovary. Previous studies using the *Cd11b-Dtr* transgenic mouse have revealed that injections of DT result in elimination of EMR1$^+$ macrophages, while numbers of peritoneal CD3$^+$ T cells are unaffected. The numbers of splenic CD3$^+$ T cells and B220$^+$ (PTPRC; protein tyrosine phosphate, receptor type, C) B cells were also unaffected by DT in transgenic mice (Duffield et al., 2005a, Cailhier et al., 2005). Cailhier et al. (2005) report that EMR1$^+$ cells are depleted from the kidney and ovary, but not from the liver or lungs (hepatic sinusoidal and alveolar macrophages).

Despite the expression of CD11B by both macrophages and granulocytes, Cailhier et al. (2005) reveal that PMN cells in the peritoneum and whole blood are not affected by DT administration after thioglycollate-induced peritonitis. Interestingly, they report that macrophage depletion before thioglycollate injection causes a marked blunting of PMN cell recruitment into the peritoneal cavity. However, they determined that this was not due to PMN cells being affected by the administration of DT as they were able to restore PMN cell number by administering macrophage-rich peritoneal cells to the macrophage-depleted peritoneum, and in the process restored the number of peritoneal PMN cells.

Clarification of the impact that the injection of DT has on granulocyte populations in *Cd11b-Dtr* mice would be insightful, as would its impact on DC populations, because these cells can express CD11B on their surface. To do this, female mice in proestrus received an i.p DT injection (25 ng/g) and were sacrificed 24 h later, during estrus. Estrous cycle stage was confirmed by daily vaginal smearing as described in Chapter 2.1.7. Uteri and ovaries were collected and enzymatically digested into single cell suspensions (Chapter 2.2). Peritoneal exudate cells (PEC) and splenocytes were also analysed. Flow cytometry was used to measure the cell surface expression of various immune cell markers. The cell surface markers analysed were CD11B, EMR1, CD11C (ITGAX; integrin alpha X), CD45 (PTPRC; protein tyrosine phosphatase, receptor type, C), RB6 (LY6G; lymphocyte antigen 6 complex, locus G), and CD3 (CD3e; CD3 antigen, epsilon polypeptide); all markers were analysed individually and in conjunction with CD11B. Propidium iodide was used to exclude all non-viable cells from analyses.

CD11B is present on a range of cell types, including macrophages, granulocytes and myeloid derived DCs. There is an upregulation of CD11B expression on neutrophils after activation. EMR1 is expressed at high levels on the cell membrane of macrophages, and is a well known macrophage differentiation marker (Austyn and Gordon, 1981, Potter et al., 2003). CD11C is a marker of DC (Metlay et al., 1990). CD45 (Ly5; leukocyte common antigen; T200) is a leukocyte differentiation antigen expressed by all leukocytes (Trowbridge, 1978, Thomas, 1989, Hermiston et al., 2003). RB6 is a myeloid differentiation antigen expressed on bone marrow granulocytes and peripheral neutrophils, and is transiently expressed on monocytes during their development in bone marrow (Hestdal et al., 1991, Fleming et al., 1993). The anti-CD3 antibody (17A2) reacts with the T cell receptor associated CD3 molecular complex, found on many thymocytes and mature T lymphocytes (Miescher et al., 1989).

Representative dotplots and data in Figures 3.1-3.6 show the expression of these markers in the uterus and PEC in wild-type and *Cd11b-Dtr* mice. Table 3.1 summarises the expression of all markers analysed in the uterus, ovary, PEC and spleen.

### 3.2.1 Macrophages

CD11B-expressing cells were significantly depleted from the uterus, ovary and peritoneal cavity. In the wild-type uterus, 6.4 ± 0.9% (mean ± SEM) of cells were $CD11B^+$ and 2.0 ± 0.6% in *Cd11b-Dtr* mice (*P* = 0.03, Mann-Whitney U test; Figure 3.1D).

There was a significant depletion of $EMR1^+$ cells from the uterus (Figure 3.2D, wild-type = 16.6 ± 2.8% and *Cd11b-Dtr* = 8.4 ± 1.6%; p = 0.03 one-tailed Mann Whitney U test). When looking at EMR1 and CD11B co-expression (Figure 3.2E), there was a significant decrease in $EMR1^+CD11B^+$ cells (in wild-type = 3.2 ± 2.5%, in *Cd11b-Dtr* = 1.3 ± 0.5%; *P* = 0.03, Mann Whitney U test). There was also a significant depletion of $EMR1^-CD11B^+$ cells, but not of $EMR1^+CD11B^-$ cells. $EMR1^+$ cells were also depleted significantly from the ovary and peritoneal cavity.

CD11B-expressing cells were not depleted from the spleen. This is because wild-type splenocytes express CD11B at very low levels. This was a consistent result observed in several experiments. $EMR1^+$ cells were not depleted from the spleen (as expected due to the low/absent expression of CD11B by splenocytes; see Table 3.1).

### 3.2.2 Dendritic cells

CD11C expression was analysed to determine whether DC populations might be affected by the administration of DT. There was a significant depletion of $CD11C^+$ cells from the uterus (Figure 3.3D; wild-type = 6.6 ± 1.0% and *Cd11b-Dtr* = 1.9 ± 0.7%; $P = 0.04$, Mann Whitney U test). For CD11C and CD11B co-expression (Figure 3.3E), there was not a significant depletion of $CD11C^+CD11B^+$ cells from the uterus ($P = 0.06$), but there was a significant depletion of $CD11C^+CD11B^-$ and $CD11C^-CD11B^+$ cells. CD11C expressing cells were also depleted in the ovary and peritoneal cavity, but not from the spleen.

### 3.2.3 Neutrophils

To determine whether neutrophil numbers were impacted by DT treatment, RB6-8C5 expression was analysed. There was no depletion of total RB6 expressing cells from the uterus (Figure 3.4D), but there was a significant depletion of $RB6^+CD11B^+$ cells ($P = 0.03$, Mann Whitney U test; Figure 3.4E), with a similar result being seen in the peritoneal cavity. In the ovary, there was a significant decrease in $RB6^+$ total cells ($P = 0.02$, Mann Whitney U test), but the reduction in expression of $RB6^+CD11B^+$ cells following DT injection did not reach significance ($P = 0.09$, Mann Whitney U test). Within the spleen, there was a significant increase in the number of $RB6^+$ expressing cells in *Cd11b-Dtr* mice compared to wild-type mice ($P = 0.01$, Mann Whitney U test).

### 3.2.4 All leukocytes

There was a significant depletion of $CD45^+$ leukocytes from the uterus, ovary and peritoneal cavity, but not the spleen.

### 3.2.5 T cells

The number of CD3-expressing T cells appeared unchanged in all tissues analysed (Table 3.1).

Table 3.1 provides a comparison of the expression of all markers analysed in each tissue.

Although there was no change in the total percentage of PEC expressing RB6, there was depletion of cells that were expressing both RB6 and CD11B (Figure 3.4J; Table 3.1). However, the work of Cailhier et al. (2005) suggested that there was no change in the PMN cell population. To further investigate this, Absolute Counting Beads (as described in Chapter 2.2.6) were employed to determine the total cell number of RB6-expressing cells in the peritoneal cavity, and in the spleen as a control tissue for the same set of mice examined above. The total number of RB6 expressing cells, as well RB6-low expressing and RB6-high expressing cells were quantified, as illustrated in Figure 3.7, in PEC (top) and the spleen (bottom). This data confirmed that there was no change in the total number of RB6 expressing PEC (in wild-type = 1546 ± 377, in *Cd11b-Dtr* = 785 ± 244), however, there was a significant depletion of RB6 high cells from DT-treated *Cd11b-Dtr* mice (in wild-type = 1263 ± 284, in *Cd11b-Dtr* = 180 ± 32; *P* = 0.02, Mann Whitney U test).

**Figure 3.1 Effect of macrophage depletion on CD11B$^+$ cell populations in the uterus and peritoneal cavity**

Representative histograms illustrate the proportion of CD11B$^+$ cells in the uterus (A-C) and PEC (E-G). Wild-type uterine cells labelled with isotype control antibody (A). CD11B$^+$ cells in the uteri of DT-treated wild-type (B) or *Cd11b-Dtr* mice (C). D, percent CD11B$^+$ cells in the uteri of DT-treated wild-type and *Cd11b-Dtr* mice. Wild-type PEC labelled with isotype control antibody (E). CD11B$^+$ PECs in DT-treated wild-type (F) or *Cd11b-Dtr* mice (G). H, percent CD11B$^+$ PECs of DT- treated wild-type and *Cd11b-Dtr* mice. Data are presented as mean ± SEM with statistical analysis using a Mann-Whitney U test. * indicates statistical significance from control. *$P$ < 0.05, **$P$ < 0.01. Uterus; n = 4 per group, PEC; wild-type n = 6, *Cd11b-Dtr* n = 4. MD; macrophage depleted. DT; diphtheria toxin. PEC; peritoneal exudate cells.

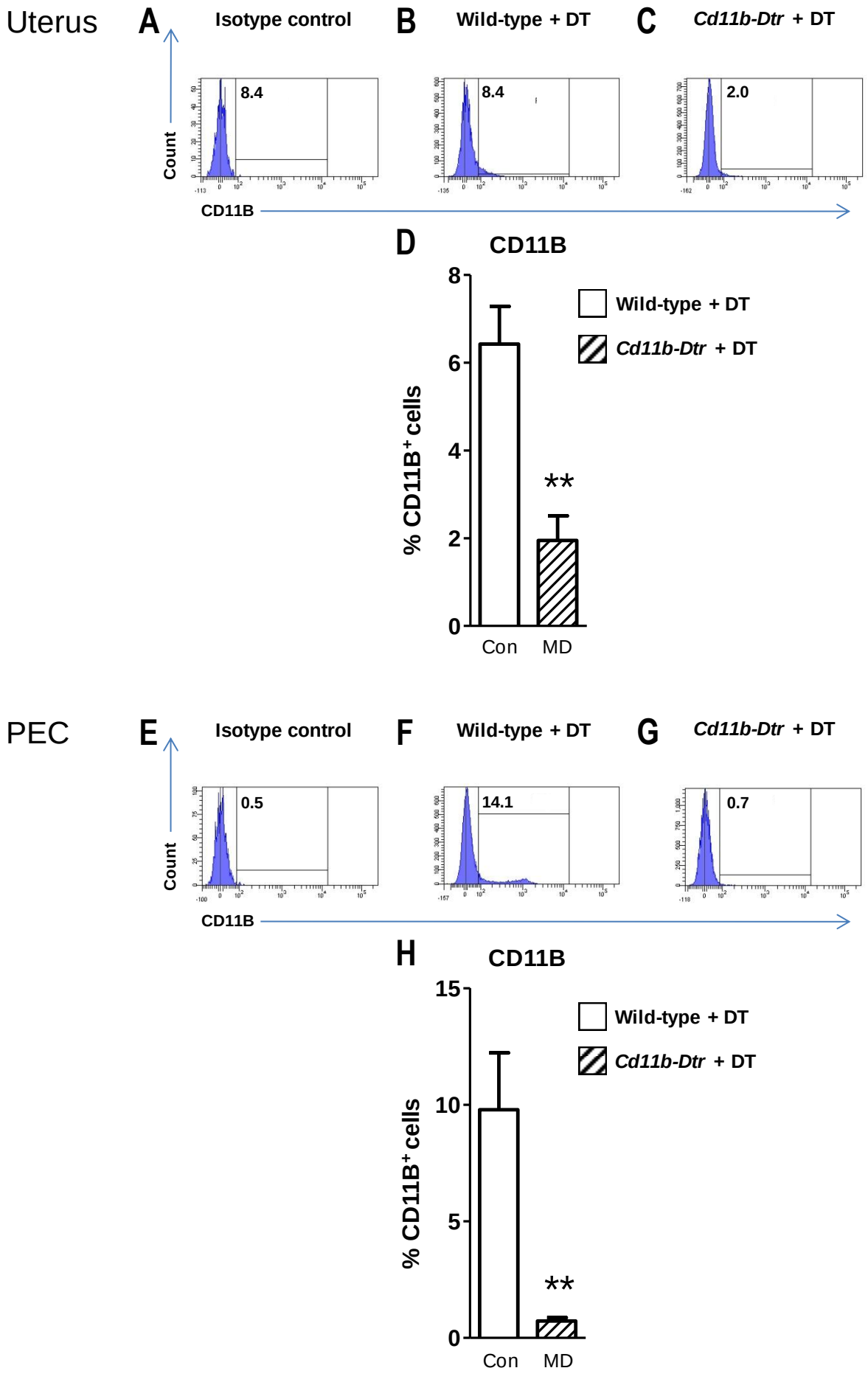

Uterus
A
Isotype control
B
Wild-type + DT
C
Cd11b-Dtr + DT
8.4
8.4
2.0
Count
CD11B
D
CD11B
Wild-type + DT
Cd11b-Dtr + DT
% CD11B+ cells
**
Con
MD
PEC
E
Isotype control
F
Wild-type + DT
G
Cd11b-Dtr + DT
0.5
14.1
0.7
Count
CD11B
H
CD11B
Wild-type + DT
Cd11b-Dtr + DT
% CD11B+ cells
**
Con
MD

**Figure 3.2 Effect of macrophage depletion on EMR1$^+$ and CD11B$^+$ cell populations in the uterus and peritoneal cavity**

Representative dotplots illustrate the proportion of EMR1$^+$ and CD11B$^+$ cells in the uterus (A-C) and PEC (F-H). Wild-type uterine tissue labelled with isotype control antibody (A). EMR1$^+$ and CD11B$^+$ cells in the uteri of DT-treated wild-type (B) or *Cd11b-Dtr* mice (C). D, percent EMR1$^+$ cells, and E, percent of EMR1$^+$ and CD11B$^+$ cells in the uteri of DT-treated wild-type and *Cd11b-Dtr* mice. Wild-type PEC labelled with isotype control antibody (F). EMR1$^+$ and CD11B$^+$ PECs in DT-treated wild-type (G) or *Cd11b-Dtr* mice (H). I, percent EMR1$^+$, and J, percent of EMR1$^+$ and CD11B$^+$ PECs in DT-treated wild-type and *Cd11b-Dtr* mice. Data are presented as mean ± SEM with statistical analysis using a Mann-Whitney U test. * indicates statistical significance from control. *$P$ < 0.05, **$P$ < 0.01. Uterus; n = 4 per group, PEC; wild-type n = 6, *Cd11b-Dtr* n = 4. MD; macrophage depleted. DT; diphtheria toxin. PEC; peritoneal exudate cells.

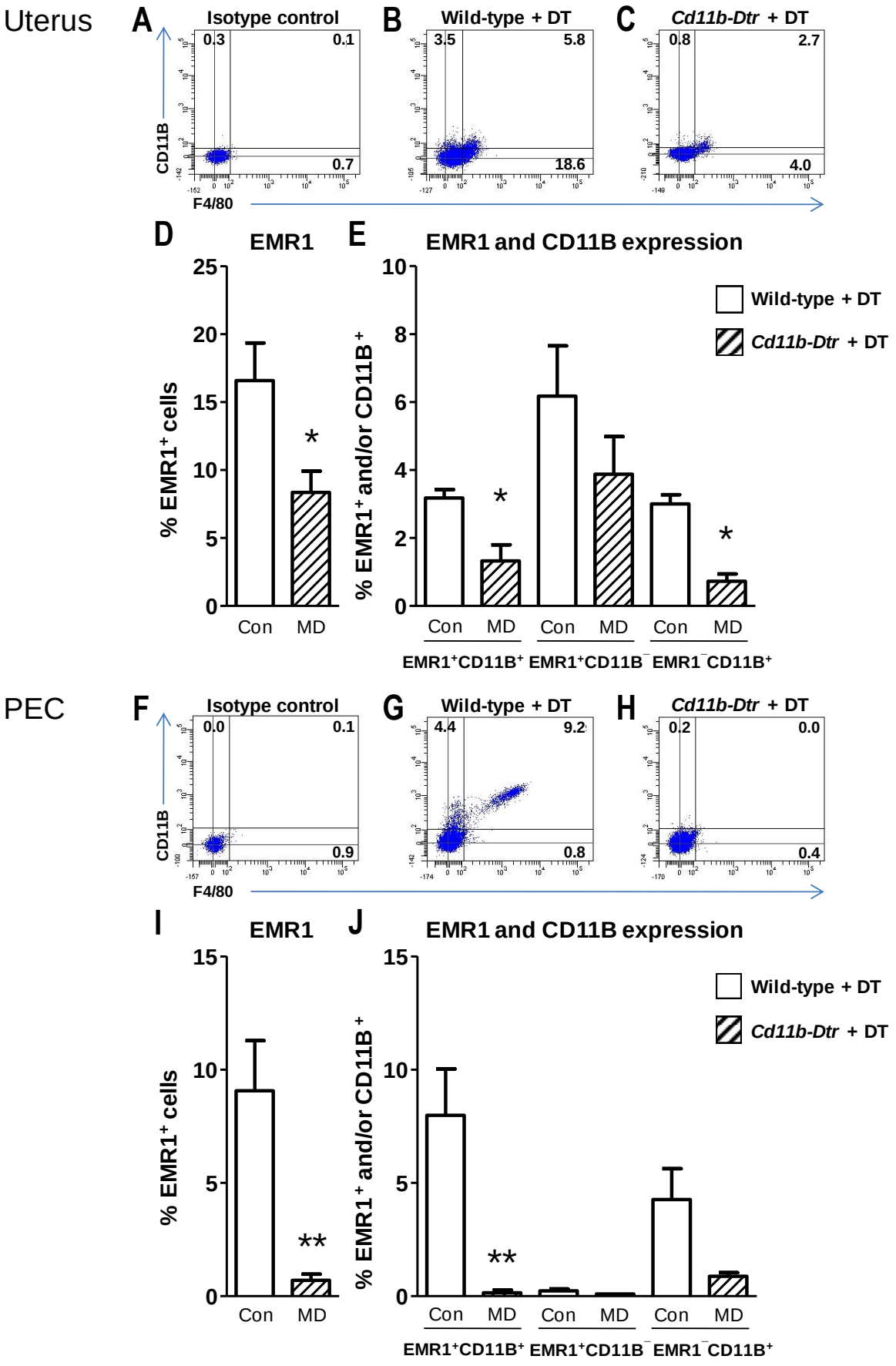

Uterus
A
Isotype control
0.3
0.1
0.7
B
Wild-type + DT
3.5
5.8
18.6
C
Cd11b-Dtr + DT
0.8
2.7
4.0
CD11B
F4/80
D
EMR1
% EMR1+ cells
Con
MD
E
EMR1 and CD11B expression
% EMR1+ and/or CD11B+
Wild-type + DT
Cd11b-Dtr + DT
EMR1+CD11B+ EMR1+CD11B- EMR1-CD11B+
PEC
F
Isotype control
0.0
0.1
0.9
G
Wild-type + DT
4.4
9.2
0.8
H
Cd11b-Dtr + DT
0.2
0.0
0.4
I
EMR1
J
EMR1 and CD11B expression

**Figure 3.3 Effect of macrophage depletion on $CD11C^+$ and $CD11B^+$ cell populations in the uterus and peritoneal cavity**

Representative dotplots illustrate the proportion of $CD11C^+$ and $CD11B^+$ cells in the uterus (A-C) and PEC (F-H). Wild-type uterine tissue labelled with isotype control antibody, A. $CD11C^+$ and $CD11B^+$ cells in the uteri of DT-treated wild-type (B) or *Cd11b-Dtr* mice (C). D, percent $CD11C^+$ cells, and E, percent of $CD11C^+$ and $CD11B^+$ cells in the uteri of DT-treated wild-type and *Cd11b-Dtr* mice. Wild-type PEC labelled with isotype control antibody (F). $CD11C^+$ and $CD11B^+$ PECs in DT-treated wild-type (G) or *Cd11b-Dtr* mice (H). I, percent $CD11C^+$, and J, percent of $CD11C^+$ and $CD11B^+$ PECs in DT-treated wild-type and *Cd11b-Dtr* mice. Data are presented as mean ± SEM with statistical analysis using a Mann-Whitney U test. * indicates statistical significance from control. $*P < 0.05$, $**P < 0.01$, # indicates a *P* value of >0.05 but <0.06. Uterus; n = 4 per group, PEC; wild-type n = 6, *Cd11b-Dtr* n = 4. MD; macrophage depleted. DT; diphtheria toxin. PEC; peritoneal exudate cells.

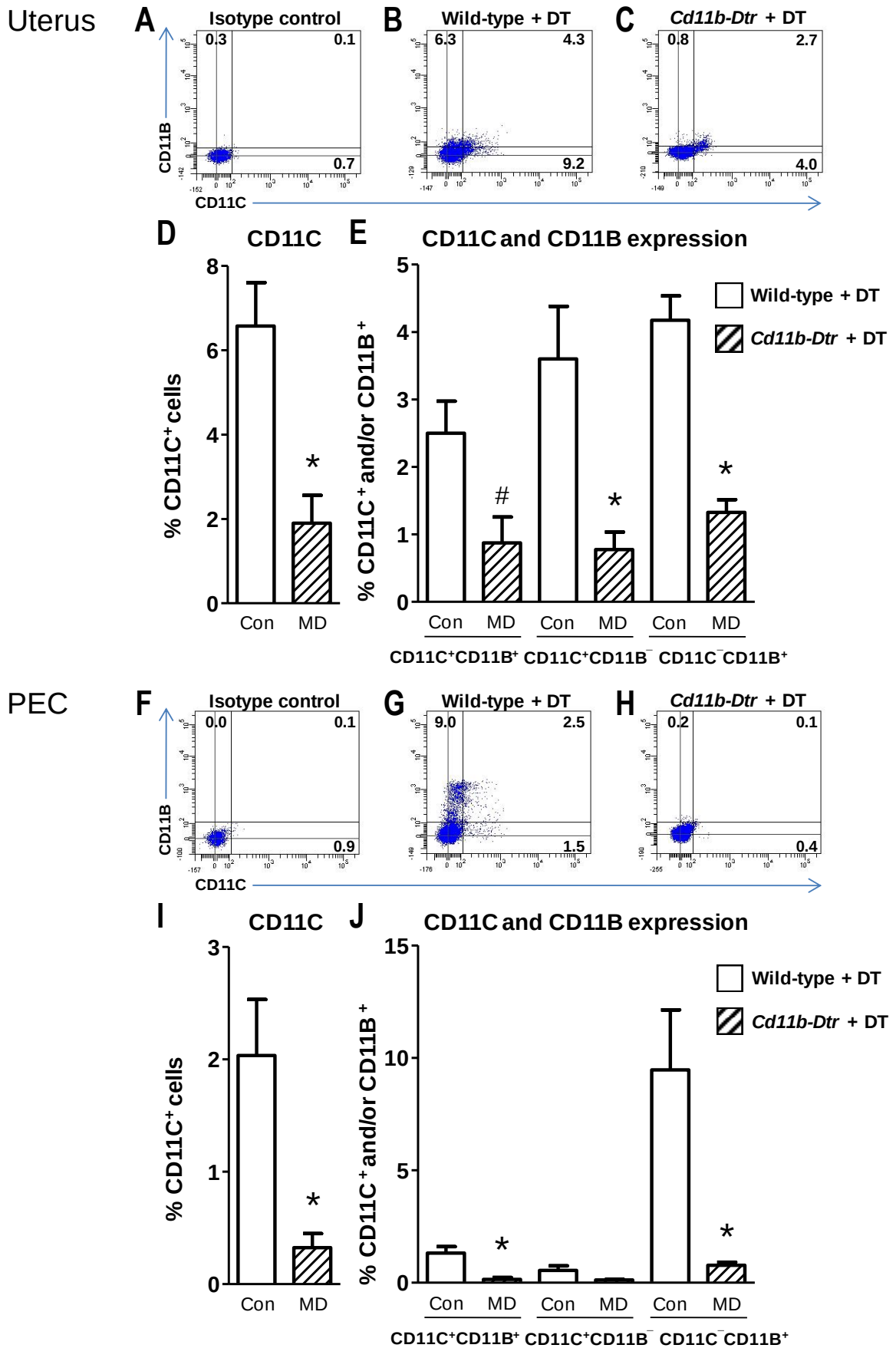
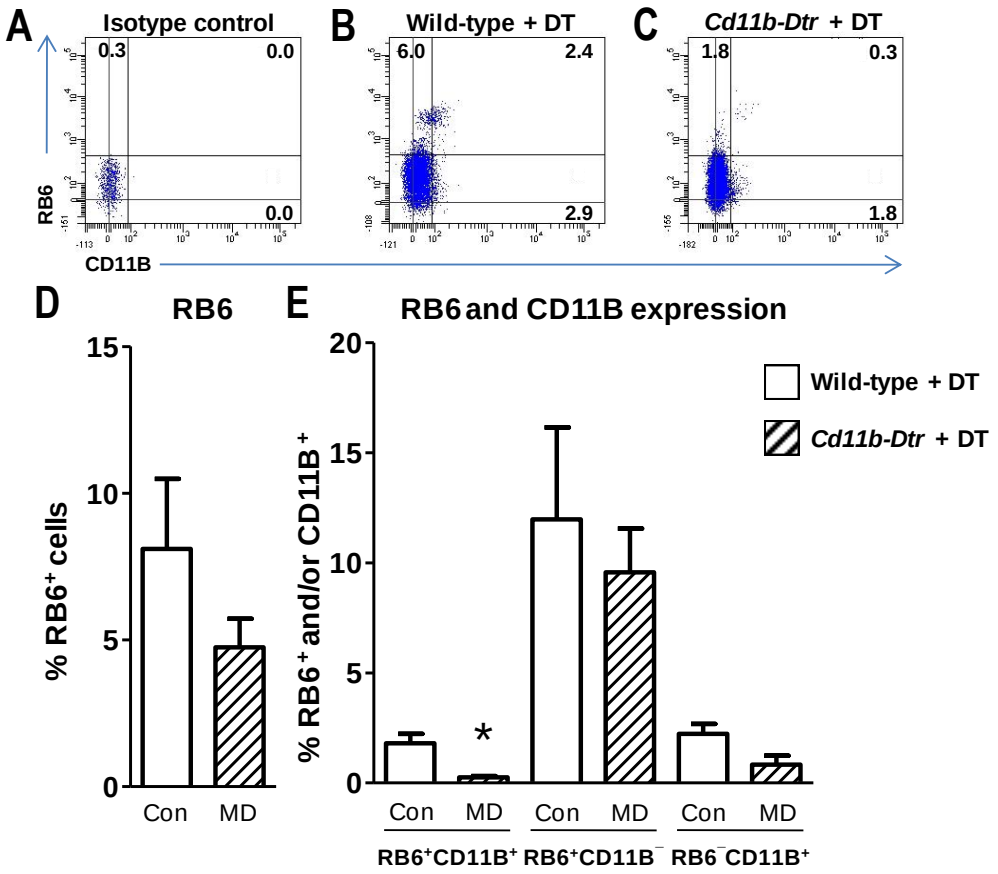

Uterus
A
Isotype control
0.3
0.1
0.7
B
Wild-type + DT
6.3
4.3
9.2
C
Cd11b-Dtr + DT
0.8
2.7
4.0
CD11B
CD11C
D
CD11C
% CD11C+ cells
Con
MD
E
CD11C and CD11B expression
% CD11C+ and/or CD11B+
Wild-type + DT
Cd11b-Dtr + DT
CD11C+CD11B+
CD11C+CD11B−
CD11C−CD11B+
PEC
F
Isotype control
0.0
0.1
0.9
G
Wild-type + DT
9.0
2.5
1.5
H
Cd11b-Dtr + DT
0.2
0.1
0.4
I
CD11C
J
CD11C and CD11B expression

**Figure 3.4 Effect of macrophage depletion on RB6$^+$ and CD11B$^+$ cell populations in the uterus and peritoneal cavity**

Representative dotplots illustrate the proportion of RB6$^+$ and CD11B$^+$ cells in the uterus (A-C) and PEC (F-H). Wild-type uterine tissue labelled with isotype control antibody (A). RB6$^+$ and CD11B$^+$ cells in the uteri of DT-treated wild-type (B) or *Cd11b-Dtr* mice (C). D, percent RB6$^+$ cells, and E, percent of RB6$^+$ and CD11B$^+$ cells in the uteri of DT-treated wild-type and *Cd11b-Dtr* mice. Wild-type PEC labelled with isotype control antibody (F). RB6$^+$ and CD11B$^+$ PECs in DT-treated wild-type (G) or *Cd11b-Dtr* mice (H). I, percent RB6$^+$, and J, percent of RB6$^+$ and CD11B$^+$ PECs in DT-treated wild-type and *Cd11b-Dtr* mice. Data are presented as mean ± SEM with statistical analysis using a Mann-Whitney U test. * indicates statistical significance from control. *$P$ < 0.05. Uterus; n = 4 per group, PEC; wild-type n = 6, *Cd11b-Dtr* n = 4. MD; macrophage depleted. DT; diphtheria toxin. PEC; peritoneal exudate cells.

Uterus


A
Isotype control
0.3
0.0
0.0
B
Wild-type + DT
6.0
2.4
2.9
C
Cd11b-Dtr + DT
1.8
0.3
1.8
RB6
CD11B
D
RB6
% RB6+ cells
15
10
5
0
Con
MD
E
RB6 and CD11B expression
% RB6+ and/or CD11B+
20
15
10
5
0
*
Con
MD
Con
MD
Con
MD
RB6+CD11B+
RB6+CD11B−
RB6−CD11B+
Wild-type + DT
Cd11b-Dtr + DT

PEC

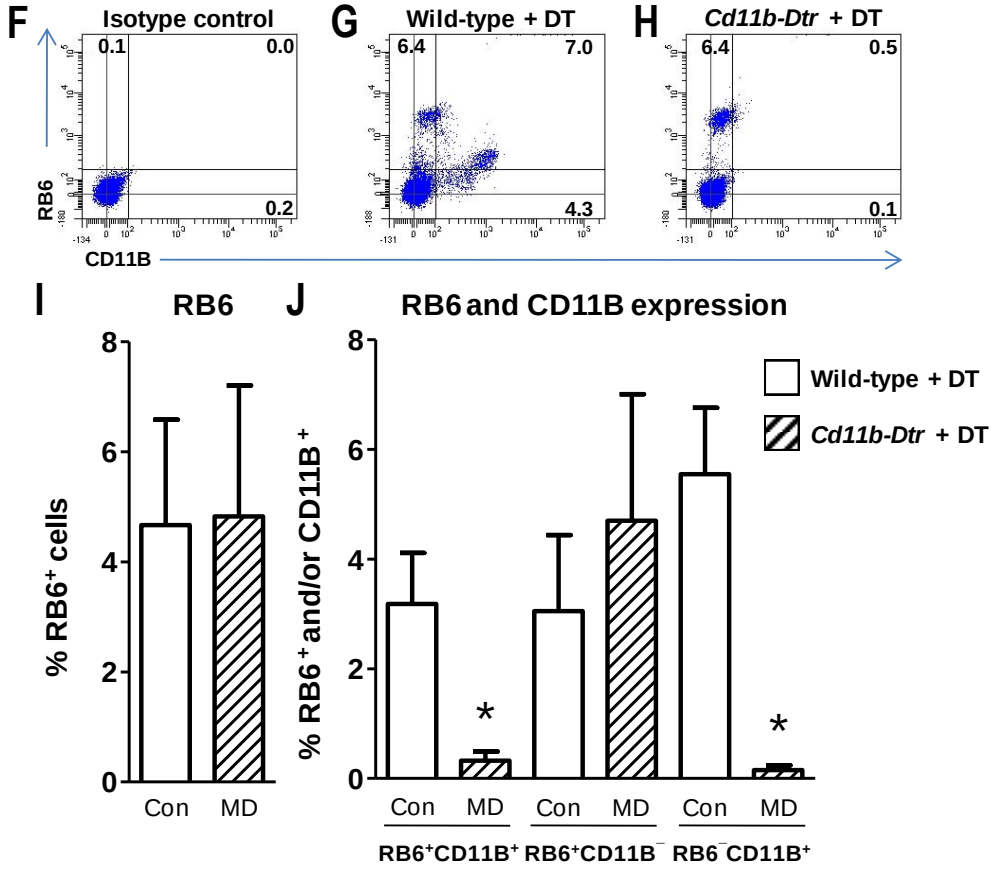
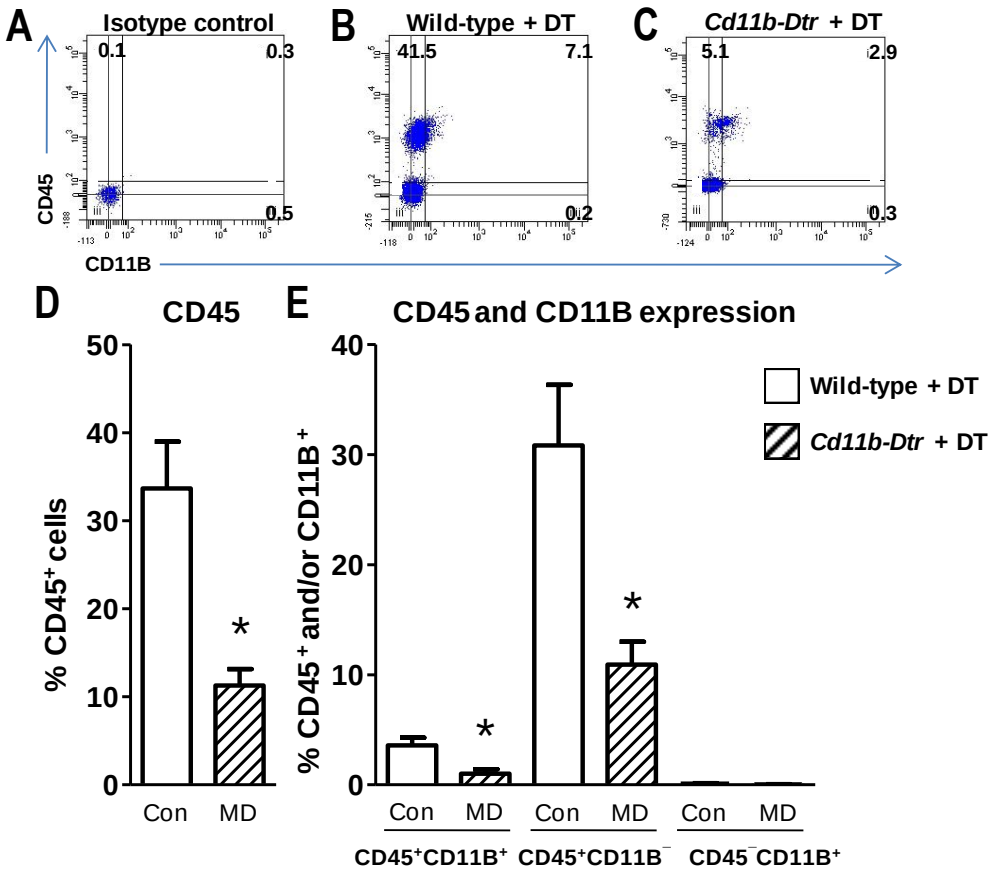

F
Isotype control
0.1
0.0
0.2
G
Wild-type + DT
6.4
7.0
4.3
H
Cd11b-Dtr + DT
6.4
0.5
0.1
RB6
CD11B
I
RB6
% RB6+ cells
8
6
4
2
0
Con
MD
J
RB6 and CD11B expression
% RB6+ and/or CD11B+
8
6
4
2
0
*
*
Con
MD
Con
MD
Con
MD
RB6+CD11B+
RB6+CD11B−
RB6−CD11B+
Wild-type + DT
Cd11b-Dtr + DT

**Figure 3.5 Effect of macrophage depletion on CD45$^+$ and CD11B$^+$ cell populations in the uterus and peritoneal cavity**

Representative dotplots illustrate the proportion of CD45$^+$ and CD11B$^+$ cells in the uterus (A-C) and PEC (F-H). Wild-type uterine tissue labelled with isotype control antibody (A). CD45$^+$ and CD11B$^+$ cells in the uteri of DT-treated wild-type (B) or *Cd11b-Dtr* mice (C). D, percent CD45$^+$ cells, and E, percent of CD45$^+$ and CD11B$^+$ cells in the uteri of DT-treated wild-type and *Cd11b-Dtr* mice. Wild-type PEC labelled with isotype control antibody (F). CD45$^+$ and CD11B$^+$ PECs in DT-treated wild-type (G) or *Cd11b-Dtr* mice (H). I, percent CD45$^+$, and J, percent of CD45$^+$ and CD11B$^+$ PECs in DT-treated wild-type and *Cd11b-Dtr* mice. Data are presented as mean ± SEM with statistical analysis using a Mann-Whitney U test. * indicates statistical significance from control. *$P$ < 0.05, **$P$ < 0.01. Uterus; n = 4 per group, PEC; wild-type n = 6, *Cd11b-Dtr* n = 4. MD; macrophage depleted. DT; diphtheria toxin. PEC; peritoneal exudate cells.

Uterus


A
Isotype control
0.1
0.3
0.5
B
Wild-type + DT
41.5
7.1
0.2
C
Cd11b-Dtr + DT
5.1
2.9
0.3
CD45
CD11B
D
CD45
% CD45+ cells
50
40
30
20
10
0
*
Con
MD
E
CD45 and CD11B expression
% CD45+ and/or CD11B+
40
30
20
10
0
*
*
Wild-type + DT
Cd11b-Dtr + DT
Con
MD
Con
MD
Con
MD
CD45+CD11B+
CD45+CD11B−
CD45−CD11B+

PEC

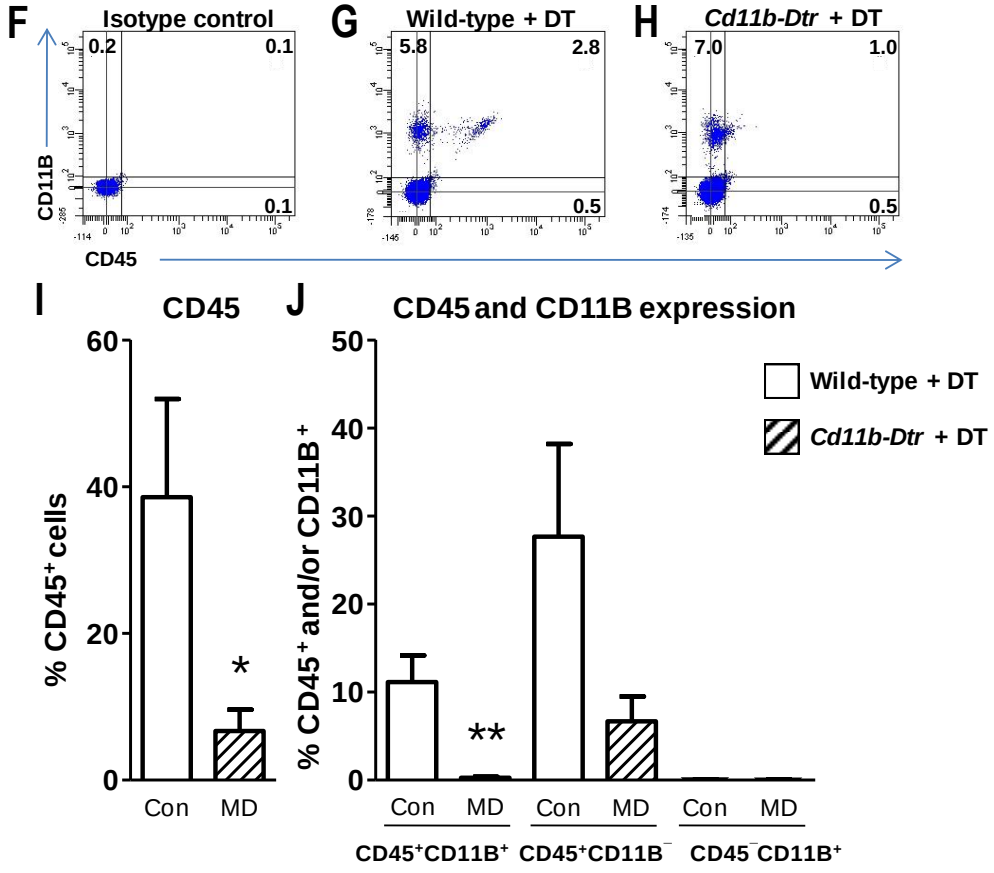
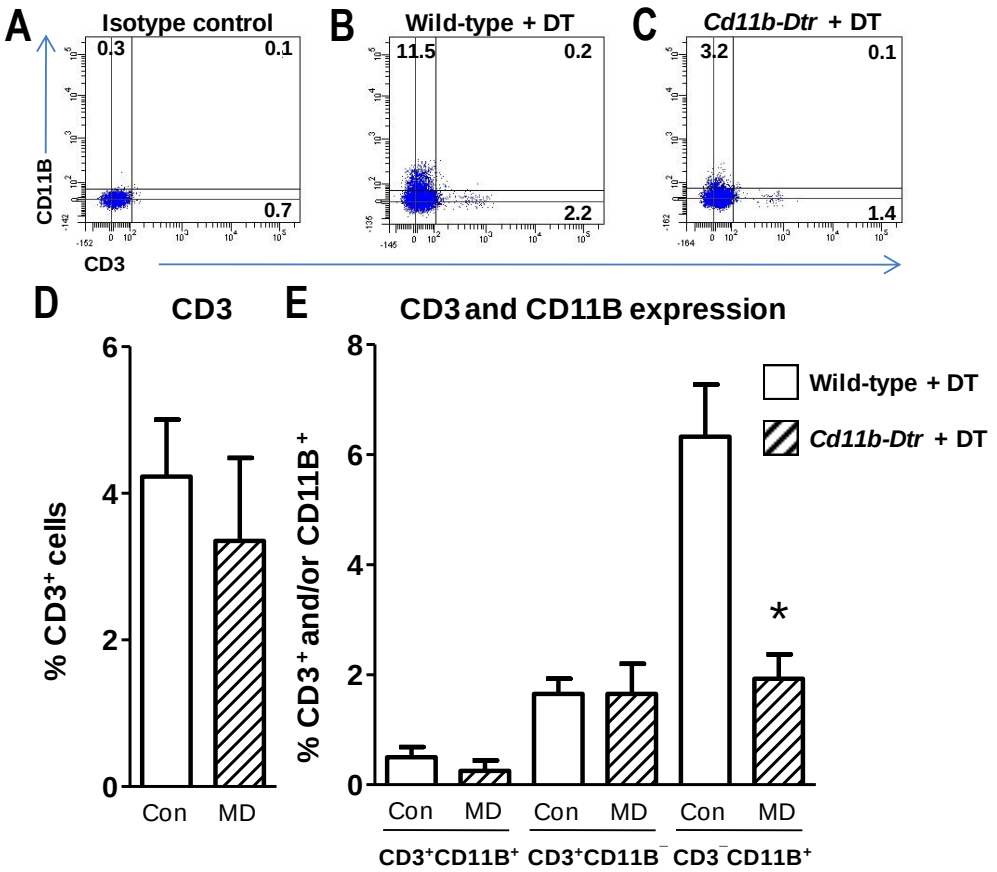

F
Isotype control
0.2
0.1
0.1
G
Wild-type + DT
5.8
2.8
0.5
H
Cd11b-Dtr + DT
7.0
1.0
0.5
CD11B
CD45
I
CD45
% CD45+ cells
60
40
20
0
*
Con
MD
J
CD45 and CD11B expression
% CD45+ and/or CD11B+
50
40
30
20
10
0
**
Wild-type + DT
Cd11b-Dtr + DT
Con
MD
Con
MD
Con
MD
CD45+CD11B+
CD45+CD11B−
CD45−CD11B+

**Figure 3.6 Effect of macrophage depletion on $CD3^+$ and $CD11B^+$ cell populations in the uterus and peritoneal cavity**

Representative dotplots illustrate the proportion of $CD3^+$ and $CD11B^+$ cells in the uterus (A-C) and PEC (F-H). Wild-type uterine tissue labelled with isotype control antibody (A). $CD3^+$ and $CD11B^+$ cells in the uteri of DT-treated wild-type (B) or *Cd11b-Dtr* mice (C). D, percent $CD3^+$ cells, and E, percent of $CD3^+$ and $CD11B^+$ cells in the uteri of DT-treated wild-type and *Cd11b-Dtr* mice. Wild-type PEC labelled with isotype control antibody (F). $CD3^+$ and $CD11B^+$ PECs in DT-treated wild-type (G) or *Cd11b-Dtr* mice (H). I, percent $CD3^+$, and J, percent of $CD3^+$ and $CD11B^+$ PECs in DT-treated wild-type and *Cd11b-Dtr* mice. Data are presented as mean ± SEM with statistical analysis using a Mann-Whitney U test. * indicates statistical significance from control. *$P < 0.05$, **$P < 0.01$. Uterus; n = 4 per group, PEC; wild-type n = 6, *Cd11b-Dtr* n = 4. MD; macrophage depleted. DT; diphtheria toxin. PEC; peritoneal exudate cells.

Uterus


A
Isotype control
0.3
0.1
0.7
B
Wild-type + DT
11.5
0.2
2.2
C
Cd11b-Dtr + DT
3.2
0.1
1.4
CD11B
CD3
D
CD3
% CD3+ cells
Con
MD
E
CD3 and CD11B expression
% CD3+ and/or CD11B+
Wild-type + DT
Cd11b-Dtr + DT
*
CD3+CD11B+
CD3+CD11B−
CD3−CD11B+

PEC

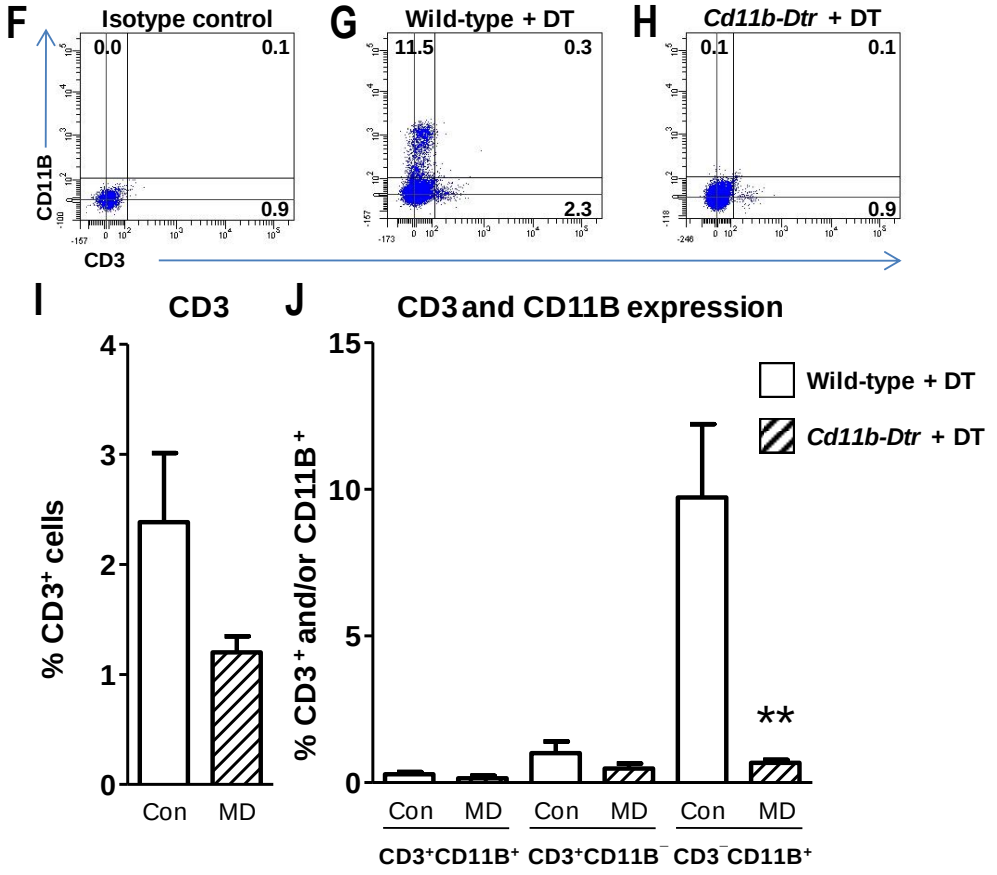

F
Isotype control
0.0
0.1
0.9
G
Wild-type + DT
11.5
0.3
2.3
H
Cd11b-Dtr + DT
0.1
0.1
0.9
CD11B
CD3
I
CD3
% CD3+ cells
Con
MD
J
CD3 and CD11B expression
% CD3+ and/or CD11B+
Wild-type + DT
Cd11b-Dtr + DT
**
CD3+CD11B+
CD3+CD11B−
CD3−CD11B+

**Table 3.1 Effect of DT administration on immune cell population percentage in the uterus, ovary, peritoneal cavity and spleen**

| | $CD11B^+$ | |
|---|---|---|
| | Wild-type | *Cd11b-Dtr* |
| Uterus | 6.4 ± 0.9 | 2.0 ± 0.6** |
| Ovary | 4.5 ± 0.4 | 1.4 ± 0.2** |
| PEC | 9.8 ± 2.4 | 0.7 ± 0.1* |
| Spleen | 0.7 ± 0.3 | 0.6 ± 0.3 |

| | $EMR1^+$ | | $EMR1^+CD11B^+$ | | $EMR1^+CD11B^-$ | | $EMR1^-CD11B^+$ | |
|---|---|---|---|---|---|---|---|---|
| | Wild-type | *Cd11b-Dtr* | Wild-type | *Cd11b-Dtr* | Wild-type | *Cd11b-Dtr* | Wild-type | *Cd11b-Dtr* |
| Uterus | 16.6 ± 2.8 | 8.4 ± 1.6* | 3.2 ± 0.2 | 1.3 ± 0.5* | 6.2 ± 1.5 | 3.9 ± 1.1 | 3.0 ± 0.3 | 0.7 ± 0.2* |
| Ovary | 7.1 ± 1.9 | 0.7 ± 0.3** | 1.4 ± 0.3 | 0.2 ± 0.2* | 4.8 ± 2.5 | 0.3 ± 0.1* | 2.7 ± 0.5 | 0.9 ± 0.1 |
| PEC | 9.1 ± 2.2 | 0.7 ± 0.3** | 8.0 ± 2.0** | 0.2 ± 0.1 | 0.2 ± 0.1 | 0.1 ± 0.0 | 4.3 ± 1.4 | 0.9 ± 0.2 |
| Spleen | 2.8 ± 0.6 | 2.5 ± 0.6 | 0.1 ± 0.0 | 0.1 ± 0.0 | 1.6 ± 0.3 | 1.4 ± 0.4 | 0.2 ± 0.0 | 0.2 ± 0.0 |

| | $CD11C^+$ | | $CD11C^+CD11B^+$ | | $CD11C^+CD11B^-$ | | $CD11C^-CD11B^+$ | |
|---|---|---|---|---|---|---|---|---|
| | Wild-type | *Cd11b-Dtr* | Wild-type | *Cd11b-Dtr* | Wild-type | *Cd11b-Dtr* | Wild-type | *Cd11b-Dtr* |
| Uterus | 6.6 ± 1.0 | 1.9 ± 0.7* | 2.5 ± 0.5 | 0.9 ± 0.4 | 3.6 ± 0.8 | 0.8 ± 0.3* | 4.2 ± 0.4 | 1.3 ± 0.2* |
| Ovary | 2.4 ± 0.4 | 0.3 ± 0.2* | 1.6 ± 0.3 | 0.2 ± 0.2* | 1.4 ± 0.4 | 0.2 ± 0.1* | 3.4 ± 0.6 | 1.0 ± 0.2* |
| PEC | 2.0 ± 0.5 | 0.3 ± 0.1* | 1.3 ± 0.3 | 0.2 ± 0.1* | 0.6 ± 0.2 | 0.1 ± 0.0 | 9.5 ± 2.7 | 0.8 ± 0.1* |
| Spleen | 4.4 ± 0.3 | 4.6 ± 0.3 | 0.1 ± 0.1 | 0.1 ± 0.0 | 4.1 ± 0.3 | 4.4 ± 0.3 | 0.2 ± 0.0 | 0.2 ± 0.0 |

| | $RB6^+$ | | $RB6^+CD11B^+$ | | $RB6^+CD11B^-$ | | $RB6^-CD11B^+$ | |
|---|---|---|---|---|---|---|---|---|
| | Wild-type | *Cd11b-Dtr* | Wild-type | *Cd11b-Dtr* | Wild-type | *Cd11b-Dtr* | Wild-type | *Cd11b-Dtr* |
| Uterus | 8.1 ± 2.4 | 4.8 ± 1.0 | 1.8 ± 0.4 | 0.3 ± 0.1* | 12.0 ± 4.2 | 9.6 ± 2.0 | 2.2 ± 0.5 | 0.8 ± 0.4 |
| Ovary | 10.2 ± 3.1 | 3.0 ± 0.9* | 2.5 ± 0.6 | 1.0 ± 0.2 | 15.1 ± 5.1 | 4.3 ± 1.7# | 1.7 ± 0.3 | 0.4 ± 0.4 |
| PEC | 4.7 ± 1.9 | 4.8 ± 2.4 | 3.2 ± 0.9 | 0.3 ± 0.2* | 3.1 ± 1.4 | 4.7 ± 2.3 | 5.6 ± 1.2 | 0.2 ± 0.1* |
| Spleen | 6.8 ± 0.5 | 11.1 ± 1.5** | 0.1 ± 0.0 | 0.2 ± 0.1 | 7.8 ± 0.5 | 12.3 ± 1.5** | 0.2 ± 0.1 | 0.2 ± 0.1 |

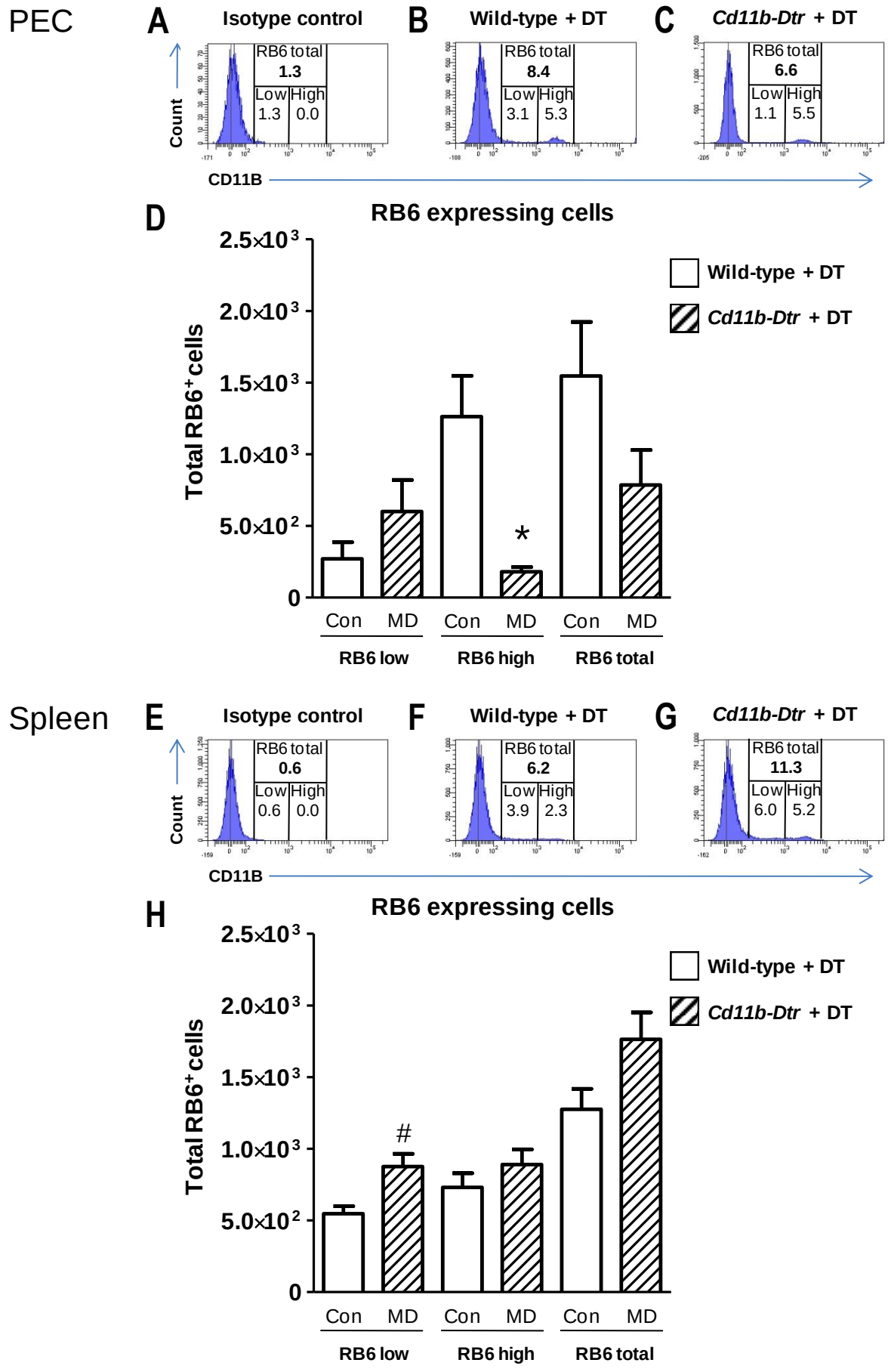
| | $CD45^+$ | | $CD45^+CD11B^+$ | | $CD45^+CD11B^-$ | | $CD45^-CD11B^+$ | |
|---|---|---|---|---|---|---|---|---|
| | Wild-type | *Cd11b-Dtr* | Wild-type | *Cd11b-Dtr* | Wild-type | *Cd11b-Dtr* | Wild-type | *Cd11b-Dtr* |
| Uterus | 33.7 ± 5.3 | 11.3 ± 1.9* | 3.6 ± 0.7 | 1.0 ± 0.4* | 30.9 ± 5.5 | 10.9 ± 2.0* | 0.1 ± 0.1 | 0.0 ± 0.0 |
| Ovary | 10.1 ± 1.2 | 2.1 ± 1.1* | 3.3 ± 0.5 | 1.3 ± 0.6* | 8.3 ± 1.8 | 1.2 ± 0.4** | 0.1 ± 0.1 | 0.1 ± 0.1 |
| PEC | 38.6 ± 13.4 | 6.7 ± 2.9* | 11.2 ± 3.0 | 0.3 ± 0.1** | 27.7 ± 10.5 | 6.7 ± 2.8 | 0.1 ± 0.0 | 0.1 ± 0.1 |
| Spleen | 94.2 ± 2.2 | 95.0 ± 1.9 | 0.3 ± 0.2 | 0.3 ± 0.2 | 94.5 ± 1.8 | 95.4 ± 1.3 | 0.0 ± 0.0 | 0.0 ± 0.0 |

| | $CD3^+$ | | $CD3^+CD11B^+$ | | $CD3^+CD11B^-$ | | $CD3^-CD11B^+$ | |
|---|---|---|---|---|---|---|---|---|
| | Wild-type | *Cd11b-Dtr* | Wild-type | *Cd11b-Dtr* | Wild-type | *Cd11b-Dtr* | Wild-type | *Cd11b-Dtr* |
| Uterus | 4.2 ± 0.8 | 3.4 ± 1.1 | 0.5 ± 0.2 | 0.3 ± 0.2 | 1.7 ± 0.3 | 1.7 ± 0.5 | 6.3 ± 0.9 | 1.9 ± 0.4* |
| Ovary | 1.3 ± 0.3 | 0.3 ± 0.2 | 0.4 ± 0.2 | 0.3 ± 0.2 | 1.5 ± 0.3 | 0.2 ± 0.1* | 4.2 ± 0.5 | 1.3 ± 0.3* |
| PEC | 2.4 ± 0.6 | 1.2 ± 0.1 | 0.3 ± 0.1 | 0.2 ± 0.1 | 1.0 ± 0.4 | 0.5 ± 0.2 | 9.7 ± 2.5 | 0.7 ± 0.1** |
| Spleen | 19.4 ± 1.8 | 27.5 ± 5.4 | 0.1 ± 0.0 | 0.1 ± 0.0 | 18.6 ± 1.8 | 27.2 ± 5.4 | 0.1 ± 0.0 | 0.2 ± 0.0 |

Data are presented as mean ± SEM with statistical analysis using a Mann-Whitney U test. * indicates statistical significance from control. *$P$ < 0.05, **$P$ < 0.01, # indicates a $P$ value of >0.05 but <0.06. Uterus; n = 4 per group. Ovary, PEC and Spleen; wild-type n = 6, *Cd11b-Dtr* n = 4. DT; diphtheria toxin. PEC; peritoneal exudate cells.

**Figure 3.7 Total number of RB6 expressing cells in the peritoneal cavity and the spleen**

Representative histograms show RB6 expression by PEC (A-C) and splenocytes (E-G). Wild-type PEC labelled with isotype-control antibody (A). RB6 expressing PEC from DT-treated wild-type (B) and *Cd11b-Dtr* mice (C). Wild-type splenocytes labelled with isotype-control antibody (E). RB6 expressing splenocytes from DT-treated wild-type (F) and *Cd11b-Dtr* mice (G). Total numbers of RB6 expressing cells were quantified in the peritoneal cavity (D) and spleen (H). Cells were also classified as either RB6 high or RB6 low. Data are presented as mean ± SEM with statistical analysis using a Mann-Whitney U test. * indicates statistical significance from control. *$P < 0.05$. # indicates a $P$ value of >0.05 but <0.06. Wild-type n = 5, *Cd11b-Dtr* n = 4. MD; Macrophage depleted. DT; diphtheria toxin PEC; peritoneal exudate cells.


PEC
A
Isotype control
RB6 total
1.3
Low
1.3
High
0.0
B
Wild-type + DT
RB6 total
8.4
Low
3.1
High
5.3
C
Cd11b-Dtr + DT
RB6 total
6.6
Low
1.1
High
5.5
Count
CD11B
D
RB6 expressing cells
Total RB6+ cells
2.5×10³
2.0×10³
1.5×10³
1.0×10³
5.0×10²
0
Wild-type + DT
Cd11b-Dtr + DT
*
Con
MD
RB6 low
RB6 high
RB6 total
Spleen
E
Isotype control
RB6 total
0.6
Low
0.6
High
0.0
F
Wild-type + DT
RB6 total
6.2
Low
3.9
High
2.3
G
Cd11b-Dtr + DT
RB6 total
11.3
Low
6.0
High
5.2
H
RB6 expressing cells
#

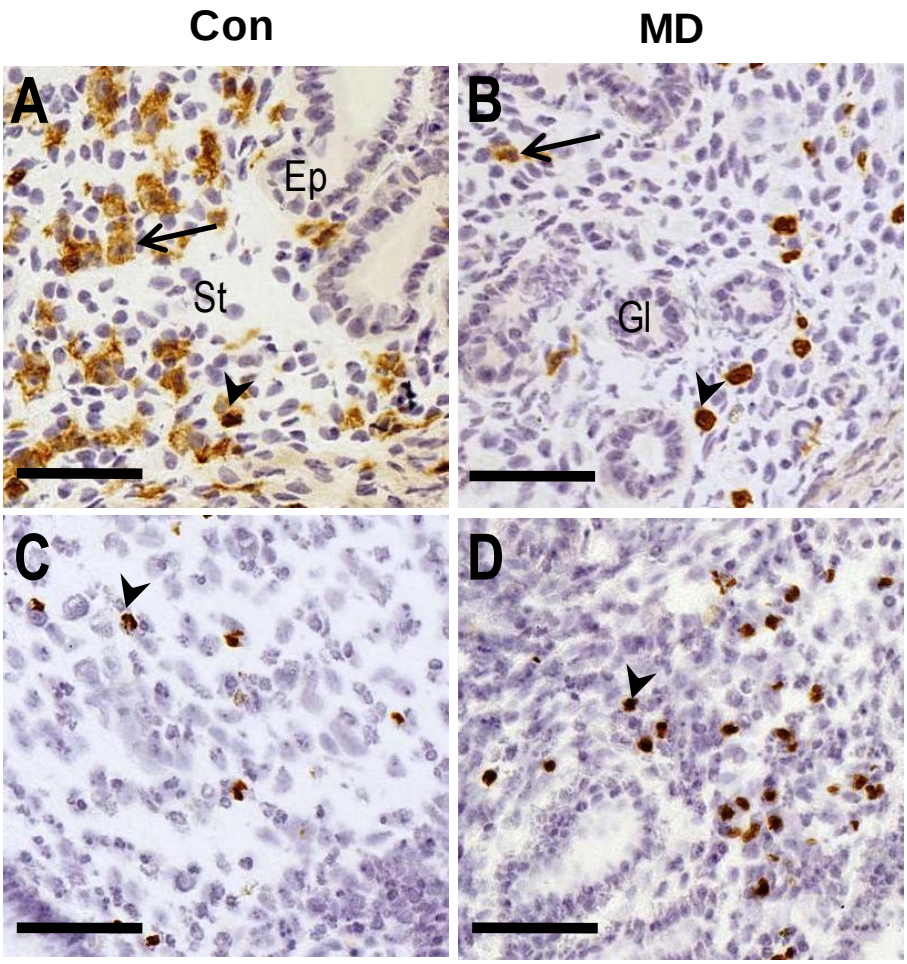
EMR1 was initially thought to be a macrophage specific marker (Austyn and Gordon, 1981, Hume et al., 1983), but has more recently been shown to be expressed on eosinophilic granulocytes (McGarry and Stewart, 1991). Thus, immunohistochemical analysis was conducted to more specifically identify macrophage specific EMR1 expression, and to determine the exact extent of macrophage depletion from the endometrium after DT administration. This method allows the identification and exclusion of EMR1-positive eosinophils from the analysis, as outlined in section 2.3.2.5. Briefly, estrus uteri were taken from PBS or DT-treated *Cd11b-Dtr* mice (where DT was injected 24 h prior, during proestrus) and frozen in an embedding medium, as described in section 2.3.1.1. Macrophages were identified using the monoclonal antibody EMR1, present on the cell surface of monocytes and macrophages, and eosinophils were identified on the basis of their endogenous peroxidase activity.

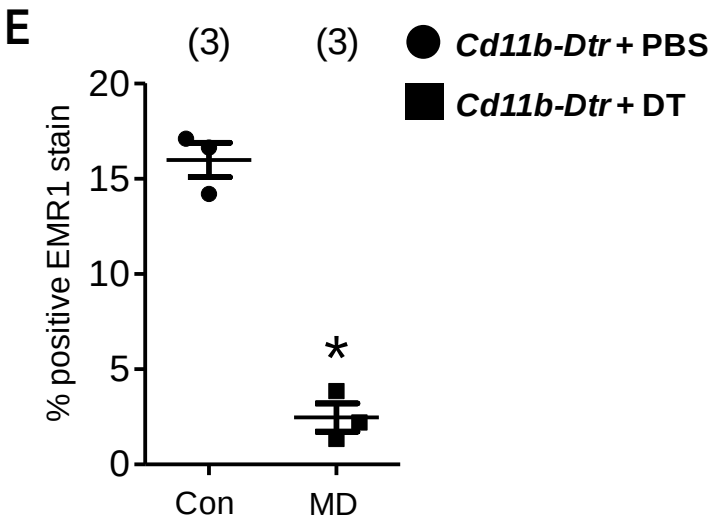
Stain positivity was quantified by video image analysis, where the percent of positive staining was normalised to total cell number (Chapter 2.3.2.5). The number of endogenous peroxidise positive eosinophils did not differ significantly between treatment groups (in PBS-treated *Cd11b-Dtr* = 1.5 ± 0.7 and in DT-treated *Cd11b-Dtr* = 1.0 ± 0.6; $P = 0.7$, two-tailed Mann-Whitney U test). Values for eosinophils were subtracted from total EMR1$^+$ values to determine staining values for EMR1 macrophage-positivity only (example shown in Figure 3.8). The final result showed that the PBS treated *Cd11b-Dtr* endometrium had an average of 16.0% EMR1-positivity, and this was significantly decreased to 2.5% in the DT-treated *Cd11b-Dtr* group ($P = 0.0003$, Unpaired t test; Figure 3.8E). Macrophage depletion was also confirmed on day 3.5 pc, 24 h following DT injection, but this result was not quantified.

Immunohistochemistry was again used to determine whether macrophages were depleted from the ovary during pregnancy. Briefly, day 3.5 pc wild-type or *Cd11b-Dtr* mice were treated with DT, and 24 h later on day 4.5 pc, mice were sacrificed, their ovaries excised and frozen in embedding medium. Macrophages were identified using EMR1 antibody as described above. The photomicrographs in Figure 3.9 shows there are substantially fewer EMR1$^+$ macrophages following treatment with DT (B and D) compared to PBS treated *Cd11b-Dtr* mice (A and C), correlating with the results obtained by flow cytometry in the ovary at estrus (Table 3.1).

**Figure 3.8 Effect of DT administration on uterine EMR1-positive cell population at estrus**

Uteri were obtained from estrus mice that had received an i.p injection of DT (25 ng/g) 24 h prior. Frozen tissue was sectioned and stained using anti-EMR1 antibody, which is present on the surface of macrophages. Representative photomicrographs of tissue from PBS- (A) and DT-treated (B) *Cd11b-Dtr* mice illustrate macrophage numbers in control and treatment groups. C and D are representative photomicrographs of tissue that has been incubated with no antibody to identify endogenous peroxidase-positive eosinophils. Approximately 10 individual fields per uterus per mouse were analysed. Percentage of EMR1-positivity in the PBS- and DT-treated uteri of *Cd11b-Dtr* mice (E). Values for endogenous peroxidase-positive eosinophils were subtracted from the total staining to determine the percent of $EMR1^+$ staining macrophages. Data are presented as mean ± SEM with statistical analysis using a one-tailed Mann-Whitney U test. * indicates statistical significance from control. *$P < 0.05$. Number of animals in each group shown in parentheses. Arrows; EMR1-positive macrophages. Arrowheads; endogenous peroxidase positive eosinophils. MD; macrophage depleted. DT; diphtheria toxin. St; stroma. Gl; glandular epithelium. Ep; luminal epithelium. Scale bar = 50μm.


Con
MD
A
Ep
St
B
Gl
C
D


E
(3)
(3)
Cd11b-Dtr + PBS
Cd11b-Dtr + DT
% positive EMR1 stain
20
15
10
5
0
*
Con
MD

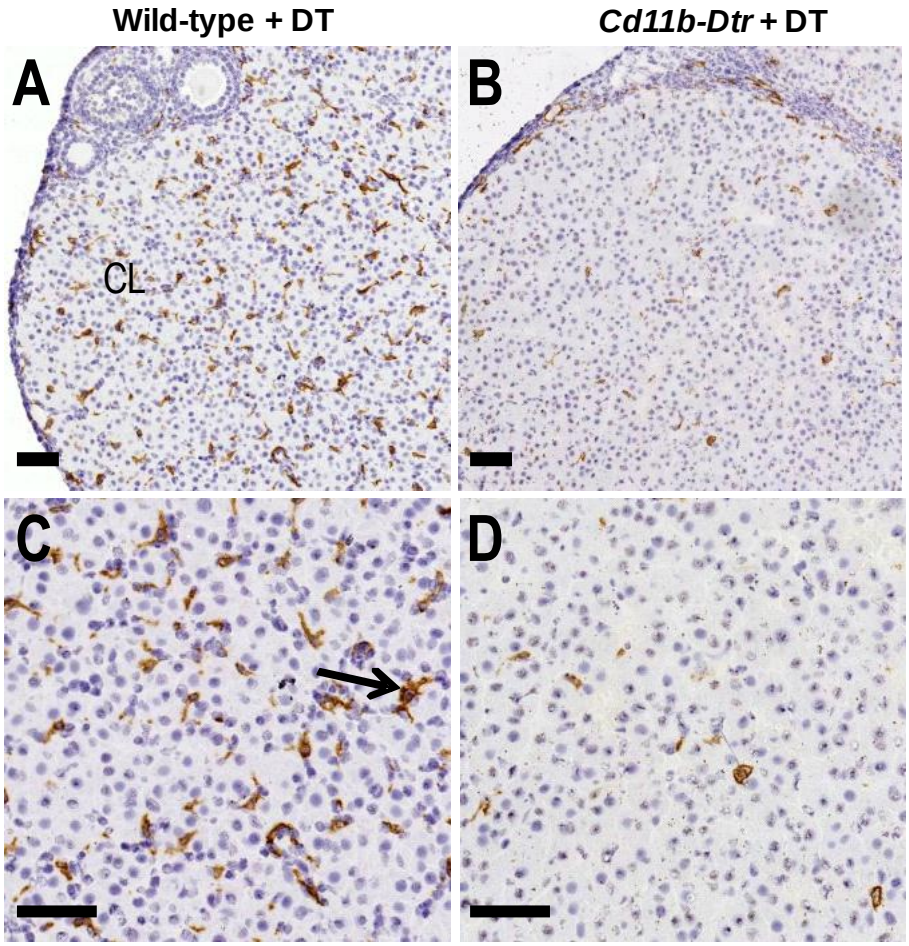



**Figure 3.9 Effect of DT administration on EMR1-positive macrophages in the corpus luteum**

Ovaries were obtained from day 4.5 pc females 24 h after i.p injection of DT (25 ng/g). Frozen sections were stained using anti-EMR1 antibody to detect the EMR1 marker on the surface of macrophages. Representative photomicrographs of tissue from DT-treated wild-type mice at low (A) and high power (C), and DT-treated *Cd11b-Dtr* mice at low (B) and high power (D). Arrows; EMR1-positive macrophages. DT; diphtheria toxin. CL; corpus luteum. Scale bar = 50 µm.

### 3.3 EFFECT OF MACROPHAGE DEPLETION ON EARLY PREGNANCY

It has been suggested that resident uterine macrophages may have key roles in endometrial tissue remodelling in preparation for embryo implantation and trophoblast invasion. After showing that macrophages were depleted from female reproductive tissues in DT-treated *Cd11b-Dtr* mice, we utilised this model to determine the importance of these cells in early pregnancy.

Briefly, wild-type and *Cd11b-Dtr* mice were housed with stud males of the same genotype (maximum of two females per stud male) and checked daily for the presence of a vaginal plug (designated day 0.5 pc). During early pregnancy (day 0.5 pc) or at implantation (day 3.5 pc) mice received DT (25 ng/g) at 9:00 h. On day 7.5 pc mice were sacrificed, their uteri excised, and implantation sites were counted. Representative photos in Figure 3.10 shows normal implantation sites in wild-type mice treated with DT on day 0.5 or day 3.5 pc (A and C, respectively). However, no implantation sites were present in the uteri of *Cd11b-Dtr* mice given DT at either of these time-points (B and D). Figure 3.11A shows the percentage of viable pregnancies for wild-type mice receiving DT on day 0.5 pc is 83.3% (10/12), and is reduced to zero (0/9) in DT-treated *Cd11b-Dtr* mice ($P$ = 0.0002, Fisher's exact test), with the number (mean ± SEM) of implantation sites being 6.6 ± 1.4 and zero, respectively ($P$ = 0.001, Unpaired t test). Similar results were seen when DT injections were given on day 3.5 pc (B), where the percent viable pregnancies in DT-treated wild-type mice was 58.3% (7/12), while there were no viable pregnancies in six DT-treated *Cd11b-Dtr* mice ($P$ = 0.04, Fisher's exact test); the average number of implantation sites was 6.3 ±1.6 and zero, respectively ($P$ = 0.016, Unpaired t test). *Cd11b-Dtr* breeder pairs breed normally and have healthy offspring. *Cd11b-Dtr* female mice treated with PBS on day 3.5 pc have normal implantations sites on day 7.5 pc (n = 2, with 10 and 11 implantation sites, respectively).

Figure 3.11C shows that *Cd11b-Dtr* mice lost weight between days 0.5 pc or 3.5 pc, and day 7.5. The change in the weight of wild-type and *Cd11b-Dtr* mice receiving DT on day 0.5 pc was significant, with wild-type mice gaining 2.4 ± 0.4 g and *Cd11b-Dtr* mice losing 3.7 ± 0.9 g ($P$ = 0.0006, Mann Whitney U test). Wild-type mice receiving DT on day 3.5 pc gained 1.4 ± 0.4 g, and *Cd11b-Dtr* mice lost 0.9 ± 1.1 g, however, the effect in this group did not reach statistical significance ($P$ = 0.11, Mann Whitney U test).

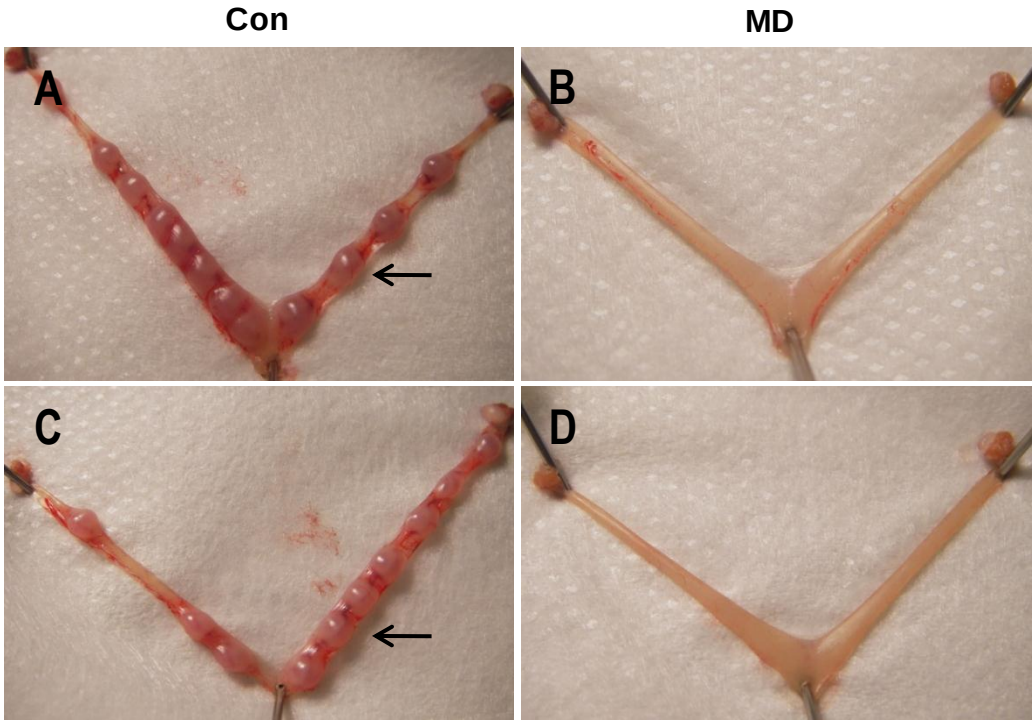
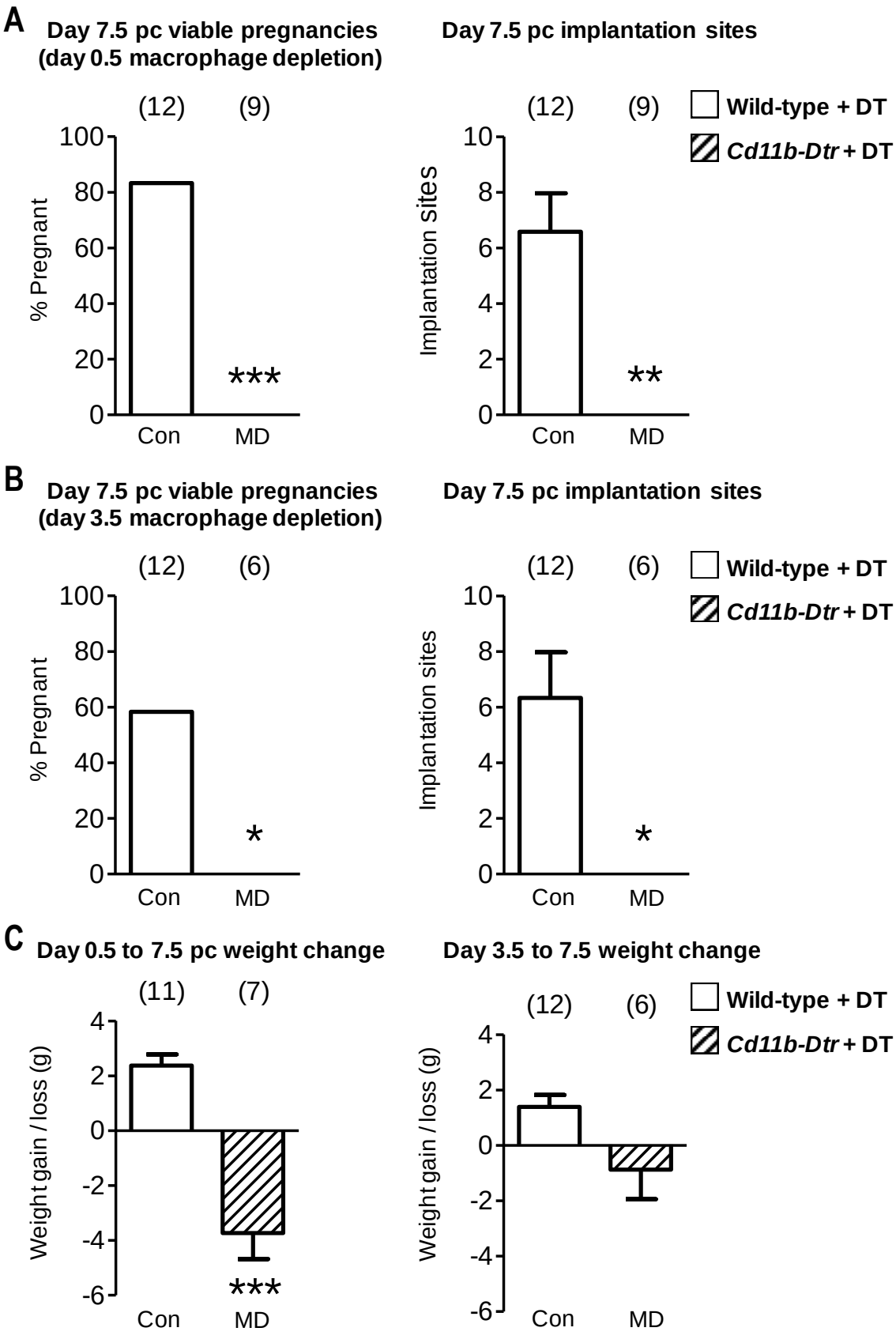



**Figure 3.10 Effect of macrophage depletion in early pregnancy and at implantation**

Wild-type (left images) and *Cd11b-Dtr* (right images) mice received an injection of DT (25 ng/g; i.p.) during early pregnancy (day 0.5 pc; A and B) or at implantation (day 3.5 pc; C and D). On day 7.5 pc mice were sacrificed and the number of implantation sites per uterus was counted. Arrows; implantation sites. MD; macrophage depleted. DT; diphtheria toxin.

**Figure 3.11 Effect of macrophage depletion on viable pregnancies and implantation rate**

DT was administered to wild-type or *Cd11b-Dtr* mice on day 0.5 pc (A, early pregnancy) or day 3.5 pc (B, implantation). The percentage of mice carrying viable pregnancies (A and B, left) and the mean number of implantation sites per mother (A and B, right) is shown. The weight gain/loss per animal was determined for mice given DT on day 0.5 pc (C, left) and of day 3.5 pc (C, right). Data are presented as mean ± SEM with statistical analysis using a Mann-Whitney U test. * indicates statistical significance from control. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Number of animals in each group shown in parentheses. MD; macrophage depleted. DT; diphtheria toxin.


A
Day 7.5 pc viable pregnancies
(day 0.5 macrophage depletion)
Day 7.5 pc implantation sites
(12)
(9)
Wild-type + DT
Cd11b-Dtr + DT
% Pregnant
Implantation sites
100
80
60
40
20
0
10
8
6
4
2
***
**
Con
MD
B
Day 7.5 pc viable pregnancies
(day 3.5 macrophage depletion)
Day 7.5 pc implantation sites
(6)
*
C
Day 0.5 to 7.5 pc weight change
Day 3.5 to 7.5 weight change
(11)
(7)
Weight gain / loss (g)
4
2
0
-2
-4
-6

### 3.4 EFFECT OF LOWER DOSES OF DIPHTHERIA TOXIN ON EARLY PREGNANCY

A second experiment was conducted where mice received a lower dose of DT, and pregnancy outcome was measured. Briefly, wild-type and *Cd11b-Dtr* females were mated with stud males of the same genotype. Mice were checked daily for the presence of a vaginal plug, and removed from males thereafter. On day 3.5 pc, females received an i.p. injection of DT at 10 ng/g, and pregnancy outcome was assessed at autopsy on day 7.5 pc.

Figure 3.12A shows that 50% of *Cd11b-Dtr* mice carried viable pregnancies on day 7.5, compared to 100% of wild-type mice ($P = 0.16$, Fisher's exact test). Figure 3.12B shows that there was a significant reduction in the number of implantations sites (Wild-type implantation sites (mean ± SEM) = 3.2 ± 1.7 and *Cd11b-Dtr* mice = 10.0 ± 0.4; $P = 0.04$, Mann Whitney U test).

A small number of mice were also given 10 ng/g of DT on day 1 pc (data not shown). In this group, two out of three (66%) wild-type mice carried viable pregnancies on day 7.5 pc, with 4.7 ± 2.3 implantation sites. Two out of four (50%) *Cd11b-Dtr* carried viable pregnancies on day 7.5, with 4.0 ± 2.3 implantation sites.

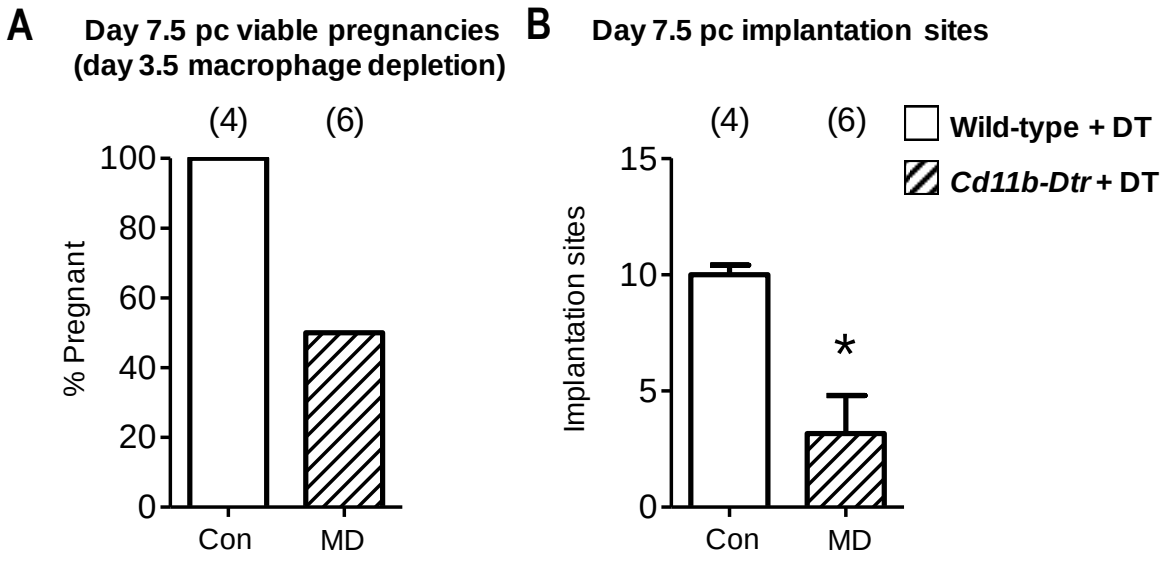



**Figure 3.12 Effect of low dose DT on viable pregnancies and implantation rate**

10 ng/g of DT was administered to wild-type or *Cd11b-Dtr* mice on day 3.5 pc. The percentage of mice carrying viable pregnancies were calculated (A) as were the number of implantation sites per mother (B). Data are presented as mean ± SEM with statistical analysis using a Mann-Whitney U test and Fisher's exact test. * indicates statistical significance from control. *$P < 0.05$. Number of animals in each group shown in parentheses. MD; macrophage depleted. DT; diphtheria toxin.

### 3.5 EFFECT OF DIPHTHERIA TOXIN ON DEVELOPING EMBRYOS

As described in the previous section, pregnancy was seen to fail following macrophage depletion. We could not rule out the possibility that the administration of DT to pregnant *Cd11b-Dtr* mice may have had direct adverse effects on the developing embryos. To test this, a series of *in vitro* embryo culture experiments were undertaken.

Briefly, *Cd11b-Dtr* females were superovulated and caged with a *Cd11b-Dtr* stud male (one female per stud male). Embryos were recovered either late in the morning on the day of the plug (day 1 pc) or on the afternoon of day 3 pc. Embryos recovered from each animal were split into two groups and cultured with or without DT (25 ng/ml). A concentration of 25 ng/ml was expected to be higher than the concentration that embryos would be exposed to *in vivo* following an i.p. injection. Embryos were scored for their developmental stage on the day of recovery from the mouse, and once daily thereafter. The embryos recovered on day 1 pc were initially cultured in G1 media ± DT, and were changed to G2 media on day 3 of culture, with no DT in either group (to assess the effect of DT on development from the one-cell to the 8-cell stage). Embryos flushed on day 3 pc were cultured with G2 media ± DT (to assess the effect of DT on development from the 8-cell stage to blastocyst stage). Figure 3.13 shows the number of viable embryos recovered from the oviduct (day 1 pc; A) or uterus (day 3 pc; B) that proceeded to develop to the blastocyst stage when cultured in control media, or with DT (Chapter 2.4.1).

There was no difference in the ability of day 1 or day 3 pc recovered embryos to develop to the blastocyst stage when cultured with or without DT (Day 1 embryos with DT (mean ± SEM) = 56.2 ± 8.7% and with no DT = 69.0 ± 8.5%; $P = 0.24$, Mann Whitney U test; Figure 3.13A. Day 3 embryos with DT = 64.5 ± 10.5% and with no DT = 70.4 ± 6.2%; $P = 0.77$, Mann Whitney U test; Figure 3.13B).

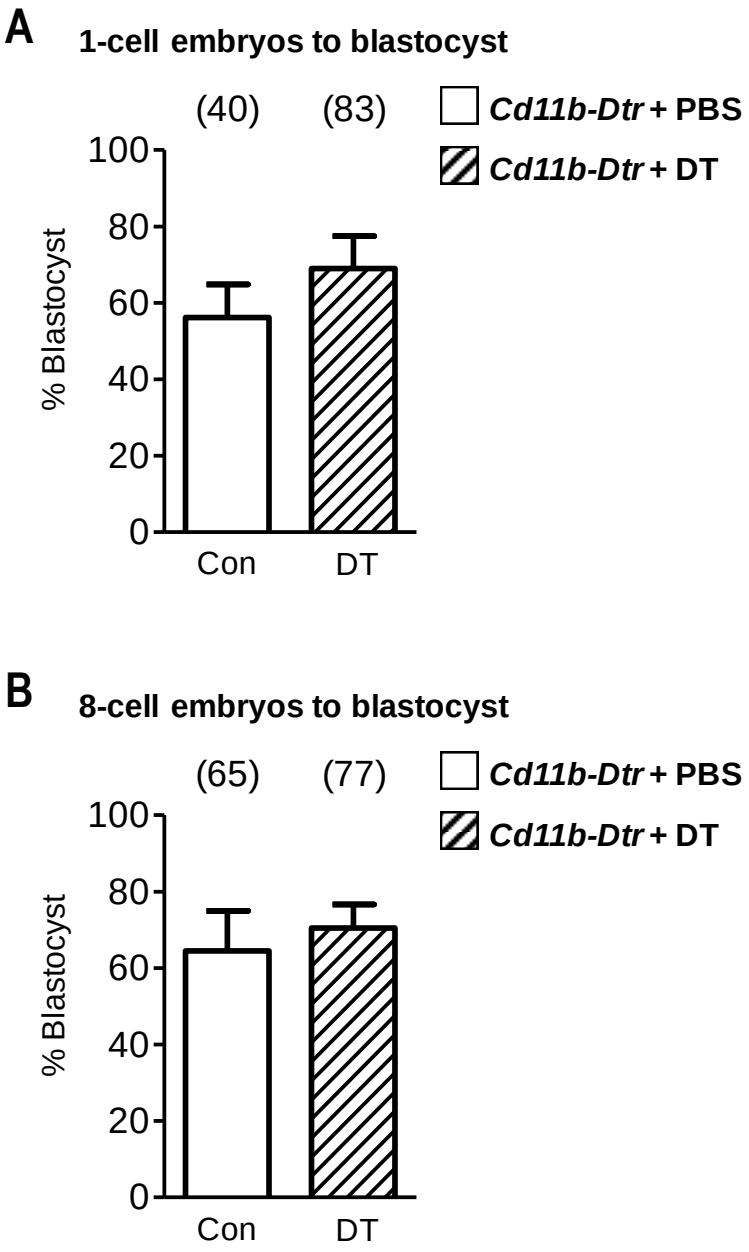



**Figure 3.13 Effect of DT on the development of 1-cell and 8-cell embryos to blastocyst stage**

Embryos were recovered from the oviduct of *Cd11b-Dtr* females mated with *Cd11b-Dtr* males on day 1 pc (1-cell embryos; A), or flushed from the uterus on day 3 pc (8-cell embryos; B). 1-cell embryos were cultured ± DT (25 ng/ml) until day 3, where the media was changed, and all embryos were then cultured in the absence of DT until they reached blastocyst stage. 8-cell embryos were cultured ± DT (25 ng/ml) until they reached blastocyst stage. Data are presented as mean ± SEM with statistical analysis using a Mann-Whitney U test. * indicates statistical significance from control. Number of embryos in each group shown in parentheses. DT; diphtheria toxin.

### 3.6 PREGNANCY FAILS AFTER IMPLANTATION IN THE *Cd11b-Dtr* MOUSE

Because of the complete absence of implantation sites or resorptions in the uteri of DT-treated *Cd11b-Dtr* mice, an earlier time-point was assessed to determine the stage at which implantation was arrested. This was evaluated by injecting DT into wild-type and *Cd11b-Dtr* mice on day 3.5 pc, and inspecting uteri for implantation sites on either day 4.5 (24 h after DT), or day 5.5 (48 h after DT). Trypan blue solution was injected intravenously to anaesthetised animals in order to visualise and count the number of implanting embryos (Chapter 2.1.9), taking advantage of the increase in mircrovascular permeability that occurs at this time in the endometrium. Trypan blue, like the Pontamine sky blue (or Evans' blue) reaction, binds to serum albumin. Tissues will be coloured blue in areas an increase in vascular permeability allows serum albumin to leak into tissues (Psychoyos, 1960, Rogers, 1992, Ni et al., 2002). The results for each of these time points are presented in Figure 3.14.

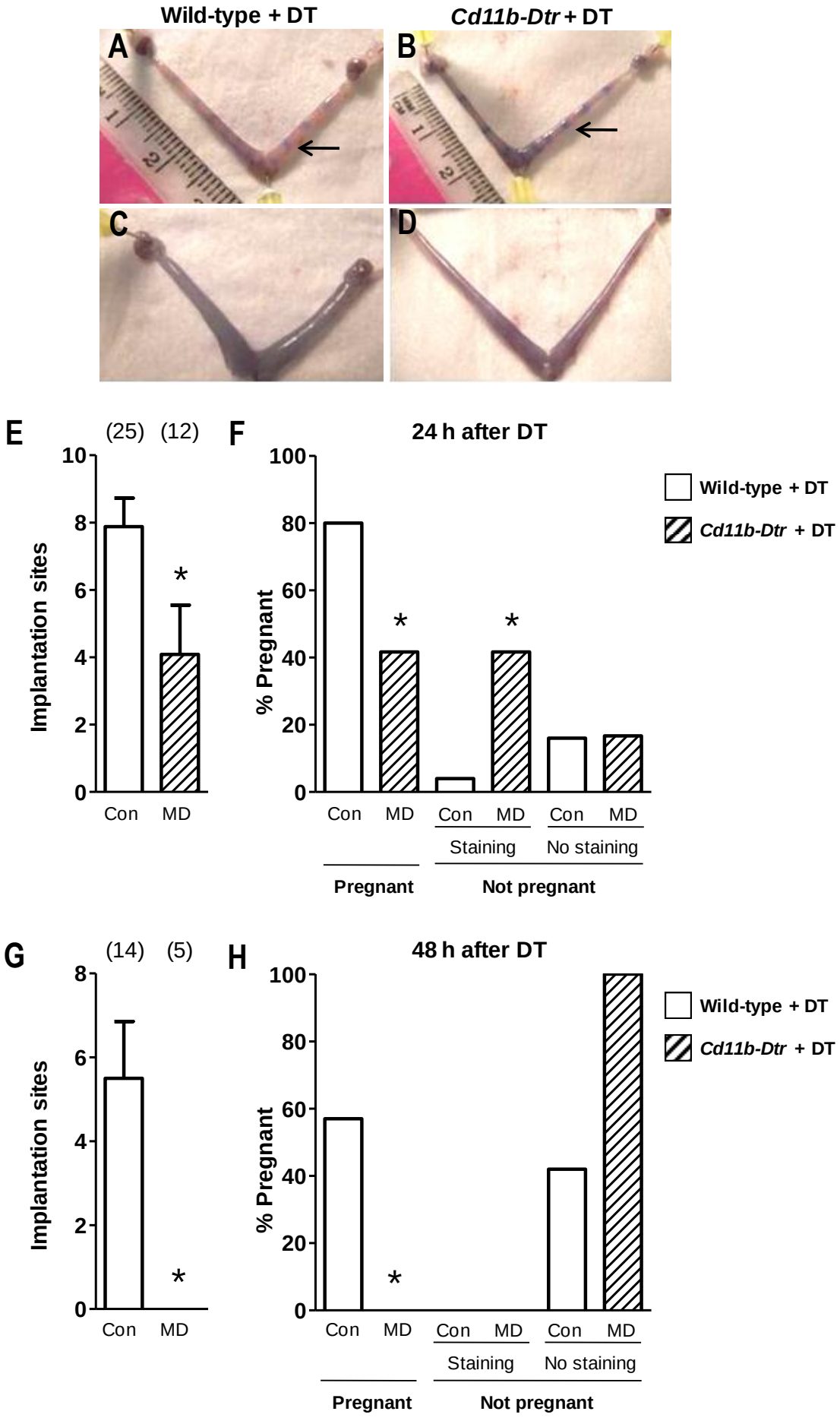
There was a significant reduction in the number of implantation sites in *Cd11b-Dtr* mice on day 4.5 pc (24h after DT). The mean ± SEM implantation sites was 7.9 ± 0.9 in wild-type animals receiving DT, and this was reduced to 4.1 ± 1.5 in *Cd11b-Dtr* ($P$ = 0.04, Mann Whitney U test; Figure 3.14E). The percentage of mice that were pregnant (with defined implantation sites; Figure 3.14A and B), or not pregnant (no defined implantation sites; Figure 3.10B and D), or undefined staining (Figure 3.14C and D) were assessed. Only 41.7% (5/12) of *Cd11b-Dtr* mice were pregnant on day 4.5 pc, compared to 80.0% (20/25) of wild-type mice ($P$ = 0.03, Fisher's exact test). Of the 58.3% of *Cd11b-Dtr* mice that were not pregnant, 42.0% showed undefined staining on day 4.5 pc, compared with 4.0% of wild-type mice ($P$ = 0.01, Fisher's exact test). 16.7% of *Cd11b-Dtr* mice and 16.0% of wild-type mice were not pregnant on day 4.5 pc, showing no evidence of any vascular changes ($P$ = 1, Fisher's exact test; Figure 3.14F). The fact that 42% of *Cd11b-Dtr* mice did have implantation sites on day 4.5 pc, 24 h after DT injection, suggests that macrophage depletion does not fully prevent the events of implantation from commencing, but rather that pregnancy fails shortly after the initiation of the implantation process, or that implantation is inadequate and unsustainable.

By day 5.5 pc, there were no implantation sites found in the uteri of *Cd11b-Dtr* mice, compared to 5.5 ± 1.4 in wild-type mice ($P$ = 0.03, Unpaired t test; Figure 3.14G). Zero out of five *Cd11b-Dtr* mice were found to be pregnant by this stage, compared to 57.1% (8/14) of wild-type animals ($P$ = 0.04, Fisher's exact test; Figure 3.14H). No mice of either genotype displayed undefined implantation sites at the day 5.5 pc time-point. The data from day 4.5 pc and day 5.5 pc (24 and 48 h after DT administration,

respectively) support the interpretation that pregnancy fails before or shortly after implantation commences.

**Figure 3.14 Effect of macrophage depletion on implantation**

Wild-type (A and C) and *Cd11b-Dtr* (B and D) mice received an injection of DT (25 ng/g; i.p.) at implantation (day 3.5 pc). 24 h later, implantation sites were counted after an intravenous injection of trypan blue dye. Mice were categorised as pregnant if implantations sites were seen (A and B; arrows), or not pregnant if there was no evidence of implantation, or if dye staining was evident in the uterus but no defined implantation sites were visible (C and D). The number of implantation sites found on day 4.5 pc in DT-treated wild-type and *Cd11b-Dtr* mice were counted (E) as was number of these mice that were characterised as pregnant, or not pregnant (F) 24 h following DT injection. Another group of mice were left until day 5.5 pc (48 h after DT), where implantation sites (G) and the percent pregnant were calculated (H). Data are presented as mean ± SEM with statistical analysis using a Mann-Whitney U test, Unpaired t test, or Fisher's exact test. * indicates statistical significance from control. *$P < 0.05$. Number of animals in each group shown in parentheses. MD; macrophage depleted. DT; diphtheria toxin.


Wild-type + DT
Cd11b-Dtr + DT
A
B
C
D
E
(25) (12)
Implantation sites
Con
MD
F
24 h after DT
% Pregnant
Wild-type + DT
Cd11b-Dtr + DT
Staining
No staining
Pregnant
Not pregnant
G
(14) (5)
H
48 h after DT

## 3.7 EFFECT OF *IN VIVO* MACROPHAGE DEPLETOIN PRECEDING *EX VIVO* CULTURE OF EMBRYOS

The *in vitro* culture of *Cd11b-Dtr* embryos in the presence of DT did not impair development to the blastocyst stage. However, the exposure of embryos to DT may have local (ovary, oviduct, uterus) and systemic effects that could impact maternal reproductive competence. To determine whether *in vivo* exposure of embryos to DT directly, or indirectly through the effects of DT, affects developmental competence, DT was injected into pregnant females (day 2.5 pc) and the embryos were recovered 24 h later by uterine flushing. The resultant embryos were scored for developmental stage, and cultured to monitor their progression to late stage blastocyst, their ability to hatch from the zona pellucida, and their attachment to the culture dish. Embryos were considered viable and normally developed if they were at morulla to blastocyst stage when flushed from the reproductive tract (Chapter 2.4.2).

Table 3.2 shows the number of embryos and their developmental stage when flushed on day 3.5 pc. Figure 3.15A shows embryos recovered from wild-type mice, where the image on the left is representative of the embryos recovered from the uterus on day 3.5 pc (mostly morulla to early blastocyst stage, see Table 3.2). Figure 3.15B shows embryos recovered from *Cd11b-Dtr* mice (mostly morulla, early blastocyst, and degenerating embryos recovered, Table 3.2). A proportion of degenerating embryos were recovered on day 3.5 pc from both *Cd11b-Dtr* and wild-type mice (16.0% in *Cd11b-Dtr* mice compared to 8.4% in wild-type mice).

Figure 3.14C shows that 77.9% of embryos recovered from wild-type mice on day 3.5 pc were viable and normally developed, compared with 72.6% from *Cd11b-Dtr* mice ($P = 0.45$, Fisher's exact test). There was a significant decrease in the number of viable and total (viable and degenerating) embryos recovered from *Cd11b-Dtr* mice on day 3.5 pc that developed successfully and were able to hatch and attach to the culture dish (Figure 3.15D and E, respectively). The mean ± SEM of viable wild type embryos = 87.6 ± 8.3%, compared to 57.8 ± 12.6% for *Cd11b-Dtr* viable embryos ($P = 0.04$, Mann Whitney U test; Figure 3.15D). When total flushed embryos were analysed, 78.0 ± 9.3% of wild-type embryos were able to develop and attach to the culture dish, compared to 47.0 ± 11.4% of *Cd11b-Dtr* embryos ($P = 0.04$, Mann Whitney U test; Figure 3.15E). Figure 3.15F shows that there was no difference in the number of viable embryos recovered from wild-type and *Cd11b-Dtr* mice that were able to develop to the blastocyst stage, where 99.1 ± 0.9% of wild-type embryos were able to do so, compared to 95.59 ± 2.2% of *Cd11b-Dtr* embryos ($P = 0.15$, Mann Whitney U test).

These data suggest that although viable embryos are recovered from the reproductive tracts of *Cd11b-Dtr* mice, and these embryos are developmentally competent (able to grow, hatch and attach to culture dish), that some of the embryos had likely been adversely affected from the intrauterine environment, and the environment within the oviduct, prior to being recovered and cultured. These adverse effects are most likely due to the environment they had been exposed to for 24 h following the DT injection, rather than a direct effect of the DT, since embryos were not impaired in their ability to develop to blastocyst stage with no impedance when cultured with DT *in vitro* (section 3.5)

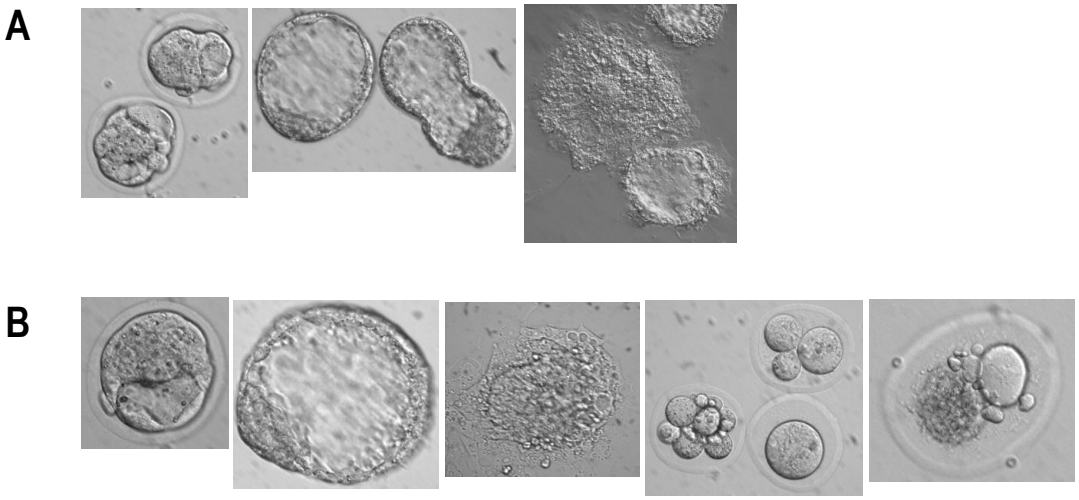
.

**Table 3.2 Percentage of embryos at various developmental stages when recovered on day 3.5 pc, 24 h after DT injection**

| | Degenerating | 1 cell | 2 cell | 3-4 cell | 4-8 cell | Morulla | Early BΦ | Late BΦ |
|---|---|---|---|---|---|---|---|---|
| Wild-type | 8.1 | 7.0 | 0.0 | 1.2 | 5.8 | 40.7 | 34.9 | 2.3 |
| *Cd11b-Dtr* | 16.2 | 4.4 | 2.9 | 1.5 | 2.9 | 45.6 | 22.1 | 4.4 |

**Figure 3.15 Effect of *in vivo* macrophage depletion on *ex vivo* developmental competence of embryos**

Embryos were flushed from the uteri of wild-type and *Cd11b-Dtr* mice on day 3.5 pc, 24 h after DT injection (25 ng/g). A, from left, early blastocysts recovered from wild-type mice on day 3.5 pc, expanding wild-type blastocysts, and wild-type blastocysts that have hatched from the zona pellucida and attached to the culture dish. B, from left, early blastocysts recovered from *Cd11b-Dtr* mice on day 3.5 pc, expanding *Cd11b-Dtr* blastocyst, *Cd11b-Dtr* blastocyst that has hatched from the zona pellucida and attached to the culture dish. The far right images in B show some of the degenerating embryos recovered from the uteri of *Cd11b-Dtr* mice. C, the percentage of normally developed "viable" embryos, of morulla to blastocyst stage, recovered from the reproductive tract on day 3.5 pc. D. The percentage of viable embryos that were able to go on to blastocyst stage and attach to culture dish. E, the percentage of total embryos recovered (viable and non-viable) that were able to develop and attach to the culture dish. F, the number of viable embryos that were able to develop to blastocyst stage. Data are presented as mean ± SEM with statistical analysis using a Mann-Whitney U test. * indicates statistical significance from control. *$P < 0.05$. Number of embryos in each group shown in parentheses. MD; macrophage depleted. DT; diphtheria toxin.


A
B
C
Normally developed embryos recovered
(86)
(68)
% Morulla/blastocyst
100
80
60
40
20
0
Con
MD
D
Viable embryos recovered that attached to culture dish
(67)
(49)
Wild-type + DT
Cd11b-Dtr + DT
% Attaching embryos
*
E
Total embryos recovered that attached to culture dish
F
Viable embryos recovered that developed to blastocyst stage
% Blastocyst

## 3.8 DISCUSSION

These studies demonstrate that macrophages can be depleted from the reproductive tissues of *Cd11b-Dtr* mice. Flow cytometry revealed that EMR1$^+$ cells were depleted from the uterus, however, the number of EMR1$^+$ cells remaining in DT-treated *Cd11b-Dtr* mice was higher than expected. The level of depletion of EMR1$^+$ cells as assessed using immunohistochemistry showed a higher level of depletion, with the percent of EMR1 positivity declining from 16% in DT-treated wild-type mice to 2.5% in DT-treated *Cd11b-Dtr* mice. This discrepancy is likely to be due to the staining of eosinophilic granulocytes with EMR1 (McGarry and Stewart, 1991). These eosinophils were removed from the analysis for the immunohistochemical studies, but this was not possible with flow cytometry, and this likely accounts for the higher number of EMR1$^+$ cells seen in DT-treated *Cd11b-Dtr* animals by flow cytometry. This is consistent with the flow cytometry result where cells were labelled with both EMR1 and CD11B; there was a statistically significant depletion of the EMR1$^+$CD11B$^+$ and the EMR1$^-$CD11B$^+$ populations, but not the EMR1$^+$CD11B$^-$ population. Gating out of eosinophils was attempted on the basis of their higher side-scatter profile, however, this removed less that 1% of EMR1 positive cells. Future experiments using an eosinophilic marker would enable correct identification and removal of this population from analysis. On the basis of the immunohistochemistry data, we conclude therefore that DT treatment is effective in depleting 85% of macrophages from the uterus.

Generally, other immune cell subsets were not substantially changed by macrophage depletion. An exception was that CD11C$^+$ cells were significantly depleted from the uterus, ovary and peritoneal cavity. In the uterus, it is the CD11C$^+$CD11B$^-$ cell population that was depleted, which is somewhat surprising. If it were the CD11C$^+$CD11B$^+$ population, it might be explained that this is a cell type that is undergoing a transition to become either a CD11C$^+$ DC, or a CD11B$^+$ macrophage, since macrophages and DC exhibit this kind of plasticity. It is recognised that clear differentiation between macrophages and DCs can be difficult, owing to their related developmental pathways, the similar functions they perform, and the often indistinguishable morphologies and distribution patterns of these cells in mucosal and lymphoid tissues (Hume et al., 2002, Wang et al., 2000, Ichikawa et al., 2003). Expression of markers such as the B2-integrins, CD11B/CD18 and CD11C/CD18 can be varied and overlapping and the expression of these markers suggests recent interaction with endothelial cell adhesion molecules during recruitment from the blood (Wang et al., 2000, Prieto et al., 1994). It is possible that CD11C$^+$CD11B$^-$ cells originate from CD11B$^+$ precursors, but this remains to be investigated further. Another possibility is that CD11B$^+$ macrophages produce chemokines that cause CD11C$^+$ cells to be recruited into tissues. The depletion of the source of these cytokines would make it

appear as though the responding cells have been depleted, when in fact they were never recruited into the tissue at all. This was seen in the work of Cailhier et al. (2005), where macrophage depletion caused a marked decrease in neutrophil recruitment into the peritoneal cavity after induced peritonitis, but not neutrophil depletion.

Chemokines secreted from uterine epithelial cells are likely to be responsible for the recruitment of monocytes and other precursor cells into the uterus. Their development into APCs is driven by exposure to local cytokines, including CSF1, CSF2, IFNG and TNFA, and the concentrations of these cytokines would inevitably alter the resultant macrophage phenotype. Alternatively, the recruited monocytes may differentiate into DCs (Robertson et al., 1994, Keenihan and Robertson, 2004, Banchereau et al., 2000). Although not the purpose of this study, to identify more specifically the phenotype of the $CD11C^{+}CD11B^{-}$ population that appeared to be depleted following DT administration, the expression of other surface markers would need to be analysed, owing to the heterogeneity of surface marker expression of cells from the MPS. Some markers that could be used to further identify this cell type are EMR1 (macrophages) and MHC-II (activated macrophages and DCs) (Keenihan and Robertson, 2004, Hume et al., 2002)

We have also shown that the neutrophil population in the ovary is depleted, and in the uterus, it is only the $RB6^{+}$ cells also expressing CD11B that are affected. It is also noteworthy that there is an increase in neutrophils in the spleen following DT treatment of *Cd11b-Dtr* mice. It appears that the $CD3^{+}$ T cell population is unaffected by DT.

The observation that there was negligible $CD11B^{+}$ cells present in the spleen of wild-type or *Cd11b-Dtr* mice is consistent with other studies showing low level expression of this marker in untreated mice, but there is an increase in expression in tumour-bearing mice following treatment with the fms-like tyrosine kinase 3-ligand (Flt3L) (Youn et al., 2008, Diener et al., 2008). It also appears that genetic background may slightly impact CD11B expression by splenocytes (Youn et al., 2008).

When DT was administered to early pregnant mice, it caused complete pregnancy failure in *Cd11b-Dtr* mice, while wild-type animals exhibited healthy pregnancies. DT caused weight loss in *Cd11b-Dtr* animals by day 7.5 pc following DT treatment on day 1 pc (compared to a weight gain in wild-type mice, where the difference in weights between the two groups on day 7.5 pc was statistically significant). It appears that the pregnancy loss occurs during early implantation, as analysis of uteri

shortly after implantation on day 4.5 pc showed that there were some implanted embryos present, albeit in fewer females than in DT-treated wild-type mice. In other *Cd11b-Dtr* females there was evidence of a generalised increase in uterine oedema but this was different to the clearly delineated implantation sites as seen in wild-type mice and some macrophage-depleted mice. When implantation sites were present in DT-treated *Cd11b-Dtr* mice on day 4.5 pc, they appeared normal. There was some variability in the timing of pregnancy arrest in *Cd11b-Dtr* mothers, with around 40% being pregnant on day 4.5 pc, and another 40% showing changes in uterine vascular permeability, but all pregnancies had arrested by day 5.5 pc. This can possibly be explained by the stage of implantation the embryo was undergoing at the time of macrophage depletion. All mice received DT on day 3.5 pc (12 noon), yet the time at which copulation took place would vary between females, and thus the development of the embryos and the stage of implantation they were undergoing at the time of macrophage depletion would also vary.

*In vitro* culture experiments revealed that embryos were not directly affected by DT, as when cultured in its presence, they were able to develop to blastocyst stage at the same rate as those cultured in its absence. The *ex vivo* culture of embryos following 24 h of *in vivo* exposure to DT and its systemic effects showed that generally healthy embryos were recovered from the uterus of DT-treated *Cd11b-Dtr* mice, and they were developmentally competent. However, a higher proportion of fragmented embryos were present in the uterus of *Cd11b-Dtr* mice on day 3.5 pc, and a lower proportion of viable embryos, as judged by their ability to hatch and attach to the culture dish, which is a marker of developmental competence. It seems likely that the process of macrophage depletion – presumably by undergoing apoptosis locally in the uterus and ovary, as well as systemically, might have an impact on the embryos present at that time.

The depletion of macrophages may alter the uterine cytokine environment. DT is a protein synthesis inhibitor (Saito et al., 2001), leading to death by the process of apoptosis. The apoptotic bodies present in the tissue may result in the release of inflammatory mediators, as apoptotic bodies are usually scavenged by macrophages. One inflammatory mediator that macrophages are known to produce is TNFA, a cytokine that has been shown to reduce the number of cells in the inner cell mass of embryos developing *in vitro*, and had a negative impact on their ability to attach to a fibronectin-coated culture dish. TNFA-pretreated blastocysts transferred to surrogate females had significantly higher rates of embryo resorption, and those fetuses that did survive were significantly lower in weight than untreated fetuses (Wuu et al., 1999). This data identifies a mechanism by which macrophage depletion could be

contributing to embryo degeneration. The release on inflammatory mediators may also impact uterine epithelial receptivity.

The issue of weight loss following macrophage depletion requires further investigation, and is likely to have a negative impact on the health of the embryos. The large weight loss that occurred in such a short space of time indicates that it is mainly due to dehydration. Consistent with this explanation is the fact that *Cd11b-Dtr* mice were observed to be thirsty following DT-treatment, and often had distended stomachs at autopsy. It has been demonstrated that macrophages are depleted from the kidney (Cailhier et al., 2005, Duffield et al., 2005b). This hypothesis may be considered conflicting in light of the work of Duffield et al. (2005b), who reported that macrophage depletion reduces histological injury in the kidney in a model of crescentic glomerulonephritis. However, this disease model is not necessarily a representation of what occurs in mice with healthy kidneys. In addition, the negative impact that DT administration has is not reported in publications using these mice. For this reason, the possibility of systemic effects contributing to implantation failure in this model cannot be disregarded. Urine analysis to determine whether there was blood or protein present would indicate whether macrophage depletion is causing kidney damage.

Our data showed that viable pregnancies can be achieved when mice receive lower doses of DT, indicating that 25 ng/g is required for the full effect and is suitable for further studies. This, taken together with the *in vitro* embryo culture experiments, further supports the fact that DT itself does not have a negative impact on the embryos and the pregnancy failure is likely attributed to the depletion of macrophages in these mice. However, further experiments will be required to elucidate the mechanism.

In conclusion, this set of experiments has shown that macrophages and DCs are depleted following DT administration to *Cd11b-Dtr* mice, and that this depletion causes failure of embryo implantation. Studies in the following chapters will attempt to decipher the mechanism for this pregnancy loss, and to gain a better understanding of the cell types involved.

# Chapter 4

# Effect of macrophage depletion on uterine parameters affecting early pregnancy

## 4.1 INTRODUCTION

The mammalian uterus is a dynamic organ, undergoing cyclic changes under the control of ovarian steroid hormones. Each cycle, the uterus undergoes a wave of proliferation, differentiation and remodelling in preparation for the possibility of an implanting blastocyst. In a cycle where mating occurs, this continues after coitus in synchrony with the development of the preimplantation embryo. Following parturition, extensive remodelling occurs in preparation for a subsequent pregnancy (Tong and Pollard, 2002). In adult mice, estradiol-17β ($E_2$) produced at estrus causes cells to undergo a wave of cell proliferation that is restricted to the luminal and glandular epithelium. In contrast, progesterone ($P_4$) synthesised following copulation enhances stromal cell proliferation while inhibiting estrogen-induced cell proliferation, and stimulates epithelial differentiation in preparation for embryo implantation (Martin & Finn 1969; Tong & Pollard 1999).

The events that occur during the murine estrous cycle and early pregnancy can be replicated in ovariectomised mice given exogenous hormones (Allen et al., 1937, Martin et al., 1973, Tong and Pollard, 2002). This discovery has given rise to much research directed at unravelling the actions of the sex steroid hormones alone, or in combination, in the murine uterus.

It is possible that the hormones act directly on uterine tissue, or alternatively, that their actions on one cell type may be mediated in a paracrine manner by their effects on another cell type. Tissue recombination experiments utilising *Esr1-/-* and *Pgr-/-* mice have given insight into the mechanisms by which steroid hormones exert their effects in the uterus. It has been demonstrated that the proliferative actions of $E_2$ on endometrial epithelial cell proliferation is likely to be mediated through its cognate receptor in the stroma (Cooke et al., 1997), indicating that epithelial cell proliferation in response to $E_2$ occurs via an indirect mechanism. The inhibitory actions $P_4$ on endometrial epithelial cell proliferation are also likely to be mediated through its cognate receptors in the stroma (Kurita et al., 1998b).

The cytokines and chemokines produced by uterine epithelial cells in response to $E_2$ cause macrophage recruitment (Pollard, 1990, Hunt, 1992, Robertson et al., 1992a), placing them in the stroma and in close juxtaposition with uterine epithelial cells. The growth factors and cytokines produced by macrophages in response to this stimulation could possibly impact epithelial and stromal proliferation events seen in the cycling and pregnant uterus, and this may alter glandular secretions and uterine epithelial cell receptivity (Tong and Pollard, 2002).

Studies in *Csf1*-null mutant mice, however, do not support this hypothesis. Even with the low number of macrophages present in the uteri of virgin *Csf1*$^{op}$/*Csf1*$^{op}$ mice, epithelial and stromal cell proliferation in response to steroid hormones appears normal (Cohen et al., 1997). However, a role for macrophages cannot be ruled out by these experiments due the perturbation in feedback regulation of the HPG axis that occur in these mice (Cohen et al., 1999). In addition, macrophage depletion in these mice is incomplete, particularly following coitus, and macrophages show a different phenotype (Pollard et al., 1991, Robertson et al., 1998). Given these caveats, the research question would be better addressed in an alternative model. Further, the finding that macrophage depletion in the mammary gland during $E_2$ and $P_4$-induced alveolar development causes a reduction in ductal epithelial cell proliferation, indicates that macrophages have a role in epithelial cell proliferation in other hormonally responsive tissues (Chua et al., 2010).

Studies by Ma et al. (2001) in the mouse uterus have shown that in early pregnancy, $E_2$ and $P_4$ regulate VEGF and its receptors to enable an increase in vascular permeability, followed by angiogenesis. In other tissues, macrophages have been identified as a source of VEGF (Yoshida et al., 1997). VEGF producing cells have been identified in the uterine stroma, and have a similar morphology to macrophages, but their identity has not been confirmed (Shweiki et al., 1993, Halder et al., 2000). In light of these studies, assessment of macrophage involvement in uterine angiogenesis would be insightful.

Expression of a variety of adhesion and anti-adhesion molecules on the surface of uterine epithelial cells is coordinated to provide a barrier to the developing embryo until the onset of receptivity. At this time, the embryo apposes uterine epithelial cells, and subsequently attaches to and displaces the epithelium to gain access to the underlying stroma. In the mouse, the window of implantation occurs on day 4 pc and this is a time where the expression of adhesion molecules on the surface of uterine epithelial cells is upregulated to enable blastocyst attachment. Some molecules that provide a means of attachment are H type 1 and Lewis blood group antigens (Aplin, 1997, Lessey et al., 1996).

Previous studies have indicated that immune cells regulate endometrial epithelial cell receptivity and improve pregnancy rates (Kosaka et al., 2003, Ideta et al., 2010). Owing to their intimate juxtaposition with endometrial epithelial cells, it is possible that macrophages may influence endometrial epithelial cell receptivity in a paracrine manner via their secretory products, and contribute to the upregulation of

adhesion molecules. *Fut2* is a gene involved in the synthesis of adhesive glycoconjugates in the uterine epithelium. *In vitro* experiments have determined that the stimulatory effects of macrophages on *Fut2* mRNA expression could be elicited by the macrophage-derived cytokines LIF, IL1B and IL12 (Jasper et al., 2011). In addition, the ability of macrophages to alter transport properties and barrier integrity of epithelial cells *in vitro* provides an additional means by which they may facilitate trophoblast breaching of the epithelial cell surface during implantation (Zareie et al., 1998, Jasper et al., 2011).

The process of decidualisation is another dramatic proliferative response in the uterine stroma, usually in response to an implanting blastocyst. The decidual reaction can also be caused by the instillation of oil into the uterine horn of a rodent that has been suitably primed with hormones (Tong and Pollard, 2002, Finn and Keen, 1963, Finn, 1966). Macrophages are a source of MMPs that may assist in the breakdown and remodelling of matrix (Goetzl et al., 1996), and may therefore contribute to the extensive remodelling of the endometrial stroma that occurs during decidualisation (Curry and Osteen, 2003, Das et al., 1997b). DCs are essential for decidualisation in mice (Plaks et al., 2008), and given the overlapping functions of DCs and macrophages (Hume et al., 2002), further investigation of macrophage contributions to this process warrants further investigation.

In the experiments described in this chapter, we utilised the *Cd11b-Dtr* mouse model to investigate the physiological role of uterine macrophages in the events of uterine tissue cyclic remodelling, expression of embryo attachment molecules and the decidual response. In these studies we elected to utilise bone marrow (BM) chimera mice, prepared by replacement of BM in wild-type FVB/N mice with BM from *Cd11b-Dtr* mice, to generate mice where BM-derived cells contained the simian DTR transgene but non-haemopoietic cell lineages did not. The purpose of this was to remove the possibility of confounding influences of off-target effects of DTR in non-BM-derived cells.

## 4.2 EFFECT OF MACROPHAGE DEPLETION ON UTERINE EPITHELIAL CELL PROLIFERATION

$E_2$ is synthesised in every estrous or menstrual cycle, causing cell proliferation in uterine luminal and glandular epithelial cells. In ovariectomised mice it can be shown that the round of DNA synthesis starts approximately six hours after the injection of $E_2$, reaching a peak at 12-15 h post-injection (Pollard et al., 1987b, Martin et al., 1973). To determine whether macrophages, through their secretory products, have a paracrine effect on uterine epithelial cell proliferation, experiments were conducted to determine whether the number of proliferating epithelial cells was reduced following macrophage depletion.

### 4.2.1 Characterisation of macrophage depletion in the *Cd11b-Dtr*-BM transgenic mouse

Because of concerns about the potential off-target effects of DT, these experiments were conducted in wild-type mice that had received a bone-marrow transplant from *Cd11b-Dtr* mice. Briefly, wild-type mice were lethally irradiated with 900 cGy of gamma irradiation, followed immediately by i.v. injection of donor bone marrow cells from *Cd11b-Dtr* mice (see Chapter 2.1.3). The resultant chimeras are referred to hereafter as *Cd11b-Dtr*-BM mice. Following recovery, mice were ovariectomised and subsequently primed with $E_2$, and then injected with $E_2$ 15 h before sacrifice, a regime previously determined to result in maximum epithelial cell proliferation (Tong and Pollard, 1999). During this regime, one group of mice were treated with DT (25 ng/g) to elicit macrophage depletion 9 h prior to the final $E_2$ injection (and 24 h prior to sacrifice, see Chapter 2.5.1), while the control group received PBS. As a control for the $E_2$ treatment, another group of mice were primed with $E_2$ only, and received no further treatment. Two hours before sacrifice, mice received an injection of BrdU to enable subsequent detection of proliferating cells.

Following sacrifice, peritoneal cells were analysed by flow cytometry to confirm depletion of $CD11B^+EMR1^+$ cells from DT treated *Cd11b-Dtr*-BM mice. One horn of the uterus was collected and frozen in OCT embedding medium, the other was fixed in paraformaldehyde for later histochemical analysis of proliferating cells and immune cells.

Depletion of $CD11B^+EMR1^+$ populations was confirmed by flow cytometry. Representative dotplots in Figure 4.1A and B show 26.3% $CD11B^+EMR1^+$ cells in the peritoneal cavity of PBS treated mice, and 0.0% $CD11B^+EMR1^+$ cells in DT-treated *Cd11b-Dtr*-BM mice. Figure 4.1C shows that the mean ± SEM

percent of peritoneal CD11B$^+$EMR1$^+$ cells was 29.4 ± 6.9% in PBS-treated mice and 1.0 ± 0.7% in DT-treated *Cd11b-Dtr*-BM mice ($P$ = 0.006, Mann Whitney U test), showing almost complete depletion of CD11B$^+$EMR1$^+$ cells following DT treatment.

Immunohistochemistry was used to analyse endometrial EMR1$^+$ macrophage populations in these mice. Figure 4.1D, E and F shows representative images in PBS-treated (D), DT-treated (E) and no hormone control *Cd11b-Dtr*-BM mice (F). The number of these cells was quantitated as a measure of EMR1 staining using video image analysis (Figure 4.1G). The percent of EMR1 positivity was 8.3 ± 1.0% in the uteri of PBS-treated mice and 2.3 ± 0.4% in DT-treated mice, and there was 2.7 ± 0.3% EMR1 positivity in the no hormone control mice. There was a significant decrease in the percentage of EMR1 positivity in DT-treated *Cd11b-Dtr*-BM mice compared to those that received PBS ($P$ = 0.02, Mann Whitney U test). Endogenous peroxidase-positive eosinophils were identified in no antibody control sections, and their numbers were also quantified (1.5 ± 0.6% positivity in PBS-treated, and 3.7 ± 0.9% positivity in DT-treated *Cd11b-Dtr* mice) and subtracted from the total EMR1 positivity values to give the results discussed above.

Macrophages are recruited into the uterus in response to $E_2$. It is noteworthy that the macrophage numbers in DT-treated mice are similar to those seen in no hormone controls, where the lack of $E_2$ causes the marked paucity in the numbers of macrophages present in the uterus (Figure 4.1G).

To ensure other immune cell populations were unaffected by the irradiation and bone marrow transfer procedures, and were similar to those in naturally cycling mice (Chapter 3), immunohistochemistry to detect CD11B, CD11C, CD45, RB6, CD3 and CD5 was used to investigate how these populations differed between PBS- and DT-treated *Cd11b-Dtr*-BM mice (Figure 4.2). CD5 (Lyt1) is a lymphocyte differentiation antigen, detected on T lymphocytes, B cells and thymocytes (Ledbetter and Herzenberg, 1979). In line with the results in Chapter 3, CD11B$^+$, CD11C$^+$ and CD45$^+$ cells were depleted from the uterus in DT-treated *Cd11b-Dtr*-BM mice. It is difficult to draw conclusions on neutrophil populations, as very few cells stained positive for RB6 in control or macrophage-depleted mice. T and B cell populations appeared similar between groups, as detected by CD3 (T cells) and CD5 (T cells and B cells) cell surface staining.

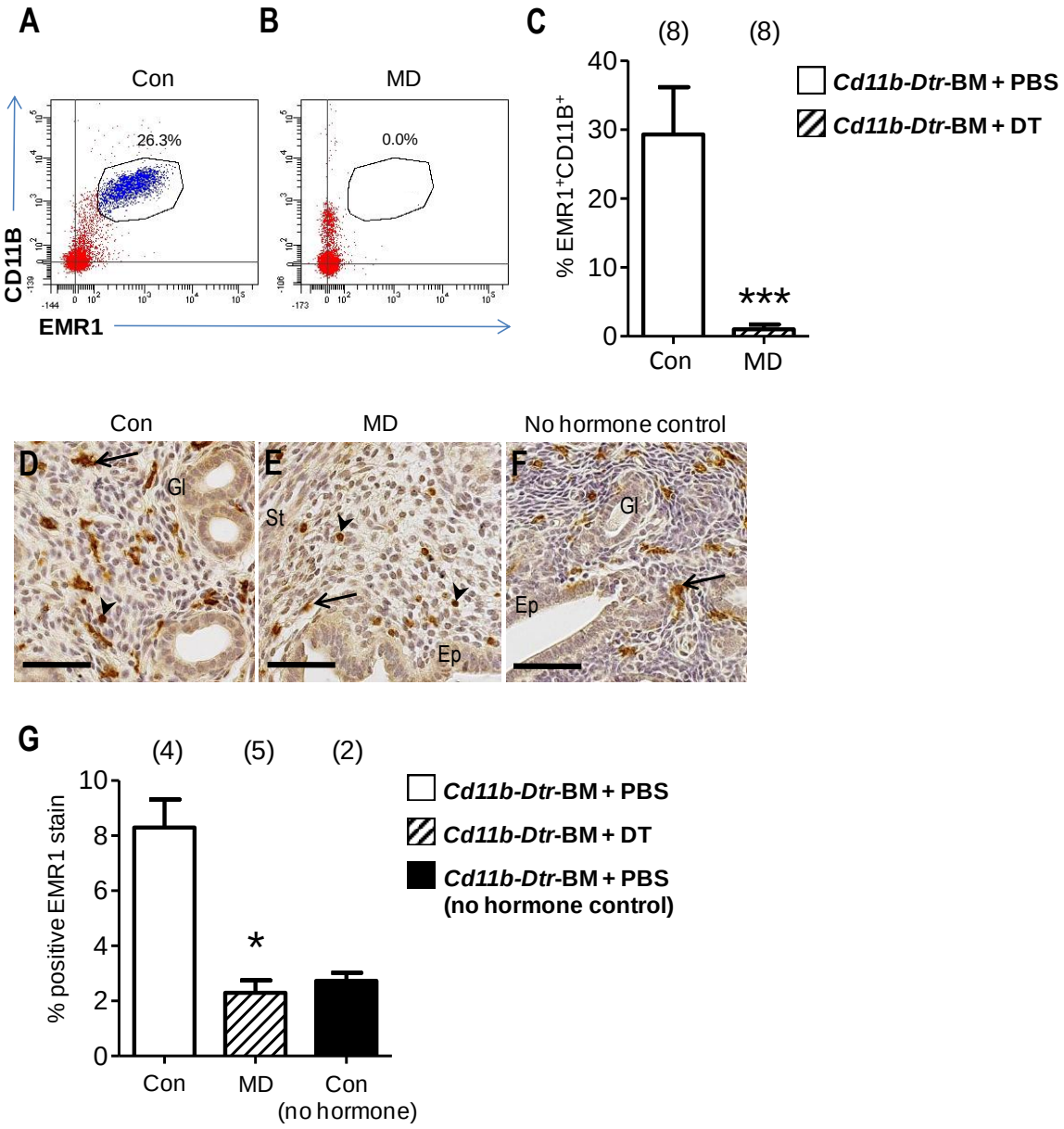



**Figure 4.1 Effect of macrophage depletion in the peritoneal cavity and uterus of the *Cd11b-Dtr* mouse**

Representative dotplots illustrate $CD11B^{+}EMR1^{+}$ cells in the peritoneal cavity in PBS-treated control (A) or DT-treated macrophage-depleted (B) *Cd11b-Dtr*-BM mice. Graph shows percentage of peritoneal $CD11B^{+}EMR1^{+}$ cells in control and macrophage-depleted mice (C). Representative photomicrographs of uterine tissue from PBS-treated (D), DT-treated (E) and no hormone control (F) *Cd11b-Dtr*-BM mice illustrate EMR1-positive macrophage populations. Percentage of EMR1-positive staining in the PBS-treated, DT-treated or no hormone control uteri (G). Approximately 10 individual fields per uterus per mouse were analysed. Values for endogenous peroxidase positive eosinophils were subtracted from the analysis. Data are presented as mean ± SEM with statistical analysis using a Mann-Whitney U test. * indicates statistical significance from control. *$P < 0.05$, ***$P < 0.001$. The number of animals in each group is shown in parentheses. Arrows; EMR1 positive macrophages. Arrowheads; endogenous peroxidase positive eosinophils. MD; macrophage depleted. DT; diphtheria toxin. St; stroma. Gl; glandular epithelium. Ep; luminal epithelium. Scale bar = 50 µm

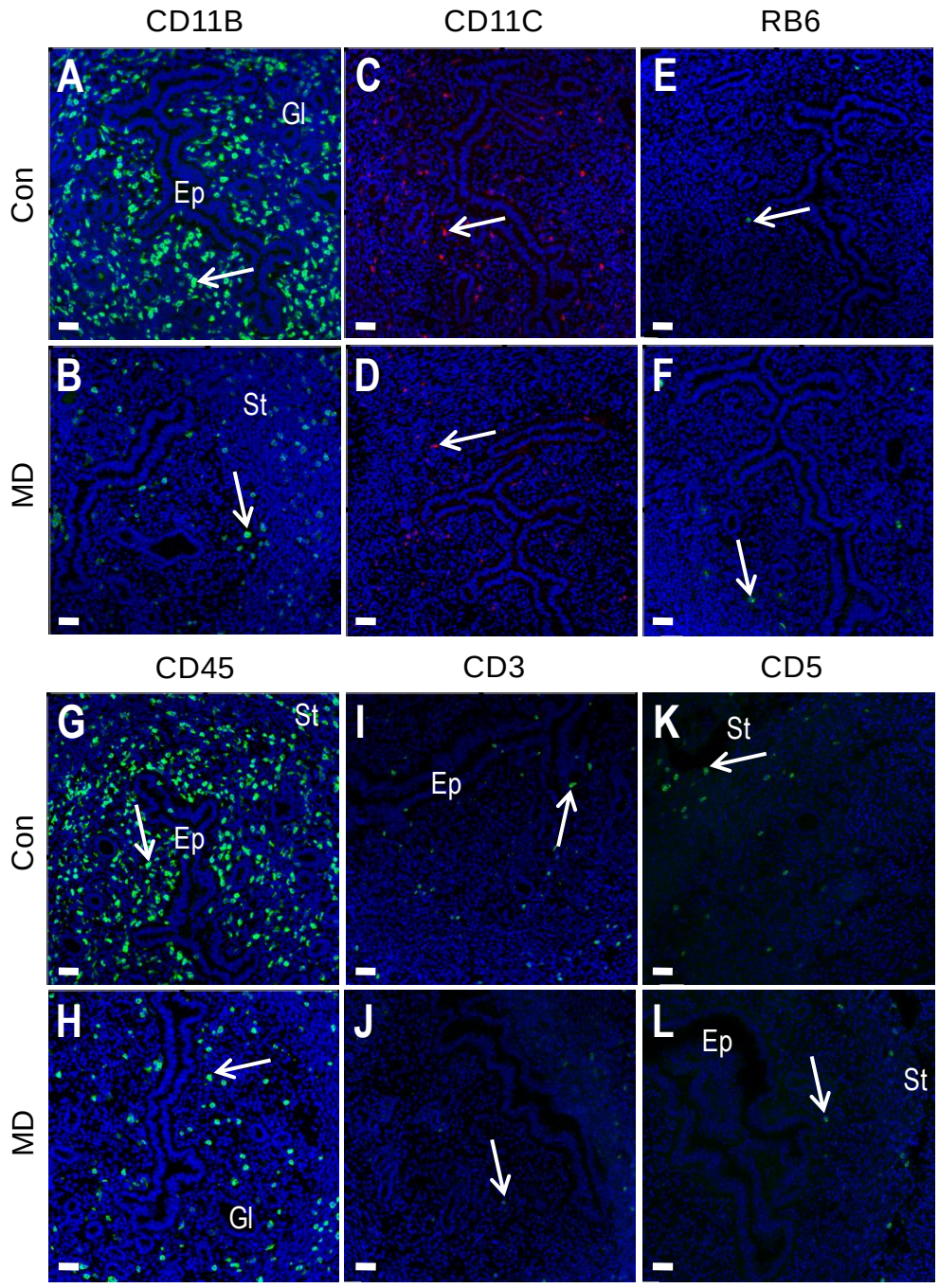



**Figure 4.2 Effect of DT on immune cell populations in *Cd11b-Dtr*-BM mice**

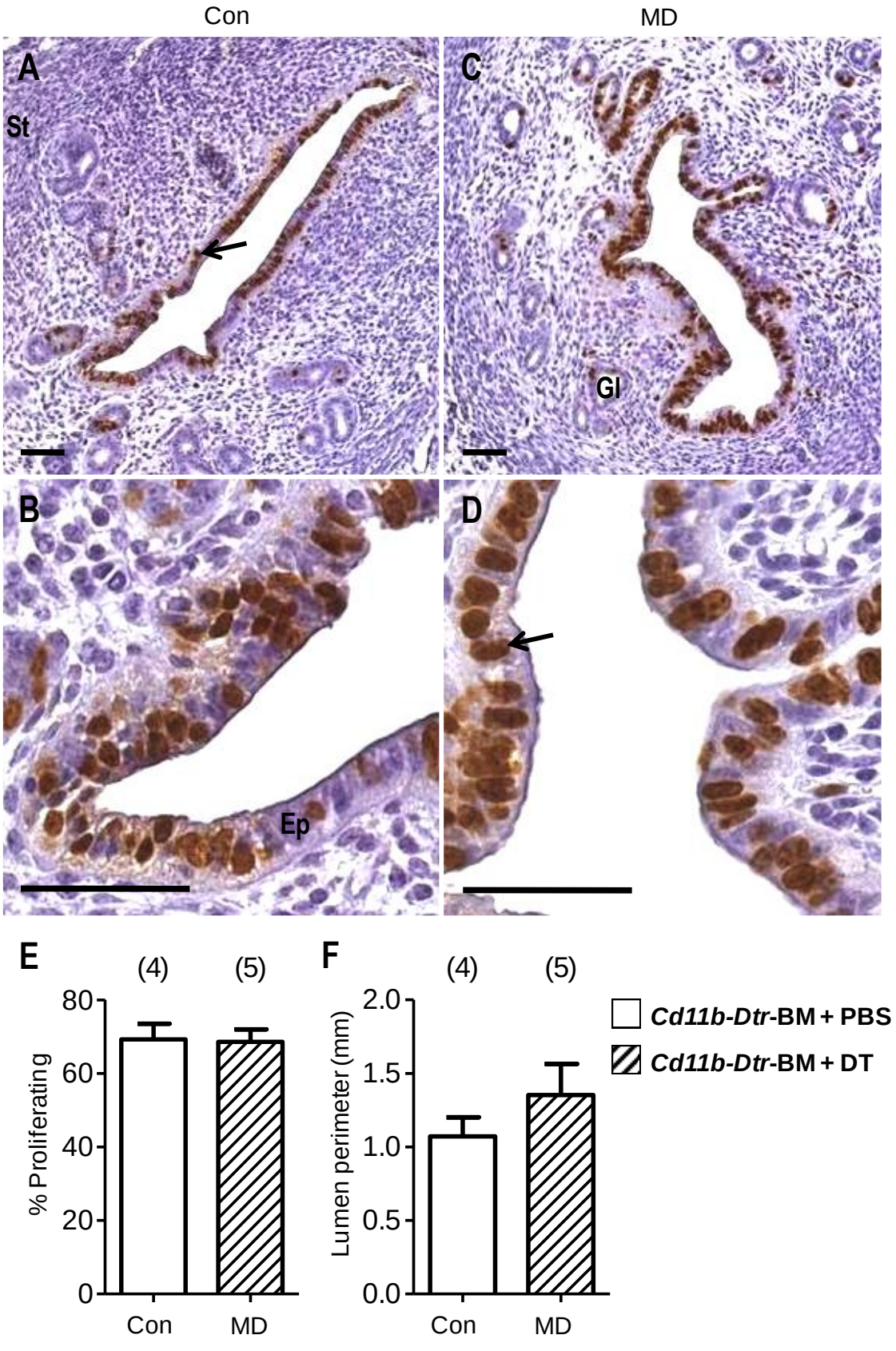
Representative photomicrographs of tissue labelled with various immune cell surface markers from PBS-treated (A, C, E, G, I, K) or DT-treated (B, D. F. H, J, L) *Cd11b-Dtr*-BM mice. Surface markers include CD11B (A, B; green), CD11C (C, D; red), RB6 (E, F; green), CD45 (G, H; green), CD3 (I, J; green) and CD5 (K, L; green). Nuclei are stained with DAPI (blue). Arrows indicate stained immune cells. MD; macrophage depleted. St; stroma. Gl; glandular epithelium. Ep; luminal epithelium. Scale bar = 50 µm.

### 4.2.2 Effect of macrophage depletion on BrdU incorporation of luminal epithelial cells in the endometrium in response to estradiol-17β

Proliferation in the $E_2$ treated uterine luminal epithelium was quantified by BrdU incorporation in PBS-treated control (Figure 3.3; A and B) and DT-treated (macrophage depleted) *Cd11b-Dtr*-BM mice (C and D). Proliferating and non-proliferating uterine epithelial cells were counted manually from stained images using the ImageJ cell counter function (Chapter 2.3.2.5). There was no significant difference in the number of proliferating uterine epithelial cells between treatment groups ((PBS-treated *Cd11b-Dtr*-BM (mean ± SEM) = 69.3 ± 4.2% and DT-treated *Cd11b-Dtr*-BM = 68.6 ± 3.4%, $P = 0.90$, Mann Whitney U test (Figure 3.3, E)). There were no proliferating epithelial cells in the uterine lumen of no hormone control animals, revealing that as expected, the hormone regime given was required to induce proliferation, and that this proliferation required the additional injection of $E_2$ following $E_2$-priming injections. The perimeter of the lumen was also measured (Figure 3.3F) with an average of 1.1 ± 0.1 mm in PBS-treated, and 1.4 ± 0.2 mm in DT-treated *Cd11b-Dtr*-BM mice ($P = 0.73$, Mann Whitney U test).

**Figure 4.3 Effect of macrophage depletion on proliferation of uterine luminal epithelial cells**

BrdU incorporation in uterine luminal epithelial cells in response to $E_2$ in PBS-treated control (A, low power; B, high power) and DT-treated macrophage-depleted (C, low power; D, high power) *Cd11b-Dtr*-BM mice. Manually counted BrdU-positive cells as a percentage of total epithelial nuclei in PBS-treated and DT-treated *Cd11b-Dtr* mice (E). Perimeter of uterine lumen (mm) between groups (F). Data are presented as mean ± SEM with statistical analysis using a Mann-Whitney U test. * indicates statistical significance from control. The number of animals in each group is shown in parentheses. Tissue was counterstained with haematoxylin. Arrows; BrdU-positive uterine epithelial cells. MD; macrophage depleted. DT; diphtheria toxin. St; stroma. Gl; glandular epithelium. Ep; luminal epithelium. L; lumen. Scale bar = 50 μm


Con
MD
A
St
C
Gl
B
Ep
D
E
(4)
(5)
% Proliferating
80
60
40
20
0
Con
MD
F
(4)
(5)
Lumen perimeter (mm)
2.0
1.5
1.0
0.5
0.0
Con
MD
Cd11b-Dtr-BM + PBS
Cd11b-Dtr-BM + DT

### 4.2.3 Effect of macrophage depletion on BrdU incorporation of glandular epithelial cells in the endometrial stroma in response to estradiol-17β

$E_2$ also causes proliferation in the glandular epithelium. To determine whether macrophage-depletion during this treatment had any impact on proliferating glandular epithelial cells, the number of glands were counted and scored manually for their approximate percentage of BrdU positive cells (Chapter 2.3.2.5). There was no significant difference in the number of glands per section in PBS-treated (29.5 ± 5.5 glands) and DT-treated (34.0 ± 7.1 glands; $P = 0.56$, Mann Whitney U test) *Cd11b-Dtr*-BM mice (Figure 4.4A). There was also no significant difference between the approximate scoring of the percentage of proliferating glandular epithelial cells between treatment groups (see Chapter 2.3.2.5), with 12.3 ± 3.2% BrdU-positive glandular epithelial cells in PBS-treated, and 17.9 ± 2.3% in DT-treated *Cd11b-Dtr*-BM mice ($P = 0.22$, Mann Whitney U test (Figure 4.4B)). There were no glands present in tissue sections obtained from no hormone control mice.

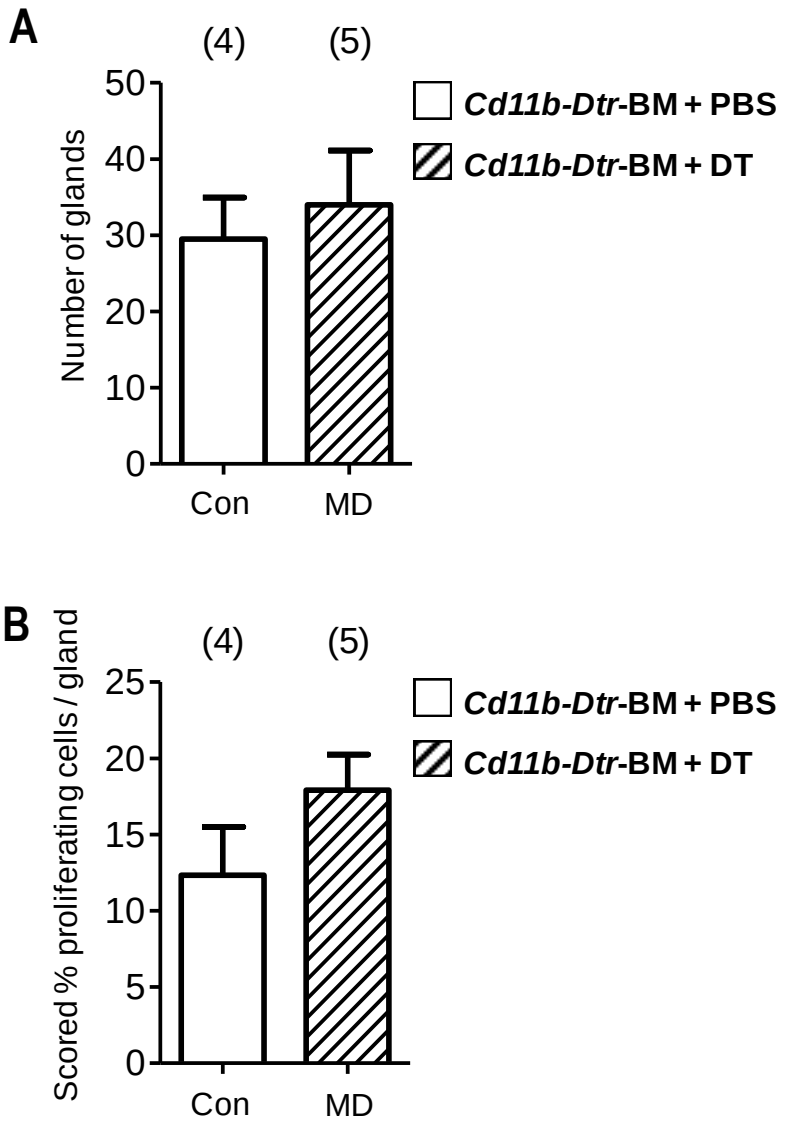



**Figure 4.4 Effect of macrophage depletion on number and proliferation in uterine endometrial glands**

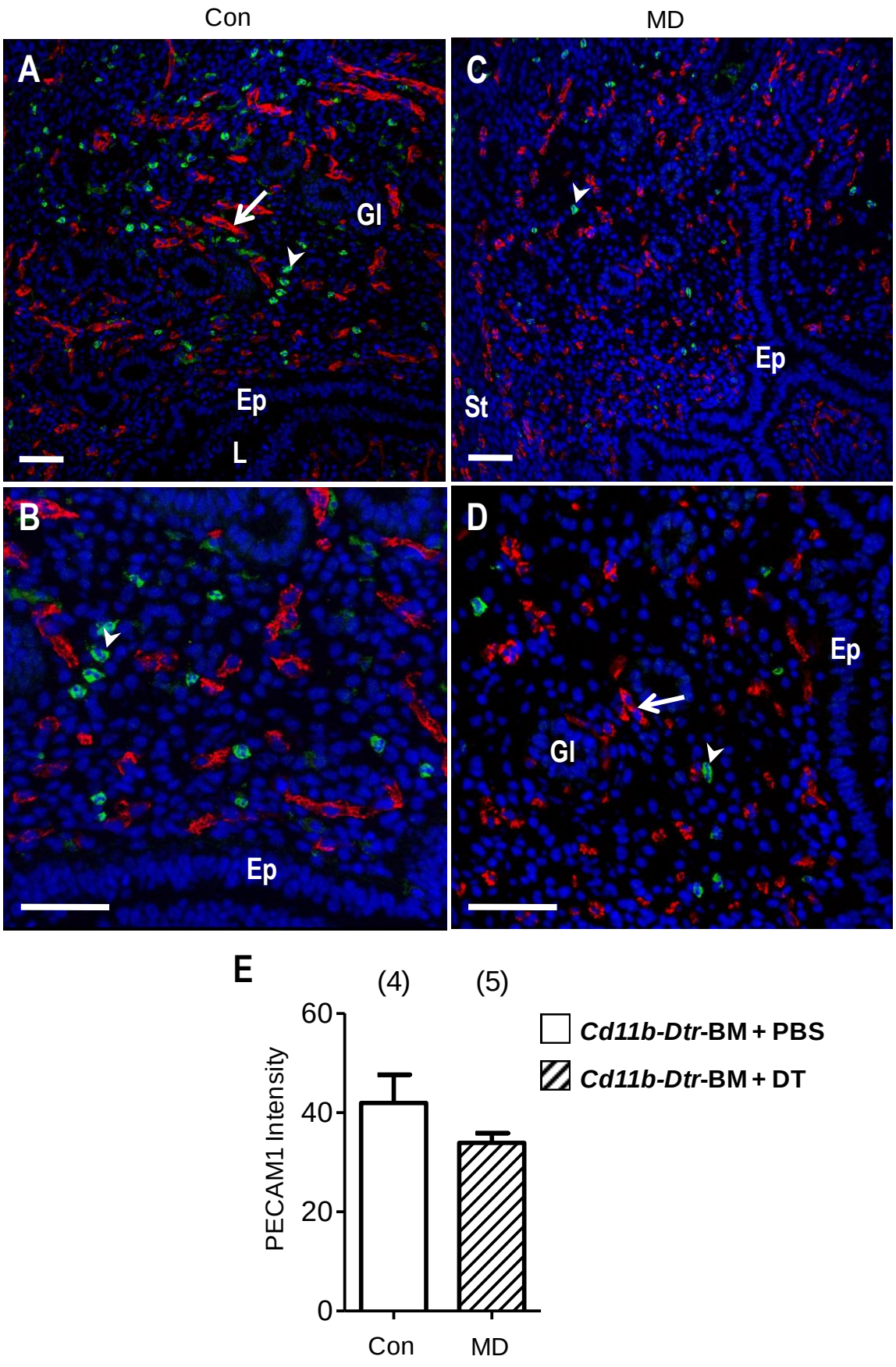
The number of uterine glands in uterine sections after $E_2$ treatment were counted in PBS-control and DT-treated macrophage-depleted mice (A). Each gland was scored for its approximate percentage of proliferating cells (B). Data are presented as mean ± SEM with statistical analysis using a Mann-Whitney U test. * indicates statistical significance from control. The number of animals in each group is shown in parentheses. MD; macrophage depleted. DT; diphtheria toxin.

### 4.2.4 Effect of macrophage depletion on stromal PECAM1-expressing endothelial cells in response to estradiol-17β

Given the potential for $E_2$ and macrophages to impact uterine angiogenesis, the effect of macrophage depletion on endothelial cell density in the endometrial stroma following treatment with $E_2$ was assessed. Briefly, tissue sections were stained with the endothelial cell specific marker, PECAM1 (CD31), and nuclei were counterstained with DAPI. Fluorescent images were obtained using confocal microscopy, and PECAM1 intensity quantified with ImageJ software (Chapter 2.3.2.5). Figure 4.4 illustrates PECAM1-expressing endothelial cells (red) and EMR1-expressing macrophages (green) in PBS-treated (A, low power; B, high power) and DT-treated (C, low power; D, high power) *Cd11b-Dtr*-BM mice. There was no significant difference in PECAM1 mean fluorescence intensity between the PBS control and macrophage-depleted groups (PBS-treated *Cd11b-Dtr*-BM (mean ± SEM) = 41.9 ± 5.7 and DT-treated *Cd11b-Dtr-BM* = 33.9 ± 2.0; $P = 0.29$, Mann Whitney U test).

**Figure 4.5 Effect of macrophage depletion on endometrial PECAM1-intensity in response to Estradiol-17β**

Anti-PECAM1 (red) and anti-EMR1 (green) immunohistochemistry illustrates endothelial cells and macrophages, respectively, in PBS-treated control (A, low power; B, high power) and DT-treated macrophage-depleted (C, low power; D, high power) *Cd11b-Dtr*-BM mice. The nuclei are stained with DAPI (blue). E, PECAM1 intensity in the endometrium of PBS-treated or macrophage-depleted *Cd11b-Dtr*-BM mice. Data are presented as mean ± SEM with statistical analysis using a Mann-Whitney U test. * indicates statistical significance between groups. The number of animals in each group is shown in parentheses. Arrows; PECAM1 positive endothelial cell. Arrow heads; EMR1 positive macrophage. MD; macrophage-depleted. DT; diphtheria toxin. St; stroma. Gl; glandular epithelium. Ep; luminal epithelium. Scale bar = 50 μm


Con
MD
A
C
Gl
Ep
L
St
B
D
E
(4)
(5)
60
40
20
0
PECAM1 Intensity
Cd11b-Dtr-BM + PBS
Cd11b-Dtr-BM + DT
Con
MD

## 4.3 EFFECT OF MACROPHAGE DEPLETION ON UTERINE STROMAL CELL PROLIFERATION

The wave of epithelial cell proliferation caused by the actions of estrus $E_2$ are inhibited by $P_4$, which instead causes stromal cell proliferation and stimulates epithelial cell differentiation in preparation for an implanting embryo. $P_4$ treatment enables uterine stromal cells to respond to $E_2$ with a single round of cell proliferation exhibiting similar kinetics to what is seen in the uterine epithelium in response to $E_2$ alone, but with lower amplitude (Tong and Pollard, 1999, Tong and Pollard, 2002). To determine whether macrophages, through their secretory products, impact stromal cell proliferation, ovariectomised *Cd11b-Dtr*-BM mice were treated with a regime of exogenous $E_2$ and $P_4$ previously shown to result in maximum proliferation of the endometrial stroma (Martin and Finn, 1969). In this regime, one group of *Cd11b-Dtr*-BM mice were treated concurrently with PBS, and the other with DT to elicit macrophage depletion and maintain depletion from 5 days prior to sacrifice (see Chapter 2.5.2). Two hours prior to sacrifice mice were injected with BrdU to enable subsequent detection of proliferating cells. Depletion of peritoneal $CD11B^+/EMR1^+$ cells was confirmed by flow cytometry.

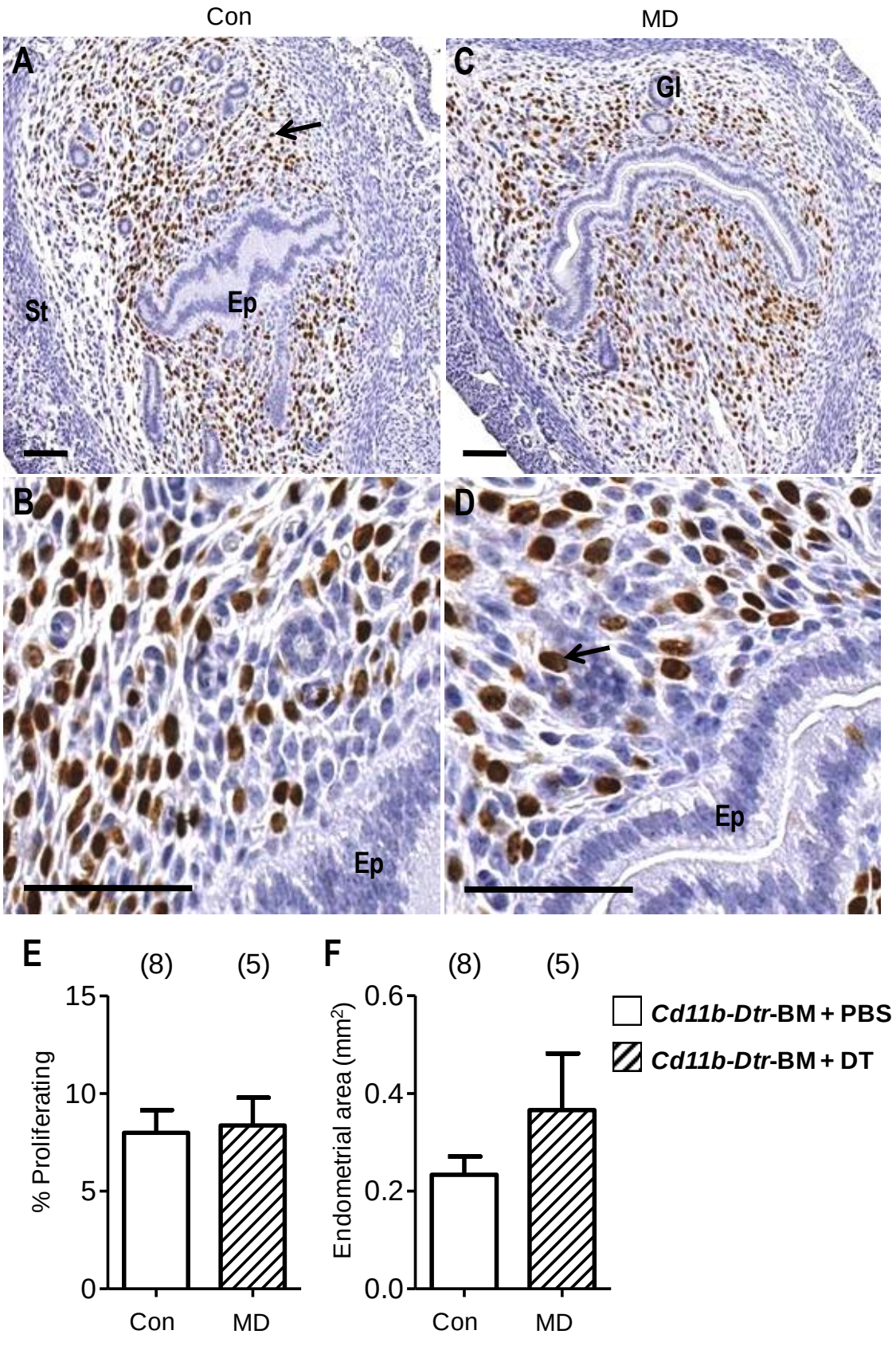
### 4.3.1 Effect of macrophage depletion on BrdU incorporation in endometrial stromal cells in the endometrium in response to estradiol-17β and progesterone

Flow cytometric analysis of PEC showed that the number of $CD11B^+EMR1^+$ cells remaining in *Cd11b-Dtr*-BM mice treated with DT to elicit macrophage depletion was significantly lower compared to PBS control mice (PBS-treated *Cd11b-Dtr*-BM (mean ± SEM) = 19.3 ± 4.5 and DT-treated *Cd11b-Dtr*-BM = 1.1 ± 0.3, $P = 0.001$, Unpaired t test; data not shown).

Proliferation of endometrial stromal cells in response to treatment with $E_2$ and $P_4$ was assessed by quantifying BrdU incorporation in PBS-treated (Figure 4.6; A, low power and B, high power) and DT-treated (C, low power and D, high power) *Cd11b-Dtr*-BM mice. The percentage of proliferating and non-proliferating stromal cells was quantified using online ImmunoRatio software (Figure 4.6E, see Chapter 2.3.2.5). There was no significant difference in the percentage of proliferating stromal cells between the PBS-control and macrophage depleted *Cd11b-Dtr*-BM mice (PBS-treated (mean ± SEM) = 8.0 ± 1.2% and DT-treated = 8.4 ± 1.4%, $P = 0.94$ Mann Whitney U test). The endometrial area was also measured, but there was no significant change following macrophage depletion (PBS-treated *Cd11b-Dtr*-BM mice = 0.23 ± 0.4 $mm^2$ and DT-treated *Cd11b-Dtr*-BM = 0.37 ± 0.1 $mm^2$, $P = 0.41$, Mann Whitney U test; Figure 4.6F).

**Figure 4.6 Effect of macrophage depletion on proliferation of uterine stromal cells**

BrdU incorporation in uterine stromal cells in response to $E_2$ and $P_4$, in PBS-treated control (A and B) and DT-treated macrophage-depleted (C and D) *Cd11b-Dtr*-BM mice. BrdU-positive cells expressed as a percentage of total nuclei in PBS-control and DT-treated *Cd11b-Dtr*-BM mice (C). Area of endometrium ($mm^2$) in PBS-treated and DT-treated *Cd11b-Dtr*-BM mice (D). Data are presented as mean ± SEM with statistical analysis using a Mann-Whitney U test. * indicates statistical significance between groups. The number of animals in each group is shown in parentheses. Arrows; BrdU-positive uterine stromal cells. MD; macrophage depleted. DT; diphtheria toxin. St; stroma. Gl; glandular epithelium. Ep; luminal epithelium. L; lumen. Scale bar = 50 μm

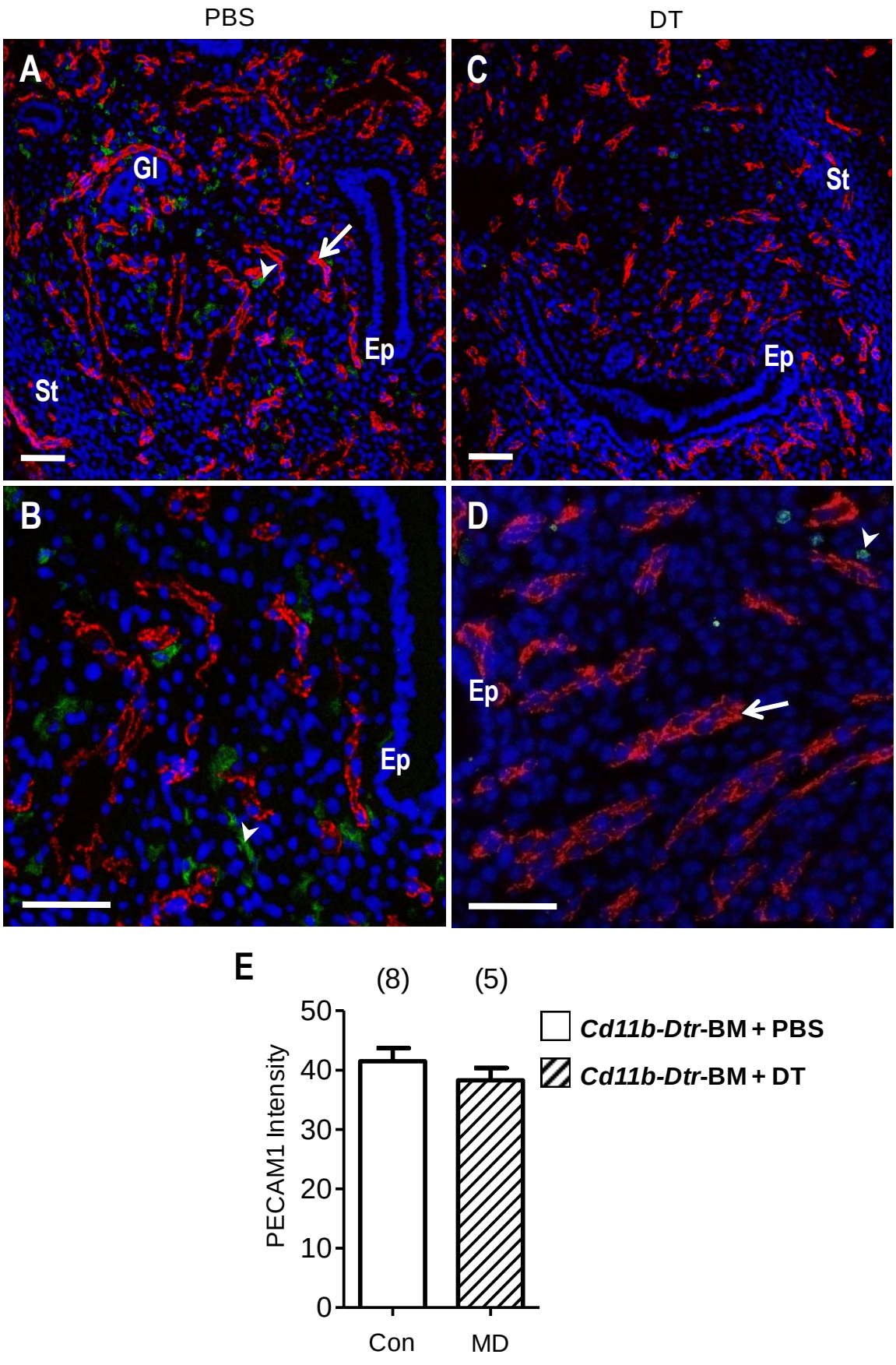

Con
MD
A
C
St
Ep
Gl
B
D
Ep
Ep
E
(8)
(5)
% Proliferating
15
10
5
0
Con
MD
F
(8)
(5)
Endometrial area (mm²)
0.6
0.4
0.2
0.0
Con
MD
Cd11b-Dtr-BM + PBS
Cd11b-Dtr-BM + DT

### 4.3.2 Effect of macrophage depletion on stromal PECAM1-expressing endothelial cells in response to estradiol-17β and progesterone

The effect of macrophage depletion on endothelial cell density in the endometrial stroma following treatment with $E_2$ and $P_4$ was assessed. Section were prepared and quantified as per section 4.1.4. Figure 4.7 illustrates PECAM1-expressing endothelial cells (red) and EMR1-expressing macrophages (green) in PBS-treated (A, low power; B, high power) and DT-treated (C, low power; D, high power) *Cd11b-Dtr*-BM mice. There was no significant difference in PECAM1 mean fluorescence intensity between the PBS control and macrophage-depleted groups (PBS-treated *Cd11b-Dtr*-BM (mean ± SEM) = 41.5 ± 2.2 and DT-treated *Cd11b-Dtr*-BM = 38.3 ± 2.1; $P = 0.35$, Mann Whitney U test).

**Figure 4.7 Effect of macrophage depletion on endometrial PECAM1-intensity in response to estradiol-17β and progesterone**

Anti-PECAM1 (red) and anti-EMR1 (green) immunohistochemistry illustrates endothelial cells and macrophages, respectively, in PBS-control (A, low power; B, high power) and DT treated macrophage-depleted (C, low power; D, high power) *Cd11b-Dtr*-BM mice. Nuclei stained with DAPI (blue). E, PECAM1 intensity in the endometrium of PBS-treated or DT-treated *Cd11b-Dtr*-BM mice. Data are presented as mean ± SEM with statistical analysis using a Mann-Whitney U test. * indicates statistical significance between groups. The number of animals in each group is shown in parentheses. Arrows; PECAM1 positive endothelial cell. Arrow heads; EMR1 positive macrophage. MD; macrophage depleted. DT; diphtheria toxin. St; stroma. Gl; glandular epithelium. Ep; luminal epithelium. Scale bar = 50 μm


PBS
DT
A
Gl
Ep
St
C
St
Ep
B
Ep
D
Ep
E
(8)
(5)
50
40
30
20
10
0
PECAM1 Intensity
Con
MD
Cd11b-Dtr-BM + PBS
Cd11b-Dtr-BM + DT

## 4.4 EFFECT OF MACROPHAGE DEPLETION ON ENDOMETRIAL EPITHELIAL RECEPTIVITY

To determine whether macrophage depletion would alter the receptivity of luminal uterine epithelial cells to implantation-competent blastocysts, *Cd11b-Dtr*-BM mice were treated with exogenous $E_2$ and $P_4$ in a regime shown to mimic the hormonal environment of early pregnancy. Mice were treated concurrently with PBS (control group) or DT (to elicit macrophage depletion) for three days prior to sacrifice (see Chapter 2.5.3). On the final day of the experiment, mice were killed four to six hours after the final injection of hormones, a time in which the uterus is maximally receptive to implanting blastocysts (Finn and Martin, 1972, Finn et al., 1989).

The expression of fucosylated structures on the uterine epithelium can be detected with lectins such as *Ulex europaeus* (UEA-1; specifically detects Fucα1-2Gal) which is used as a marker of H type 1 antigen expression, and *Lotus tetragonolobus purpureas* (LTP; detects a broader range of Fucα1-2Gal, Fucα1-3GlcNAc, and Fucα1-4GlcNAccl), or by measuring LewisX immunoreactivity. Lectin staining and immunohistochemistry for LewisX was performed on fresh-frozen uterine tissue sections obtained from PBS- or DT-treated *Cd11b-Dtr*-BM mice and analysed by confocal microscopy to determine whether there was a difference in the mean fluorescence intensity (MFI) of staining on luminal epithelial cells following macrophage depletion.

The percent of PEC expression of CD11B and EMR1 was examined by flow cytometry to ensure macrophage depletion after DT treatment in this cohort of animals (PBS-treated *Cd11b-Dtr*-BM (mean ± SEM) = 44.1 ± 3.1% and DT-treated = 1.1 ± 0.37%, $P$ = 0.0001 Mann Whitney U test; data not shown). To assess the importance of macrophages in the synthesis of these fucosylated glycoconjugates, uterine tissue from PBS- or DT-treated *Cd11b-Dtr*-BM mice was stained with UEA-1 and LTP lectin, or antibody reactive to LewisX. In this experiment, some mice were primed with $E_2$ and then received no further hormone treatment. This group served as a control to confirm the success of the hormone priming regime.

Images were captured using confocal microscopy, and the MFI of the apical half of the uterine luminal epithelial cells was measured using ImageJ software (Chapter 2.3.2.5). Figure 4.8A and B shows representative photomicrographs showing staining of the lectin UEA-1 in PBS-treated (A) and DT-treated (B) *Cd11b-Dtr*-BM mice. Figure 4.1C shows that there is a significant decrease in the staining intensity of the lectin UEA-1 after macrophage depletion was elicited by DT (PBS-treated *Cd11b-Dtr*-

BM = 12.3 ± 1.1 and DT-treated = 9.2 ± 1.2, *P* = 0.04, one-tailed Unpaired t test). The intensity of UEA-1 expression in no hormone control mice was 2.9 ± 1.3, significantly less than in the PBS control or macrophage-depleted group that received the full hormone regime (*P* = 0.01 and 0.02, respectively, Mann Whitney U test), confirming that the regime of exogenous hormone injection did result in increased uterine receptivity, as expected.

Figure 4.8D and E shows representative photomicrographs to illustrate the staining of the lectin LTP in PBS-treated (D) and DT-treated (E) *Cd11b-Dt*r-BM mice. There was no change in the intensity of LTP staining following macrophage depletion (Figure 4.8F; PBS-treated *Cd11b-Dtr*-BM = 9.7 ± 0.8 and DT-treated = 9.7 ± 1.1, *P* = 0.48, one-tailed Unpaired t test). There was a significant decrease in LTP staining intensity between the PBS-treated group and the no hormone control group (no hormone control *Cd11b-Dtr*-BM = 6.5 ± 1.1, *P* = 0.03, two-tailed Mann Whitney U test).

Figure 4.8G and H shows representative photomicrographs to illustrated LewisX staining in PBS-treated (G) and DT-treated (H) *Cd11b-Dt*r-BM mice. Figure 4.8I shows that there was a significant 67% reduction in LewisX staining intensity following DT elicited macrophage depletion (PBS-treated *Cd11b-Dtr*-BM = 62.9 ± 11.7 and DT-treated = 20.85 ± 2.4, *P* = 0.02, two-tailed Mann Whitney U test). The intensity of LewisX staining in no hormone control mice was not assessed in this experiment.

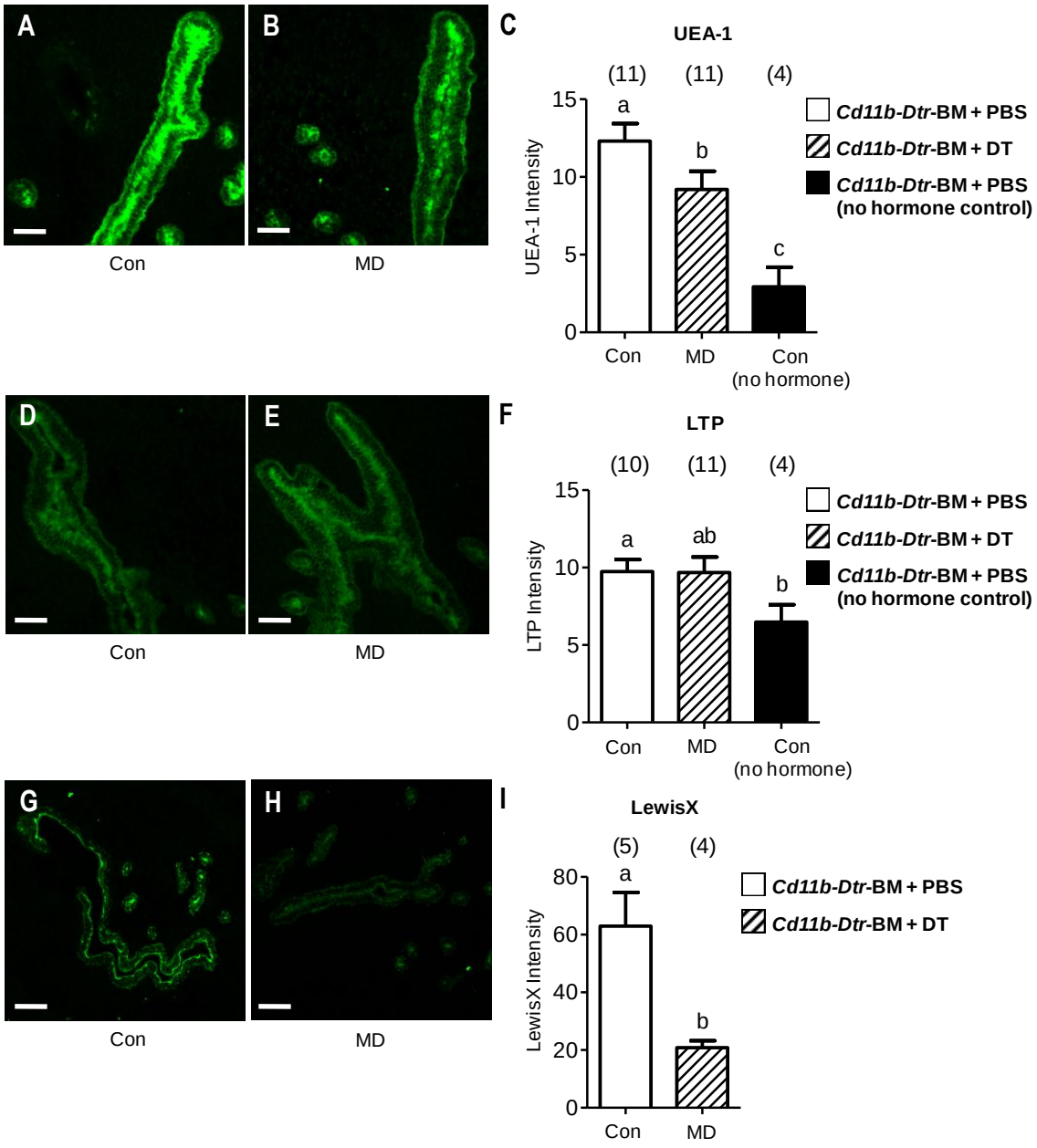



**Figure 4.8 Effect of macrophage depletion on uterine epithelial cell expression of markers of receptivity**

Representative photomicrographs of tissue from PBS-treated control (A, D and G) and DT-treated macrophage-depleted (B, E and H) *Cd11b-Dtr*-BM mice show expression of the lectins UEA-1 (A and B) and LTP (D and E), and the immunoreactive LewisX antibody (G and H). The intensity of staining on the apical surface of the uterine epithelium was quantified for UEA-1 (C), LTP (F) and anti-LewisX (I). Staining of lectins UEA-1 and LTP in no hormone control animals was also quantified (C and F). Data are presented as mean ± SEM. Statistical analysis using Unpaired t test or Mann-Whitney U test depending on whether the data was normally distributed, as determined by Shapiro-Wilk test. Different letters indicate statistical significance between groups. The number of animals in each group is shown in parentheses. MD; macrophage depleted. DT; diphtheria toxin. Scale bar = 50 µm

## 4.5 EFFECT OF MACROPHAGE DEPLETION ON THE DECIDUAL RESPONSE

As mentioned in Chapter 3, macrophages have been proposed to be involved in the endometrial tissue remodelling that occurs in preparation for the implanting blastocyst. It is therefore of interest to find out whether the process of decidualisation can take place following macrophage depletion. To test this, *Cd11b-Dtr*-BM mice were primed with the same hormonal regime mentioned in section 4.4, while being treated concurrently with either PBS (control group) or DT, to elicit macrophage depletion. Oil was injected into one horn of the uterus to induce deciduoma formation 4-6 h following hormone treatment, when the uterus would be maximally receptive to a blastocyst (Finn and Martin, 1972, Finn et al., 1989), while the contralateral horn was a sham control. Mice received hormone treatments for two days following the induction of deciduoma, before being sacrificed on the afternoon of the second day (see Chapter 2.5.4 for timeline) and the wet weight of each horn of the uterus was recorded.

PEC were collected and examined by flow cytometry to determine the number of $CD11B^+/EMR1^+$ cells following treatment with either PBS or DT (PBS-treated *Cd11b-Dtr*-BM (mean ± SEM) = 11.3 ± 2.1 and DT-treated = 2.5 ± 0.4, $P = 0.0003$ Unpaired t test; data not shown).

Figure 4.9 shows photographs of uteri that have been injected with oil to induce deciduoma formation while being treated with PBS (A) or DT (B). There was no significant effect of macrophage depletion on the weight change in the treated horn (determined by wet weight of treated horn minus wet weight of control horn), as shown in Figure 4.9C (PBS-treated *Cd11b-Dtr*-BM = 30.4 ± 7.8 mg and DT-treated = 22.2 ± 4.5 mg, $P = 0.53$ Mann Whitney U test).

Uterine stroma associated with the decidual cell reaction has an associated increase in alkaline phosphatase activity (Finn and Hinchliffe, 1964, Yee and Kennedy, 1988). Incubation with substrate can allow differentiation of decidualised uteri from non-decidualised uteri (Chapter 2.3.2.1.3). As shown in Figure 4.9D-F, tissue from the oil induced deciduoma from a PBS-treated (D) or from a DT-treated (E) mouse showed the expected staining with alkaline phosphatase, while tissue from the untreated contralateral horn of a *Cd11b-Dtr*-BM mouse (F) did not.

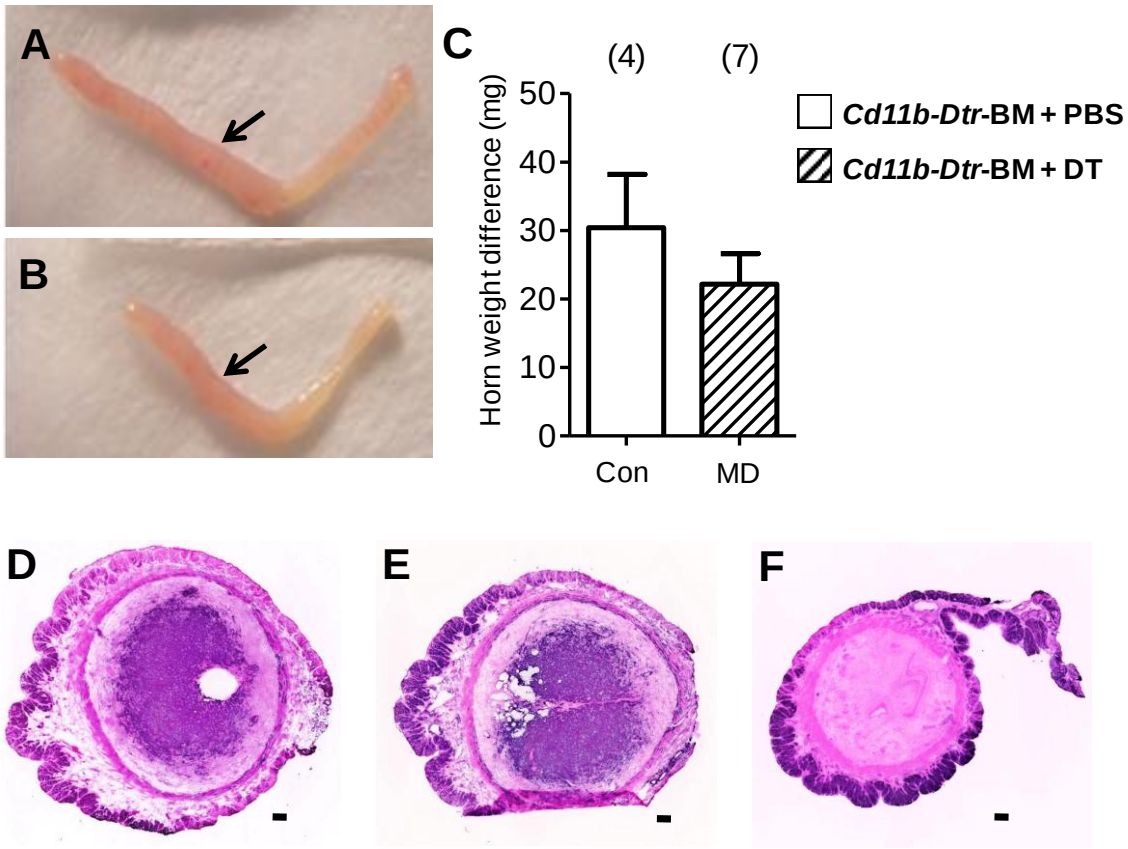



**Figure 4.9 Effect of macrophage depletion on the uterine deciduoma formation**

Uteri recovered from PBS-treated control (A) or DT-treated macrophage-depleted (B) *Cd11b-Dtr*-BM mice following the instillation of oil in one horn to induce deciduoma formation (left; arrow) and the other horn was a sham control (right). The difference in weight (mg) of the decidualised horn compared to the sham control horn was measured (C). Alkaline phosphatase activity is a marker of cells that have undergone decidualisation (purple) in uterine horns that have been injected with oil from PBS-treated (D), DT-treated (E) *Cd11b-Dtr*-BM mice. F is the sham treated contralateral horn of a PBS-treated mouse. Tissue is counterstained with eosin (pink). Data are presented as mean ± SEM with statistical analysis using a Mann-Whitney U test. * indicates statistical significance between groups. The number of animals in each group is shown in parentheses. MD; macrophage depleted. DT; diphtheria toxin. Scale bar = 50 µm

## 4.6 Discussion

The experiments presented in this chapter show that macrophages are not necessary for uterine epithelial or stromal cell proliferation in response to exogenous ovarian steroid hormones. Macrophages did not affect the endothelial cell density in the uterine stroma following treatment with either $E_2$ alone, or $E_2$ and $P_4$ treatment. Macrophages appear to have a role in the optimal expression of fucosylated structures on the uterine epithelium that are involved in embryo attachment and implantation. However, they are not required for artificially induced deciduoma formation in response to oil injected at the uterotubal junction.

Given the involvement of macrophages in the proliferation of ductal epithelium and tissue remodelling events that occur in mammary gland development (Chua et al., 2010), it seemed reasonable to consider a role for these cells in proliferation and remodelling events that occur in the uterus, since both tissues are under the influence of ovarian steroid hormones and remodelling events occur with each cycle. However, in light of the experiments presented in this chapter, it appears that macrophages are unlikely to be required for the proliferation events occurring in the uterus. The contrast between the mammary gland and the uterus highlights the differing roles these cells can have in different tissues, supporting the view that macrophages tailor their activities and behaviours to their local microenvironment (McCormick et al., 2000, Gordon, 2003).

Given the differential actions of the selective estrogen receptor modulator (SERM) tamoxifen in different reproductive tissues, where it behaves as an ER antagonist in breast tissue, but an agonist in uterus where it stimulates epithelial cell proliferation, it appears that proliferation events in these tissues are controlled by different mechanisms (Zhang et al., 2005). Since macrophages are very sensitive to their local microenvironment, it seems likely the signals they would receive in the breast and endometrium are different, and this taken together with the apparent different machinery to regulate proliferation in these tissues, may provide some explanation for their ability to influence epithelial cell proliferation in the mammary gland, but not in the endometrium.

It is important to consider whether there may be another interpretation of the data – that is, the extent to which a role for macrophages can be dismissed conclusively. In the experiments where macrophage involvement in uterine epithelial cell proliferation was examined, mice received a single $E_2$ injection following priming, and this was administered 9 h after the injection of DT to elicit macrophage

depletion. 15 h following the $E_2$ injection, mice were sacrificed, a time where maximal epithelial cell proliferation should be occurring (Tong and Pollard, 1999). However, 9 h after DT administration, there is likely to be macrophages still present in the uterus. Although they should be mostly depleted by 24 h, it is possible that their secreted products may already have had a paracrine effect on epithelial cell proliferation. To more conclusively exclude a role for macrophages in this process, it would be helpful to repeat the experiment and have macrophages depleted for a day or two prior to the final $E_2$ injection. For the experiments in which the effect of macrophages on stromal cell proliferation were assessed, macrophage depletion was maintained for 5 days prior to sacrifice, throughout the injection of exogenous hormones following priming, and this is therefore a more robust experiment that supports a strong conclusion for no requirement for macrophages in stromal cell proliferation.

Paracrine signals released from cells in the uterine stroma, under the influence of ovarian steroid hormones, have a well established influence on uterine epithelial cells (Pollard, 1990, Bigsby, 2002). Stromal macrophages have contact with epithelial cells, but their potential role in these paracrine signalling events is not well understood. The *in vitro* macrophage-epithelial coculture experiments reported by Jasper et al. (2011) indicated that macrophages, through their secretory products LIF and IL1B, were able to upregulate the mRNA expression of the fucosyltransferase *Fut2*, an enzyme that leads to increased expression of fucosylated glycoproteins implicated in initial attachment of the blastocyst to the epithelium . The experiments reported in this chapter further support these observations, indicating that macrophages are necessary for the normal α1,2 fucosylation of glycans of the endometrial epithelium. There was a 25% reduction in UEA-1 staining intensity following DT-elicited macrophage depletion in *Cd11b-Dtr*-BM mice; UEA-1 lectin detects terminal Fucα1-2 structures involved in embryo attachment (Sidhu and Kimber, 1999). There was also a 67% reduction in the intensity of LewisX staining following macrophage depletion. There was no change in intensity of the lectin LTP, possibly explained by its broader reactivity (Fucα1-2Gal, Fucα1-3GlcNAc, and Fucα1-4GlcNAccl) and it therefore less likely to be sensitive enough to detect changes in specific fucose structures.

Despite this finding, it is questionable whether the reduced expression of these particular fucosylated structures on uterine epithelial cells would translate to implantation defects in a pregnant mouse. FUT2 is a major regulator of α1,2 fucosyltransferase activity in mouse uterine epithelial cells. However, mice with a null mutation in the *Fut2* gene display no abnormality in their fertility, despite an absence of UEA-1 and H type 1 staining on uterine epithelial cells (Domino et al., 2001).

The overall intensity of UEA-1 and LTP lectin expression is far lower than that seen for LewisX reactivity (see Figure 4.8C and F, compared to I). This is due to the scanning of the confocal images being conducted differently. The image collection and analysis of UEA-1 and LTP intensity were carried out at 20X magnification by A.S. Care, and the collection and analysis of LewisX expression was carried out at 10X magnification by M.J.Jasper, on a different confocal microscope. However, for each group there were PBS-treated and DT-treated mice, so the difference in intensity between these groups remains valid.

Further experiments to measure the expression of other adhesion and anti-adhesion molecules involved in the window of implantation would further our understanding of macrophage involvement in uterine epithelial receptivity. Molecules such as the integrins and LIF are involved in defining endometrial receptivity, and their expression following macrophage depletion would be insightful. In the *Lif* null mutant mouse implantation fails to occur (Stewart et al., 1992), and uterine macrophages are reduced in number (Schofield and Kimber, 2005). Further, oviductal LIF expression is reduced following mating with SV- mice, following reduced endometrial macrophage recruitment occurring in this mating model, and this reduction may also be seen in the uterus (Jasper et al., 2005, Bromfield, 2006). It would also be of interest to determine whether the expression of molecules that provide a barrier between the blastocyst and the uterine epithelium until the onset of receptivity, such as the mucins, may be regulated by macrophages (Nakamura et al., 2008). The expression of mucins has been shown *in vitro* to down-regulate at the precise site of embryo implantation, but not at other sites on the uterine epithelial cell surface (Meseguer et al., 2001). Further, given the reduced expression of adhesion molecules by endometrial epithelial cells following macrophage depletion, one could speculate that increased macrophage density beyond normal levels, perhaps due to infection, may be associated with increased aberrant expression of such adhesion molecules in the oviduct, and this could possibly be a causative factor in EP.

Finally, the experiments assessing the role of macrophages in the process of deciduoma formation clearly demonstrate that macrophages are not required. In these experiments, macrophage depletion was maintained throughout hormonal treatment following priming, for 5 days prior to sacrifice. The alkaline phosphatase reaction does not differ between deciduomas in PBS-treated and DT-treated *Cd11b-Dtr*-BM mice. This, taken together with the data showing no significant difference between treatment groups with regard to the proportion of mice undergoing a decidual reaction, or the extent of

that reaction, suggests that macrophages are not required for decidualisation in the mouse. This contrasts with experiments that have indicated an essential role for uterine DCs in decidualisation and deciduoma formation in a study that utilised the *Cd11c-Dtr* mouse to depleted DCs (Plaks et al., 2008). Interestingly, as described in Chapter 3, $CD11C^{+}CD11B^{-}$ cells are significantly depleted from the uterus in the *Cd11b-Dtr* mouse, however, there was only a trend towards a difference in the proportion of cells expressing $CD11C^{+}CD11B^{+}$ between DT-treated wild-type and *Cd11b-Dtr* mice. This, taken together with the studies by Plaks et al. (2008) suggest it is the $CD11C^{+}CD11B^{+}$ cells that may be important for the decidual response, although further investigation would be required to confirm this. Further studies are required to determine whether the vascularisation of the decidua is affected in the macrophage depleted *Cd11b-Dtr* mouse, or its ability to accept an implanting blastocyst, questions that would be best answered in embryo transfer experiments into pseudopregnant mice that have been treated with DT to deplete their macrophages.

In conclusion, the experiments in this chapter reveal only a limited potential role for macrophages in the uterine changes required to establish pregnancy, with some impact on the regulation of uterine epithelial cell receptivity in preparation for embryo attachment. It appears that $CD11B^{+}$ macrophages are not required for the process of deciduoma formation. Together the studies in this chapter fail to provide an explanation for the pregnancy failure described in Chapter 3.

# Chapter 5

# Effect of macrophage depletion on ovarian function in early pregnancy

## 5.1 INTRODUCTION

The cause of pregnancy failure in *Cd11b-Dtr* mice following macrophage depletion is unresolved. The experiments in Chapter 4 suggest that it is not due to failure of the uterus to proliferate and decidualise in preparation for the implanting embryo, and experiments in Chapter 3 revealed that the early stages of implantation begin, but pregnancies are lost shortly thereafter.

An issue not addressed thus far is the possible impact of macrophage depletion on ovarian function. When characterising the model in Chapter 3, it was shown that macrophages could be depleted from the ovary of pregnant *Cd11b-Dtr* mice (Figure 3.9), concurring with studies by Cailhier et al. (2005) showing macrophage depletion from the cycling ovary. It is well established that leukocytes and cytokines play key roles in ovarian physiology. There is traffic of leukocytes into and out of the ovary, and associated changes in morphology and function occur throughout the cycle, including roles in tissue remodelling and steroidogenesis. Macrophages are recruited to the thecal layer of developing follicles, their numbers being greatest just prior to ovulation in mice, rats and humans (Brannstrom et al., 1994b, Brannstrom et al., 1993, Cohen et al., 1997, Cohen et al., 1999). Macrophages accumulate at the apical region of the follicle just prior to ovulation, indicating a possible role in follicle rupture and/or luteinisation (Brannstrom et al., 1993, Brannstrom and Norman, 1993). Macrophages are also abundant in the corpus luteum (Brannstrom et al., 1994a, Cohen et al., 1997), where their precise role is unclear. It is possible that they secrete proteolytic enzymes involved in angiogenesis and tissue remodelling (Brannstrom and Norman, 1993) and may enhance steroidogenesis by luteal cells.

Studies have described increased $P_4$ output from granulosa or luteal cells following coculture with peritoneal macrophages in the mouse and human (Kirsch et al., 1981, Halme et al., 1985), and in the rat such coculture enhanced granulosa cell proliferation (Fukumatsu et al., 1992). A further increase in $P_4$ occurs following luteal cell culture with ovarian macrophages (Kirsch et al., 1981).

As mentioned previously, mating female mice with SV- males causes reduced recruitment of leukocytes, including macrophages, into the endometrium, when compared to matings with intact males (Robertson et al., 1996a). Following such matings, fewer macrophages were observed within corpora lutea on day 1 pc, although this did not impact $P_4$ levels (Gangnuss et al., 2004). Studies in pigs revealed that the addition of SP at artificial insemination caused significantly increased in

macrophage recruitment to ovarian tissue, with increases in corpora lutea weight and plasma $P_4$ (O'Leary et al., 2006).

Studies in *Csf2*$^{-/-}$ mice reported altered phenotype of ovarian leukocytes (reduced MHC II expression) and increased estrous cycle length, but no change in leukocyte numbers. *In vitro* perfusion experiments showed that significantly less $P_4$ was produced by *Csf2*$^{-/-}$ ovaries compared to those from wild-type mice (Jasper et al., 2000).

Cycling *Csf1*$^{op}$*/Csf1*$^{op}$ mice are severely depleted of ovarian macrophages (Cohen et al., 1999). Dysfunction at the level of the HPG axis impairs ovarian function in *Csf1*$^{op}$*/Csf1*$^{op}$ mice, causing extended estrous cycle length and a lower ovulation rate when compared to wild-type controls. Despite an almost complete absence of normally abundant EMR1$^+$ macrophages surrounding developing follicles in estrus *Csf1*$^{op}$*/Csf1*$^{op}$ mice, there is a marked increase in the number of corpora lutea-associated EMR1$^+$ macrophages on day 1 pc, with the density reaching approximately 35% of that seen in wild-type mice. There appears to be a slight delay in the ability of the *Csf1*$^{op}$*/Csf1*$^{op}$ ovary to produce $P_4$, but levels are not significantly different from wild-type mice by day 8 pc (Cohen et al., 1997).

This research provides evidence that macrophages, and the cytokines which regulate them, are important in ovarian function. Further investigation of their role using the *Cd11b-Dtr* mouse model was undertaken to better define their precise role in this complex organ.

## 5.2 EFFECT OF MACROPHAGE DEPLETION ON CIRCULATING STEROID HORMONE LEVELS IN EARLY PREGNANCY

To elucidate the cause of pregnancy failure after macrophage depletion in *Cd11b-Dtr* mice, we investigated whether the corpora lutea in the ovary were functioning normally and producing sufficient amounts of $P_4$ to support a pregnancy. Briefly, wild-type and *Cd11b-Dtr* females were mated with males of the same genotype, and checked daily for the presence of a vaginal plug (designated day 0.5 pc). Circulating plasma $P_4$ was measured after three different protocols for administration of DT to elicit macrophage depletion in transgenic mice;

- Day 4.0 pc (17:00 h), 8 h after DT injection (on day 3.5 pc)
- Day 3.5 pc (12:00 h), 24 h after DT injection (on day 2.5 pc)

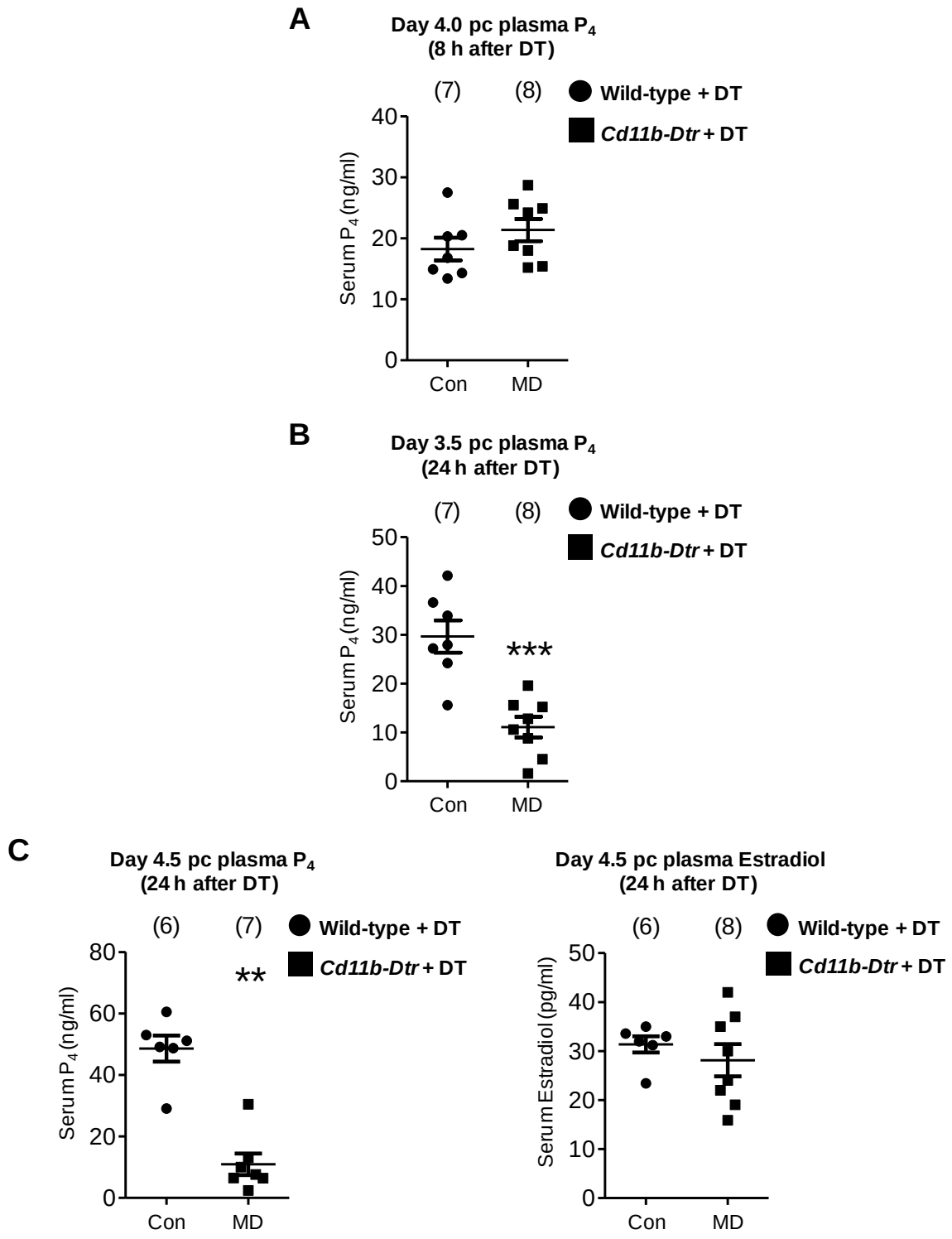
- Day 4.5 pc (12:00 h), 24 h after DT injection (on day 3.5 pc)

Circulating plasma estradiol was measured at the day 4.5 pc time point only, 24 h following DT injection.

Plasma $P_4$ levels were significantly decreased 24 h after DT-elicited macrophage depletion. As illustrated in Figure 5.1, there was no significant difference in the level of circulating plasma $P_4$ between groups on day 4.0 pc, 8h following the injection with DT (wild-type (mean ± SEM) = 18.2 ± 1.9 ng/ml and *Cd11b-Dtr* = 21.4 ± 1.8 ng/ml, *P* = 0.26, Unpaired t test)). However, on day 3.5 pc, 24 h following DT-elicited macrophage depletion, there was a significant 62.5% reduction in the level of circulating plasma $P_4$ in transgenic mice (wild-type = 29.6 ± 3.3 ng/ml and *Cd11b-Dtr* = 11.1 ± 2.1 ng/ml, *P* = 0.0003, Unpaired t test). There was also a significant 77.6% reduction in circulating plasma $P_4$ in *Cd11b-Dtr* mice on day 4.5 pc, 24 h after DT treatment (wild-type = 48.6 ± 4.3 ng/ml and *Cd11b-Dtr* = 10.9 ± 3.5 ng/ml, *P* = 0.002, Mann Whitney U test), where the levels in wild-type mice had risen compared to earlier time points measured. There was no significant difference in circulating estradiol on day 4.5 pc, following 24 h of treatment with DT (wild-type = 31.4 ± 1.7 pg/ml and *Cd11b-Dtr* = 28.1 ± 3.3 pg/ml, *P* = 0.61, Mann Whitney U test).

**Figure 5.1 Effect of macrophage depletion on ovarian steroid hormone secretions**

DT-elicited macrophage depletion was carried out on day 2.5 pc, with circulating plasma $P_4$ measured 24 h later (B), or was carried out on day 3.5 pc, and circulating plasma $P_4$ levels were measured 8 h (A) or 24 h (C) later. Circulating plasma estradiol was also measured on day 4.5 pc, 24 h following DT-elicited macrophage depletion on day 3.5. Data are presented as mean ± SEM with statistical analysis using an Unpaired t test or a Mann-Whitney U test. * indicates statistical significance from control. ** $P < 0.01$, *** $P < 0.001$. The number of animals in each group is shown in parentheses. MD; macrophage-depleted. DT; diphtheria toxin


A
Day 4.0 pc plasma P4
(8 h after DT)
(7)
(8)
Wild-type + DT
Cd11b-Dtr + DT
Serum P4 (ng/ml)
40
30
20
10
0
Con
MD
B
Day 3.5 pc plasma P4
(24 h after DT)
(7)
(8)
Wild-type + DT
Cd11b-Dtr + DT
Serum P4 (ng/ml)
50
40
30
20
10
0
***
Con
MD
C
Day 4.5 pc plasma P4
(24 h after DT)
(6)
(7)
Wild-type + DT
Cd11b-Dtr + DT
Serum P4 (ng/ml)
80
60
40
20
0
**
Con
MD
Day 4.5 pc plasma Estradiol
(24 h after DT)
(6)
(8)
Wild-type + DT
Cd11b-Dtr + DT
Serum Estradiol (pg/ml)
50
40
30
20
10
0
Con
MD

## 5.3 EFFECT OF EXOGENOUS PROGESTERONE ADMINISTRATION FOLLOWING MACROPHAGE DEPLETION IN EARLY PREGNANCY

To determine whether the reduced circulating plasma $P_4$ levels were a cause of pregnancy loss in DT-treated *Cd11b-Dtr* mice, rescue experiments were initiated. Briefly, wild-type and *Cd11b-Dtr* mice were mated with males of the same genotype. On day 3.5 pc, mice received an i.p. injection of DT (25 ng/g) to elicit macrophage depletion. Mice simultaneously received a subcutaneous (s.c.) injection of either $P_4$ (2 mg) or sesame seed oil vehicle, which continued daily until day 6.5 pc. An autopsy to examine implantation site numbers was conducted on day 7.5 pc. In addition, a small number of *Cd11b-Dtr* mice received DT injections on day 0.5 pc, as well as daily injections of $P_4$ until day 6.5 pc and uteri were examined on day 7.5 pc.

Representative photographs in Figure 5.2A and B show normal implantation sites in wild-type mice treated with DT and sesame seed oil vehicle (A) and *Cd11b-Dtr* mice treated with DT and $P_4$ (B). Strikingly, 91.7% (11/12) of *Cd11b-Dtr* mice treated with DT and $P_4$ carried viable pregnancies on day 7.5 pc (Figure 5.2C), while there were no viable pregnancies (0/6) in *Cd11b-Dtr* mice treated with DT and vehicle ($P$ = 0.0000, Fisher's exact test). $P_4$ supplemented *Cd11b-Dtr* mice carried 8.1 ± 1.1 implantation sites on day 7.5 pc, while *Cd11b-Dtr* mice supplemented with sesame seed oil vehicle carried no implantation sites ($P$ = 0.0000, Unpaired t test; Figure 5.2C).

In control experiments, 100% (6/6) of wild-type mice treated with DT and $P_4$ were pregnant on day 7.5 pc, and 77.8% (7/9) of wild-type mice treated with DT and sesame seed oil vehicle were pregnant ($P$ = 0.34, Fisher's exact test). The number of implantation sites was 6.3 ± 1.7 for wild-type mice treated with DT and vehicle, and 9.7 ± 0.9 for wild-type mice receiving DT and $P_4$ ($P$ = 0.28, Mann Whitney U test; Figure 5.2D).

Figure 5.2D shows that there was no significant difference in the number of implantation sites carried by wild-type mice treated with DT and sesame seed oil vehicle and *Cd11b-Dtr* mice treated with DT and $P_4$ ($P$ = 0.38, Unpaired t test) or in the number of implantations carried by wild-type or *Cd11b-Dtr* mice treated with DT and $P_4$ ($P$ = 0.51, Mann Whitney U test).

Seven *Cd11b-Dtr* mice received DT on day 0.5 pc and daily injections of $P_4$ until day 6.5 pc. On day 7.5 pc, 71.4% (5/7) of these mice were carrying viable pregnancies, with the number of implantation

sites being 6.1 ± 1.8 (data not shown). This is a substantial increase compared to *Cd11b-Dtr* females receiving DT on day 0.5 pc and no $P_4$ (sesame seed oil vehicle), where 0% (0/7) mice were pregnant by day 7.5 pc (as reported in Chapter 3, Figure 3.11A).

Another experiment was conducted to determine whether pregnancy could be rescued to day 17.5 pc, following macrophage depletion on day 3.5 pc. Briefly, wild-type and *Cd11b-Dtr* females were mated with males of the same genotype, and were administered DT (25 ng/g) on day 3.5 pc. Wild-type mice either received no further treatment (Wild-type + DT) or received s.c. sesame seed oil vehicle injections daily from day 3.5-16.5 pc (Wild-type + DT + vehicle). *Cd11b-Dtr* mice received $P_4$ (2 mg; s.c) daily from day 3.5-16.5 pc (*Cd11b-Dtr* + DT + $P_4$). Females were sacrificed and the total, viable and resorbing implantation sites were counted. Viable fetuses and placentae were weighed. Scoring outcomes for day 17.5 pc are described in section 2.1.10 and results are shown in Table 5.1. It must be highlighted that autopsy took place earlier than day 17.5 pc if mice were moribund, and this was the case for 4 out of 11 *Cd11b-Dtr* mice receiving DT and $P_4$ treatment. This must be taken into account when considering the implantation site numbers in Table 5.1; the remains of resorbing implants that were detected at autopsy would most likely no longer be evident if the mice were to survive until day 17.5 pc. This would lower the number of *Cd11b-Dtr* mice considered pregnant (viable and non-viable implants) at autopsy.

Pregnancy was able to be rescued following DT-elicited macrophage depletion until day 17.5 pc in 4 out of 11 *Cd11b-Dtr* mice. There were significantly less DT-treated *Cd11b-Dtr* females carrying viable pregnancies at autopsy when compared to wild-type females treated with DT only (Wild-type + DT versus *Cd11b-Dtr* + DT + $P_4$, $P = 0.009$, Fisher's exact test). There were significantly more DT-treated *Cd11b-Dtr* mother's where all implantation sites were resorbing at autopsy when compared to wild-type + DT treated mother's (wild-type + DT versus *Cd11b-Dtr* + DT + $P_4$, $P = 0.046$, Fisher's exact test). When assessing the number or resorptions as a percentage of total implants, there were significantly more in DT-treated *Cd11b-Dtr* when compared to wild-type mice treated with DT only or wild-type mice treated with both DT and sesame seed oil vehicle (wild-type + DT versus *Cd11b-Dtr* + DT + $P_4$, $P = 0.0001$, Fisher's exact test. Wild-type + DT + vehicle versus *Cd11b-Dtr* + DT + $P_4$, $P = 0.0001$, Fisher's exact test). There was no difference between any of the treatment groups when assessing the number of implantation sites per mother (viable and non-viable implantations). However, when comparing the number of viable implantations per mother, there were significantly less in DT-treated *Cd11b-Dtr* females compared to wild-type females treated with DT only or DT and sesame seed oil vehicle.

In *Cd11b-Dtr* mice where pregnancy was rescued, there were no adverse effects of macrophage depletion on fetal and placental weights, or the fetal:placental weight ratio (a measure of placental efficiency). Table 5.1 shows that there was no difference in fetal weight across all three treatment groups, and there was no difference in the weights of placentae obtained from DT-treated *Cd11b-Dtr* mother's compared to wild-type mother's receiving DT only or DT and sesame seed oil vehicle. There was no effect on placental efficiency in DT-treated *Cd11b-Dtr* mice when compared to wild-type mice treated with DT only or DT and sesame seed oil vehicle.

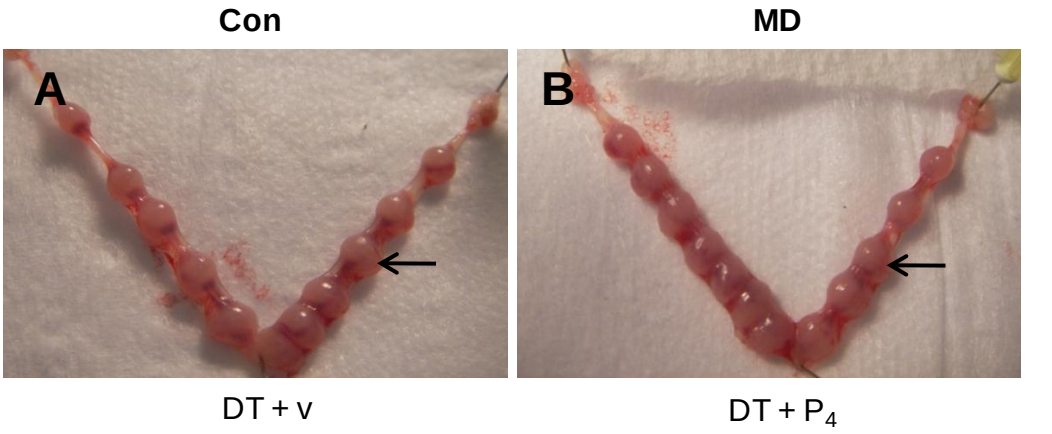
**Table 5.1 The effect of macrophage depletion on day 17.5 pc pregnancy outcomes**

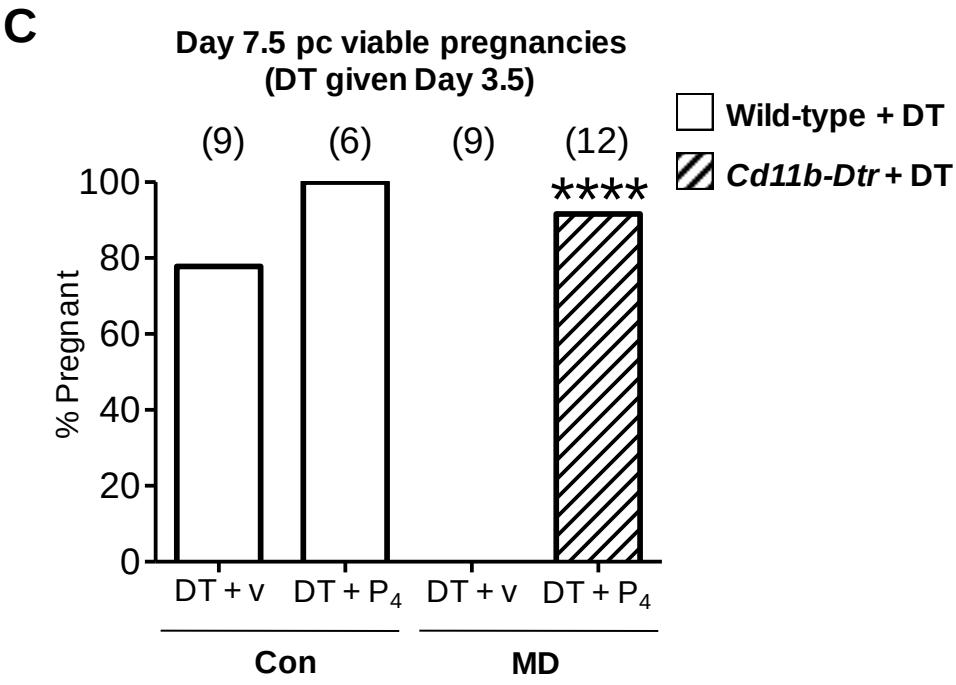
| | Wild-type (DT only) | Wild-type (DT + vehicle) | *Cd11b-Dtr* (DT + $P_4$) |
|---|---|---|---|
| Plugged females | 14 | 10 | 11 |
| Females pregnant at autopsy [#*] (%) | 11/14 (79%) [a] | 6/10 (60%) [a] | 9/11 (82%) [a] |
| Females with viable pregnancies at autopsy [#*] (%) | 11/14 (79%) [a] | 6/10 (60%) [ab] | 4/11 (36%) [b] |
| All implantations resorbing at autopsy [#*] | 0/11 [a] | 0/6 [ab] | 5/9 [b] |
| Total resorptions (% implants) * | 13/95 (14) [a] | 3/48 (6) [a] | 30/50 (60) [b] |
| Implantation sites/pregnant mother (total and viable) [†] | 8.6 ± 0.9 [a] | 8.0 ± 0.5 [a] | 5.6 ± 0.9 [a] |
| Implantation sites/pregnant mother (viable) [†] | 7.5 ± 0.8 [a] | 7.5 ± 0.5 [a] | 2.2 ± 1.0 [b] |
| Fetal weight (mean ± SEM) [†] | 834 ± 16 [a] | 780 ± 18 [a] | 727 ± 38 [a] |
| Placental weight (mean ± SEM) [†] | 68 ± 2 [a] | 56 ± 1 [b] | 60 ± 2 [ab] |
| Fetal:placental weight ratio (mean ± SEM) [†] | 12.7 ± 0.4 [a] | 14 .1 ± 0.3 [b] | 12.4 ± 0.8 [ab] |

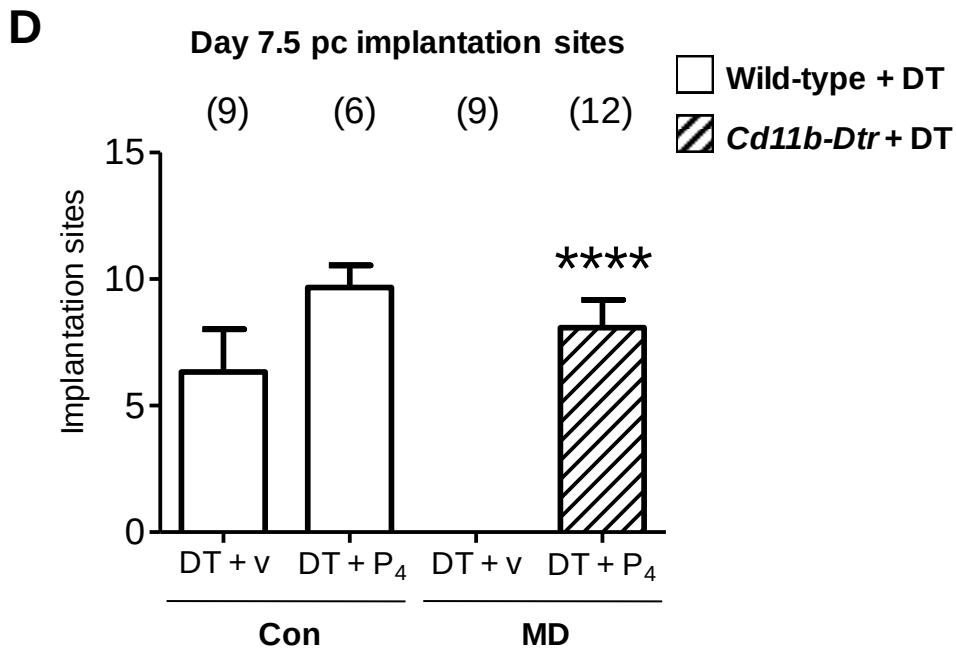
Implantation sites defined as total implantations, viable and non viable. Pregnant mother defined as a mouse with implantation sites.
[#] Autopsy was conducted at day 17.5 pc or earlier (day 7.5 - 10.5) if mice were moribund.
* Categorical data was analysed using Fisher's exact test.
[†] All data is mean ± SEM and was analysed by Kruskal-Wallis test and post-hoc Dunns test.
[a, b] Different superscripts denote significant differences between treatment groups.
DT; diphtheria toxin. Vehicle; sesame seed oil vehicle.

**Figure 5.2 Effect of exogenous $P_4$ administration following macrophage depletion in early pregnancy**

Representative photomicrograph showing implantation sites on day 7.5 pc in wild-type mice treated with DT and sesame seed oil vehicle from day 3.5 pc (A). Representative photomicrograph showing implantation sites on day 7.5 pc in *Cd11b-Dtr* mice treated with DT and $P_4$ from day 3.5 pc (B). The percentage of pregnant mice on day 7.5 pc was evaluated, where wild-type and *Cd11b-Dtr* mice were treated with either DT and sesame seed oil vehicle, or DT and $P_4$ from day 3.5 pc (C). The number of implantation sites per mother was quantified (D). Data are presented as percentages or mean ± SEM with statistical analysis using an Unpaired t test and Fisher's exact test. * indicates statistical significance from control. ****$P < 0.0001$. The number of animals in each group is shown in parentheses. Arrows; implantation sites. MD; macrophage-depleted. DT; diphtheria toxin. V; sesame seed oil vehicle.


Con
MD
A
B
DT + v
DT + P4


C
Day 7.5 pc viable pregnancies
(DT given Day 3.5)
Wild-type + DT
Cd11b-Dtr + DT
(9)
(6)
(9)
(12)
****
% Pregnant
100
80
60
40
20
0
DT + v
DT + P4
DT + v
DT + P4
Con
MD


D
Day 7.5 pc implantation sites
Wild-type + DT
Cd11b-Dtr + DT
(9)
(6)
(9)
(12)
****
Implantation sites
15
10
5
0
DT + v
DT + P4
DT + v
DT + P4
Con
MD

## 5.4 EFFECT OF MACROPHAGE DEPLETION ON OVARIAN STRUCTURE

Given the lower levels of circulating plasma $P_4$ detected in pregnant *Cd11b-Dtr* females following macrophage depletion, the ovarian tissue was assessed histologically to investigate the cause. Briefly, on day 4.5 pc, 24 h following DT administration, ovaries were harvested and frozen in OCT embedding medium for sectioning. At autopsy, ovaries recovered from DT-treated *Cd11b-Dtr* mice appeared red and haemorrhagic, while those obtained from DT-treated wild-type mice appeared normal, with only corpora lutea being red in colour, indicative of the dense blood vessel network within functioning corpora lutea. When sections were cut from ovaries of DT-treated *Cd11b-Dtr* mice, blood was evident in the OCT embedded tissue. However, red blood cells (RBC) were not evident in sections because they were washed away. Sections were stained with haematoxylin to visualise the overall structure of the ovary, or with haematoxylin and eosin for a more detailed view of the cells within the ovary. The same was true for *Cd11b-Dtr* ovaries harvested on day 3.5 pc, 24 h following DT treatment.

Figure 5.3B shows a representative image of the day 4.5 pc ovaries from macrophage-depleted *Cd11b-Dtr* mice. While the ovary displayed an overtly normal structure, within some of the corpora lutea there was evidence of serum/blood exudation into the stroma (arrow head), which was not present in sections obtained from wild-type animals (Figure 5.3A). The haematoxylin and eosin stained sections shown in Figure 5.3C and D, obtained from DT-treated wild-type and *Cd11b-Dtr* mice respectively, show a more detailed view of the cellular structure within the corpus luteum. It appears there is a network of nuclei that interdigitate between the luteal cells in the photomicrograph from the wild-type mouse (Figure 5.3C), yet in the image from the *Cd11b-Dtr* mouse, this network of cells seems to be largely absent, and likely accounts for the interstitial spaces that are present (Figure 5.3D; arrow); the luteal cells also appear distended when compared to those of the wild-type mouse.

To determine whether the reduced circulating plasma $P_4$ detected in macrophage-depleted *Cd11b-Dtr* mice (Section 5.2) was due to reduced corpora lutea number or altered corpora lutea function in these mice, sections were analysed and follicles and corpora lutea were counted from tissue obtained from day 4.5 pc mice. One haematoxylin stained tissue section was counted per animal (n = 6 wild-type; n =7 *Cd11b-Dtr;* Chapter 2.3.2.6). Follicles were classified as preantral, antral or atretic. Preantral included primordial, primary and secondary follicles that had no visible antrum. Antral follicles included early antral, antral and pre-ovulatory follicles. Corpora lutea were counted, including those that displayed a disorganised cellular structure and/or evidence of serum/blood exudates. The number of

corpora albicantia was also assessed. Figure 5.4 provides an example of how the follicles and corpora lutea were categorised in a wild-type mouse. Only the follicles with a clearly defined developmental stage and a visible oocyte were counted. Although there are more accurate ways of counting follicles, the primary purpose of this analysis was to look at corpora lutea number, and this method provided a reasonable assessment of corpora lutea numbers in pregnant, DT-treated wild-type and *Cd11b-Dtr* mice, and an insight into follicle numbers and stages of development. The data presented in Table 5.1 reveals that there was no significant difference between the number of corpora lutea following DT treatment of wild-type or *Cd11b-Dtr* mice (*P* = 0.47, Mann Whitney U test). However, when looking closer at the structure of the CL, macrophage-depleted mice consistently had corpora lutea exhibiting a disorganised structure, as illustrated in Figure 5.3. For the sections analysed from seven *Cd11b-Dtr* mice, there was a total of 50 corpora lutea, all of which exhibited structural abnormalities when compared to corpora lutea from wild-type mice. All of the corpora lutea from wild-type mice (total 38) appeared normal (P = 0.000, Unpaired t test).

**Table 5.2 Effect of macrophage depletion on ovarian follicle and corpora lutea number**

| | **Wild-type** | ***Cd11b-Dtr*** |
|---|---|---|
| **Preantral** | 5.3 ± 1.7 | 4.9 ± 1.6 |
| **Antral** | 1.2 ± 0.5 | 0.4 ± 0.2 |
| **Atretic** | 1.5 ± 0.6 | 1.1 ± 0.6 |
| **CL** | 6.3 ± 1.2 | 7.1 ± 0.6 |
| **Disorganised CL** | 0.0 ± 0.0 | 7.1 ± 0.6*** |
| **CA** | 0.2 ± 0.2 | 0.0 ± 0.0 |

Haematoxylin stained ovarian sections were obtained from day 4.5 pc wild-type and *Cd11b-Dtr* mice, following DT injection 24 h prior. Sections were analysed and follicle stages classified and counted for one section per mouse. Data are presented as mean ± SEM with statistical analysis using a Mann-Whitney U test. * indicates statistical significance between groups. ****P* < 0.001 CL; corpora lutea. CA; corpus albicans. n = 6 wild-type and n = 7 *Cd11b-Dtr*.

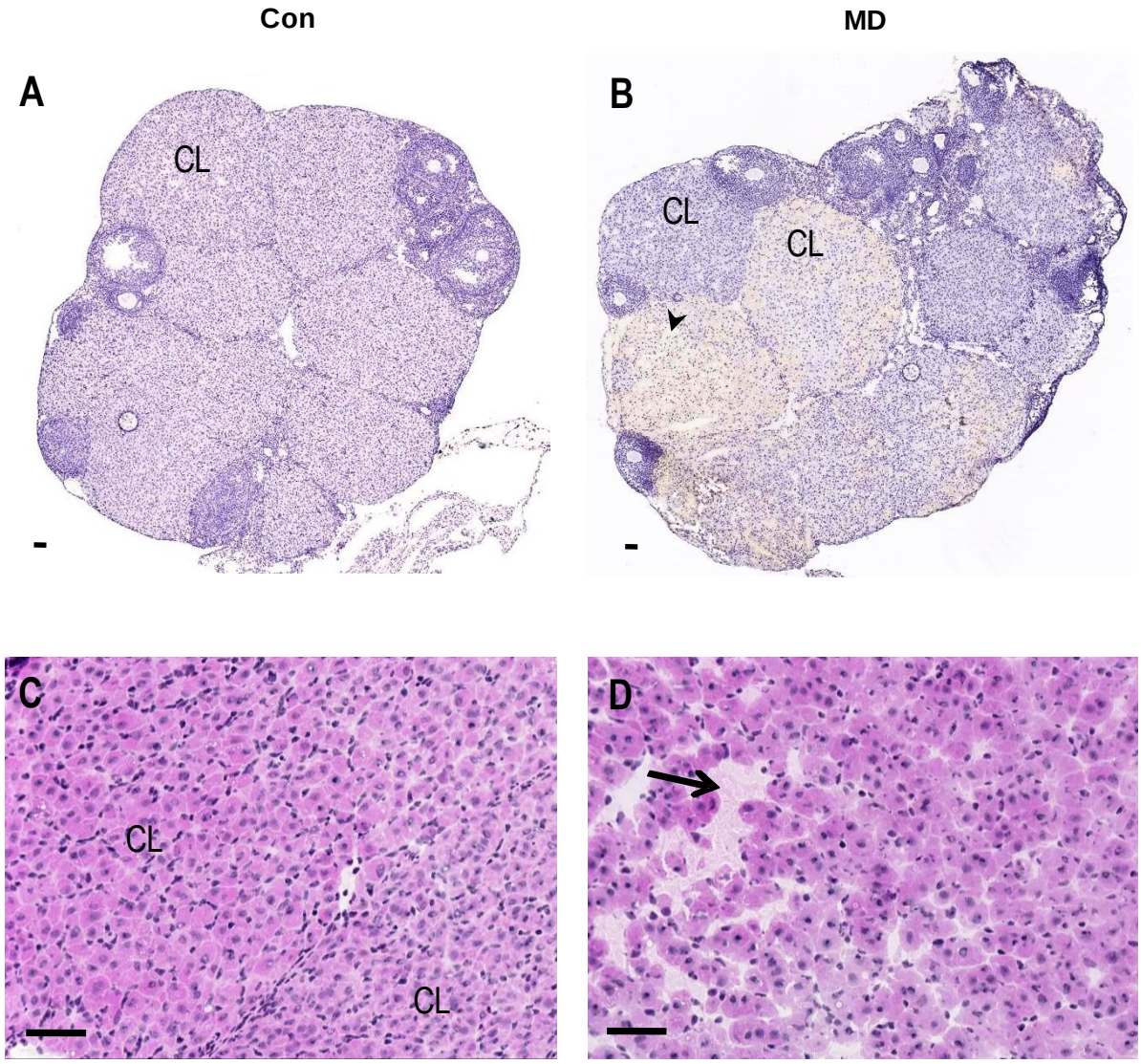



**Figure 5.3 Effect of macrophage depletion on the structural integrity of corpora lutea**

Ovaries were obtained from day 4.5 pc mice that received an i.p. injection of DT (25 ng/g) 24 h prior and frozen in OCT. Sections (6 µm) were stained with haematoxylin only (A and B; low power) or with haematoxylin and eosin (C and D; high power). Representative photomicrographs of tissue from DT-treated wild-type (A and C) and *Cd11b-Dtr* mice (B and D) are shown. Arrowhead; blood/serum exudate in CL. Arrow; abnormal interstitial spaces in CL. CL; corpus luteum. MD; macrophage-depleted. DT; diphtheria toxin. Scale bar = 50 µm.

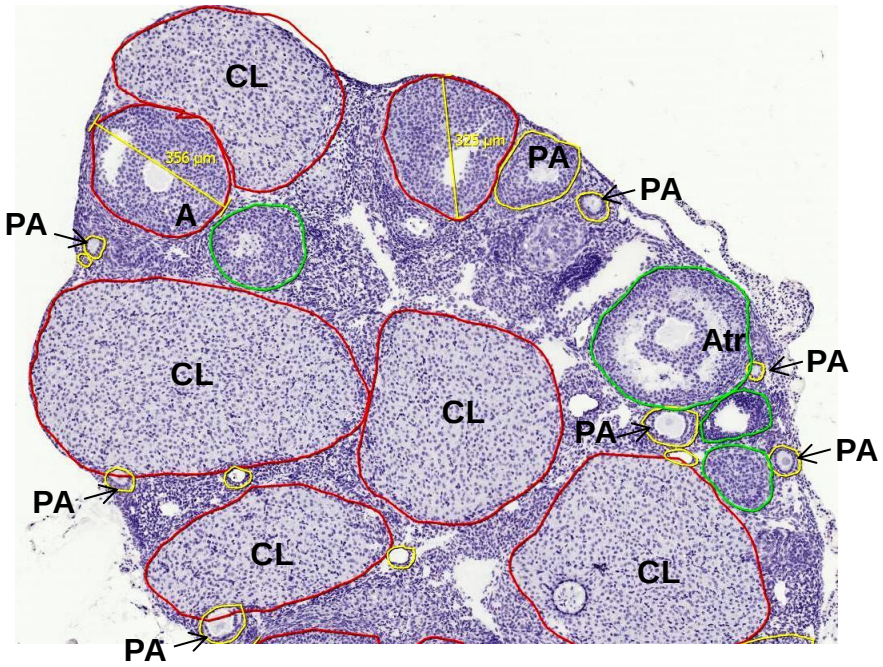



**Figure 5.4 Demonstration of follicle and corpora lutea classification in ovarian sections**

6 µm ovarian sections were prepared and stained with haematoxylin as described previously, from day 4.5 pc wild-type and *Cd11b-Dtr* mice that had received DT (25 ng/g) 24 h prior. Follicles were counted and classified as either preantral (PA), antral (A) or atretic (Atr). Corpora lutea (CL) and corpora albicantia (not shown in this figure) were counted. Only follicles in which the oocyte is visible in the section were included in the analysis. For this reason, in this example, only the follicles which are labelled were counted, although there are others visible. DT; diphtheria toxin.

## 5.5 EFFECT OF MACROPHAGE DEPLETION ON ENDOTHELIAL CELLS WITHIN THE CORPUS LUTEUM

There is a dense network of blood vessels within functioning corpora lutea that are essential for substrate supply and oxygen exchange for luteal cells. It is possible that macrophage depletion from *Cd11b-Dtr* mice damaged this fine blood vessel network and this could account for the abnormal interstitial spaces around the luteal cells, shown in Figure 5.3D. To assess the impact of macrophage depletion on the blood vessel network within corpora lutea, frozen tissue from day 4.5 pc wild-type and *Cd11b-Dtr* mice was stained with the endothelial cell specific antibody, MTS-12 (Section 2.3.2.1.1). The mice in this analysis had received DT to elicit macrophage depletion 24 h prior to tissue collection. Because of tissue haemorrhage in DT-treated *Cd11b-Dtr* mice (Figure 5.3B), whole corpora lutea were staining positive for DAB. To overcome this, ovarian tissue from DT-treated wild-type and *Cd11b-Dtr* mice was blocked for endogenous peroxidase activity prior to the application of the primary antibody (Section 2.3.2.1.1).

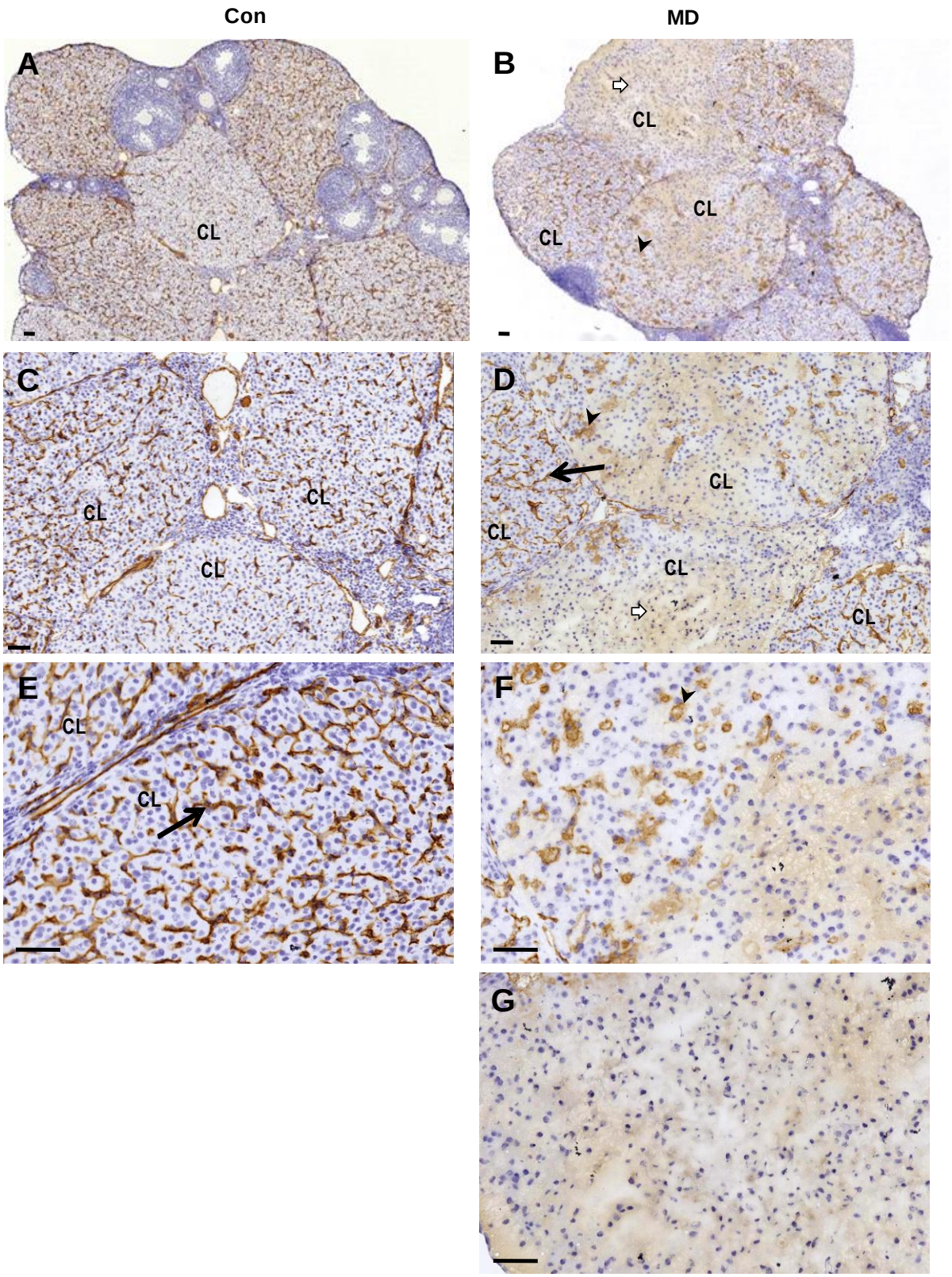
The photomicrographs in Figure 5.5 show the intricate network of blood vessels within day 4.5 pc corpora lutea, and within the ovarian stroma, from DT-treated wild-type (A, C and E) and *Cd11b-Dtr* mice (B, D, F and G). Images A and C show that the density of endothelial cells varies between corpora lutea within the same ovary, but there is a well developed lattice of blood vessels present by day 4.5 pc. In macrophage-depleted *Cd11b-Dtr* mice, while some corpora lutea have this lattice of endothelial cells, others appear to have none at all (G; high power), or patchy distribution of endothelial cells (F; high power). The same results were obtained for *Cd11b-Dtr* ovaries harvested on day 3.5 pc (24 h following DT treatment; data not shown).

Ovarian tissue sections were also stained with the endothelial cell marker PECAM1 in conjunction with EMR1 to show the spatial relationship between endothelial cells and macrophages within the corpus luteum of wild-type mice (Figure 5.6A and C) and how this relationship is affected following macrophage depletion in *Cd11b-Dtr* mice (Figure 5.6B and D) Also, due to the disruption of endothelial cell vasculature following macrophage depletion, sections were stained with the lymphatic endothelial cell marker LYVE1 (Lymphatic Vessel Endothelial Hyaluronan Receptor-1) to determine whether the lymphatic vasculature was affected in a similar manner following macrophage depletion. Lymphatic vasculature has been identified as an important means for $P_4$ efflux from the ovary during the luteal phase (Lindner et al., 1964, Staples et al., 1982, Hein et al., 1988), and in the mouse the lymphatic

vasculature surrounds the corpus luteum (Brown et al., 2010). Figure 5.6E and F illustrates the lymphatic vasculature in wild-type and macrophage depleted mice, respectively.

**Figure 5.5 Effect of macrophage depletion on blood vessel network within the corpus luteum**

Representative photomicrographs of sections that were stained with the endothelial cell specific antibody, MTS-12. Day 4.5 pc ovarian sections were prepared from wild-type (A, C and E; 5X, 10X and 20X magnification, respectively) and *Cd11b-Dtr* mice (B, D, F and G; 5X, 10X, 20X and 20X magnification, respectively) that had received DT (25 ng/g) 24 h prior. In macrophage-depleted *Cd11b-Dtr* mice, it is clear that some CL have a dense network of MTS-12 positive endothelial cells, while others have patchy coverage/a broken network of endothelial cells (F) or no endothelial cells at all (G). Arrows; MTS-12 positive endothelial cells. Arrow heads; patchy staining of endothelial cells. White arrows; CL with no endothelial cell positive staining. DT; diphtheria toxin. CL; corpus luteum. Scale bar 50 μm.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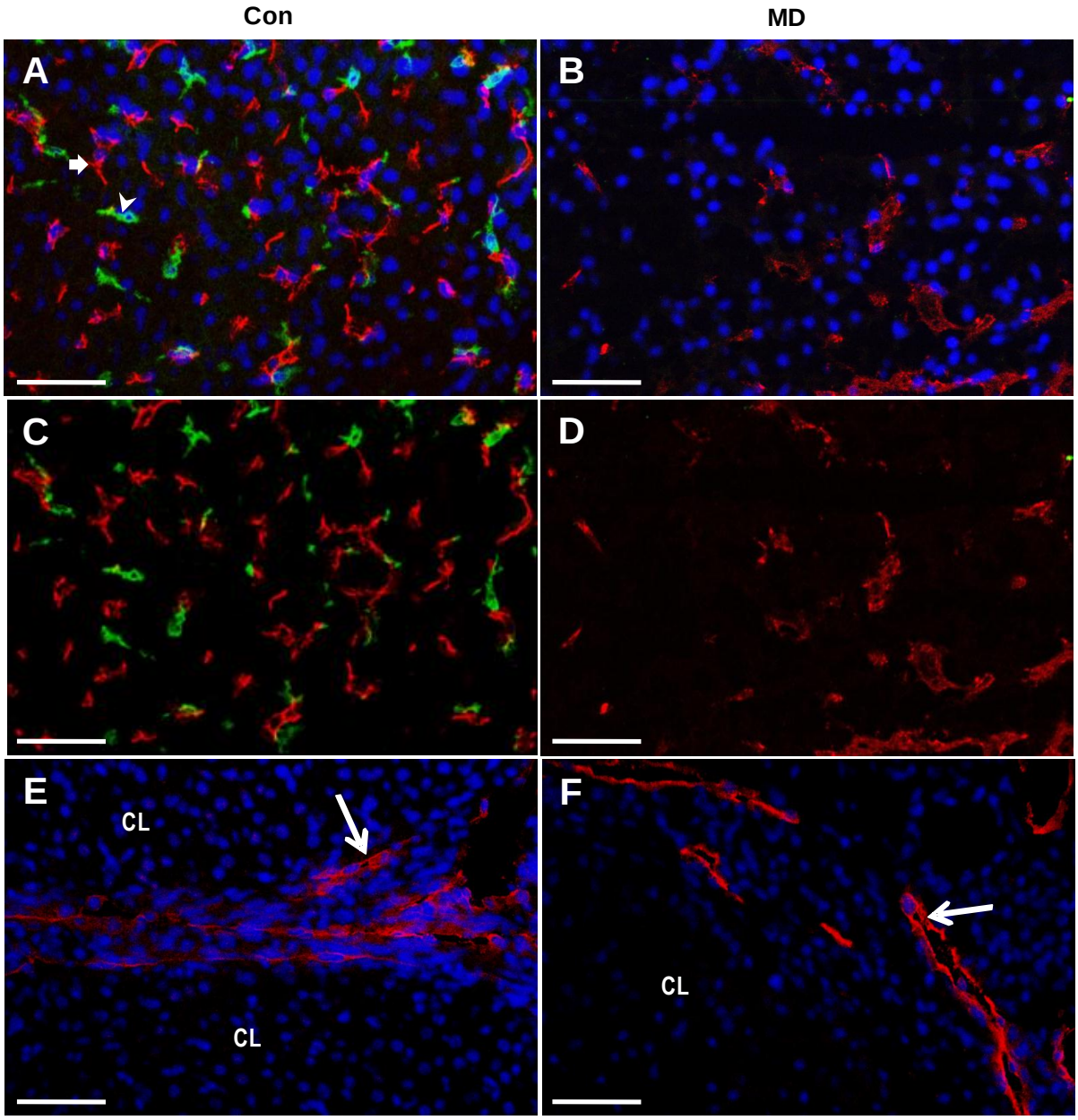



**Figure 5.6 Effect of macrophage depletion on macrophage and endothelial cell interactions within the corpus luteum and on ovarian lymphatic vasculature**

Representative photomicrographs of showing endothelial cell and macrophage interactions within corpora lutea (A-D). Tissue sections were stained with the endothelial cell specific antibody, PECAM1 (Red; fat arrow) in conjunction with EMR1 (Green; arrow head) with (A and B) or without (C and D) DAPI counterstain (blue). Day 4.5 pc ovarian tissue sections were prepared from wild-type (A and C) and *Cd11b-Dtr* mice 24 h following DT injection. In the same tissue, lymphatic vasculature was identified using the lymphatic marker LYVE1 (red; white arrow) in wild-type (E) and *Cd11b-Dtr* mice (F). DT; diphtheria toxin. CL; corpus luteum. Scale bar 50 µm.

## 5.6 EFFECT OF MACROPHAGE DEPLETION ON CORPORA LUTEA STEROIDOGENIC FUNCTION

Although the number or corpora lutea in the ovaries of macrophage-depleted *Cd11b-Dtr* mice are not significantly different to the number contained in wild-type ovaries, histology revealed alterations in the structural integrity, and compromised function is apparent due to decreased output of progesterone. To further assess the impact that macrophage depletion has had on corpora lutea function, the expression of genes involved in the synthesis of progesterone were assessed in mRNA extracted from whole ovaries. Genes analysed included *Star* (steroidogenic acute regulatory protein), which encodes the protein STAR, that transports cholesterol to the inner mitochondrial membrane; *Cyp11a1* (cytochrome P450, family 11, subfamily a, polypeptide 1), which encodes the protein CYP11A1, responsible for the conversion of cholesterol to pregnenolone; and *Hsd3b1* (hydroxyl-delta-5-steroid dehydrogenase, 3 beta- and steroid isomerase 1), which encodes the protein HSD3B1, which converts pregnenolone to progesterone (See flow diagram in Figure 5.6A).

Briefly, ovaries were collected and snap frozen in liquid nitrogen from wild-type and *Cd11b-Dtr* mice at the following time-points:

- Day 4.0 pc, 8 h following DT (DT injected day 3.5 pc)
- Day 4.5 pc, 24 h following DT (DT and sesame seed oil (vehicle) injected day 3.5 pc)
- Day 4.5 pc, 24 h following DT (DT and $P_4$ injected day 3.5 pc)
- Day 7.5 pc (DT injected day 3.5 pc)

Tissue collection and qRT-PCR was performed as described in section 2.6.1 and 2.6.4. Sequence specific primers for *Star*, *Cyp11a1* and *Hsd3b1* were utilised to identify changes in the resultant cDNA between treatment groups at each of these time-points. Expression of each of these genes was normalised to the house-keeping gene *Actb* for all time-points assessed. Expression of these genes was also normalised to 18S ribosomal RNA (rRNA) for the day 4.5 pc time-point only (24 h following DT), where the 18S primers were mixed with competimers in a previously determined optimal ratio (see section 2.6.4). However, for the data in this chapter, *Actb* was selected as the preferred housekeeper gene. This is because when the individual Ct was plotted for wild-type and *Cd11b-Dtr* mice, there was more spread in the data points for the 18S housekeeper (day 4.5 pc time-point: *Actb*; wild-type Ct (mean ± SEM) = 14.7 ± 0.1 and *Cd11b-Dtr* = 14.3 ± 0.1. 18S; wild-type = 15.9 ± 0.4 and *Cd11b-Dtr* = 15.9 ± 0.3). Further, when the data was normalised to the 18S, this resulted in more outlying datum points that required removal from the analysis than was seen when data was normalised to *Actb*.

Results obtained from normalisation to 18S rRNA are presented in Table 1 in Appendix 1, along with the results obtained from *Actb* normalisation for comparison.

*Star* mRNA was detectable in all samples analysed. There was no significant difference in *Star* mRNA expression between control and macrophage-depleted mice at any of the time-points assessed, nor was there any difference in *Star* mRNA expression between the different treatment groups (Figure 5.7B).

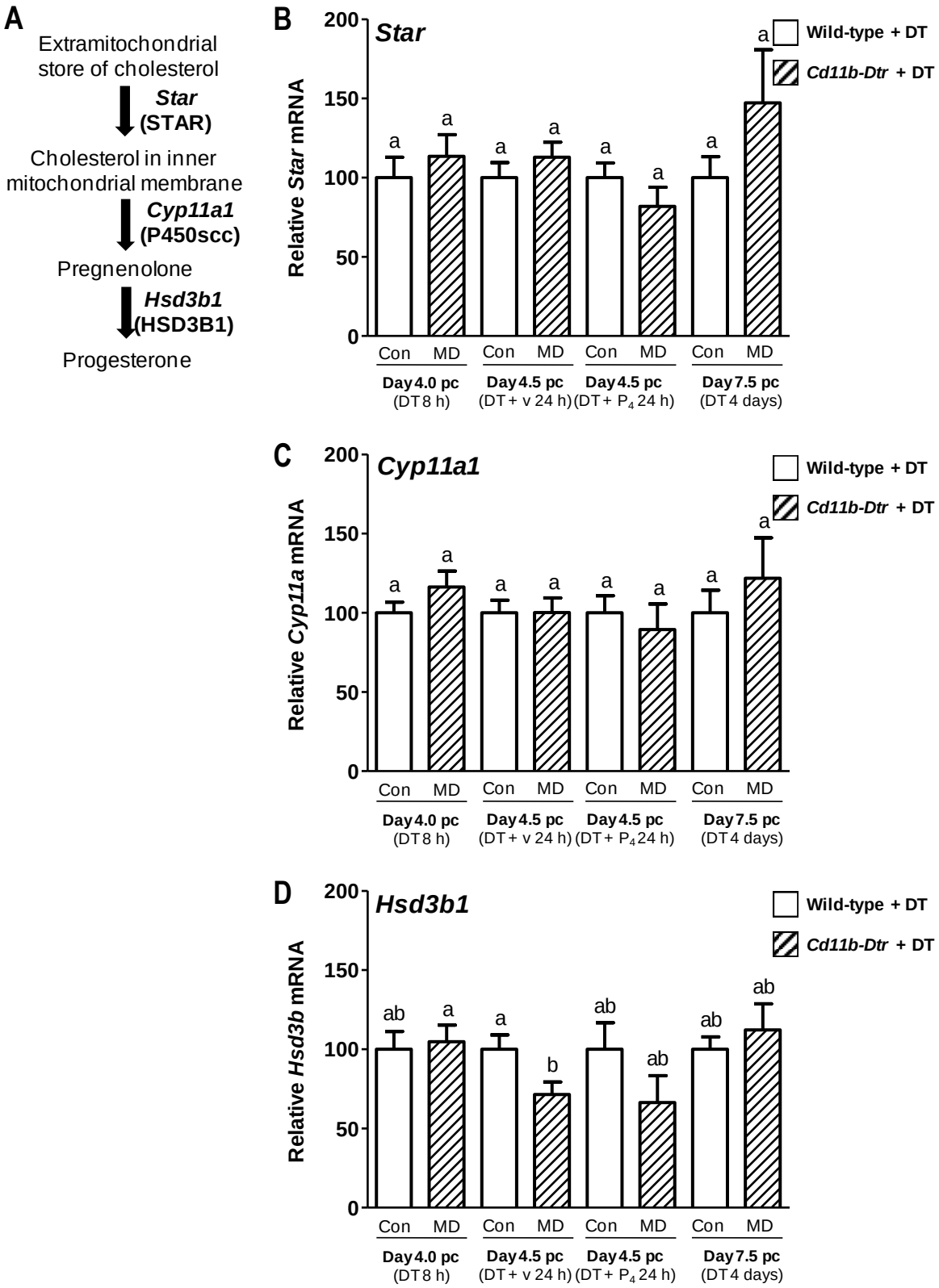
*Cyp11a* mRNA was detectable in all samples analysed. Again, there was no significant difference in *Cyp11a* mRNA expression between control and macrophage-depleted mice at any of the time-points, and no difference between different treatment groups (Figure 5.7C).

*Hsd3b* mRNA was detectable in all samples analysed. There was a significant 28.6% decrease in *Hsd3b* mRNA expression in macrophage-depleted *Cd11b-Dtr* mice on day 4.5 pc, compared to DT-treated wild-type control mice ($P$ = 0.03, Unpaired t test), where animals were treated with DT for 24 h prior to sacrifice. This was also significantly less than the *Hsd3b* mRNA expression detected in ovaries *Cd11b-Dtr* mice on day 4.0 pc, following 8 h of DT treatment ($P$ = 0.02, respectively, Unpaired t test). There was a trend toward a significant decrease when comparing the expression of *Hsd3b* mRNA in ovaries from wild-type mice on day 4.0 pc (following 8 h of DT treatment) with expression in ovaries from *Cd11b-Dtr* mice on day 4.5 pc (following 24 h of DT treatment ($P$ = 0.055, Unpaired t test; Figure 5.7D)).

Tissue was collected on day 4.5 pc from wild-type and *Cd11b-Dtr* mice that had received an injection of DT and $P_4$ on day 3.5 pc. It was not anticipated that gene expression would vary in wild-type mice supplemented with $P_4$ compared to wild-type mice receiving vehicle injections. The effect of exogenous $P_4$ administration to macrophage-depleted *Cd11b-Dtr* mice was analysed to investigate whether there was any negative feedback regulation of genes involved in the synthesis of $P_4$ following exogenous supplementation of this hormone. However, there was no significant difference in the expression of *Star*, *Cyp11a1* or *Hsd3b1* (normalised to *Actb*) between wild-type and *Cd11b-Dtr* mice supplemented with vehicle or $P_4$, or compared to other treatment groups (Figure 5.7). The significant downregulation of *Hsd3b1* seen between wild-type and *Cd11b-Dtr* mice treated with DT and vehicle was not seen between wild-type and *Cd11b-Dtr* mice treated with DT and $P_4$, although the data appeared to show a similar trend ($P$ = 0.08, Unpaired t test; Figure 5.7C).

**Figure 5.7 Effect of macrophage depletion on expression of steroidogenic enzymes involved in progesterone production within the corpus luteum**

Genes that encode various enzymes involved in the production of ovarian $P_4$ (*Star, Cyp11a, Hsd3b1*; A). Expression of these enzymes was analysed in DT-treated wild-type and *Cd11b-Dtr* mice on day 4.0 pc following 8 h of DT treatment; on day 4.5 pc, following 24 h of DT and sesame seed oil vehicle treatment; on day 4.5 pc, 24 following DT and $P_4$ (2 mg) treatment, and on day 7.5 pc following DT injection on day 3.5 pc. Expression of *Star* (B), *Cyp11a1* (C) and *Hsd3b1* (D), was quantified using quantitative real-time PCR following RNA extraction and cDNA preparation from excised ovaries. Data are normalised to *Actb* expression and are presented as the mean ± SEM, with statistical analysis using an Unpaired t test. Different letters indicate statistical significance between groups, where n = 7-8 mice per group. MD; macrophage-depleted. DT; diphtheria toxin. V; sesame seed oil vehicle.


A
Extramitochondrial store of cholesterol
Star (STAR)
Cholesterol in inner mitochondrial membrane
Cyp11a1 (P450scc)
Pregnenolone
Hsd3b1 (HSD3B1)
Progesterone
B
Star
Relative Star mRNA
Wild-type + DT
Cd11b-Dtr + DT
C
Cyp11a1
Relative Cyp11a mRNA
D
Hsd3b1
Relative Hsd3b mRNA
Con MD
Day 4.0 pc (DT 8 h)
Day 4.5 pc (DT + v 24 h)
Day 4.5 pc (DT + P4 24 h)
Day 7.5 pc (DT 4 days)

## 5.7 EFFECT OF MACROPHAGE DEPLETION ON OTHER GENES INVOLVED IN FUCTIONING CORPORA LUTEA

Because macrophage depletion appeared to have a limited impact on the expression of genes involved in the synthesis of $P_4$, further analysis was done on other genes expressed in the ovary that might give some insight into how macrophage depletion was affecting the ovary's functional capacity. For this set of experiments, only tissue from the day 4.5 pc time-point was analysed, 24 h following DT injection to wild-type or *Cd11b-Dtr mice*. Gene expression was normalised to *Actb* and 18S. Again, results referred to in this chapter are those obtained after normalisation to *Actb*. Results obtained from normalisation to 18S rRNA are presented alongside *Actb* data in Appendix 1. Genes analysed were *Cyp17a1* (cytochrome P450, family 17, subfamily a, polypeptide 1); the protein CYP17A1 is involved in the conversion of progesterone to androstendione, and the conversion of pregnenolone to DHEA dihydroepiandrosterone (DHEA). *Emr1* was also analysed; it encodes the protein EMR1 (F4/80), expressed on the cell surface of macrophages. Due to the impact of macrophage depletion on the vascular network within the corpora lutea, expression of genes involved in endothelial cell angiogenesis and survival were analysed. These included *Pecam1* (Platelet/endothelial cell adhesion molecule 1), and the vascular endothelial growth factors, *Vegfa, Vegfb* and *Vegfc*, and *Figf* (c-fos induced growth factor; also known as *Vegfd)*. Expression of *Flt1* (FMS-like tyrosine kinase, or Vegf receptor 1) and *Kdr* (kinase insert domain protein receptor, or Vegf receptor 2) was also analysed.

*Cyp17a1* mRNA was detectable in all samples (Figure 5.8A). There was no significant difference in the expression of *Cyp17a1* between DT-treated wild-type and *Cd11b-Dtr* mice when normalised to *Actb* ($P = 0.12$, Unpaired t test).

*Emr1* mRNA was detectable in all samples analysed (Figure 5.8B). There was a 56.8% decrease in *Emr1* mRNA expression in DT-treated *Cd11b-Dtr* mice compared to DT-treated wild-type mice ($P = 0.0001$, Unpaired t test).

*Pecam1* mRNA was detectable in all samples (Figure 5.8C), and there was no significant difference in the expression of this gene following macrophage depletion in *Cd11b-Dtr* mice when data was normalised to *Actb* ($P = 0.68$, Unpaired t test).

*Vegfa* mRNA was detectable in all samples analysed (Figure 5.8D). Ovarian expression of *Vegfa* was increased following macrophage depletion (3.2-fold increase in DT-treated *Cd11b-Dtr* mice compared

to DT-treated wild-type mice, *P* = 0.003, Unpaired t test). The result was similar when normalised to 18S rRNA with a 1.7-fold increase in *Vegfa* mRNA expression in DT treated *Cd11b-Dtr* mice compared to wild-type controls, although this result did not reach significance (*P* = 0.12, Mann Whitney U test).

*Vegfb* mRNA was detectable in all samples (Figure 5.8E). There was no difference in the expression of *Vegfb* mRNA between DT-treated wild-type and *Cd11b-Dtr* mice when normalised to *Actb* (*P* = 0.94, Unpaired t test).

Vegfc mRNA was detectable in all samples analysed (Figure 5.8F). There was no significant difference in the expression of *Vegfc* mRNA between DT treated wild-type and *Cd11b-Dtr* mice when normalised to *Actb* (*P* = 0.13, Unpaired t test).

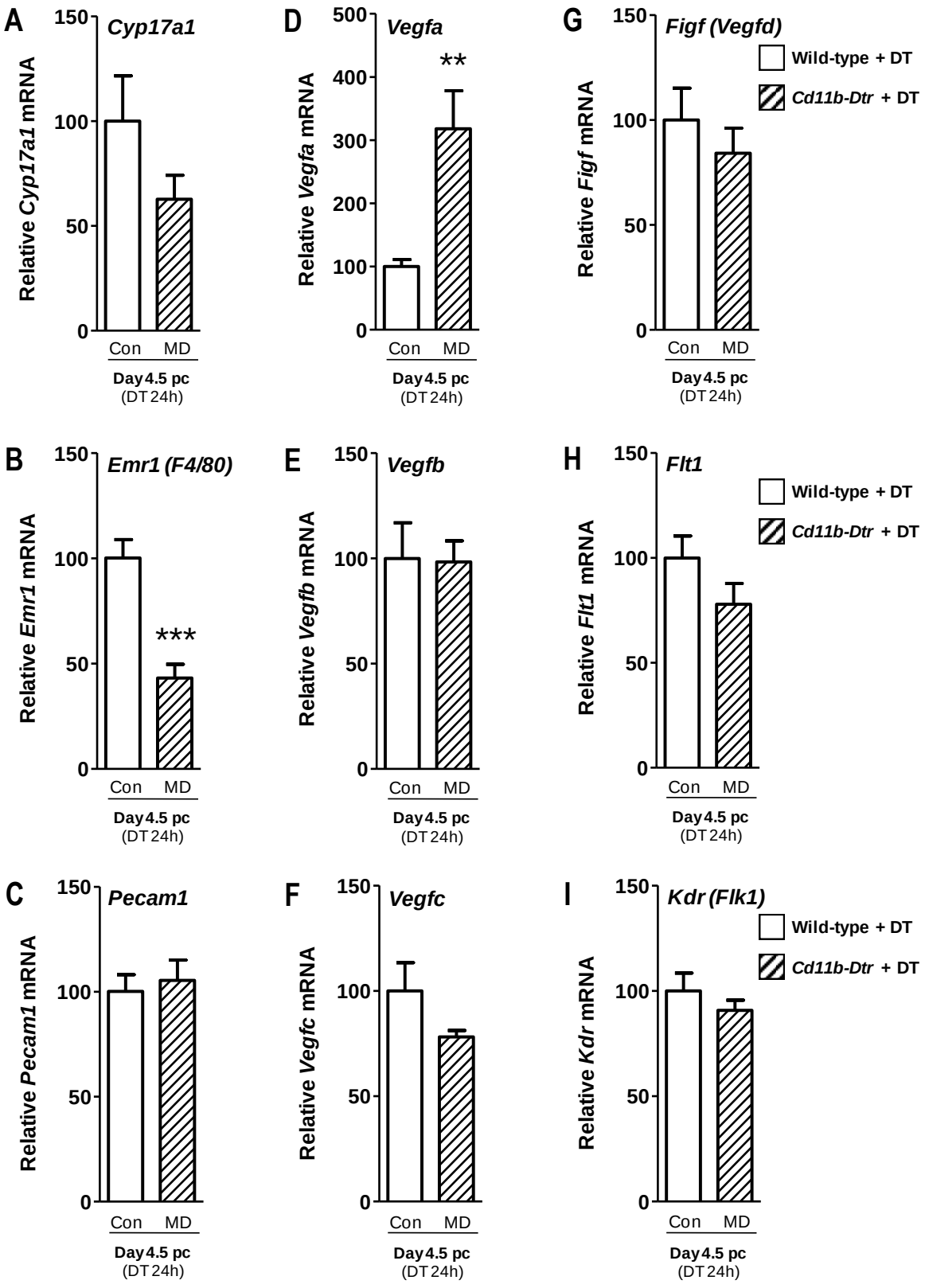
*Figf (Vegfd)* mRNA was detectable in all samples (Figure 5.8F). There was no difference in expression of this gene between DT-treated wild-type and *Cd11b-Dtr* mice when data was normalised to *Actb* (*P* = 0.42, Unpaired t test).

*Flt1* mRNA was detectable in all samples analysed (Figure 5.8G). When data were normalised to *Actb*, there was no significant difference in the expression of this gene between DT-treated wild-type and *Cd11b-Dtr* mice (*P* = 0.15, Unpaired t test).

*Kdr* mRNA was also detectable in all samples analysed (Figure 5.8H). There was no difference in the expression of this gene between DT-treated wild-type and *Cd11b-Dtr* mice when data was normalised to *Actb* (*P* = 0.38, Unpaired t test).

**Figure 5.8 Effect of macrophage depletion on other parameters regulating corpora lutea function**

Whole ovaries were obtained from day 4.5 pc wild-type and *Cd11b-Dtr* mice following an i.p. injection of DT (25 ng/g) 24 h prior. Following RNA extraction and cDNA preparation, the expression of various genes was examined to determine how they were affected by macrophage depletion. The genes included *Cyp17a1* (A) and *Emr1* (F4/80; B), as well as genes involved in endothelial cell function, angiogenesis and survival (*Pecam1*, C; *Vegfa*, D; *Vegfb*, E; *Vegfc*, F; *Figf (Vegfd)*, G; *Flt1*, H and *Kdr (Flk1)*, I). Data are normalised to *Actb* expression and are presented as the mean ± SEM, with statistical analysis using an Unpaired t test. * indicates statistical significance from control; ** $P < 0.01$. *** $P < 0.001$. n = 7-8 mice per group. MD; macrophage-depleted. DT; diphtheria toxin.


A
Cyp17a1
Relative Cyp17a1 mRNA
150
100
50
0
Con
MD
Day 4.5 pc
(DT 24h)
D
Vegfa
**
Relative Vegfa mRNA
500
400
300
200
100
0
G
Figf (Vegfd)
Relative Figf mRNA
Wild-type + DT
Cd11b-Dtr + DT
B
Emr1 (F4/80)
***
Relative Emr1 mRNA
E
Vegfb
Relative Vegfb mRNA
H
Flt1
Relative Flt1 mRNA
C
Pecam1
Relative Pecam1 mRNA
F
Vegfc
Relative Vegfc mRNA
I
Kdr (Flk1)
Relative Kdr mRNA

## 5.8 EFFECT OF MACROPHAGE DEPLETION ON ANGIOGENIC PARAMETERS IN THE NEWLY FORMED CORPUS LUTEUM

The results in the previous section indicated that macrophage depletion may be perturbing angiogenesis, causing a significant upregulation of *Vegfa* mRNA expression. To further investigate the impact of macrophage depletion on angiogenic parameters, an earlier time-point in corpora lutea formation was examined. This is because angiogenesis in the ovary occurs rapidly after ovulation, and at the time-point assessed in the previous section (day 4.5 pc) the corpus luteum is already functioning at its full potential. Therefore, we chose to analyse day 1.5 and day 2.5 pc, a time where the fine capillary network within corpora lutea would still be under construction, and thus a time where macrophage regulation of endothelial cell parameters can be better assessed. Briefly, wild-type and *Cd11b-Dtr* mice were mated with males of the same genotype; the presence of a vaginal plug was designated day 0.5 pc. Mice received an i.p injection of DT (25 ng/g) on either day 0.5 or day 1.5 pc to elicit macrophage depletion, and 24 h following this injection (day 1.5 or day 2.5 pc, respectively) mice were sacrificed, and one ovary was snap frozen in liquid nitrogen for RNA extraction at a later time. The other ovary was frozen in OCT embedding medium. Again, due to less spread in the data, the data shown in the chapter is that obtained after normalisation to *Actb* (Day 1.5 pc time-point: *Actb*; wild-type Ct (mean ± SEM) = 14.2 ± 0.04 and *Cd11b-Dtr* = 14.1 ± 0.06. 18S; wild-type = 14.0 ± 0.07 and *Cd11b-Dtr* = 14.2 ± 0.1. Day 2.5 pc time-point: *Actb*; wild-type Ct = 14.4 ± 0.04 and *Cd11b-Dtr* = 14.2 ± 0.09. 18S; wild-type = 14.2 ± 0.08 and *Cd11b-Dtr* = 14.5 ± 0.2). In addition, there were more outliers after normalisation to 18S that required removal from the data set. The results obtained from normalisation to 18S are presented alongside the results obtained from *Actb* normalisation in Appendix 1.

The expression of mRNA encoded by the *Vegf* genes (*Vegfa, Vegfb, Vegfc* and *Figf (Vegfd))* and their receptors (*Flt1 and Kdr*) were quantified using qRT-PCR, as described previously (Section 2.6.4). Very similar results were obtained for both time-points analysed.

*Vegfa* mRNA was detectable in all samples analysed. On day 1.5 pc, there was a 3.6-fold increase in *Vegfa* mRNA expression in DT-treated *Cd11b-Dtr* mice compared to DT-treated wild-type animals, when the data was normalised to *Actb* (*P* = 0.0007 Mann Whitney U test; Figure 5.9A). On day 2.5 pc there was a 3.7-fold increase in *Vegfa* mRNA expression in DT-treated *Cd11b-Dtr*, compared to DT-treated wild-type mice when the data was normalised to *Actb* (*P* = 0.004, Unpaired t test; Figure 5.10A).

*Vegfb* mRNA was detectable in all samples analysed. There was no significant difference in the expression of *Vegfb* mRNA normalised to *Actb* between DT-treated wild-type and *Cd11b-Dtr* mice on either day 1.5 pc ($P$ = 0.66, Mann Whitney U test; Figure 5.9B) or day 2.5 pc ($P$ = 0.62, Unpaired t test; Figure 5.10B).

*Vegfc* mRNA was detectable in all samples analysed. On day 1.5 pc, there was a decrease in *Vegfc* mRNA expression in DT-treated *Cd11b-Dtr* mice compared to DT-treated wild-type mice, when normalised to *Actb* (41.2% decrease, $P$ = 0.008, Mann Whitney U test; Figure 5.9C). On day 2.5 pc there was a 35.5% decrease in *Vegfc* mRNA expression in DT-treated *Cd11b-Dtr* mice when data was normalised to *Actb* ($P$ = 0.01, Unpaired t test; Figure 5.10C).

*Figf (Vegfd)* mRNA was detectable in all samples analysed. On day 1.5 pc there was a 30.2% decrease in *Figf* mRNA expression in DT-treated *Cd11b-Dtr* mice following normalisation to *Actb* ($P$ = 0.03, Mann Whitney U test; Figure 5.9D. On day 2.5 pc, when data was normalised to *Actb*, there was a 28.6% decrease in *Figf* mRNA expression in DT-treated *Cd11b-Dtr* mice compared to DT-treated wild-type mice ($P$ = 0.03, Unpaired t test; Figure 5.10D).

*Flt1* mRNA was detectable in all samples. On day 1.5 pc, for data normalised to *Actb*, there was a 45.7% decrease in *Flt1* mRNA expression in *Cd11b-Dtr* mice following DT treatment, compared to DT-treated wild-type control mice ($P$ = 0.008, Mann Whitney U test; Figure 5.9E). On day 2.5 pc, there was a 53.4% decrease in *Flt1* mRNA expression in DT-treated *Cd11b-Dtr* mice compared to DT-treated wild-type mice (data normalised to *Actb*, $P$ = 0.0006, Unpaired t test; Figure 5.10E).

*Kdr (Flk1)* mRNA was also detectable in all samples analysed. On day 1.5 pc, there was a 32.4% decrease in expression in DT-treated *Cd11b-Dtr* mice, compared to DT-treated wild-type mice when data was normalised to *Actb* ($P$ = 0.03, Mann Whitney U test; Figure 5.9F). On day 2.5 pc, *Kdr* mRNA expression was decreased by 33.3% in DT-treated *Cd11b-Dtr* mice compared to DT-treated wild-type mice (normalised to *Actb*; $P$ = 0.01, Unpaired t test; Figure 5.10F).

**Figure 5.9 Effect of macrophage depletion on genes involved in angiogenesis in the day 1.5 pc corpus luteum**

Whole ovaries were obtained from day 1.5 pc wild-type and *Cd11b-Dtr* mice following an i.p. injection of DT (25 ng/g) 24 h prior. Following RNA extraction and cDNA preparation, the expression of various genes involved in angiogenesis and lymphangiogenesis were examined to determine how they were affected by macrophage depletion. The genes included *Vegfa*, A; *Vegfb*, B; *Vegfc*, C; *Figf (Vegfd)*, D; *Flt1*, E and *Kdr (Flk1)*, F. Data are normalised to *Actb* expression and are presented as the mean ± SEM, with statistical analysis using an Unpaired t test. * indicates statistical significance from control; **P* < 0.05. ***P* < 0.01. ****P* < 0.001. n = 6 wild-type, n = 8 *Cd11b-Dtr*. MD; macrophage-depleted. DT; diphtheria toxin.

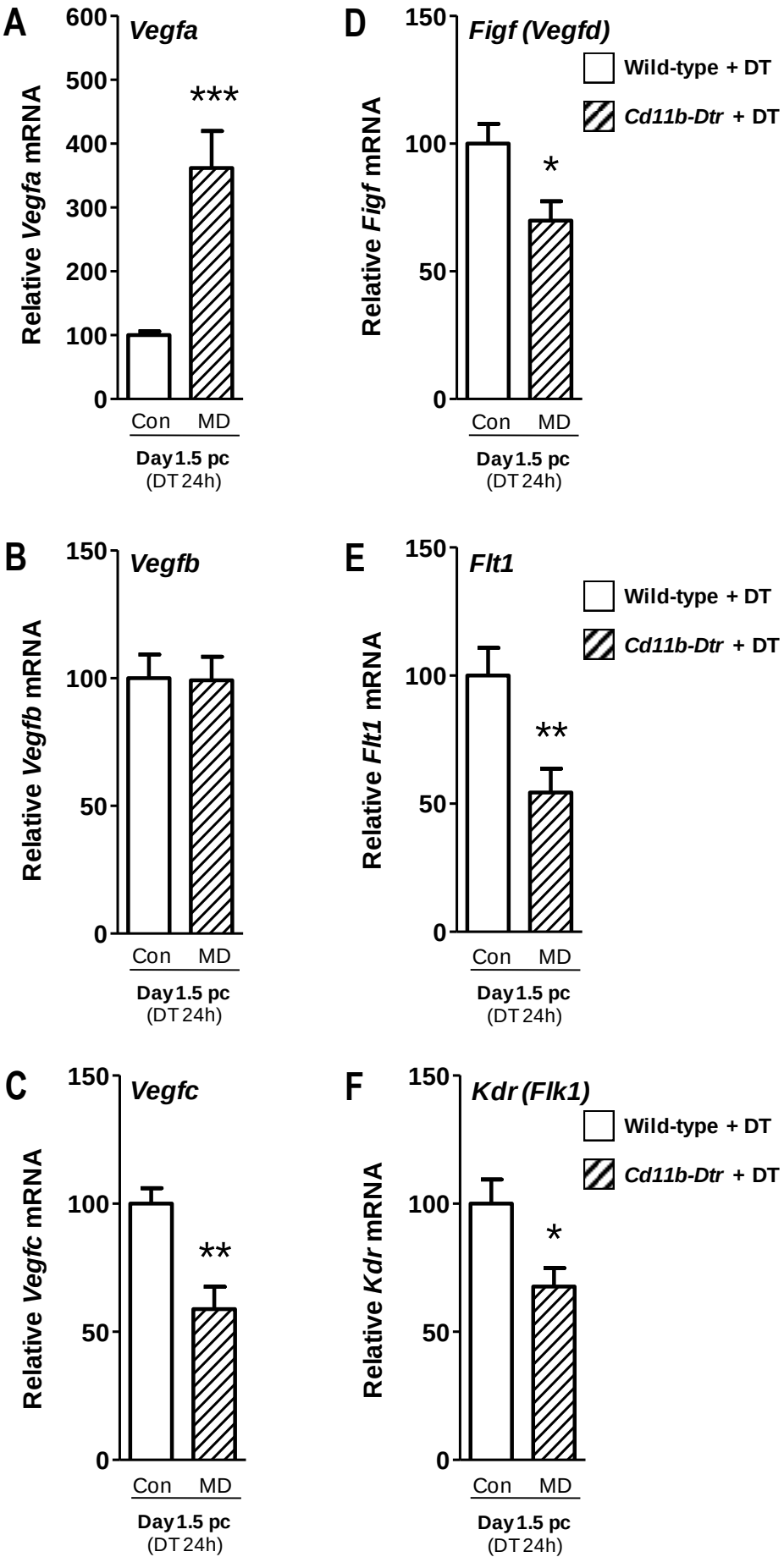
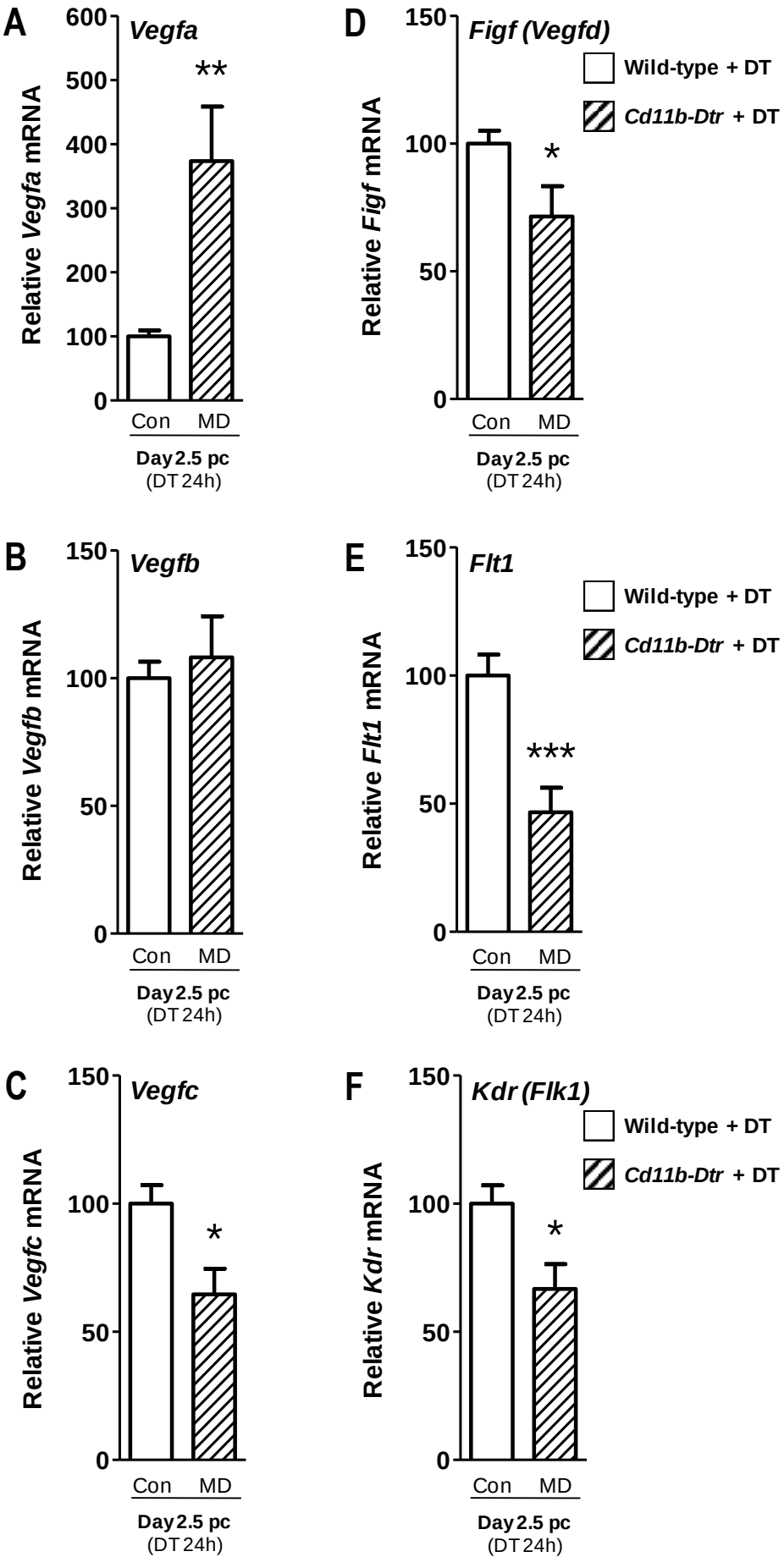

A
Vegfa
Relative Vegfa mRNA
600
500
400
300
200
100
0
***
Con
MD
Day 1.5 pc
(DT 24h)
B
Vegfb
Relative Vegfb mRNA
150
100
50
0
Con
MD
Day 1.5 pc
(DT 24h)
C
Vegfc
Relative Vegfc mRNA
150
100
50
0
**
Con
MD
Day 1.5 pc
(DT 24h)
D
Figf (Vegfd)
Relative Figf mRNA
150
100
50
0
*
Con
MD
Day 1.5 pc
(DT 24h)
Wild-type + DT
Cd11b-Dtr + DT
E
Flt1
Relative Flt1 mRNA
150
100
50
0
**
Con
MD
Day 1.5 pc
(DT 24h)
Wild-type + DT
Cd11b-Dtr + DT
F
Kdr (Flk1)
Relative Kdr mRNA
150
100
50
0
*
Con
MD
Day 1.5 pc
(DT 24h)
Wild-type + DT
Cd11b-Dtr + DT

**Figure 5.10 Effect of macrophage depletion on genes involved in angiogenesis in the day 2.5 pc corpus luteum**

Whole ovaries were obtained from day 2.5 pc wild-type and *Cd11b-Dtr* mice following an i.p. injection of DT (25 ng/g) 24 h prior. Following RNA extraction and cDNA preparation, the expression of various genes involved in angiogenesis and lymphangiogenesis were examined to determine how they were affected by macrophage depletion. The genes included *Vegfa*, A; *Vegfb*, B; *Vegfc*, C; *Figf (Vegfd)*, D; *Flt1*, E and *Kdr (Flk1)*, F. Data are normalised to *Actb* expression and are presented as the mean ± SEM, with statistical analysis using an Unpaired t test. * indicates statistical significance between groups; **P* < 0.05. ***P* < 0.01. ****P* < 0.001. n = 10 wild-type, n = 8 *Cd11b-Dtr*. MD; macrophage-depleted. DT; diphtheria toxin.


A
Vegfa
Relative Vegfa mRNA
600
500
400
300
200
100
0
**
Con
MD
Day 2.5 pc
(DT 24h)
D
Figf (Vegfd)
Relative Figf mRNA
150
100
50
0
*
Wild-type + DT
Cd11b-Dtr + DT
B
Vegfb
Relative Vegfb mRNA
E
Flt1
Relative Flt1 mRNA
***
C
Vegfc
Relative Vegfc mRNA
F
Kdr (Flk1)
Relative Kdr mRNA

## 5.9 DISCUSSION

### 5.9.1 Macrophage depletion perturbs corpora lutea structure and function

It is well established that the corpus luteum is a transient reproductive gland that produces $P_4$, which is essential for the establishment and maintenance of pregnancy (Davis and Rueda, 2002). The process of luteolysis can be subdivided into two categories; functional luteolysis and structural luteolysis. Functional luteolysis is defined as a decline in progesterone synthesis, and structural luteolysis as the structural involution of the corpus luteum (Davis and Rueda, 2002). The results in this chapter reveal the cause of pregnancy loss after macrophage depletion from *Cd11b-Dtr* mice is due to both functional and structural luteolysis.

Analysis of serum $P_4$ levels showed a significant reduction in the level of circulating plasma $P_4$ 24 h DT-elicited macrophage depletion in *Cd11b-Dtr* mice, with a 62.5% decrease on day 3.5 pc, and a 77.6% decrease on day 4.5 pc. Histology of ovaries following macrophage depletion also revealed that the structural integrity of corpora lutea had been compromised. There were abnormal interstitial spaces observed throughout the corpora lutea of macrophage-depleted *Cd11b-Dtr* ovaries on day 4.5 pc. Although it appears that the luteal cells may be still in contact with one another, the network of blood vessels that would normally surround them was absent, and this is likely to be the cause of these unusual spaces. The fact that the luteal cells contact one another suggests that the integrity of the gap junctions that exist between these cells is not yet compromised (Albertini and Anderson, 1975). Although difficult to discern at the magnification shown, it is likely that the nuclei from some of the luteal cells from the *Cd11b-Dtr* mouse are undergoing the process of pyknosis, as there is some evidence of nuclear shrinkage. This would likely be followed by karyorrhexis and necrosis. Ovarian tissue sections from day 4.5 pc DT-treated wild-type and *Cd11b-Dtr* mice stained with TUNEL (TdT-mediated dUTP-X nick end labelling) to identify apoptosis (DNA strand breaks). However, at this time-point, there were no apoptotic cells identified within corpora lutea, and it is likely that they have progressed to secondary necrosis. The only apoptotic cells identified were within atretic follicles. An earlier time-point would need to be assessed to identify cell apoptosis, and this could possibly be done with anti-EMR1 and anti-PECAM1 antibodies to determine the identity of the apoptotic cells.

There have been a number of *in vitro* studies implicating macrophages in $P_4$ production within the ovary (Kirsch et al., 1981, Halme et al., 1985). *Ex-vivo* analysis of ovaries from *Csf2*$^{-/-}$ mice indicates this cytokine may be involved in $P_4$ production, as levels of this hormone are reduced in null mutant

mice (Jasper et al., 2000). *In vivo* studies in *Csf1$^{op}$/Csf1$^{op}$* mice also suggest a role for macrophages in $P_4$ production, owing to the initially low levels of $P_4$ in early pregnancy, but recovery to normal levels as more macrophages populate the corpora lutea (Cohen et al., 1997). Unlike the studies in cytokine deficient mice, where the absence of the cytokine consistently impacts availability and/or function of cells of the myeloid lineage, the experiments presented in this chapter show that acute macrophage depletion causes decreased $P_4$ production and associated pregnancy loss. Further, the *Cd11b-Dtr* mice are not plagued with problems with feedback regulation of the hypothalamic-pituitary axis evident in *Csf1$^{op}$/Csf1$^{op}$* (Cohen et al., 1999). For these reasons, the studies presented in this chapter provide compelling evidence of an essential role of macrophages for progesterone production by the corpus luteum during pregnancy. However, there are other possibilities that need to be explored. It is well established that in most mammals including the mouse (with the exception of higher primates), that luteolysis is induced by the production of the luteolytic factor PGF2A, which is secreted from epithelial and glandular cells in the endometrium. To reach the ipsilateral ovary, it first passes to the uterine vein, and then up the ovarian artery via a local countercurrent (Johnson and Everitt, 2007a). PGF2A is known to stimulate activity of the enzyme, 20α-HSD, which is involved in the catabolism of $P_4$, and analysis of the levels of both of these factors would offer insight into the mechanism causing reduced plasma $P_4$ levels in macrophage-depleted mice (Albarracin et al., 1994, Bachelot et al., 2009, Leung and Adashi, 2004). Given that macrophages are also depleted from the endometrium, it would be interesting to compare the levels of PGF2A in DT-treated *Cd11b-Dtr* mice with those seen in DT-treated wild type mice. This would determine whether macrophage depletion from the endometrium caused the premature release of PGF2A, leading to the demise of the corpus luteum. The simplest way of measuring an effect of PG production would be to look at the mRNA expression of *Ptgst* (prostaglandin-endoperoxide synthase 2; COX2) which is involved in the biosynthesis of prostaglandins. However, even if there is a premature release of PGF2A following macrophage depletion in *Cd11b-Dtr* mice, this wouldn't explain the rapid destruction of the microvascular endothelial cells within corpora lutea, because these cells do not express significant amounts of the PGF2A-receptor (FP; (Davis and Rueda, 2002).

Another possibility is that macrophage depletion deprives the corpus luteum of luteotrophic factors. Some luteotrophic factors important for the maintenance of the corpus luteum in the mouse include LH and prolactin (PRL)(Johnson and Everitt, 2007a). In the rodent, the surge of LH at proestrus is responsible for the formation of the corpus luteum at estrus. It is the release of pituitary PRL in response to coital stimulation of the cervix that rescues the corpus luteum and enables it to continue its

production of $P_4$ (Gunnet and Freeman, 1983). PRL also induces an upregulation of LHR expression during corpus luteum formation, and LH in turn can increase transcription of the rate limiting enzyme STAR, increasing the quantity of substrate available at the site of CYP11A1 (Gåfvels et al., 1992, Segaloff et al., 1990). PRL and placental lactogens, acting through PRL-receptor (PRLR), have been shown to inhibit the synthesis of 20-alpha-HSD (Albarracin et al., 1994, Bachelot et al., 2009). A null mutation in the prolactin receptor renders mice infertile (Ormandy et al., 1997). In addition, PGF2A stimulates 20-alpha-HSD activity (Ferrara et al., 1992).

A measure of the levels of serum LH and PRL before and after DT-elicited macrophage depletion would give an insight into whether the production of these factors is perturbed following macrophage depletion, and whether the perturbation is occurring at the pre- or post-receptor level. Further, analysis of their cognate receptors by immunohistochemistry and/or quantitative real-time PCR would be insightful. It is difficult to conceptualise how PRL signalling might be affected by macrophage depletion. However, since LH synthesis does appear to depend on macrophage function (or at least is dysregulated by depletion of cytokines that control macrophages (Cohen et al., 2002, Ingman et al., 2006)) this is a possibility worthy of further exploration. Further, it is recognised that during the first week of pregnancy in the rodent, prolactin is capable of maintaining basal levels of $P_4$ secretion, and inhibition of the PRLR curtails $P_4$ production, and this is further justification for a more in depth analysis of PRL levels and constituents of its common signal transduction pathway (Jak/Stat5) (Leung and Adashi, 2004).

### 5.9.2 Macrophage depletion and ovarian tissue injury

It is not clear whether the effect on the endothelial cell network within the corpus luteum is caused directly by macrophage depletion, or is secondary to another cause. Because DT is a protein synthesis inhibitor, macrophages die by apoptosis in *Cd11b-Dtr* mice, and the low dose required has no substantial adverse effects on other cells in the body (Saito et al., 2001). However, because there are no macrophages present to clear the debris of those that have undergone apoptosis, or to clear any infiltrating neutrophils, it is likely that there is some secondary necrosis within the affected tissues as there are no macrophages to remove the apoptotic bodies. Delayed clearance of apoptotic bodies, or high levels of apoptosis, can result in secondary necrosis and the associated loss of plasma membrane integrity (Silva et al., 2008, Chen and Nunez, 2010).

Sterile inflammation can occur in the absence of any microorganisms. Similar to inflammation resulting from a microbial infection, sterile inflammation is marked by neutrophil recruitment, and macrophage production of pro-inflammatory cytokines and chemokines, in particular, TNF and IL1 (Chen and Nunez, 2010). Macrophages would not all undergo apoptosis at the same time following DT administration. While some would be undergoing apoptosis/secondary necrosis, others may be responding to the sterile inflammation and producing the pro-inflammatory cytokines mentioned above. Importantly, it has been shown that TNFA caused increased secretion of PGF2A, which then causes luteolysis, and has also been shown to inhibit gonadotropin-supported $P_4$ accumulation (Wang et al., 1992, Brannstrom et al., 1995, Adashi et al., 1990).

Traditionally it was thought that in response to a microbial infection, several receptors, known as pattern recognition receptors (PRRs) are activated to induce a pro-inflammatory response. PRRs include the TLRs, NOD-like receptors, RIG-1-like receptors, C-type lectin receptors, and absence in melanoma 2-like receptors. These PRRs recognise structural moieties found on microorganisms called PAMPs. However, it is now evident that PRRs are also able to recognise non infectious material that has the ability to cause tissue damage as well as endogenous molecules that are released during cellular injury. These endogenous molecules have been termed DAMPs. DAMPs are host-derived, non-microbial stimuli that are released following cell death and are able to activate pro-inflammatory pathways in a similar manner to PAMPs (Chen and Nunez, 2010).

DAMPs are endogenous factors normally sequestered within the cell and therefore unable to cause immune recognition. However, under conditions of cellular stress or injury, these factors can be released into the extracellular environment. This may occur during necrosis, or when apoptosis fails to occur. DAMPs that can be derived from necrotic cells include chromatin-associated protein high-mobility group box 1 (HMGB1), heat shock proteins (HSPs) and purine metabolites (ATP and uric acid). These toxic mediators released following necrosis would be expected to activate TLR4-mediated inflammation (Piccinini and Midwood, 2010) and may be another cause of the breakdown of the endothelial cell network. DAMPs can also be located within the ECM. ECM fragments such as hyaluronan and heparan sulphate can be generated by proteases that are activated to promote tissue repair and remodelling, or by proteolysis by enzymes released from dying cells. In addition to intracellular molecules, intracellular stores of biologically active pro-inflammatory cytokines and chemokines can be released by necrotic cells. Macrophages are a source of many pro-inflammatory

cytokines (e.g. IL1), but in addition, endothelial cells are a source of pro-inflammatory IL33 (Chen and Nunez, 2010).

Given the histological evidence of tissue injury in corpora lutea, it is likely that sterile inflammation could be occurring from the release of DAMPs and the release of inflammatory cytokines. This could likely be implicated in the cause of pregnancy loss in macrophage-depleted *Cd11b-Dtr* mice. It is thought that in chronic or acute illness, inflammatory cytokines can inhibit the production of gonadotropin from the hypothalamus and pituitary. They are also thought to inhibit $P_4$ production and initiate luteal regression during a normal ovulatory cycle (Rivest and Rivier, 1995, Davis and Rueda, 2002, Erlebacher et al., 2004). An experiment whereby pregnant macrophage-depleted *Cd11b-Dtr* mice were supplemented with PRL instead of $P_4$ would determine whether pregnancy failure was occurring due to an inability of the luteal cells to produce sufficient $P_4$ to maintain pregnancy, or alternatively whether inflammation was causing ovarian resistance towards the gonadotropic effects of pituitary-derived PRL, with the $P_4$ decrease secondary to this. Such a pathway resulting in corpora lutea demise has been shown to account for the effects of inflammation induced fetal loss through inhibition of the reproductive endocrine system (Erlebacher et al., 2004), although the mechanism of fetal demise is likely to be somewhat different. This model caused DC activation by forced ligation of CD40 (an immunostimulatory molecule present on DCs) with agonistic anti-CD40 antibody. However, this subsequently caused NK cell activation and increased serum concentrations of TNFA and its downstream target IL6. Neutralisation of TNFA prevented fetal loss following CD40 ligation (Erlebacher et al., 2004).

With the potential activation of macrophages that are yet to succumb to the effect of DT, and the release of intracellular cytokines from dying macrophages, it is likely that inflammatory cytokines such as TNFA and IFNG could also be released into the interstitium. TNFA is largely considered to be derived from monocytes or macrophages, but there is also a significant amount found within and secreted by endothelial cells of functional corpora lutea in the pig, where it is thought to have a role in vascular development. However, after functional luteolysis has occurred, there is no TNFA detected within the corpus luteum, which is consistent with the dramatic reduction in endothelial cells at the time of luteolysis (Hehnke-Vagnoni et al., 1995, Davis and Rueda, 2002).

### 5.9.3 Macrophage depletion has minimal effects on steroidogenic enzyme mRNA expression

Assessment of the impact of macrophage depletion on the mRNA levels of enzymes involved in the production of $P_4$ revealed a significant 28.6% decrease in the levels of *Hsd3b1* on day 4.5 pc (24 h following macrophage depletion). It was a surprise that the levels of the other enzymes (*Star* and *Cyp11a1*) were not affected by macrophage depletion. The intracellular location of these enzymes may be a reason for this. STAR protein binds cholesterol in the cytosol and transports it to the outer mitochondrial membrane (Niswender, 2002). The cytochrome P450 side chain cleavage enzyme complex (encoded by *Cyp11a1*) converts cholesterol to pregnenolone within the inner-mitochondrial membrane, and 3β-hydroxysteroid dehydrogenase (encoded by *Hsd3b1*) then converts pregnenolone to progesterone within the smooth endoplasmic reticulum (ER); perhaps the smooth ER is more susceptible to damage following macrophage depletion.

There was no effect on the expression of steroidogenic enzymes on day 4.0 pc, following only 8 h of DT-elicited macrophage depletion; it is likely that this time-point is too early for any effect to be seen, although macrophage apoptosis is occurring, the cells are not fully depleted after 8 h of DT treatment (Cailhier et al., 2005). There was no change in the expression of any of the enzymes measured on day 7.5 pc, 4 days after the injection of DT to elicit macrophage depletion. In this group, it is possible that the mice have resumed cycling. The experiments in Chapter 3 revealed that when DT was administered on day 3.5 pc, no *Cd11b-Dtr* mice were pregnant on day 5.5 pc. On day 4.5 pc, only around 40% of DT-treated *Cd11b-Dtr* mice were pregnant. Based on these results, it is possible that by the time the tissue was recovered on day 7.5 pc in these experiments, the mice had resumed estrous cyclicity. Jasper (1998) reported that after PGF2A-initiated luteal regression in pseudopregnancy, the onset of estrus was seen within two days in wild-type and *Csf2*$^{-/-}$ mice. This seems the more likely explanation, the other being that there is extensive tissue damage as a result of macrophage depletion, which would lead to failure to resume cycling after pregnancy loss. However, because the mRNA levels involved in steroidogenesis are not different to those in wild-type, pregnant mice, this seems somewhat unlikely. Given the minimal changes seen in expression of the steroidogenic enzymes studied in this chapter, the expression of 20α-HSD needs to be investigated, as if elevated following macrophage depletion, could further explain the decline in circulating serum $P_4$ seen in DT-treated *Cd11b-Dtr* mice.

### 5.9.4 Macrophage depletion affects the endothelial cell network within the corpus luteum

The impact of macrophage depletion on endothelial cells is a novel result for the ovary, with the only other report to show this being published very recently (Turner et al., 2011). However, the concept of macrophage involvement in angiogenic processes has been extant for some time (Sunderkotter et al., 1994). In some tissues, macrophages have been identified as a prevalent source of VEGF, and key angiogenic factors are recognised as macrophage secretory products (Aharinejad et al., 2004, Yoshida et al., 1997, Naidu et al., 2003).

Just as macrophages have an intimate association with endothelial cells in the corpus luteum (Figure 5.6A and C) the same is true for DCs and endothelial cells in the mouse decidua (Plaks et al., 2008). Plaks et al. (2008) have demonstrated that ablation of $CD11C^+$ DCs results in embryo resorption due to severely impaired decidualisation, even in a model of artificial deciduoma formation, where an impact from the embryo can be excluded. Further investigation found this to be due to impaired proliferation of decidual cells, and impaired decidual differentiation. When the impact of DC depletion on vascular expansion was examined, there was a significant reduction in capillary density. This was associated with a reduced blood volume and capillary leak, suggesting a role for DCs in vascular expansion, vascular maturation (less anti-α-smooth muscle actin after DC depletion), and attenuation of vascular permeability. Some similarities were seen following the depletion of $CD11B^+$ cells from the ovary, resulting in an impairment in ovarian vasculature. It was also demonstrated in this model that DT-elicited macrophage depletion results in depletion of both $CD11C^+$ and $CD11B^+CD11C^+$ cells from the ovary (flow cytometry, Table 3.1). This provides strong evidence of a role for $CD11C^+$ mononuclear phagocytes in vascular regulation and suggests that $CD11B^+$ and perhaps $CD11C^+$ cells may be important for ovarian vasculature.

Ovarian lymph is important in the transport of $P_4$ and is an important source of $P_4$ in peripheral plasma (Hein et al., 1988). Importantly, ovarian lymphatic vasculature was found to be unchanged following macrophage depletion, indicating that reduced plasma $P_4$ levels were not due to impaired lymphatic transport of this hormone.

Pericytes (perivascular cells closely associated with capillaries; also called Rouget or mural cells) in some species support cells that supply angiogenic factors and control the development, maintenance and regression of the corpus luteum (Knobil and Neill, 2006, Redmer et al., 2001, Collett and Canfield, 2005). Due to the rapid formation of the microvascular network in the corpus luteum, and the intimate

association between macrophages and endothelial cells, it is possible that macrophages play a pericyte-like role and support these immature vessels. Macrophage ablation could result in the rapid destabilisation and destruction of the microvascular endothelial cells.

A reduction in *Emr1* (F4/80) mRNA was observed following DT-elicited macrophage depletion in *Cd11b-Dtr* mice on day 4.5 pc (24h post-DT administration), and supports the flow cytometry and immunohistochemistry data to show that macrophages are depleted in these mice. The fact that the mRNA expression of *Pecam1* on day 4.5 pc remained the same after macrophage depletion came as a surprise given the depletion of PECAM1-expressing cells shown by immunohistochemistry. However, not all corpora lutea seemed to be affected equally, and the fact that some corpora lutea still had an existing microvascular network, while others has some endothelial cells (albeit a regressing network), may provide an explanation for this result (see Figure 5.5). However, even in CLs still containing a microvascular network, there was disruption of the CL structure (Table 5.2). The down-regulation of *Cyp17a* mRNA expression following macrophage depletion in *Cd11b-Dtr* mice may be in an attempt to prevent the catabolism of the already declining $P_4$ levels.

### 5.9.5 Macrophage depletion and the expression of angiogenic and lymphangiogenic factors

VEGFA (or VEGF) belongs to a gene family that is inclusive of placental growth factor (PLGF) and VEGFB, VEGFC and FIFG (VEGFD). VEGFA is the key regulator of blood vessel growth, acting as a mitogenic growth factor that specifically targets endothelial cells (Ferrara et al., 2003, Gospodarowicz et al., 1989, Conn et al., 1990, Shweiki et al., 1993). VEGFA acts via its tyrosine kinase receptors, FLT1 and KDR. It is thought that activation of KDR enables specific stimulation of endothelial cell proliferation, and is considered the major receptor of VEGFA (Ferrara and Davis-Smyth, 1997, Ferrara et al., 2003, Coultas et al., 2005). FLT-1 is considered to play an inhibitory role by sequestering VEGF and preventing its interaction with KDR, and is thought to be unable to transduce signals when stimulated with VEGF (Ferrara, 2004, Douglas et al., 2005, Landgren et al., 1998). An alternatively spliced form of FLT1 (sFLT) has also been shown to inhibit VEGF activity (Kendall and Thomas, 1993). VEGF is reported to be produced by lutein cells (Ferrara et al., 1992), and *in situ* analysis has revealed that endothelial cells comprising the vascular network within the corpus luteum possess VEGF-binding activity (Phillips et al., 1990). In the marmoset, *Vegf* mRNA was found to be intensely expressed by granulosa luteal cells by *in situ* hybridisation, but was absent from thecal and endothelial cells (Wulff et

al., 2000). Shweiki et al. (1993) have reported that VEGF receptors are constitutively expressed in the endothelial cells of the reproductive tract, regardless of the proliferative status.

In the experiments presented herein, it is not possible to identify the source of the increased *Vegfa* mRNA. However, given this information, it seems likely that the luteal cells are the source. The destruction of the microvascular network surrounding the luteal cells would have caused a hypoxic environment, and hypoxia is the stimulus for synthesis of angiogenic factors (Ferrara and Davis-Smyth, 1997), possibly explaining the 3.6-, 3.7-, and 3.2-fold increases in *Vegfa* mRNA expression detected on day 1.5, 2.5 and 4.5 pc respectively, following macrophage depletion. However, this would need to be confirmed by measuring *Hif1a* mRNA expression. It is reported that angiogenic factors (VEGF, bFGF and the angiopoietins) are attached to the ECM; a loss of integrity of the ECM will result in an increased concentration of these factors (Douglas et al., 2005). It also fits with reports suggesting that VEGF may promote monocyte chemotaxis (Clauss et al., 1990). VEGF is also a potent inducer of vascular permeability (Senger et al., 1983), so the large increase in *Vegfa* mRNA may also play a role in the haemorrhage and serum exudate evident in the ovaries of macrophage-depleted *Cd11b-Dtr* mice.

Studies in the mouse have shown an essential role for VEGFA in corpus luteum angiogenesis. Administration of a truncated form of the sFLT1, known to inhibit the biological activity of VEGF, caused almost complete inhibition of angiogenesis when given prior to gonadotropin stimulated ovulation (Ferrara et al., 1998). Corpus luteum development and $P_4$ production were also inhibited, and within the corpora lutea of treated animals, ischemic necrosis was evident, but there was no impact on pre-existing vasculature. Associated with this treatment was the failure of the endometrium to mature (Ferrara et al., 1998). Fraser et al. (2000) demonstrated the effects of administering an antibody to VEGF at ovulation in the marmoset. Anti-VEGF treatment suppressed the intense endothelial cell proliferation that would normally occur in the early luteal phase, prevented the development of the extensive capillary bed, and was associated with a 60% decrease in $P_4$ secretion. It is reported that the luteal cells post-treatment were largely unaltered, although there was lipid accumulation, and small pockets of cells showing nuclear condensation and basophilia. Treatment of animals with the anti-VEGF antibody in the mid-luteal phase also interrupted ongoing angiogenesis, with a reduction in $P_4$ production, decreased endothelial cell proliferation, and endothelial cell apoptosis (Fraser et al., 2000, Douglas et al., 2005).

Further studies in the marmoset investigated the impact of inhibiting the actions of VEGF using VEGF-trap, a soluble truncated form of the FLT1 receptor. Animals treated at the time of ovulation, or 3 days post-ovulation, presented with decreased endothelial cell proliferation, failed development of the microvascular tree, and reduced serum $P_4$ (Wulff et al., 2001). *In situ* hybridisation experiments revealed an increase in *Vegf* mRNA expression in luteal cells, and a decrease in its *Flt1* receptor. Messenger RNA levels of *Angiopoietin-2 (Ang2)* and its receptor, *Tie2*, were also increased. Experiments were also conducted to determine the impact of VEGF-trap treatment in the mid-luteal phase of the marmoset, a time at which vascularisation of the corpus luteum is largely complete (Fraser et al., 2006). Treatment resulted in a rapid decline in $P_4$ levels. These results came as a surprise, and indicated that treatment with VEGF-trap was affecting luteal cell function by mechanisms other than just the inhibition of angiogenesis. It was found that inhibition of VEGF caused an increase in caspase-3 positive endothelial cells, and a decreased endothelial cell area (Fraser et al., 2006).

The studies showing the impact of inhibiting the actions of VEGF are insightful and provide clear evidence of an essential role of VEGF in corpus luteum function. In the experiments presented in this chapter however, there is a marked increase in the production of *Vegfa* mRNA, yet we still see the decrease in plasma $P_4$, and endothelial cell death. It is possible that macrophage depletion affects the binding of VEGFA to its receptor. The main receptor required for VEGFA to exert its angiogenic actions is KDR, and following macrophage depletion, there was a significant decrease in the expression of *Kdr* mRNA on day 1.5 and 2.5 pc. The experiments by Wulff et al. (2001) discussed above, show a similar situation. They showed an up-regulation of *VEGF* mRNA detected in luteal cells by *in situ* hybridisation, and in addition, they report a decrease in *FLT1* mRNA expression, which we have observed following macrophage depletion. *KDR* mRNA was not measured in the experiment by Wulff et al. (2001), although it would have been interesting to see if *KDR* was up-regulated at the mRNA level in these studies to enable the actions of VEGFA.

A possible reason for the decrease in *Flt1* mRNA expression following macrophage depletion in *Cd11b-Dtr* mice is the presence of this receptor on monocytes/macrophage and hematopoietic cells, where it transduces signals for the migration of these cells (Sato, 2008). Endothelial cells express all of the VEGF-receptors, while macrophages predominantly express FLT1 (VEGFR-1)(Barleon et al., 1996)

VEGFB and its functions are less well described; however, it is reported to be important for blood vessel survival, but dispensable for blood vessel growth (Zhang et al., 2009). This is supported by

studies in *Vegfb*-null mice, which retain normal fertility (Bellomo et al., 2000). Since there were no alterations in VEGFB expression following DT-mediated macrophage depletion, it appears not to be essential and will not be discussed any further.

VEGFC and FIGF (VEGFD) are best known for their role in lymphangiogenesis, which is defined as the formation of new lymphatic vessels from pre-existing vasculature (Alitalo et al., 2005). For their role in lymphangiogenesis, VEGFC and FIGF bind their receptor FLT4 (VEGFR-3), as well as neuropilin-2 (NRP2). In addition, proteolytically processed VEGFC and FIGF can also activate KDR and induce blood vessel growth (Alitalo et al., 2005, Achen et al., 1998, Joukov et al., 1997, Cao et al., 1998, Stacker et al., 1999, Rissanen et al., 2003). VEGFC, like VEGFA, is able to increase vascular permeability and induce endothelial cell proliferation (Joukov et al., 1997). In addition, macrophages are reported to be a likely source of VEGFC and FIGF (Granata et al., 2010, Cursiefen et al., 2004, Schoppmann et al., 2002) and macrophage depletion is likely to cause the removal of trophic support for microvascular development and maintenance.

Thus, the decrease in *Vegfc* and *Flgf* mRNA, taken together with the decrease in the mRNA expression of their receptor *Kdr,* could further explain the negative impact that macrophage depletion had on endothelial cell populations within the corpus luteum.

### 5.9.6 Overall conclusions and future directions

The experiments in this chapter demonstrate an essential role for corpora lutea associated macrophages in maintaining its structural and functional integrity during pregnancy. Macrophage depletion resulted in decreased circulating plasma $P_4$ and a decline in the mRNA expression of an enzyme (*Hsd3b1*) involved in its synthesis. The observation that macrophage depletion impacted endothelial cell populations within the corpus luteum lead to experiments aimed at further understanding the impact of macrophages on angiogenesis in the ovary. These studies have shown that macrophage depletion causes a marked upregulation of *Vegfa* mRNA, possibly derived from the luteal cells that become hypoxic due to endothelial cell death. There is also a decrease in the mRNA expression of *Vegfc* and *Figf*, which are known to be produced by macrophages (Granata et al., 2010, Schoppmann et al., 2002, Cursiefen et al., 2004). In addition to their role in lymphangiogenesis (mediated by FLT4), these factors can also interact with KDR and be mitogenic to endothelial cells. Macrophage depletion also causes a down-regulation of *Kdr* expression, indicating that there was less

receptor present through which the apparently reduced levels of VEGFC and FIGF can signal through to promote angiogenesis. The decline in *Flt1* mRNA expression is likely attributed to macrophage depletion, as they express this receptor.

Another point that must be considered is the immune-modulatory effects of $P_4$; it is possible that the *in vivo* administration of $P_4$ to macrophage-depleted animals may have an immunosuppressive effect. Siiteri et al. (1977) showed that $P_4$ was able to inhibit T lymphocyte-mediated responses involved in tissue rejection (Jaffe, 1999). The administration of $P_4$ to rats was able to prolong the survival of xenogenic hamster skin implants and limit inflammatory responses. $P_4$ was also suggested to promote the survival of human trophoblastic tissue in rodents. Thus the authors proposed that the high uterine concentration of $P_4$ may inhibit an immune response to foreign antigens (Siiteri et al., 1977, Jaffe, 1999). More recently, several effects of $P_4$ in pregnancy have been attributed to lymphocyte derived progesterone-induced blocking factor (PIBF), which is able to influence cytokine synthesis. PIBF increases the production of Th2 type cytokines (Szekeres-Bartho and Wegmann, 1996, Szekeres-Bartho and Polgar, 2010). The high resorption rates caused by high NK cell activity (Szekeresbartho et al., 1990b) or blockade of the $P_4$ receptor (Szekeresbartho et al., 1990a) can be overcome by administration of PIBF in mice (Szekeres-Bartho and Polgar, 2010). However, given that there was no change in $CD3^+$ T lymphocyte numbers in either the uterus, ovary, peritoneal cavity or spleen (Chapter 3, Table 3.1), it is apparent that T lymphocytes are not activated following macrophage depletion and thus it seems unlikely that the results obtained are due to $P_4$-induced immunosupression.

Further studies are required to fully elucidate the role of macrophages in angiogenesis in the ovary. Analysis of other genes, such as the MMPs and their inhibitors (TIMPs), and the angiopoietins and their receptor will give further insight into the stability of the vessels within the ovary. Expression of MMPs by macrophages has been described previously, but importantly, angiogenesis cannot occur without the required remodelling of the ECM to enable endothelial cell migration and proliferation. Studies have shown that MMP expression increases during luteinisation, declines in the fully functional corpus luteum, before increasing again during luteolysis (Leung and Adashi, 2004). Macrophages are also a source of angiopoietin 2 (Ang2) and its receptor TIE2 (Hubbard et al., 2005, Murdoch et al., 2007, De Palma and Naldini, 2011). Further, it has been demonstrated that Ang1 can prevent vessel leakiness caused by VEGFA without interfering with angiogenic processes, and thus if *Ang1* expression is found to be reduced following macrophage-depletion, it could possibly explain the

ovarian haemorrhage seen at autopsy (Thurston et al., 1999, Thurston et al., 2000, Leung and Adashi, 2004).

Another possibility worthy of exploration is whether macrophages that surround follicles at ovulation are able to transdifferentiate into endothelial cells as the corpus luteum forms. It has been suggested in previous studies that macrophages are able to express endothelial cell markers under certain conditions (Sharifi et al., 2006, Ninomiya et al., 2006, Yan et al., 2011). Given this, an experimental protocol has been established to test this. Day 4.5 pc ovarian tissue from wild-type mice had been labelled with EMR1 and PECAM1 specific antibodies and analysed using confocal microscopy to look for any cells possibly expressing both markers (see Appendix 2 for representative images). This experimental technique now needs to be applied to tissue where the corpora lutea are still being formed to determine whether macrophage transdifferentiation has any role in corpus luteum formation. Flow cytometry will be used to quantify any cells expressing both macrophage and endothelial cell markers. In addition, it has been shown that $CD11B^+$ macrophages are critical for the development of inflammation-dependent lymphangiogenesis in the eye, and that bone marrow-derived $CD11B^+$ macrophages expressed the lymphatic markers LYVE-1 and PROX-1 (Maruyama et al., 2005). It may therefore be worth looking at co-localisation of macrophage and lymphatic endothelial cell specific markers during corpus luteum formation also. Although macrophage to endothelial/lymphatic endothelial cell transdifferentiation remains a possibility, it is more likely that macrophages within the corpus luteum are there for the provision of trophic support.

# Chapter 6

# General discussion and conclusions

## 6.1 DISCUSSION AND CONCLUSION

Macrophages are ubiquitous cells with functions within the body extending far beyond host defence. Macrophages are involved in the maintenance of immune regulation, tissue remodelling, wound healing and homeostasis. The remarkable plasticity of these cells enables them to respond appropriately to signals they receive from their local microenvironment. The diversity of phenotypes possessed by these cells enables them to respond differently to the same signal within the same tissue. Numerous *in vitro* and *in vivo* studies point to a role for these cells in reproductive function, with compelling evidence emerging from genetically modified mice. Many such studies show altered macrophage phenotype in mice deficient in macrophage regulating growth factors such as CSF1, CSF2 and TGFB1, and the mice have many reproductive defects, some of which can be directly attributed to macrophage deficiency. It is becoming increasingly apparent that environmental and lifestyle factors can change the macrophage microenvironment, and may influence the way these cells behave. Given their extensive role in tissue remodelling and development in early pregnancy, such alterations may modify macrophage function to the detriment of pregnancy outcome.

The studies described in this thesis are the first to investigate the adverse impact of acute macrophage depletion during early pregnancy in the mouse. These experiments provide compelling evidence of an essential supporting role of macrophages in the corpus luteum to maintain sufficient vascularity and $P_4$ production and circulation. Macrophages also appear to play a supporting role in embryo attachment events during implantation.

In Chapter 3, a detailed characterisation was undertaken to identify the cell types affected by DT administration to *Cd11b-Dtr* mice. Flow cytometric and immunohistochemical analysis revealed that macrophages were sufficiently depleted from reproductive tissues, as well as the peritoneal cavity 24 h following DT injection. In addition to macrophages, we identified that CD11C-expressing cells were depleted from the ovary and peritoneal cavity, and there were significantly less neutrophils in the ovary following DT treatment. Whether these $CD11C^+$ cells are actually depleted as a consequence of their co-expression the CD11B marker, or whether macrophage depletion impairs their recruitment into the tissue due to the absence of macrophage-derived chemokines, remains to be investigated.

These studies demonstrate an absolute requirement for macrophages in the success of early pregnancy. Macrophage depletion between day 0.5 and day 3.5 pc caused complete pregnancy failure. The intravenous injection of trypan blue dye into mice following macrophage depletion revealed

that although early stages of the implantation cascade begin, with changes in vascular permeability observed 24 h following DT injection, embryo implantation ultimately fails by 48 h post-DT. The histological analysis of blastocysts within uteri 24 h following macrophage depletion would be insightful as it would enable us to understand whether blastocysts appeared healthy and had begun the implantation process, and the condition of the surrounding tissue. Use of markers for trophoblast cells (cytokeratin) and endothelial cells (PECAM1) would provide further information on the state of the implantation site.

*In vitro* experiments identified that DT had no apparent direct effect on the development of embryos to blastocyst stage. *In vivo* experiments highlighted that there may be indirect effects of DT on embryo developmental competence, but this appeared to be secondary to their exposure to a suboptimal intrauterine environment and diminished $P_4$. To more definitively decipher whether DT exposure directly impacts developmental competence, embryos would need to be cultured *in vitro* in the presence of DT, and then transferred to pseudopregnant mice where their implantation rates and development can be directly assessed.

Importantly, macrophage depletion does not appear to impair the uterine proliferative response to $E_2$ and $P_4$ that occurs as the endometrium prepares for the implanting blastocyst, nor is the ability to form deciduoma affected. This is in line with studies using the most well established model of macrophage depletion, the *Csf1*$^{op}$*Csf1*$^{op}$ mouse, where uterine proliferative responses to $E_2$ and $P_4$ also take a normal course (Cohen et al., 1997). Data from tissue recombinant studies demonstrate that the actions of $E_2$ in the uterus are via the stromal ER, and the authors propose that this results in the secretion of a paracrine factor that stimulates uterine epithelial cell mitogenesis (Cooke et al., 1997). In light of the preliminary experiments presented in Chapter 4 and the studies in the *Csf1*$^{op}$*Csf1*$^{op}$ mouse (Cohen et al., 1997), it appears that neither macrophages nor their secretory products play a role in this process, although a further experiment where macrophages are depleted for a longer period would provide more conclusive evidence. $E_2$ causes increased vascular permeability and uterine oedema, which means that the local synthesis of growth factors may not be necessary for the resultant mitogenic response. It is possible that the altered permeability and increased oedema allows the influx of a multitude of serum growth factors into the uterus which may cause cell proliferation (Pollard, 1990), although it appears unlikely that such factors are of macrophage origin.

It is known that stromal expression of PR is required for the anti-mitogenic effects of $P_4$-pretreatment on $E_2$-induced epithelial cell proliferation (Kurita et al., 1998b). However, less detail is understood about the ways in which stromal cell proliferation occur in response to both $E_2$ and $P_4$. The studies herein conclusively rules out a paracrine factor of macrophage origin in this proliferative response.

Depletion of macrophages was observed to affect uterine parameters of implantation receptivity. Specifically, the expected upregulation in early pregnancy of adhesive glycoconjugates is impaired by macrophage depletion. There was a significant decrease in the epithelial expression of fucosylated structures detected with the lectin UEA-1 and with LewisX immunostaining, implying that macrophages contribute to the increased expression of adhesive structures during the implantation phase of pregnancy (Figure 6.1B). It is likely that macrophages exert such effects in a paracrine manner, and candidate molecules identified include LIF and IL1B (Jasper et al., 2011).

Thus the work presented in Chapter 4 demonstrated that macrophages are not essential for the preparatory proliferative events that occur in the uterus in response to steroid hormones, nor do they impact the vascular response measured by endothelial cell density following hormonal stimulation. However, they do appear to have an important role in embryo attachment to endometrial epithelial cells. This finding has important clinical implications for understanding causes of implantation failure. Previous studies have demonstrated improved pregnancy outcome in IVF patients following exposure to seminal fluid (Tremellen et al., 2000, Bromfield, 2006, Pang et al., 1979, Queen et al., 1981, Peitz and Olds-Clarke, 1986, Carp et al., 1984). Given that macrophage recruitment is reduced in matings or pregnancies that occur in the absence of seminal fluid, it appears that the seminal fluid-induced improvement in pregnancy outcome may be in part mediated by macrophage secretory products. In addition, studies have revealed that subfertile women have altered leukocyte numbers (Klentzeris et al., 1994) and the extent of colocalisation of macrophages with IL1A, IL1B and IL6 was affected by the fertility status of the patient (Dechaud et al., 1998). In addition, women afflicted by recurrent miscarriage show lowered expression of *IL6* and *IL1A* mRNA during the secretory phase of their cycle (Jasper et al., 2007). IL1A is likely to be secreted by macrophages, but IL6 could also be of uterine epithelial cell origin (Robertson et al., 1992b, Jacobs et al., 1992).

The experiments in Chapter 5 demonstrate that macrophage depletion exerts profound effects during early pregnancy on the corpus luteum. Macrophage depletion caused structural and functional luteolysis of the corpus luteum, leading to luteal insufficiency and pregnancy failure. That pregnancy

could be rescued by exogenous $P_4$ supplementation following macrophage depletion demonstrates that the primary cause of pregnancy failure following macrophage depletion is luteal insufficiency. This result demonstrates that macrophage depletion does not impact other progesterone-independent events, and alleviates the initial concern (Chapter 4) that off-target effects of DT might contribute to the infertility seen in the *Cd11b-Dtr* model. These results concur with previous studies showing that macrophage coculture with granulosa cells increased progesterone secretion in the mouse (Kirsch et al., 1981), pig (Standaert et al., 1990) and human (Halme et al., 1985). Further support of a role for macrophages in progesterone production comes from studies utilising *Csf1*$^{op}$*Csf1*$^{op}$ (Cohen et al., 1997), *Csf2*$^{-/-}$ (Jasper et al., 2000), and *Tgfb1*$^{-/-}$ mice (Ingman et al., 2006).

Macrophage depletion disrupted the integrity of the vascular network within corpora lutea, and caused haemorrhage within ovarian tissue, consistent with a previous report that macrophage depletion from the cycling, non-pregnant mouse results in vascular impairment within corpora lutea (Turner et al., 2011). Such obvious vascular disruption is not reported in other tissues in previous studies utilising the macrophage-depleted *Cd11b-Dtr* model investigating kidney, peritoneum, skin, or mammary gland function (Cailhier et al., 2005, Duffield et al., 2005b, Mirza et al., 2009, Chua et al., 2010). The corpus luteum vasculature is distinguished by the cyclical nature of its development and demise; emerging rapidly over the course of the ovarian cycle and during pregnancy, before regressing to form the corpus albicans. This is in stark contrast to the mature and long-lived vasculature present in organs such as the liver and kidney. By virtue of its transitory appearance and rapid development each reproductive cycle, the vasculature of the corpus luteum may depend on macrophages for trophic or structural support in a manner unparalleled in other less dynamic tissues. As depicted in Figure 6.1A, the studies herein demonstrate that macrophages may provide trophic support in the form of VEGFC and FIGF, as the ovarian gene expression of these growth factors was significantly decreased following macrophage depletion.

Despite results indicating a crucial role for macrophages in corpora lutea development and function, the mechanism by which this occurs still needs to be addressed. At the present time it is unclear whether the effects of macrophage depletion on corpus luteum vasculature is a direct effect due to the removal of trophic support, or due to inflammation resulting from uncleared apoptotic macrophages. Figure 6.2 shows a schematic representation of the relationship between macrophages, endothelial cells and luteal cells in a healthy corpus luteum, and the known and proposed mechanism by which macrophage depletion affects this. To address the unknown links within this proposed mechanism,

peritoneal macrophages from wild-type (resistant) and *Cd11b-Dtr* (susceptible) mice could be co-cultured with corpora lutea tissue dissected from pregnant wild-type and *Cd11b-Dtr* mice. DT could then be added to the culture to deplete all susceptible ovarian and peritoneal macrophages. Following this, $P_4$ and PGF2A levels in the media could be sampled, the tissue homogenized and the mRNA extracted to quantitate the expression of steroidogenic enzymes involved in the synthesis of $P_4$. The advantage of an *in vitro* culture system is that it removes the need for vascular transport of $P_4$, and enables isolation of the tissue of interest without any other systemic and potentially confounding effects. If $P_4$ levels are reduced in this culture system, it could be concluded that macrophage depletion directly impacts the capacity of luteal cells to acquire substrate or synthesise the hormone, and RT-PCR analysis of steroidogenic enzyme expression would likely corroborate. If the concentration of $P_4$ is not substantially affected following macrophage depletion *in vitro*, it may be that macrophage depletion impacts corpora lutea vasculature required for acquiring substrate and circulating $P_4$ peripherally. This could also be assessed *in vivo*. To determine whether $P_4$ is still being synthesised at normal levels (given only minor changes in the expression of *Hsd3b1*), ovaries from wild-type and macrophage-depleted *Cd11b-Dtr* mice could be excised, the tissue homogenised, and the levels of $P_4$ and PG measured by EIA. Activated macrophages are a known source of TNFA, and TNFA has been shown to increase luteolytic PGF2A secretion, which in turn stimulates 20α-HSD activity and thus $P_4$ catabolism (Wang et al., 1992, Brannstrom et al., 1995, Leung and Adashi, 2004)(Figure 6.2B), and thus in vitro and in vivo assessment of the levels of PGF2A and 20α-HSD would provide great mechanistic insight.

The $P_4$-rescue experiments suggest that the main effect of macrophage depletion is luteal insufficiency. But to directly address whether macrophage depletion is the cause of the reduced $P_4$ output, macrophage add-back experiments would be required. To do this, elicited peritoneal macrophages could be recovered from wild-type mice and purified using lymphoprep, leaving only macrophages and lymphocytes (no neutrophils) or MACS columns or a complement depletion protocol could be used to remove T and B lymphocytes and NK cells. The resultant preparation could be injected intravenously into pregnant *Cd11b-Dtr* mice at the time of DT injection, and possibly 8 h following DT treatment. This treatment protocol would ensure that the DT-treated *Cd11b-Dtr* mouse was never macrophage depleted, and if this treatment protocol could rescue the pregnancy failure normally seen following DT-treatment in these mice, it would show that macrophages were directly and specifically responsible. Flow cytometry could be used to examine the purity and viability of macrophages prior to the add-back experiment.

Our observations in mice raise the question of whether perturbations in ovarian macrophage populations contribute to insufficient luteal phase progesterone production in some infertile women (Hinney et al., 1996). Progesterone supplementation is routinely provided in assisted reproductive technology treatment cycles to counteract the inhibitory effects of gonadotropins on corpus luteum function (Pritts and Atwood, 2002) and is viewed as a promising treatment for recurrent miscarriage (Coomarasamy et al., 2011). A combination of endogenous and environmental factors is thought to impact the number and phenotypes of macrophages in human peripheral tissues. Resident tissue macrophages display marked local heterogeneity and have a diverse range of phenotypes in different tissues, and in different compartments within the same tissue (Hume et al., 2002). Environmental factors that have been associated with infertility, including infection, diet, obesity and stress, are all likely to impact the cytokine microenvironment and could well operate through altered macrophage phenotype (Hudson et al., 1999, Lumeng et al., 2007, Joachim et al., 2003). It would be of interest to determine how such factors alter ovarian macrophage phenotypes and numbers, and in particular whether any changes in the vascular density and blood flow within the corpus luteum can be detected in humans, and whether this could be correlated to ovarian progesterone output. Samples of luteinised follicle wall, containing granulosa and theca cells could be obtained from patients undergoing IVF at the time of oocyte aspiration. Corpora lutea tissue segments could possibly be obtained from patients having gynaecologic indications for hysterectomy or surgical treatment for endometriosis.

In conclusion, these studies reveal that macrophages appear to be dispensable for the uterine proliferative responses to steroid hormones in preparation for pregnancy, and that macrophages are not required for deciduoma formation. Macrophages appear to be involved in the upregulation of adhesive glycoconjugates on the apical surface of endometrial epithelial cells, and thus may be important in promoting embryo attachment events occurring before trophoblast invasion begins. Most striking was the discovery that macrophages are essential for normal progesterone synthesis by the ovary, and their absence leads to complete pregnancy failure. The paramount function of macrophages appears to be exerted in the corpus luteum where they provide trophic support to the developing vasculature. Collectively, the current work and previous studies suggest that fine control of the numbers and phenotypes of macrophages, exerted through macrophage-regulating cytokines including TGFB1, CSF1 and CSF2 in the ovarian microenvironment, exert a major influence on corpus luteum steroidogenic function and capacity to circulate $P_4$ necessary to establish pregnancy. Perturbation of the cytokine-macrophage crosstalk within the ovary during early pregnancy is likely to be a pivotal feature of luteal insufficiency and there may be therapeutic opportunities to improve $P_4$

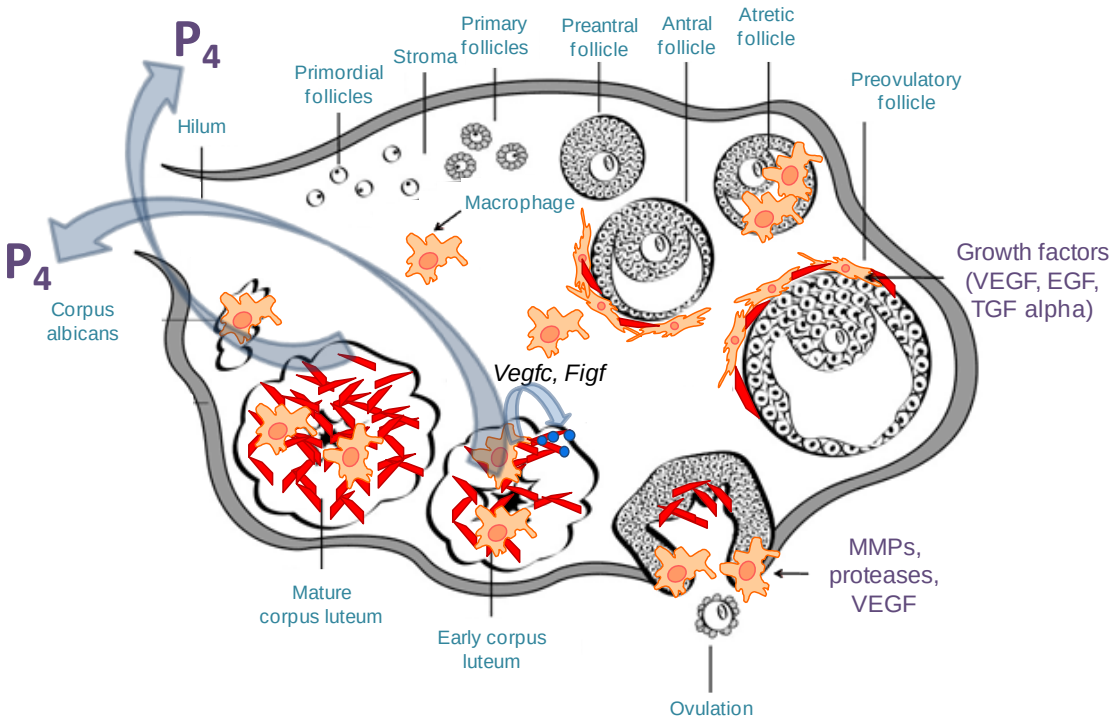
production through promotion of better macrophage function. Further studies are required to establish the link between macrophage dysfunction within the ovary and human infertility.

**Figure 6.1 Schematic illustration of mechanisms involving macrophage regulation of ovarian function, endometrial remodelling and embryo implantation**

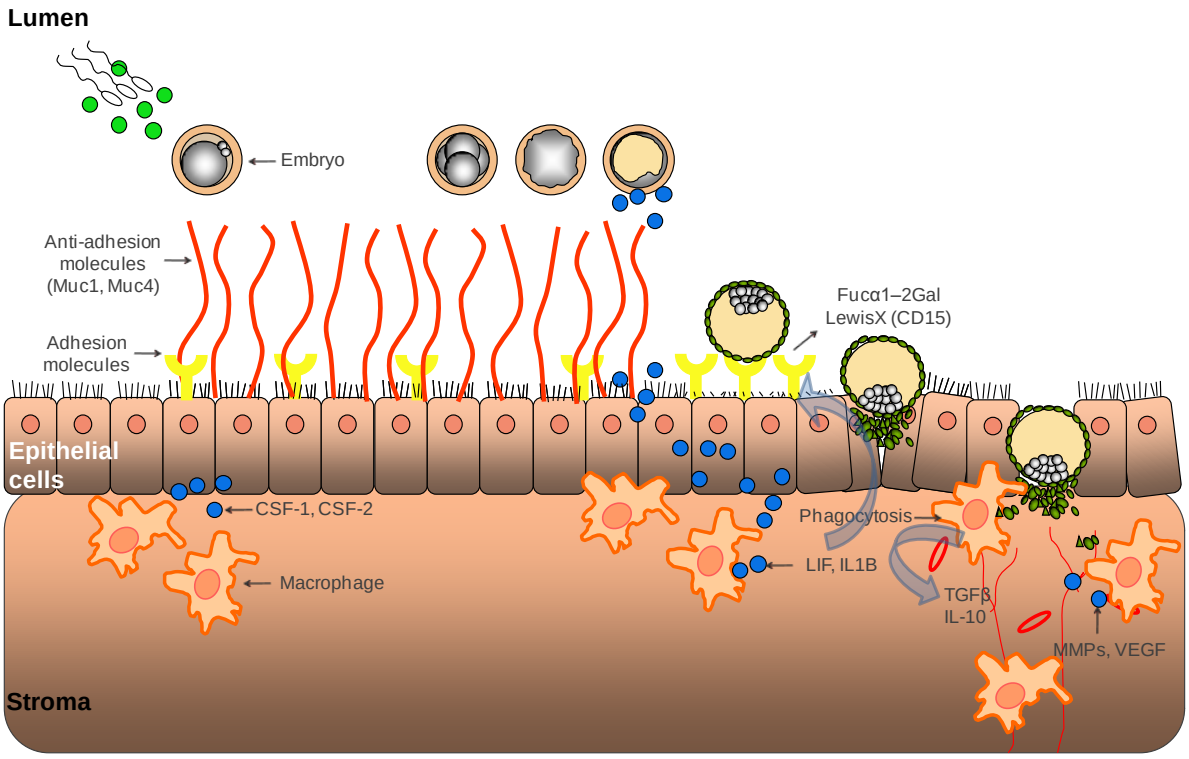
A. This illustration depicts the known and proposed roles of macrophages in ovarian function. Studies described in this thesis have demonstrated an essential *in vivo* role for macrophages in the production of sufficient $P_4$ to maintain early pregnancy. Macrophages also appear to support the newly formed vasculature within the corpus luteum, and this may be due to their production of factors including VEGFC and FIGF, as the mRNA expression of these genes was decreased following macrophage depletion.
Image used with permission and adapted from (Buse et al., 2008)

B. This illustration depicts known and proposed roles of macrophages in endometrial remodelling in preparation for embryo implantation. The studies described in this thesis demonstrate a role for macrophages in the upregulation of glycosylated structures on the apical surface of endometrial epithelial cells. Studies described by Jasper et al. (2011) suggest that this upregulation could be mediated by macrophage secreted products LIF and IL6, however, other candidate molecules exist. It is likely that macrophage secretory products cause the upregulation of Fucα1–2Gal (detected by the lectin UEA1) and LewisX by increasing the expression of the fucosyltransferase enzymes, FUT2 and FUT4, respectively. Although a role for macrophages in the remodelling of endometrial stroma in preparation for an implanting blastocyst remains likely, macrophages are not essential for the remodelling that occurs to enable deciduoma formation.

A


P4
Hilum
Primordial follicles
Stroma
Primary follicles
Preantral follicle
Antral follicle
Atretic follicle
Preovulatory follicle
Macrophage
P4
Corpus albicans
Growth factors (VEGF, EGF, TGF alpha)
Vegfc, Figf
MMPs, proteases, VEGF
Mature corpus luteum
Early corpus luteum
Ovulation

B


Lumen
Embryo
Anti-adhesion molecules (Muc1, Muc4)
Adhesion molecules
Fucα1–2Gal LewisX (CD15)
Epithelial cells
CSF-1, CSF-2
Macrophage
Phagocytosis
LIF, IL1B
TGFβ IL-10
MMPs, VEGF
Stroma

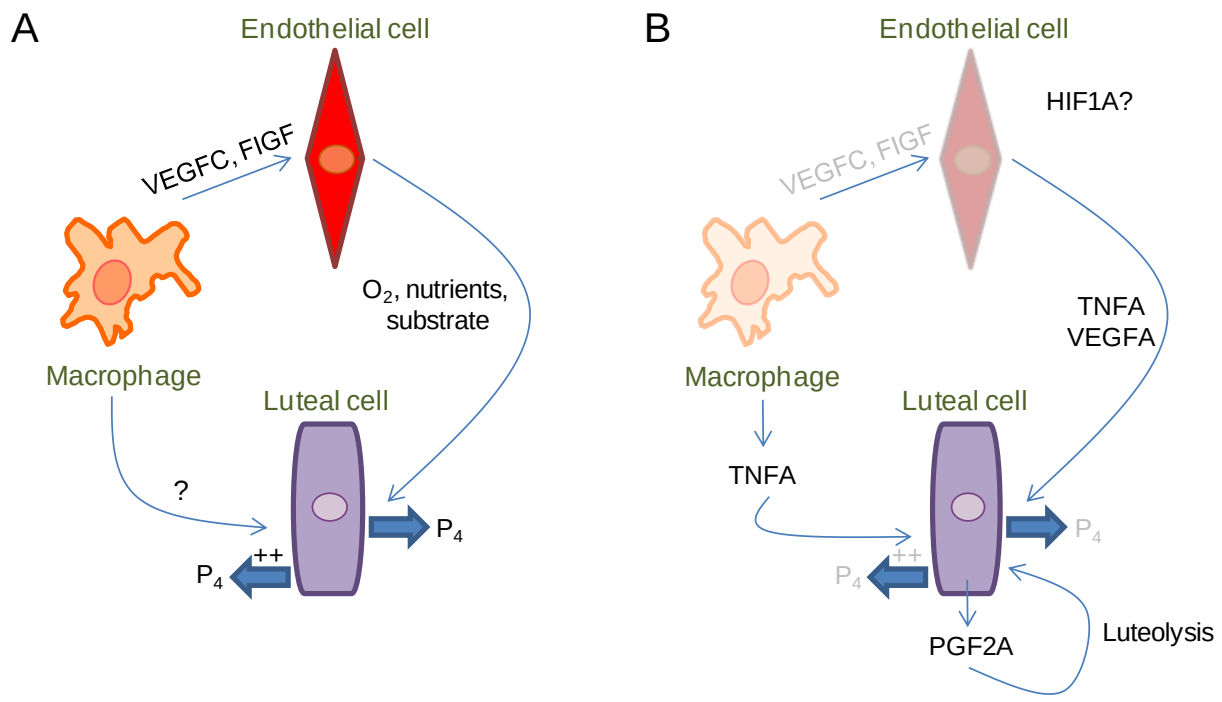



**Figure 6.2 A proposed mechanism by which macrophages provide luteal support**

A. In a normally functioning corpus luteum, macrophages may support the newly formed vasculature by providing trophic support in the form of VEGFC and FIGF. The vasculature in-turn delivers the required nutrient and substrate to enable luteal cells to synthesise and secrete $P_4$, which makes its way back into the vasculature and exerts its effects on the uterus. *In vitro* studies have shown that macrophages increase luteal cell $P_4$ output in the absence of vasculature, but the secretory factor responsible is unknown.
B. Following macrophage-depletion in the *Cd11b-Dtr* mouse, the trophic support provided to endothelial cells is significantly reduced. This may cause endothelial cell apoptosis, causing a hypoxic environment. This could possibly lead to HIF1A production, which would explain the increase in *Vegfa* mRNA expression, possibly coming from both the endothelial cells and luteal cells. TNFA may come from macrophages that are yet to succumb to the effects of DT, or from macrophages that have progressed into a state of post-apoptotic necrosis, or additionally it may come from the endothelial cells. TNFA is known to cause the release of luteal prostaglandins, in particular, luteolytic PGF2A, causing both functional and structural luteolysis.

# Appendices

**Table 3 Quantitative Real-time PCR data following normalisation to *Actb* or 18S housekeepers**

| | Time of sacrifice | Day 1.5 pc | | Day 2.5 pc | | Day 4.5 pc | |
|---|---|---|---|---|---|---|---|
| | DT treatment | DT 24 h | | DT 24 h | | DT 24 h | |
| | Other treatment | | | | | | |
| **Gene** | **Housekeeper** | **Wild-type** | ***Cd11b-Dtr*** | **Wild-type** | ***Cd11b-Dtr*** | **Wild-type** | ***Cd11b-Dtr*** |
| *Star* | *Actb* | | | | | 100.0 ± 9.6 | 127.5 ± 16.5 |
| | 18S | | | | | 100.0 ± 15.1 | 101.2 ± 12.0 |
| *Cyp11a1* | *Actb* | | | | | 100.0 ± 8.0 | 100.1 ± 9.3 |
| | 18S | | | | | 100.0 ± 17.5 | 82.4 ± 10.8 |
| *Hsd3b1* | *Actb* | | | | | 100.0 ± 9.0 | 71.5 ± 7.9 * |
| | 18S | | | | | 100.0 ± 22.8 | 50.1 ± 7.9 # |
| *Cyp17a1* | *Actb* | | | | | 100.0 ± 21.6 | 62.7 ± 11.4 |
| | 18S | | | | | 100.0 ± 25.2 | 39.1 ± 9.2 * |
| *Emr1* | *Actb* | | | | | 100.0 ± 8.7 | 43.2 ± 6.6 *** |
| | 18S | | | | | 100.0 ± 27.2 | 20.9 ± 3.9 ** |
| *Pecam1* | *Actb* | | | | | 100.0 ± 8.0 | 105.5 ± 9.7 |
| | 18S | | | | | 100.0 ± 27.9 | 56.7 ± 7.3 |
| *Vegfa* | *Actb* | 100.0 ± 5.8 | 361.5 ± 58.2 *** | 100.0 ± 9.2 | 373.5 ± 85.1 ** | 100.0 ± 11.1 | 318.0 ± 60.1 ** |
| | 18S | 100.0 ± 8.3 | 253.1 ± 20.9 ** | 100.0 ± 11.0 | 306.6 ± 86.0 * | 100.0 ± 24.5 | 169.5 ± 30.9 |
| *Vegfb* | *Actb* | 100.0 ± 9.2 | 99.1 ± 9.2 | 100.0 ± 6.5 | 108.1 ± 16.1 | 100.0 ± 17.0 | 98.4 ± 10.1 |
| | 18S | 100.0 ± 12.6 | 85.4 ± 14.4 | 100.0 ± 8.2 | 89.77 ± 19.02 | 100.0 ± 26.0 | 77.9 ± 14.4 |
| *Vegfc* | *Actb* | 100.0 ± 6.0 | 58.8 ± 8.7 ** | 100.0 ± 7.2 | 64.6 ± 10.0 * | 100.0 ± 13.4 | 78.1 ± 3.0 |
| | 18S | 100.0 ± 7.4 | 52.5 ± 11.7 * | 100.0 ± 9.8 | 50.2 ± 10.8 ** | 100.0 ± 25.5 | 58.1 ± 7.8 |
| *Figf* | *Actb* | 100.0 ± 7.7 | 69.8 ± 7.6 * | 100.0 ± 5.1 | 71.4 ± 11.9 * | 100.0 ± 15.1 | 84.1 ± 12.0 |
| | 18S | 100.0 ± 10.6 | 60.5 ± 10.8 | 100.0 ± 8.3 | 52.0 ± 11.0 ** | 100.0 ± 22.3 | 59.8 ± 13.6 |
| *Flt1* | *Actb* | 100.0 ± 10.7 | 54.3 ± 9.3 ** | 100.0 ± 8.1 | 46.6 ± 9.6 *** | 100.0 ± 10.5 | 77.9 ± 10.0 |
| | 18S | 100.0 ± 11.5 | 48.9 ± 12.0 * | 100.0 ± 10.1 | 38.1 ± 8.9 *** | 100.0 ± 22.5 | 45.9 ± 10.2 * |
| *Kdr* | *Actb* | 100.0 ± 9.4 | 67.6 ± 7.2 * | 100.0 ± 7.1 | 66.7 ± 9.7 * | 100.0 ± 8.5 | 90.7 ± 4.9 |
| | 18S | 100.0 ± 7.9 | 59.0 ± 9.9 * | 100.0 ± 8.8 | 54.7 ± 10.6 ** | 100.0 ± 21.8 | 57.2 ± 6.3 |

Whole ovaries were obtained from wild-type and *Cd11b-Dtr* mice on day 1.5, 2.5 or 4.5 pc following an i.p. injection of DT (25 ng/g) 24 h prior. Following RNA extraction and cDNA preparation, the expression of various genes was examined to determine how they were affected by macrophage depletion. Data are normalised to *Actb* or 18S mRNA expression and are presented as the mean ± SEM, with statistical analysis using an Unpaired t test or Mann Whitney U test. * indicates statistical significance from control; *$P < 0.05$. **$P < 0.01$. ***$P < 0.001$. n = 6-10 mice per group. DT; diphtheria toxin.

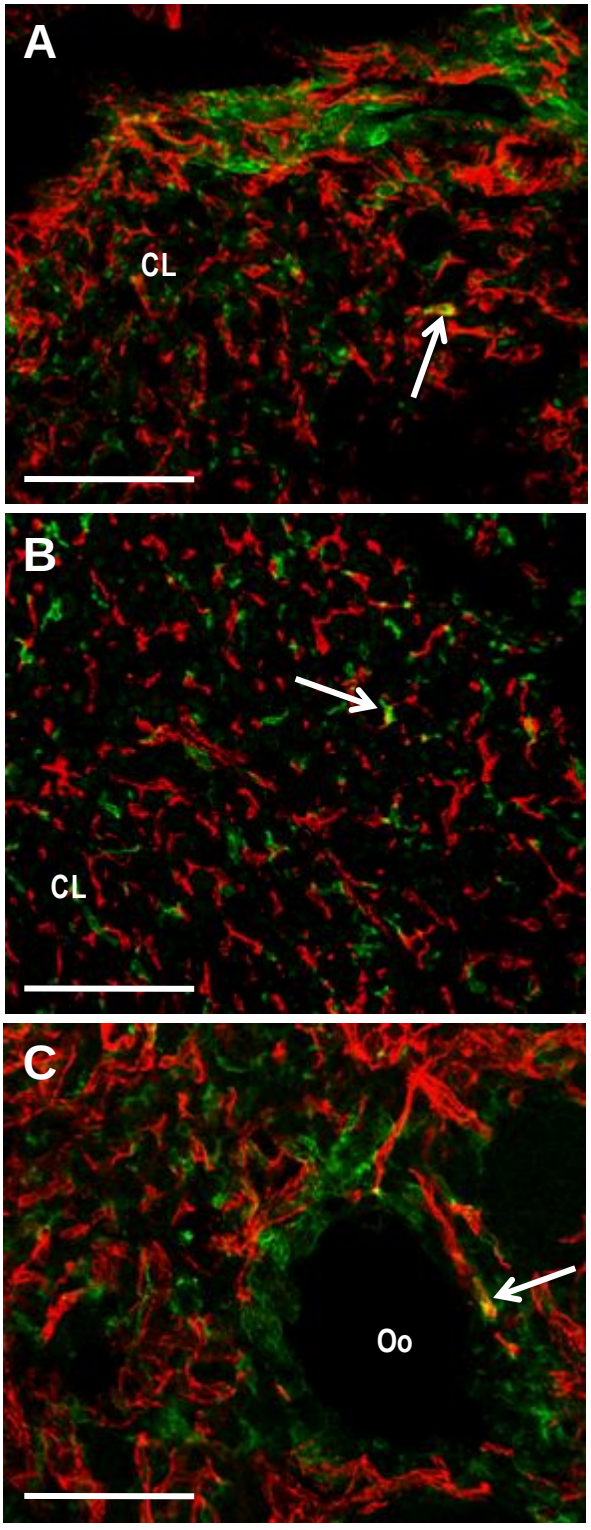



**Figure.1 Colocalisation of macrophage and endothelial cell markers on day 4.5 pc**

Representative photomicrographs of sections that were stained with the macrophage specific marker EMR1 (green) and the endothelial cell specific marker PECAM1 (red). Day 4.5 pc ovarian sections were prepared from wild-type mice. Colocalisation of EMR1 and PECAM1 (yellow) staining is evident in the CL (A and B) and surrounding follicles (C). Arrows; Co-localisation of EMR1 and PECAM. CL; corpus luteum. Oo; oocyte. Scale bar = 50 µm

# Chapter 7

# Bibliography

## 7.1 References


ABBONDANZO, S. J., CULLINAN, E. B., MCINTYRE, K., LABOW, M. A. & STEWART, C. L. 1996. Reproduction in mice lacking a functional type 1 IL-1 receptor. *Endocrinology,* 137**,** 3598-601.

ABRAHAMS, V. M., KIM, Y. M., STRASZEWSKI, S. L., ROMERO, R. & MOR, G. 2004. Macrophages and Apoptotic Cell Clearance During Pregnancy.

ACHEN, M. G., JELTSCH, M., KUKK, E., MÄKINEN, T., VITALI, A., WILKS, A. F., ALITALO, K. & STACKER, S. A. 1998. Vascular endothelial growth factor D (VEGF-D) is a ligand for the tyrosine kinases VEGF receptor 2 (Flk1) and VEGF receptor 3 (Flt4). *Proceedings of the National Academy of Sciences,* 95**,** 548-553.

ADAMS WALDORF, K. M. & NELSON, J. L. 2008. Autoimmune Disease During Pregnancy and the Microchimerism Legacy of Pregnancy*. *Immunological Investigations,* 37**,** 631-644.

ADASHI, E. Y., RESNICK, C. E., HURWITZ, A., RICCIARELLI, E., HERNANDEZ, E. R., ROBERTS, C. T., LEROITH, D. & ROSENFELD, R. 1991. Insulin-like growth factors: the ovarian connection *Human Reproduction,* 6**,** 1213-1219.

ADASHI, E. Y., RESNICK, C. E., PACKMAN, J. N., HURWITZ, A. & PAYNE, D. W. 1990. Cytokine-mediated regulation of ovarian function: tumor necrosis factor alpha inhibits gonadotropin-supported progesterone accumulation by differentiating and luteinized murine granulosa cells. *Am J Obstet Gynecol,* 162**,** 889-96; discussion 896-9.

AHARINEJAD, S., PAULUS, P., SIOUD, M., HOFMANN, M., ZINS, K., SCHAFER, R., STANLEY, E. R. & ABRAHAM, D. 2004. Colony-stimulating factor-1 blockade by antisense oligonucleotides and small interfering RNAs suppresses growth of human mammary tumor xenografts in mice. *Cancer Res,* 64**,** 5378-84.

ALBARRACIN, C. T., PARMER, T. G., DUAN, W. R., NELSON, S. E. & GIBORI, G. 1994. Identification of a major prolactin-regulated protein as 20 alpha-hydroxysteroid dehydrogenase: coordinate regulation of its activity, protein content, and messenger ribonucleic acid expression. *Endocrinology,* 134**,** 2453-60.

ALBERTINI, D. F. & ANDERSON, E. 1975. Structural modifications of lutein cell gap junctions during pregnancy in the rat and the mouse. *The Anatomical Record,* 181**,** 171-194.

ALITALO, K., TAMMELA, T. & PETROVA, T. V. 2005. Lymphangiogenesis in development and human disease. *Nature,* 438**,** 946-953.

ALLAIRE, A. D., BALLENGER, K. A., WELLS, S. R., MCMAHON, M. J. & LESSEY, B. A. 2000. Placental apoptosis in preeclampsia. *Obstet Gynecol,* 96**,** 271-6.

ALLEN, E. 1922. The oestrous cycle in the mouse. *American Journal of Anatomy,* 30**,** 297-371.

ALLEN, E., SMITH, G. M. & GARDNER, W. U. 1937. Accentuation of the growth effect of theelin on genital tissues of the ovariectomized mouse by arrest of mitosis with colchicine. *American Journal of Anatomy,* 61**,** 321-341.

ANDREANI, C. L., PAYNE, D. W., PACKMAN, J. N., RESNICK, C. E., HURWITZ, A. & ADASHI, E. Y. 1991. Cytokine-mediated regulation of ovarian function. Tumor necrosis factor alpha inhibits gonadotropin-supported ovarian androgen biosynthesis. *J Biol Chem,* 266**,** 6761-6.

APLIN, J. 1997. Adhesion molecules in implantation. *Rev Reprod,* 2**,** 84-93.

APLIN, J. D. 2000. The cell biological basis of human implantation. *Best Practice & Research Clinical Obstetrics & Gynaecology,* 14**,** 757-764.

APLIN, J. D. & KIMBER, S. J. 2004. Trophoblast-uterine interactions at implantation. *Reprod Biol Endocrinol,* 2**,** 48.

ARAKI, M., FUKUMATSU, Y., KATABUCHI, H., SHULTZ, L. D., TAKAHASHI, K. & OKAMURA, H. 1996. Follicular development and ovulation in macrophage colony-stimulating factor-deficient mice homozygous for the osteopetrosis (op) mutation. *Biol Reprod,* 54**,** 478-84.

ARCECI, R. J., PAMPFER, S. & POLLARD, J. W. 1992. Role and Expression of Colony Stimulating Factor-1 and Steel Factor Receptors and their Ligands during Pregnancy in the Mouse. *Reproduction, Fertility and Development,* 4**,** 619–632.

AUSTYN, J. M. & GORDON, S. 1981. F4/80, a monoclonal antibody directed specifically against the mouse macrophage. *European Journal of Immunology,* 11**,** 805-815.

BACHELOT, A., BEAUFARON, J., SERVEL, N., KEDZIA, C., MONGET, P., KELLY, P. A., GIBORI, G. & BINART, N. 2009. Prolactin independent rescue of mouse corpus luteum life span: identification of prolactin and luteinizing hormone target genes. *American Journal of Physiology - Endocrinology And Metabolism,* 297**,** E676-E684.

BAGAVANDOSS, P., WIGGINS, R. C., KUNKEL, S. L., REMICK, D. G. & KEYES, P. L. 1990. Tumor necrosis factor production and accumulation of inflammatory cells in the corpus luteum of pseudopregnancy and pregnancy in rabbits. *Biol Reprod,* 42**,** 367-376.

BANCHEREAU, J., BRIERE, F., CAUX, C., DAVOUST, J., LEBECQUE, S., LIU, Y.-J., PULENDRAN, B. & PALUCKA, K. 2000. Immunobiology of Dendritic Cells. *Annual Review of Immunology,* 18**,** 767-811.

BARLEON, B., SOZZANI, S., ZHOU, D., WEICH, H., MANTOVANI, A. & MARME, D. 1996. Migration of human monocytes in response to vascular endothelial growth factor (VEGF) is mediated via the VEGF receptor flt-1. *Blood,* 87**,** 3336-3343.

BARTOCCI, A., POLLARD, J. W. & STANLEY, E. R. 1986. Regulation of colony-stimulating factor 1 during pregnancy. *J Exp Med,* 164**,** 956-61.

BELLOMO, D., HEADRICK, J. P., SILINS, G. U., PATERSON, C. A., THOMAS, P. S., GARTSIDE, M., MOULD, A., CAHILL, M. M., TONKS, I. D., GRIMMOND, S. M., TOWNSON, S., WELLS, C., LITTLE, M., CUMMINGS, M. C., HAYWARD, N. K. & KAY, G. F. 2000. Mice lacking the vascular endothelial growth factor-B gene (Vegfb) have smaller hearts, dysfunctional coronary vasculature, and impaired recovery from cardiac ischemia. *Circ Res,* 86**,** E29-35.

BENYO, D. F. & PATE, J. L. 1992. Tumor necrosis factor-alpha alters bovine luteal cell synthetic capacity and viability. *Endocrinology,* 130**,** 854-60.

BEST, C. L., PUDNEY, J., WELCH, W. R., BURGER, N. & HILL, J. A. 1996. Localization and characterization of white blood cell populations within the human ovary throughout the menstrual cycle and menopause. *Human Reproduction,* 11**,** 790-797.

BIGSBY, R. M. 2002. Control of Growth and Differentiation of the Endometrium: The Role of Tissue Interactions. *Annals of the New York Academy of Sciences,* 955**,** 110-117.

BLACKWELL, P. M. & FRASER, I. S. 1981. Superficial lymphatics in the functional zone of normal human endometrium. *Microvascular Research,* 21**,** 142-152.

BOWEN, J. M., KEYES, P. L., WARREN, J. S. & TOWNSON, D. H. 1996. Prolactin-induced regression of the rat corpus luteum: expression of monocyte chemoattractant protein-1 and invasion of macrophages. *Biol Reprod,* 54**,** 1120-1127.

BRANNSTROM, M., BONELLO, N., WANG, L. J. & NORMAN, R. J. 1995. Effects of tumour necrosis factor alpha (TNF alpha) on ovulation in the rat ovary. *Reprod Fertil Dev,* 7**,** 67-73.

BRANNSTROM, M., GIESECKE, L., MOORE, I. C., VAN DEN HEUVEL, C. J. & ROBERTSON, S. A. 1994a. Leukocyte subpopulations in the rat corpus luteum during pregnancy and pseudopregnancy. *Biol Reprod,* 50**,** 1161-7.

BRANNSTROM, M., MAYRHOFER, G. & ROBERTSON, S. A. 1993. Localization of leukocyte subsets in the rat ovary during the periovulatory period. *Biol Reprod,* 48**,** 277-86.

BRANNSTROM, M. & NORMAN, R. J. 1993. Involvement of leukocytes and cytokines in the ovulatory process and corpus luteum function. *Hum Reprod,* 8**,** 1762-75.

BRÄNNSTRÖM, M., NORMAN, R. J., SEAMARK, R. F. & ROBERTSON, S. A. 1994. Rat ovary produces cytokines during ovulation. *Biol Reprod,* 50**,** 88-94.

BRANNSTROM, M., PASCOE, V., NORMAN, R. J. & MCCLURE, N. 1994b. Localization of leukocyte subsets in the follicle wall and in the corpus luteum throughout the human menstrual cycle. *Fertil Steril,* 61**,** 488-95.

BROMFIELD, J. J. 2006. *The physiological significance of insemination in programming pregnancy outcome.* Doctor of Philosophy, The University of Adelaide.

BRONSON, F. H., DAGG, C. P. & SNELL, G. D. 1966. Reproduction. *In:* GREEN, E. L. (ed.) *Biology of the Laboratory Mouse.* 2 ed. New York: McGraw-Hill Book Company.

BROWN, H. M., DUNNING, K. R., ROBKER, R. L., PRITCHARD, M. & RUSSELL, D. L. 2006. Requirement for ADAMTS-1 in extracellular matrix remodeling during ovarian folliculogenesis and lymphangiogenesis. *Developmental Biology,* 300**,** 699-709.

BROWN, H. M., ROBKER, R. L. & RUSSELL, D. L. 2010. Development and Hormonal Regulation of the Ovarian Lymphatic Vasculature. *Endocrinology,* 151**,** 5446-5455.

BUKOVSKY, A., PRESL, J., KRABEC, Z. & BEDNARIK, T. 1977. Ovarian function in adult rats treated with antithymocyte serum. *Experientia,* 33**,** 280-1.

BULMER, J. N., PACE, D. & RITSON, A. 1988. Immunoregulatory cells in human decidua: morphology, immunohistochemistry and function. *Reprod Nutr Dev,* 28**,** 1599-613.

BUSE, E., ZÖLLER, M. & VAN ESCH, E. 2008. The Macaque Ovary, with Special Reference to the Cynomolgus Macaque (Macaca fascicularis). *Toxicologic Pathology,* 36**,** 24S-66S.

CAILHIER, J. F., PARTOLINA, M., VUTHOORI, S., WU, S., KO, K., WATSON, S., SAVILL, J., HUGHES, J. & LANG, R. A. 2005. Conditional Macrophage Ablation Demonstrates That Resident Macrophages Initiate Acute Peritoneal Inflammation. *The Journal of Immunology,* 174**,** 2336-2342.

CAO, Y., LINDEN, P., FARNEBO, J., CAO, R., ERIKSSON, A., KUMAR, V., QI, J.-H., CLAESSON-WELSH, L. & ALITALO, K. 1998. Vascular endothelial growth factor C induces angiogenesis in vivo. *Proceedings of the National Academy of Sciences,* 95**,** 14389-14394.

CARP, H. J., SERR, D. M., MASHIACH, S. & NEBEL, L. 1984. Influence of insemination on the implantation of transferred rat blastocysts. *Gynecol Obstet Invest,* 18**,** 194-8.

CASTRO, A., CASTRO, O., TRONCOSO, J. L., KOHEN, P., SIMÓN, C., VEGA, M. & DEVOTO, L. 1998. Luteal leukocytes are modulators of the steroidogenic process of human mid-luteal cells. *Human Reproduction,* 13**,** 1584-1589.

CAVENDER, J. L. & MURDOCH, W. J. 1988. Morphological studies of the microcirculatory system of periovulatory ovine follicles. *Biol Reprod,* 39**,** 989-97.

CHAKRABORTY, I., DAS, S. K. & DEY, S. K. 1995. Differential expression of vascular endothelial growth factor and its receptor mRNAs in the mouse uterus around the time of implantation. *Journal of Endocrinology,* 147**,** 339-352.

CHANG, Z. L. 2009. Recent development of the mononuclear phagocyte system: in memory of Metchnikoff and Ehrlich on the 100th Anniversary of the 1908 Nobel Prize in Physiology or Medicine. *Biol Cell,* 101**,** 709-21.
CHEN, G. Y. & NUNEZ, G. 2010. Sterile inflammation: sensing and reacting to damage. *Nat Rev Immunol,* 10**,** 826-37.
CHOW, P. H., JIANG, H. Y., POON, H. K., LEE, K. H. & O, W. S. 2003. Embryos sired by males without accessory sex glands induce failure of uterine support: a study of VEGF, MMP and TGF expression in the golden hamster. *Anatomy and Embryology,* 206**,** 203-213.
CHUA, A. C., HODSON, L. J., MOLDENHAUER, L. M., ROBERTSON, S. A. & INGMAN, W. V. 2010. Dual roles for macrophages in ovarian cycle-associated development and remodelling of the mammary gland epithelium. *Development,* 137**,** 4229-38.
CLAUSS, M., GERLACH, M., GERLACH, H., BRETT, J., WANG, F., FAMILLETTI, P. C., PAN, Y. C., OLANDER, J. V., CONNOLLY, D. T. & STERN, D. 1990. Vascular permeability factor: a tumor-derived polypeptide that induces endothelial cell and monocyte procoagulant activity, and promotes monocyte migration. *J Exp Med,* 172**,** 1535-45.
COHEN, P. E., NISHIMURA, K., ZHU, L. & POLLARD, J. W. 1999. Macrophages: important accessory cells for reproductive function. *J Leukoc Biol,* 66**,** 765-72.
COHEN, P. E., ZHU, L., NISHIMURA, K. & POLLARD, J. W. 2002. Colony-stimulating factor 1 regulation of neuroendocrine pathways that control gonadal function in mice. *Endocrinology,* 143**,** 1413-22.
COHEN, P. E., ZHU, L. & POLLARD, J. W. 1997. Absence of colony stimulating factor-1 in osteopetrotic (csfmop/csfmop) mice disrupts estrous cycles and ovulation. *Biol Reprod,* 56**,** 110-8.
COLLETT, G. D. M. & CANFIELD, A. E. 2005. Angiogenesis and Pericytes in the Initiation of Ectopic Calcification. *Circ Res,* 96**,** 930-938.
CONN, G., SODERMAN, D. D., SCHAEFFER, M. T., WILE, M., HATCHER, V. B. & THOMAS, K. A. 1990. Purification of a glycoprotein vascular endothelial cell mitogen from a rat glioma-derived cell line. *Proceedings of the National Academy of Sciences,* 87**,** 1323-1327.
COOKE, P. S., BUCHANAN, D. L., YOUNG, P., SETIAWAN, T., BRODY, J., KORACH, K. S., TAYLOR, J., LUBAHN, D. B. & CUNHA, G. R. 1997. Stromal estrogen receptors mediate mitogenic effects of estradiol on uterine epithelium. *Proceedings of the National Academy of Sciences,* 94**,** 6535-6540.
COOMARASAMY, A., TRUCHANOWICZ, E. G. & RAI, R. 2011. Does first trimester progesterone prophylaxis increase the live birth rate in women with unexplained recurrent miscarriages? *BMJ,* 342.
CORPA, J. M. 2006. Ectopic pregnancy in animals and humans. *Reproduction,* 131**,** 631-640.
COULTAS, L., CHAWENGSAKSOPHAK, K. & ROSSANT, J. 2005. Endothelial cells and VEGF in vascular development. *Nature,* 438**,** 937-945.
CRANE-GODREAU, M. A. & WIRA, C. R. 2004. Effect of Escherichia coli and Lactobacillus rhamnosus on Macrophage Inflammatory Protein 3{alpha}, Tumor Necrosis Factor Alpha, and Transforming Growth Factor {beta} Release by Polarized Rat Uterine Epithelial Cells in Culture. *Infect. Immun.,* 72**,** 1866-1873.
CURRY, T. E., JR. & OSTEEN, K. G. 2003. The matrix metalloproteinase system: changes, regulation, and impact throughout the ovarian and uterine reproductive cycle. *Endocr Rev,* 24**,** 428-65.

CURSIEFEN, C., CHEN, L., BORGES, L. P., JACKSON, D., CAO, J., RADZIEJEWSKI, C., D'AMORE, P. A., DANA, M. R., WIEGAND, S. J. & STREILEIN, J. W. 2004. VEGF-A stimulates lymphangiogenesis and hemangiogenesis in inflammatory neovascularization via macrophage recruitment. *J Clin Invest,* 113**,** 1040-1050.

DAS, S. K., CHAKRABORTY, I., WANG, J., DEY, S. K. & HOFFMAN, L. H. 1997a. Expression of vascular endothelial growth factor (VEGF) and VEGF-receptor messenger ribonucleic acids in the peri-implantation rabbit uterus. *Biol Reprod,* 56**,** 1390-1399.

DAS, S. K., YANO, S., WANG, J., EDWARDS, D. R., NAGASE, H. & DEY, S. K. 1997b. Expression of matrix metalloproteinases and tissue inhibitors of metalloproteinases in the mouse uterus during the peri-implantation period. *Dev Genet,* 21**,** 44-54.

DAVIS, J. S. & RUEDA, B. R. 2002. The corpus luteum: an ovarian structure with maternal instincts and suicidal tendencies. *Front Biosci,* 7**,** d1949-78.

DE, M., CHOUDHURI, R. & WOOD, G. W. 1991. Determination of the number and distribution of macrophages, lymphocytes, and granulocytes in the mouse uterus from mating through implantation. *J Leukoc Biol,* 50**,** 252-62.

DE, M., SANFORD, T. & WOOD, G. W. 1993. Relationship between macrophage colony-stimulating factor production by uterine epithelial cells and accumulation and distribution of macrophages in the uterus of pregnant mice. *J Leukoc Biol,* 53**,** 240-8.

DE, M. & WOOD, G. W. 1990. Influence of oestrogen and progesterone on macrophage distribution in the mouse uterus. *Journal of Endocrinology,* 126**,** 417-NP.

DE PALMA, M. & NALDINI, L. 2011. Angiopoietin-2 TIEs up macrophages in tumor angiogenesis. *Clin Cancer Res,* 17**,** 5226-32.

DECHAUD, H., MAUDELONDE, T., DAURES, J. P., ROSSI, J. F. & HEDON, B. 1998. Evaluation of endometrial inflammation by quantification of macrophages, T lymphocytes, and interleukin-1 and -6 in human endometrium. *J Assist Reprod Genet,* 15**,** 612-8.

DIENER, K. R., MOLDENHAUER, L. M., LYONS, A. B., BROWN, M. P. & HAYBALL, J. D. 2008. Human Flt-3 ligand-mobilized dendritic cells require additional activation to drive effective immune responses. *Experimental hematology,* 36**,** 51-60.

DIFEDERICO, E., GENBACEV, O. & FISHER, S. J. 1999. Preeclampsia is associated with widespread apoptosis of placental cytotrophoblasts within the uterine wall. *Am J Pathol,* 155**,** 293-301.

DOMINO, S. E., ZHANG, L., GILLESPIE, P. J., SAUNDERS, T. L. & LOWE, J. B. 2001. Deficiency of reproductive tract alpha(1,2)fucosylated glycans and normal fertility in mice with targeted deletions of the FUT1 or FUT2 alpha(1,2)fucosyltransferase locus. *Mol Cell Biol,* 21**,** 8336-45.

DONOGHUE, J. F., LEDERMAN, F. L., SUSIL, B. J. & ROGERS, P. A. W. 2007. Lymphangiogensis of normal endometrium and endometrial adenocarcinoma. *Human Reproduction,* 22**,** 1705-1713.

DOUGLAS, D., NAKHUDA, G., SAUER, M. & ZIMMERMANN, R. 2005. Angiogenesis and Ovarian Function. *Journal für Fertilität und Reproduktion,* 15**,** 7-15.

DUFFIELD, J. S., FORBES, S. J., CONSTANDINOU, C. M., CLAY, S., PARTOLINA, M., VUTHOORI, S., WU, S., LANG, R. & IREDALE, J. P. 2005a. Selective depletion of macrophages reveals distinct, opposing roles during liver injury and repair. *J Clin Invest,* 115**,** 56-65.

DUFFIELD, J. S., TIPPING, P. G., KIPARI, T., CAILHIER, J. F., CLAY, S., LANG, R., BONVENTRE, J. V. & HUGHES, J. 2005b. Conditional ablation of macrophages halts progression of crescentic glomerulonephritis. *Am J Pathol,* 167**,** 1207-19.

ERLEBACHER, A., ZHANG, D., PARLOW, A. F. & GLIMCHER, L. H. 2004. Ovarian insufficiency and early pregnancy loss induced by activation of the innate immune system. *J Clin Invest,* 114**,** 39-48.

ESPEY, L. L. 1980. Ovulation as an inflammatory reaction--a hypothesis. *Biol Reprod,* 22**,** 73-106.

ESPEY, L. L. 1994. Current status of the hypothesis that mammalian ovulation is comparable to an inflammatory reaction. *Biol Reprod,* 50**,** 233-8.

FARR, K. L., MAU, V. J., SEAMARK, R. F. & ROBERTSON, S. A. 1995. Leukocyte populations in the mouse oviduct during early pregnancy. *Society of Reproductive Biology.* Melbourne, Australia.

FELIX, R., CECCHINI, M. G. & FLEISCH, H. 1990. Macrophage colony stimulating factor restores in vivo bone resorption in the op/op osteopetrotic mouse. *Endocrinology,* 127**,** 2592-4.

FERRARA, N. 2004. Vascular endothelial growth factor: basic science and clinical progress. *Endocr Rev,* 25**,** 581-611.

FERRARA, N., CHEN, H., DAVIS-SMYTH, T., GERBER, H.-P., NGUYEN, T.-N., PEERS, D., CHISHOLM, V., HILLAN, K. J. & SCHWALL, R. H. 1998. Vascular endothelial growth factor is essential for corpus luteum angiogenesis. *Nat Med,* 4**,** 336-340.

FERRARA, N. & DAVIS-SMYTH, T. 1997. The Biology of Vascular Endothelial Growth Factor. *Endocrine Reviews,* 18**,** 4-25.

FERRARA, N., GERBER, H. P. & LECOUTER, J. 2003. The biology of VEGF and its receptors. *Nat Med,* 9**,** 669-76.

FERRARA, N., HOUCK, K., JAKEMAN, L. & LEUNG, D. W. 1992. Molecular and biological properties of the vascular endothelial growth factor family of proteins. *Endocr Rev,* 13**,** 18-32.

FEST, S., ALDO, P. B., ABRAHAMS, V. M., VISINTIN, I., ALVERO, A., CHEN, R., CHAVEZ, S. L., ROMERO, R. & MOR, G. 2007. Trophoblast-macrophage interactions: a regulatory network for the protection of pregnancy. *Am J Reprod Immunol,* 57**,** 55-66.

FINDLAY, J. K., TSONIS, C. G., STAPLES, L. D. & CAHILL, R. N. P. 1986. Inhibin secretion by the sheep ovary. *Journal of Reproduction and Fertility,* 76**,** 751-761.

FINN, C. A. 1966. Endocrine control of endometrial sensitivity during the induction of the decidual cell reaction in the mouse. *J Endocrinol,* 36**,** 239-48.

FINN, C. A. 1985. Uterine responses to the corpus luteum. *In:* JEFFCOATE, S. L. (ed.) *The Luteal Phase.* Norwich, Great Britain: John Wiley and Sons Ltd.

FINN, C. A. & HINCHLIFFE, J. R. 1964. Reaction of the Mouse Uterus during Implantation and Deciduoma Formation as Demonstrated by Changes in the Distribution of Alkaline Phosphatase. *J Reprod Fertil,* 8**,** 331-8.

FINN, C. A. & KEEN, P. M. 1963. The Induction of Deciduomata in the Rat. *J Embryol Exp Morphol,* 11**,** 673-82.

FINN, C. A. & MARTIN, L. 1969. Hormone secretion during early pregnancy in the mouse. *J Endocrinol,* 45**,** 57-65.

FINN, C. A. & MARTIN, L. 1972. Endocrine Control of the Timing of Endometrial Sensitivity to a Decidual Stimulus.

FINN, C. A., POPE, M. D. & MILLIGAN, S. R. 1989. A study of the early morphological changes initiated in the uterine luminal epithelium by substances (oil and carrageenan) which induce the decidual cell reaction in mice. *J Reprod Fertil,* 86**,** 619-26.

FLEMING, T., FLEMING, M. & MALEK, T. 1993. Selective expression of Ly-6G on myeloid lineage cells in mouse bone marrow. RB6-8C5 mAb to granulocyte-differentiation antigen (Gr-1) detects members of the Ly-6 family. *The Journal of Immunology,* 151**,** 2399-2408.

FORMBY, B. 1995. Immunologic response in pregnancy. Its role in endocrine disorders of pregnancy and influence on the course of maternal autoimmune diseases. *Endocrinol Metab Clin North Am,* 24**,** 187-205.

FOSTER, M. H. & KELLEY, V. R. 1999. Lupus nephritis: update on pathogenesis and disease mechanisms. *Semin Nephrol,* 19**,** 173-81.

FRASER, H. M. 2006. Regulation of the ovarian follicular vasculature. *Reprod Biol Endocrinol,* 4**,** 18.

FRASER, H. M., DICKSON, S. E., LUNN, S. F., WULFF, C., MORRIS, K. D., CARROLL, V. A. & BICKNELL, R. 2000. Suppression of Luteal Angiogenesis in the Primate after Neutralization of Vascular Endothelial Growth Factor. *Endocrinology,* 141**,** 995-1000.

FRASER, H. M. & LUNN, S. F. 2000. Angiogenesis and its control in the female reproductive system. *Br Med Bull,* 56**,** 787-97.

FRASER, H. M., WILSON, H., WULFF, C., RUDGE, J. S. & WIEGAND, S. J. 2006. Administration of vascular endothelial growth factor Trap during the 'post-angiogenic' period of the luteal phase causes rapid functional luteolysis and selective endothelial cell death in the marmoset. *Reproduction,* 132**,** 589-600.

FUKUMATSU, Y., KATABUCHI, H., NAITO, M., TAKEYA, M., TAKAHASHI, K. & OKAMURA, H. 1992. Effect of macrophages on proliferation of granulosa cells in the ovary in rats. *J Reprod Fertil,* 96**,** 241-9.

GÅFVELS, M., BJURULF, E. & SELSTAM, G. 1992. Prolactin stimulates the expression of luteinizing hormone/chorionic gonadotropin receptor messenger ribonucleic acid in the rat corpus luteum and rescues early pregnancy from bromocriptine-induced abortion. *Biol Reprod,* 47**,** 534-540.

GANGNUSS, S., SUTTON-MCDOWALL, M. L., ROBERTSON, S. A. & ARMSTRONG, D. T. 2004. Seminal Plasma Regulates Corpora Lutea Macrophage Populations During Early Pregnancy in Mice. *Biol Reprod,* 71**,** 1135-1141.

GARDNER, D. K. & LANE, M. 1997. Culture and selection of viable blastocysts: a feasible proposition for human IVF? *Hum Reprod Update,* 3**,** 367-82.

GARDNER, D. K. & LANE, M. 2004. *A Laboratory Guide to the Mammalian Embryo,* New York, Oxford University Press.

GARDNER, L. & MOFFETT, A. 2003. Dendritic Cells in the Human Decidua. *Biol Reprod,* 69**,** 1438-1446.

GASSON, J. 1991. Molecular physiology of granulocyte-macrophage colony-stimulating factor. *Blood,* 77**,** 1131-1145.

GAYTAN, F., MORALES, C., BELLIDO, C., AGUILAR, E. & SANCHEZ-CRIADO, J. E. 1998. Ovarian follicle macrophages: is follicular atresia in the immature rat a macrophage-mediated event? *Biol Reprod,* 58**,** 52-9.

GAYTÁN, F., MORALES, C., GARCÍA-PARDO, L., REYMUNDO, C., BELLIDO, C. & SÁNCHEZ-CRIADO, J. E. 1998. Macrophages, Cell Proliferation, and Cell Death in the Human Menstrual Corpus Luteum. *Biol Reprod,* 59**,** 417-425.

GEVA, E. & JAFFE, R. B. 2000. Role of vascular endothelial growth factor in ovarian physiology and pathology. *Fertility and sterility,* 74**,** 429-438.

GLENNY, A. T. & ALLEN, K. 1922. The Action of Diphtheria Toxin on Mice. *J Hyg (Lond),* 21**,** 96-9.

GOETZL, E., BANDA, M. & LEPPERT, D. 1996. Commentary: Matrix metalloproteinases in immunity. *The Journal of Immunology,* 156**,** 1-4.
GORDON, S. 2003. Alternative activation of macrophages. *Nat Rev Immunol,* 3**,** 23-35.
GORDON, S. 2007. The macrophage: past, present and future. *Eur J Immunol,* 37 Suppl 1**,** S9-17.
GOSPODAROWICZ, D., ABRAHAM, J. A. & SCHILLING, J. 1989. Isolation and characterization of a vascular endothelial cell mitogen produced by pituitary-derived folliculo stellate cells. *Proceedings of the National Academy of Sciences,* 86**,** 7311-7315.
GOTTSCHALL, P. E., KATSUURA, G. & ARIMURA, A. 1989. Interleukin-1 suppresses follicle-stimulating hormone-induced estradiol secretion from cultured ovarian granulosa cells. *J Reprod Immunol,* 15**,** 281-90.
GOTTSCHALL, P. E., KATSUURA, G., HOFFMANN, S. T. & ARIMURA, A. 1988. Interleukin 1: an inhibitor of luteinizing hormone receptor formation in cultured rat granulosa cells. *FASEB J,* 2**,** 2492-6.
GRANATA, F., FRATTINI, A., LOFFREDO, S., STAIANO, R. I., PETRAROLI, A., RIBATTI, D., OSLUND, R., GELB, M. H., LAMBEAU, G., MARONE, G. & TRIGGIANI, M. 2010. Production of Vascular Endothelial Growth Factors from Human Lung Macrophages Induced by Group IIA and Group X Secreted Phospholipases A2. *The Journal of Immunology,* 184**,** 5232-5241.
GUERIN, L. R. 2010. *Seminal Fluid and Cytokine Control of Regulatory T-Cells in Murine Pregnancy.* Doctor of Philosophy, University of Adelaide.
GUERIN, L. R., MOLDENHAUER, L. M., PRINS, J. R., BROMFIELD, J. J., HAYBALL, J. D. & ROBERTSON, S. A. 2011. Seminal Fluid Regulates Accumulation of FOXP3+ Regulatory T Cells in the Preimplantation Mouse Uterus Through Expanding the FOXP3+ Cell Pool and CCL19-Mediated Recruitment. *Biol Reprod*.
GUNNET, J. W. & FREEMAN, M. E. 1983. The Mating-induced Release of Prolactin: A Unique Neuroendrocine Response. *Endocrine Reviews,* 4**,** 44-61.
HALDER, J. B., ZHAO, X., SOKER, S., PARIA, B. C., KLAGSBRUN, M., DAS, S. K. & DEY, S. K. 2000. Differential expression of VEGF isoforms and VEGF164-specific receptor neuropilin-1 in the mouse uterus suggests a role for VEGF164 in vascular permeability and angiogenesis during implantation. *genesis,* 26**,** 213-224.
HALME, J., HAMMOND, M. G., SYROP, C. H. & TALBERT, L. M. 1985. Peritoneal macrophages modulate human granulosa-luteal cell progesterone production. *J Clin Endocrinol Metab,* 61**,** 912-6.
HANNA, J., GOLDMAN-WOHL, D., HAMANI, Y., AVRAHAM, I., GREENFIELD, C., NATANSON-YARON, S., PRUS, D., COHEN-DANIEL, L., ARNON, T. I., MANASTER, I., GAZIT, R., YUTKIN, V., BENHARROCH, D., PORGADOR, A., KESHET, E., YAGEL, S. & MANDELBOIM, O. 2006. Decidual NK cells regulate key developmental processes at the human fetal-maternal interface. *Nat Med,* 12**,** 1065-1074.
HARRIS, L. K. & APLIN, J. D. 2007. Vascular Remodeling and Extracellular Matrix Breakdown in the Uterine Spiral Arteries During Pregnancy. *Reproductive Sciences,* 14**,** 28-34.
HEHNKE-VAGNONI, K. E., CLARK, C. L., TAYLOR, M. J. & FORD, S. P. 1995. Presence and localization of tumor necrosis factor alpha in the corpus luteum of nonpregnant and pregnant pigs. *Biol Reprod,* 53**,** 1339-1344.

HEIN, W. R., SHELTON, J. N., SIMPSON-MORGAN, M. W., SEAMARK, R. F. & MORRIS, B. 1988. Flow and composition of lymph from the ovary and uterus of cows during pregnancy. *Journal of Reproduction and Fertility,* 83**,** 309-323.

HELLBERG, P., THOMSEN, P., JANSON, P. O. & BRÄNNSTRÖM, M. 1991. Leukocyte supplementation increases the luteinizing hormone-induced ovulation rate in the in vitro-perfused rat ovary. *Biol Reprod,* 44**,** 791-797.

HENSON, P. M., BRATTON, D. L. & FADOK, V. A. 2001. The phosphatidylserine receptor: a crucial molecular switch? *Nat Rev Mol Cell Biol,* 2**,** 627-633.

HERMISTON, M. L., XU, Z. & WEISS, A. 2003. CD45: a critical regulator of signaling thresholds in immune cells. *Annu Rev Immunol,* 21**,** 107-37.

HERYANTO, B., GIRLING, J. E. & ROGERS, P. A. W. 2004. Intravascular neutrophils partially mediate the endometrial endothelial cell proliferative response to oestrogen in ovariectomised mice. *Reproduction,* 127**,** 613-620.

HERYANTO, B., LIPSON, K. & ROGERS, P. 2003. Effect of angiogenesis inhibitors on oestrogen-mediated endometrial endothelial cell proliferation in the ovariectomized mouse. *Reproduction,* 125**,** 337-346.

HESTDAL, K., RUSCETTI, F., IHLE, J., JACOBSEN, S., DUBOIS, C., KOPP, W., LONGO, D. & KELLER, J. 1991. Characterization and regulation of RB6-8C5 antigen expression on murine bone marrow cells. *The Journal of Immunology,* 147**,** 22-28.

HINNEY, B., HENZE, C., KUHN, W. & WUTTKE, W. 1996. The corpus luteum insufficiency: a multifactorial disease. *J Clin Endocrinol Metab,* 81**,** 565-70.

HOGAN, B., CONSTANTINI, F. & LACY, E. 1986. *Manipulating the Mouse Embryo,* Cold Spring Harbor, NY, Cold Spring Harbor Lab.

HOSANG, K., KNOKE, I., KLAUDINY, J., WEMPE, F., WUTTKE, W. & SCHEIT, K. H. 1994. Porcine Luteal Cells Express Monocyte Chemoattractant Protein-2 (MCP-2): Analysis by cDNA Cloning and Northern Analysis. *Biochemical and Biophysical Research Communications,* 205**,** 148-153.

HOUSER, B. L., TILBURGS, T., HILL, J., NICOTRA, M. L. & STROMINGER, J. L. 2011. Two Unique Human Decidual Macrophage Populations. *The Journal of Immunology,* 186**,** 2633-2642.

HUANG, H., HE, J., YUAN, Y., AOYAGI, E., TAKENAKA, H., ITAGAKI, T., SANNOMIYA, K., TAMAKI, K., HARADA, N., SHONO, M., SHIMIZU, I. & TAKAYAMA, T. 2008. Opposing effects of estradiol and progesterone on the oxidative stress-induced production of chemokine and proinflammatory cytokines in murine peritoneal macrophages. *J Med Invest,* 55**,** 133-41.

HUBBARD, N. E., LIM, D., MUKUTMONI, M., CAI, A. & ERICKSON, K. L. 2005. Expression and regulation of murine macrophage angiopoietin-2. *Cell Immunol,* 234**,** 102-9.

HUDSON, S. N., SEAMARK, R. F. & ROBERTSON, S. A. 1999. The effect of restricted nutrition on uterine macrophage populations in mice. *J Reprod Immunol,* 45**,** 31-48.

HUME, D. A., HALPIN, D., CHARLTON, H. & GORDON, S. 1984. The mononuclear phagocyte system of the mouse defined by immunohistochemical localization of antigen F4/80: macrophages of endocrine organs. *Proc Natl Acad Sci U S A,* 81**,** 4174-7.

HUME, D. A., ROBINSON, A. P., MACPHERSON, G. G. & GORDON, S. 1983. The mononuclear phagocyte system of the mouse defined by immunohistochemical localization of antigen F4/80. Relationship between macrophages, Langerhans cells, reticular cells, and dendritic cells in lymphoid and hematopoietic organs.

HUME, D. A., ROSS, I. L., HIMES, S. R., SASMONO, R. T., WELLS, C. A. & RAVASI, T. 2002. The mononuclear phagocyte system revisited. *J Leukoc Biol,* 72**,** 621-627.
HUNT, J. S. 1989. Cytokine networks in the uteroplacental unit: Macrophages as pivotal regulatory cells. *J Reprod Immunol,* 16**,** 1-17.
HUNT, J. S. 1992. Immunobiology of pregnancy. *Current Opinion in Immunology,* 4**,** 591-596.
HUNT, J. S. & ROBERTSON, S. A. 1996. Uterine macrophages and environmental programming for pregnancy success. *J Reprod Immunol,* 32**,** 1-25.
HURWITZ, A., RICCIARELLI, E., BOTERO, L., ROHAN, R. M., HERNANDEZ, E. R. & ADASHI, E. Y. 1991. ENDOCRINE- AND AUTOCRINE-MEDIATED REGULATION OF RAT OVARIAN (THECA-INTERSTITIAL) INTKRLEUKIN-β GENE EXPRESSION: GONADOTROPIN-DEPENDENT PREOVIILATORY ACQUISITION. *Endocrinology,* 129**,** 3427-3429.
ICHIKAWA, M., SUGITA, M., TAKAHASHI, M., SATOMI, M., TAKESHITA, T., ARAKI, T. & TAKAHASHI, H. 2003. Breast milk macrophages spontaneously produce granulocyte–macrophage colony-stimulating factor and differentiate into dendritic cells in the presence of exogenous interleukin-4 alone. *Immunology,* 108**,** 189-195.
IDETA, A., SAKAI, S., NAKAMURA, Y., URAKAWA, M., HAYAMA, K., TSUCHIYA, K., FUJIWARA, H. & AOYAGI, Y. 2010. Administration of peripheral blood mononuclear cells into the uterine horn to improve pregnancy rate following bovine embryo transfer. *Anim Reprod Sci,* 117**,** 18-23.
INABA, K., INABA, M., ROMANI, N., AYA, H., DEGUCHI, M., IKEHARA, S., MURAMATSU, S. & STEINMAN, R. M. 1992. Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J Exp Med,* 176**,** 1693-702.
INGMAN, W. V. & ROBERTSON, S. A. 2002. Defining the actions of transforming growth factor beta in reproduction. *BioEssays,* 24**,** 904-914.
INGMAN, W. V., ROBKER, R. L., WOITTIEZ, K. & ROBERTSON, S. A. 2006. Null Mutation in Transforming Growth Factor β1 Disrupts Ovarian Function and Causes Oocyte Incompetence and Early Embryo Arrest. *Endocrinology,* 147**,** 835-845.
JACOBS, A. L., SEHGAL, P. B., JULIAN, J. & CARSON, D. D. 1992. Secretion and hormonal regulation of interleukin-6 production by mouse uterine stromal and polarized epithelial cells cultured in vitro. *Endocrinology,* 131**,** 1037-46.
JAFFE, R. B. 1999. Neuroendocrine-metabolic regulation of pregnancy. *In:* YEN, S. S. C., JAFFE, R. B. & BALRBIERI, R. L. (eds.) *Reproductive Endocrinology.* 4 ed. Philidelphia, Pennsylvania: W.B. Saunders Company.
JASPER, M. J. 1998. *Paracrine regulation of ovarian function by Granulocyte-Macrophage colony-Stimulating-Factor (GM-CSF) and Colony Stimulating Factor-1 (CSF-1).* Doctor of Philosophy, The University of Adelaide.
JASPER, M. J., BRANNSTROM, M., OLOFSSON, J. I., PETRUCCO, O. M., MASON, H., ROBERTSON, S. A. & NORMAN, R. J. 1996. Granulocyte-macrophage colony-stimulating factor: presence in human follicular fluid, protein secretion and mRNA expression by ovarian cells. *Mol Hum Reprod,* 2**,** 555-62.
JASPER, M. J., BROMFIELD, J. J., SKINNER, S. A. & ROBERTSON, S. A. 2005. LIF expression is induced in the mouse oviduct following activation by seminal factors. *Society for Reproductive Biology.* Perth, Australia.
JASPER, M. J., CARE, A. S., SULLIVAN, B., INGMAN, W. V., APLIN, J. D. & ROBERTSON, S. A. 2011. Macrophage-derived LIF and IL1B regulate

alpha(1,2)fucosyltransferase 2 (Fut2) expression in mouse uterine epithelial cells during early pregnancy. *Biol Reprod,* 84**,** 179-88.
JASPER, M. J., ROBERTSON, S. A., VAN DER HOEK, K. H., BONELLO, N., BRANNSTROM, M. & NORMAN, R. J. 2000. Characterization of ovarian function in granulocyte-macrophage colony-stimulating factor-deficient mice. *Biol Reprod,* 62**,** 704-13.
JASPER, M. J., TREMELLEN, K. P. & ROBERTSON, S. A. 2007. Reduced expression of IL-6 and IL-1alpha mRNAs in secretory phase endometrium of women with recurrent miscarriage. *J Reprod Immunol,* 73**,** 74-84.
JOACHIM, R., ZENCLUSSEN, A. C., POLGAR, B., DOUGLAS, A. J., FEST, S., KNACKSTEDT, M., KLAPP, B. F. & ARCK, P. C. 2003. The progesterone derivative dydrogesterone abrogates murine stress-triggered abortion by inducing a Th2 biased local immune response. *Steroids,* 68**,** 931-40.
JOHNSON, M. A. & EVERITT, B. J. 2007a. Ovarian function in the adult. *Essential Reproduction.* 6 ed. Massachusetts, USA. : Blackwell Publishing.
JOHNSON, M. A. & EVERITT, B. J. 2007b. Regulation of gonadal function. *Essential Reproduction.* 6 ed. Massachusetts, USA.: Blackwell Publishing.
JOUKOV, V., SORSA, T., KUMAR, V., JELTSCH, M., CLAESSON-WELSH, L., CAO, Y., SAKSELA, O., KALKKINEN, N. & ALITALO, K. 1997. Proteolytic processing regulates receptor specificity and activity of VEGF-C. *EMBO J,* 16**,** 3898-3911.
KABAWAT, S. E., MOSTOUFI-ZADEH, M., DRISCOLL, S. G. & BHAN, A. K. 1985. Implantation site in normal pregnancy. A study with monoclonal antibodies. *Am J Pathol,* 118**,** 76-84.
KACHKACHE, M., ACKER, G. M., CHAOUAT, G., NOUN, A. & GARABEDIAN, M. 1991. Hormonal and local factors control the immunohistochemical distribution of immunocytes in the rat uterus before conceptus implantation: effects of ovariectomy, fallopian tube section, and injection. *Biol Reprod,* 45**,** 860-8.
KAIPIA, A., CHUN, S. Y., EISENHAUER, K. & HSUEH, A. J. 1996. Tumor necrosis factor-alpha and its second messenger, ceramide, stimulate apoptosis in cultured ovarian follicles. *Endocrinology,* 137**,** 4864-70.
KASSON, B. G. & GOROSPE, W. C. 1989. Effects of interleukins 1, 2 and 3 on follicle-stimulating hormone-induced differentiation of rat granulosa cells. *Molecular and Cellular Endocrinology,* 62**,** 103-111.
KEENIHAN, S. N. & ROBERTSON, S. A. 2004. Diversity in phenotype and steroid hormone dependence in dendritic cells and macrophages in the mouse uterus. *Biol Reprod,* 70**,** 1562-72.
KENDALL, R. L. & THOMAS, K. A. 1993. Inhibition of vascular endothelial cell growth factor activity by an endogenously encoded soluble receptor. *Proc Natl Acad Sci U S A,* 90**,** 10705-9.
KERJASCHKI, D. 2005. The crucial role of macrophages in lymphangiogenesis. *J Clin Invest,* 115**,** 2316-9.
KIM, J. H., SEIBEL, M. M., MACLAUGHLIN, D. T., DONAHOE, P. K., RANSIL, B. J., HAMETZ, P. A. & RICHARDS, C. J. 1992. The inhibitory effects of müllerian-inhibiting substance on epidermal growth factor induced proliferation and progesterone production of human granulosa-luteal cells. *Journal of Clinical Endocrinology & Metabolism,* 75**,** 911-7.
KIM, Y. M., CHAIWORAPONGSA, T., GOMEZ, R., BUJOLD, E., YOON, B. H., ROTMENSCH, S., THALER, H. T. & ROMERO, R. 2002. Failure of physiologic

transformation of the spiral arteries in the placental bed in preterm premature rupture of membranes. *American journal of obstetrics and gynecology,* 187**,** 1137-1142.
KIRSCH, T. M., FRIEDMAN, A. C., VOGEL, R. L. & FLICKINGER, G. L. 1981. Macrophages in corpora lutea of mice: characterization and effects on steroid secretion. *Biol Reprod,* 25**,** 629-38.
KLENTZERIS, L. D., BULMER, J. N., WARREN, M. A., MORRISON, L., LI, T. C. & COOKE, I. D. 1994. Lymphoid tissue in the endometrium of women with unexplained infertility: morphometric and immunohistochemical aspects. *Human Reproduction,* 9**,** 646-652.
KNOBIL, E. & NEILL, J. D. (eds.) 2006. *Knobil and Neill's physiology of reproduction,* St. Louis, MO: Elsevier Academic Press.
KODAMA, H., YAMASAKI, A., NOSE, M., NIIDA, S., OHGAME, Y., ABE, M., KUMEGAWA, M. & SUDA, T. 1991. Congenital osteoclast deficiency in osteopetrotic (op/op) mice is cured by injections of macrophage colony-stimulating factor. *J Exp Med,* 173**,** 269-72.
KOSAKA, K., FUJIWARA, H., TATSUMI, K., YOSHIOKA, S., HIGUCHI, T., SATO, Y., NAKAYAMA, T. & FUJII, S. 2003. Human peripheral blood mononuclear cells enhance cell–cell interaction between human endometrial epithelial cells and BeWo-cell spheroids. *Human Reproduction,* 18**,** 19-25.
KRITIKOU, E. A., SHARKEY, A., ABELL, K., CAME, P. J., ANDERSON, E., CLARKSON, R. W. E. & WATSON, C. J. 2003. A dual, non-redundant, role for LIF as a regulator of development and STAT3-mediated cell death in mammary gland. *Development,* 130**,** 3459-3468.
KURITA, T., YOUNG, P., BRODY, J. R., LYDON, J. P., O'MALLEY, B. W. & CUNHA, G. R. 1998a. Stromal Progesterone Receptors Mediate the Inhibitory Effects of Progesterone on Estrogen-Induced Uterine Epithelial Cell Deoxyribonucleic Acid Synthesis. *Endocrinology,* 139**,** 4708-4713.
KURITA, T., YOUNG, P., BRODY, J. R., LYDON, J. P., O'MALLEY, B. W. & CUNHA, G. R. 1998b. Stromal Progesterone Receptors Mediate the Inhibitory Effects of Progesterone on Estrogen-Induced Uterine Epithelial Cell Deoxyribonucleic Acid Synthesis. *Endocrinology,* 139**,** 4708-4713.
LANDGREN, E., SCHILLER, P., CAO, Y. & CLAESSON-WELSH, L. 1998. Placenta growth factor stimulates MAP kinase and mitogenicity but not phospholipase C-gamma and migration of endothelial cells expressing Flt 1. *Oncogene,* 16**,** 359-67.
LASKIN, D. L., WEINBERGER, B. & LASKIN, J. D. 2001. Functional heterogeneity in liver and lung macrophages. *J Leukoc Biol,* 70**,** 163-70.
LEDBETTER, J. A. & HERZENBERG, L. A. 1979. Xenogeneic Monoclonal Antibodies to Mouse Lymphoid Differentiation Antigens*. *Immunological Reviews,* 47**,** 63-90.
LEI, Z. M., CHEGINI, N. & RAO, C. V. 1991. Quantitative cell composition of human and bovine corpora lutea from various reproductive states. *Biol Reprod,* 44**,** 1148-1156.
LESSEY, B. A., ILESANMI, A. O., LESSEY, M. A., RIBEN, M., HARRIS, J. E. & CHWALISZ, K. 1996. Luminal and glandular endometrial epithelium express integrins differentially throughout the menstrual cycle: implications for implantation, contraception, and infertility. *Am J Reprod Immunol,* 35**,** 195-204.
LESSIN, D. L., HUNT, J. S., KING, C. R. & WOOD, G. W. 1988. Antigen expression by cells near the maternal-fetal interface. *Am J Reprod Immunol Microbiol,* 16**,** 1-7.
LEUNG, P. C. K. & ADASHI, E. Y. (eds.) 2004. *The Ovary,* California, USA: Elsevier Academic Press.

LI, X. Q., ITOH, M., YANO, A., XIE, Q., MIYAMOTO, K. & TAKEUCHI, Y. 1998. Distribution of F4/80-positive cells in developing ovaries in the mouse. *Arch Histol Cytol,* 61**,** 353-60.

LINDNER, H. R., SASS, M. B. & MORRIS, B. 1964. Steroids in the ovarian lymph and blood of conscious ewes. *Journal of Endocrinology,* 30**,** 361-376.

LUMENG, C. N., BODZIN, J. L. & SALTIEL, A. R. 2007. Obesity induces a phenotypic switch in adipose tissue macrophage polarization. *The Journal of Clinical Investigation,* 117**,** 175-184.

MA, W., TAN, J., MATSUMOTO, H., ROBERT, B., ABRAHAMSON, D. R., DAS, S. K. & DEY, S. K. 2001. Adult Tissue Angiogenesis: Evidence for Negative Regulation by Estrogen in the Uterus. *Mol Endocrinol,* 15**,** 1983-1992.

MANTOVANI, A. 2006. Macrophage diversity and polarization: in vivo veritas. *Blood,* 108**,** 408-409.

MARTIN, L. & FINN, C. A. 1969. Duration of progesterone treatment required for a stromal response to oestradiol-17-beta in the uterus of the mouse. *J Endocrinol,* 44**,** 279-80.

MARTIN, L., FINN, C. A. & TRINDER, G. 1973. Hypertrophy and hyperplasia in the mouse uterus after oestrogen treatment: an autoradiographic study. *J Endocrinol,* 56**,** 133-44.

MARUYAMA, K., II, M., CURSIEFEN, C., JACKSON, D. G., KEINO, H., TOMITA, M., VAN ROOIJEN, N., TAKENAKA, H., D'AMORE, P. A., STEIN-STREILEIN, J., LOSORDO, D. W. & STREILEIN, J. W. 2005. Inflammation-induced lymphangiogenesis in the cornea arises from CD11b-positive macrophages. *J Clin Invest,* 115**,** 2363-72.

MATSUMOTO, H. & SATO, E. 2006. Uterine angiogenesis during implantation and decidualization in mice. *Reproductive Medicine and Biology,* 5**,** 81-86.

MCCORMICK, T. S., STEVENS, S. R. & KANG, K. 2000. Macrophages and cutaneous inflammation. *Nat Biotech,* 18**,** 25-26.

MCGARRY, M. & STEWART, C. 1991. Murine eosinophil granulocytes bind the murine macrophage-monocyte specific monoclonal antibody F4/80. *Journal of Leukocyte Biology,* 50**,** 471-478.

MESEGUER, M., APLIN, J. D., CABALLERO-CAMPO, P., O'CONNOR, J. E., MARTÍN, J. C., REMOHÍ, J., PELLICER, A. & SIMÓN, C. 2001. Human Endometrial Mucin MUC1 Is Up-Regulated by Progesterone and Down-Regulated In Vitro by the Human Blastocyst. *Biol Reprod,* 64**,** 590-601.

METLAY, J. P., WITMER-PACK, M. D., AGGER, R., CROWLEY, M. T., LAWLESS, D. & STEINMAN, R. M. 1990. The distinct leukocyte integrins of mouse spleen dendritic cells as identified with new hamster monoclonal antibodies. *The Journal of Experimental Medicine,* 171**,** 1753-1771.

MEYER, G. T. & MCGEACHIE, J. K. 1988. Angiogenesis in the developing corpus luteum of pregnant rats: a stereologic and autoradiographic study. *Anat Rec,* 222**,** 18-25.

MIESCHER, G. C., SCHREYER, M. & MACDONALD, H. R. 1989. Production and characterization of a rat monoclonal antibody against the murine CD3 molecular complex. *Immunology Letters,* 23**,** 113-118.

MIRZA, R., DIPIETRO, L. A. & KOH, T. J. 2009. Selective and specific macrophage ablation is detrimental to wound healing in mice. *Am J Pathol,* 175**,** 2454-62.

MOLDENHAUER, L. M., KEENIHAN, S. N., HAYBALL, J. D. & ROBERTSON, S. A. 2010. GM-CSF Is an Essential Regulator of T Cell Activation Competence in Uterine Dendritic Cells during Early Pregnancy in Mice. *The Journal of Immunology,* 185**,** 7085-7096.

MONGET, P. & BONDY, C. 2000. Importance of the IGF system in early folliculogenesis. *Molecular and Cellular Endocrinology,* 163**,** 89-93.
MOR, G. & ABRAHAMS, V. M. 2006. Trophoblast Cells as Immune Regulators. *In:* MOR, G. (ed.) *Immunology of Pregnancy.* Springer New York.
MOR, G. & CARDENAS, I. 2010. REVIEW ARTICLE: The Immune System in Pregnancy: A Unique Complexity. *American Journal of Reproductive Immunology,* 63**,** 425-433.
MOR, G., CARDENAS, I., ABRAHAMS, V. & GULLER, S. 2011. Inflammation and pregnancy: the role of the immune system at the implantation site. *Annals of the New York Academy of Sciences,* 1221**,** 80-87.
MORRIS, B. & SASS, M. B. 1966. The Formation of Lymph in the Ovary. *Proceedings of the Royal Society of London. Series B. Biological Sciences,* 164**,** 577-591.
MOSSER, D. M. & EDWARDS, J. P. 2008. Exploring the full spectrum of macrophage activation. *Nat Rev Immunol,* 8**,** 958-969.
MURDOCH, C., TAZZYMAN, S., WEBSTER, S. & LEWIS, C. E. 2007. Expression of Tie-2 by human monocytes and their responses to angiopoietin-2. *J Immunol,* 178**,** 7405-11.
NAIDU, B. V., KRISHNADASAN, B., FARIVAR, A. S., WOOLLEY, S. M., THOMAS, R., VAN ROOIJEN, N., VERRIER, E. D. & MULLIGAN, M. S. 2003. Early activation of the alveolar macrophage is critical to the development of lung ischemia-reperfusion injury. *The Journal of thoracic and cardiovascular surgery,* 126**,** 200-207.
NAKAMURA, H., JASPER, M. J. & ROBERTSON, S. A. 2008. Macrophage regulation of embryo adhesion molecule expression in human endometrial epithelial cells. *Society for the Study of Reproduction.* Kailua-Kona, Hawaii.
NI, H., SUN, T., DING, N. Z., MA, X. H. & YANG, Z. M. 2002. Differential expression of microsomal prostaglandin e synthase at implantation sites and in decidual cells of mouse uterus. *Biol Reprod,* 67**,** 351-8.
NINOMIYA, K., TAKAHASHI, A., FUJIOKA, Y., ISHIKAWA, Y. & YOKOYAMA, M. 2006. Transforming growth factor-beta signaling enhances transdifferentiation of macrophages into smooth muscle-like cells. *Hypertension research : official journal of the Japanese Society of Hypertension,* 29**,** 269-76.
NISHIMURA, K., TANAKA, N., KAWANO, T., MATSUURA, K. & OKAMURA, H. 1998. Changes in macrophage colony-stimulating factor concentration in serum and follicular fluid in in-vitro fertilization and embryo transfer cycles. *Fertil Steril,* 69**,** 53-7.
NISHIMURA, K., TANAKA, N., OHSHIGE, A., FUKUMATSU, Y., MATSUURA, K. & OKAMURA, H. 1995. Effects of macrophage colony-stimulating factor on folliculogenesis in gonadotrophin-primed immature rats. *Journal of Reproduction and Fertility,* 104**,** 325-330.
NISHIZUKA, Y. & SAKAKURA, T. 1969. Thymus and reproduction: sex-linked dysgenesia of the gonad after neonatal thymectomy in mice. *Science,* 166**,** 753-5.
NISWENDER, G. D. 2002. Molecular control of luteal secretion of progesterone. *Reproduction,* 123**,** 333-9.
O'LEARY, S., JASPER, M. J., ROBERTSON, S. A. & ARMSTRONG, D. T. 2006. Seminal plasma regulates ovarian progesterone production, leukocyte recruitment and follicular cell responses in the pig. *Reproduction,* 132**,** 147-58.
OLIVER, G. 2004. Lymphatic vasculature development. *Nat Rev Immunol,* 4**,** 35-45.
OLSON, M. E. & BRUCE, J. 1986. Ovariectomy, ovariohysterectomy and orchidectomy in rodents and rabbits. *Can Vet J,* 27**,** 523-7.

ORMANDY, C. J., CAMUS, A., BARRA, J., DAMOTTE, D., LUCAS, B., BUTEAU, H., EDERY, M., BROUSSE, N., BABINET, C., BINART, N. & KELLY, P. A. 1997. Null mutation of the prolactin receptor gene produces multiple reproductive defects in the mouse. *Genes Dev,* 11**,** 167-178.

PALMARINI, M., GRAY, C. A., CARPENTER, K., FAN, H., BAZER, F. W. & SPENCER, T. E. 2001. Expression of Endogenous Betaretroviruses in the Ovine Uterus: Effects of Neonatal Age, Estrous Cycle, Pregnancy, and Progesterone. *J. Virol.*, 75**,** 11319-11327.

PALTIELI, Y., EIBSCHITZ, I., ZISKIND, G., OHEL, G., SILBERMANN, M. & WEICHSELBAUM, A. 2000. High Progesterone Levels and Ciliary Dysfunction—A Possible Cause of Ectopic Pregnancy. *Journal of Assisted Reproduction and Genetics,* 17**,** 103-106.

PANG, S. F., CHOW, P. H. & WONG, T. M. 1979. The role of the seminal vesicles, coagulating glands and prostate glands on the fertility and fecundity of mice. *Journal of Reproduction and Fertility,* 56**,** 129-132.

PATE, J. & LANDIS KEYES, P. 2001. Immune cells in the corpus luteum: friends or foes? *Reproduction,* 122**,** 665-676.

PATE, J. L., TOYOKAWA, K., WALUSIMBI, S. & BRZEZICKA, E. 2010. The interface of the immune and reproductive systems in the ovary: lessons learned from the corpus luteum of domestic animal models. *Am J Reprod Immunol,* 64**,** 275-86.

PEITZ, B. & OLDS-CLARKE, P. 1986. Effects of seminal vesicle removal on fertility and uterine sperm motility in the house mouse. *Biol Reprod,* 35**,** 608-617.

PENNY, L. A., ARMSTRONG, D. G., BAXTER, G., HOGG, C., KINDAHL, H., BRAMLEY, T., WATSON, E. D. & WEBB, R. 1998. Expression of Monocyte Chemoattractant Protein-1 in the Bovine Corpus Luteum Around the Time of Natural Luteolysis. *Biol Reprod,* 59**,** 1464-1469.

PETROVSKA, M., DIMITROV, D. G. & MICHAEL, S. D. 1996. Quantitative changes in macrophage distribution in normal mouse ovary over the course of the estrous cycle examined with an image analysis system. *Am J Reprod Immunol,* 36**,** 175-83.

PHILLIPS, H. S., HAINS, J., LEUNG, D. W. & FERRARA, N. 1990. Vascular endothelial growth factor is expressed in rat corpus luteum. *Endocrinology,* 127**,** 965-7.

PIACENTINI, M. & AUTUORI, F. 1994. Immunohistochemical localization of tissue transglutaminase and Bcl-2 in rat uterine tissues during embryo implantation and post-partum involution. *Differentiation,* 57**,** 51-61.

PICCININI, A. M. & MIDWOOD, K. S. 2010. DAMPening inflammation by modulating TLR signalling. *Mediators Inflamm,* 2010.

PIOLI, P. A., WEAVER, L. K., SCHAEFER, T. M., WRIGHT, J. A., WIRA, C. R. & GUYRE, P. M. 2006. Lipopolysaccharide-Induced IL-1β Production by Human Uterine Macrophages Up-Regulates Uterine Epithelial Cell Expression of Human β-Defensin 2. *The Journal of Immunology,* 176**,** 6647-6655.

PLAKS, V., BIRNBERG, T., BERKUTZKI, T., SELA, S., BENYASHAR, A., KALCHENKO, V., MOR, G., KESHET, E., DEKEL, N., NEEMAN, M. & JUNG, S. 2008. Uterine DCs are crucial for decidua formation during embryo implantation in mice. *J Clin Invest,* 118**,** 3954-65.

POLLARD, J. W. 1990. Regulation of polypeptide growth factor synthesis and growth factor-related gene expression in the rat and mouse uterus before and after implantation. *J Reprod Fertil,* 88**,** 721-731.

POLLARD, J. W., BARTOCCI, A., ARCECI, R., ORLOFSKY, A., LADNER, M. B. & STANLEY, E. R. 1987a. Apparent role of the macrophage growth factor, CSF-1, in placental development. *Nature,* 330**,** 484-6.
POLLARD, J. W., HUNT, J. S., WIKTOR-JEDRZEJCZAK, W. & STANLEY, E. R. 1991. A pregnancy defect in the osteopetrotic (op/op) mouse demonstrates the requirement for CSF-1 in female fertility. *Dev Biol,* 148**,** 273-83.
POLLARD, J. W., LIN, E. Y. & ZHU, L. 1998. Complexity in uterine macrophage responses to cytokines in mice. *Biol Reprod,* 58**,** 1469-75.
POLLARD, J. W., PACEY, J., CHENG, S. V. & JORDAN, E. G. 1987b. Estrogens and cell death in murine uterine luminal epithelium. *Cell Tissue Res,* 249**,** 533-40.
POTTER, P. K., CORTES-HERNANDEZ, J., QUARTIER, P., BOTTO, M. & WALPORT, M. J. 2003. Lupus-prone mice have an abnormal response to thioglycolate and an impaired clearance of apoptotic cells. *J Immunol,* 170**,** 3223-32.
PRIETO, J., EKLUND, A. & PATARROYO, M. 1994. Regulated Expression of Integrins and Other Adhesion Molecules during Differentiation of Monocytes into Macrophages. *Cellular Immunology,* 156**,** 191-211.
PRITTS, E. A. & ATWOOD, A. K. 2002. Luteal phase support in infertility treatment: a meta-analysis of the randomized trials. *Human Reproduction,* 17**,** 2287-2299.
PSYCHOYOS, A. 1960. [The decidual reaction is preceded by early changes in the capillary permeability of the uterus]. *C R Seances Soc Biol Fil,* 154**,** 1384-7.
QUEEN, K., DHABUWALA, C. B. & PIERREPOINT, C. G. 1981. The effect of the removal of the various accessory sex glands on the fertility of male rats. *Journal of Reproduction and Fertility,* 62**,** 423-426.
RABBANI, M. & ROGERS, P. 2001. Role of vascular endothelial growth factor in endometrial vascular events before implantation in rats. *Reproduction,* 122**,** 85-90.
REBAR, R. W., MORANDINI, I. C., BENIRSCHKE, K. & PETZE, J. E. 1980. REDUCED GONADOTROPINS IN ATHYMIC MICE: PREVENTION BY THYMIC TRANSPLANTATION. *Endocrinology,* 107**,** 2130-2132.
REDLINE, R. W. & LU, C. Y. 1988. Specific defects in the anti-listerial immune response in discrete regions of the murine uterus and placenta account for susceptibility to infection. *J Immunol,* 140**,** 3947-55.
REDMER, D. A., DORAISWAMY, V., BORTNEM, B. J., FISHER, K., JABLONKA-SHARIFF, A., GRAZUL-BILSKA, A. T. & REYNOLDS, L. P. 2001. Evidence for a role of capillary pericytes in vascular growth of the developing ovine corpus luteum. *Biol Reprod,* 65**,** 879-89.
REISTER, F., FRANK, H. G., KINGDOM, J. C., HEYL, W., KAUFMANN, P., RATH, W. & HUPPERTZ, B. 2001. Macrophage-induced apoptosis limits endovascular trophoblast invasion in the uterine wall of preeclamptic women. *Lab Invest,* 81**,** 1143-52.
REYNOLDS, L., KILLILEA, S. & REDMER, D. 1992. Angiogenesis in the female reproductive system. *The FASEB Journal,* 6**,** 886-892.
REYNOLDS, L. P. & REDMER, D. A. 1999. Growth and development of the corpus luteum. *J Reprod Fertil Suppl,* 54**,** 181-91.
RICHARDS, J. S. & PANGAS, S. A. 2010. The ovary: basic biology and clinical implications. *J Clin Invest,* 120**,** 963-72.
RICHARDS, J. S., RUSSELL, D. L., OCHSNER, S., HSIEH, M., DOYLE, K. H., FALENDER, A. E., LO, Y. K. & SHARMA, S. C. 2002. Novel Signaling Pathways That Control Ovarian Follicular Development, Ovulation, and Luteinization. *Recent Prog Horm Res,* 57**,** 195-220.

RISSANEN, T. T., MARKKANEN, J. E., GRUCHALA, M., HEIKURA, T., PURANEN, A., KETTUNEN, M. I., KHOLOVA, I., KAUPPINEN, R. A., ACHEN, M. G., STACKER, S. A., ALITALO, K. & YLA-HERTTUALA, S. 2003. VEGF-D Is the Strongest Angiogenic and Lymphangiogenic Effector Among VEGFs Delivered Into Skeletal Muscle via Adenoviruses. *Circ Res,* 92**,** 1098-1106.

RIVEST, S. & RIVIER, C. 1995. The Role of Corticotropin-Releasing Factor and Interleukin-1 in the Regulation of Neurons Controlling Reproductive Functions. *Endocrine Reviews,* 16**,** 177-199.

ROBERTSON, S. A. 2010. Immune regulation of conception and embryo implantation—all about quality control? *J Reprod Immunol,* 85**,** 51-57.

ROBERTSON, S. A., ALLANSON, M. & MAU, V. J. 1998. Molecular regulation of uterine leukocyte recruitment during early pregnancy in the mouse. *Placenta,* 19**,** 101-119.

ROBERTSON, S. A., BRÄNNSTRÖM, M. & SEAMARK, R. F. 1992a. Cytokines in rodent reproduction and the cytokine-endocrine interaction. *Current Opinion in Immunology,* 4**,** 585-590.

ROBERTSON, S. A., JASPER, M. J., BROMFIELD, J. J., CARE, A. S., NAKAMURA, H. & INGMAN, W. V. 2008. The role of macrophages in implantation and early pregnancy success. *Society for the Study of Reproduction.* Kailua-Kona, Hawaii.

ROBERTSON, S. A., MAU, V. J., TREMELLEN, K. P. & SEAMARK, R. F. 1996a. Role of high molecular weight seminal vesicle proteins in eliciting the uterine inflammatory response to semen in mice. *J Reprod Fertil,* 107**,** 265-77.

ROBERTSON, S. A., MAYRHOFER, G. & SEAMARK, R. F. 1992b. Uterine epithelial cells synthesize granulocyte-macrophage colony-stimulating factor and interleukin-6 in pregnant and nonpregnant mice. *Biol Reprod,* 46**,** 1069-1079.

ROBERTSON, S. A., MAYRHOFER, G. & SEAMARK, R. F. 1996b. Ovarian steroid hormones regulate granulocyte-macrophage colony-stimulating factor synthesis by uterine epithelial cells in the mouse. *Biol Reprod,* 54**,** 183-196.

ROBERTSON, S. A., ROBERTS, C. T., FARR, K. L., DUNN, A. R. & SEAMARK, R. F. 1999. Fertility impairment in granulocyte-macrophage colony-stimulating factor-deficient mice. *Biol Reprod,* 60**,** 251-61.

ROBERTSON, S. A. & SEAMARK, R. F. 1990. Granulocyte macrophage colony stimulating factor (GM-CSF) in the murine reproductive tract: stimulation by seminal factors. *Reprod Fertil Dev,* 2**,** 359-68.

ROBERTSON, S. A., SEAMARK, R. F., GUILBERT, L. J. & WEGMANN, T. G. 1994. The role of cytokines in gestation. *Crit Rev Immunol,* 14**,** 239-92.

ROCKWELL, L. C., PILLAI, S., OLSON, C. E. & KOOS, R. D. 2002. Inhibition of Vascular Endothelial Growth Factor/Vascular Permeability Factor Action Blocks Estrogen-Induced Uterine Edema and Implantation in Rodents. *Biol Reprod,* 67**,** 1804-1810.

ROGERS, P. A. 1992. Early endometrial microvascular response during implantation in the rat. *Reprod Fertil Dev,* 4**,** 261-4.

ROGERS, P. A., DONOGHUE, J. F., WALTER, L. M. & GIRLING, J. E. 2009. Endometrial angiogenesis, vascular maturation, and lymphangiogenesis. *Reprod Sci,* 16**,** 147-51.

ROLLINS, B., WALZ, A. & BAGGIOLINI, M. 1991. Recombinant human MCP-1/JE induces chemotaxis, calcium flux, and the respiratory burst in human monocytes. *Blood,* 78**,** 1112-1116.

SAITO, M., IWAWAKI, T., TAYA, C., YONEKAWA, H., NODA, M., INUI, Y., MEKADA, E., KIMATA, Y., TSURU, A. & KOHNO, K. 2001. Diphtheria toxin receptor-mediated conditional and targeted cell ablation in transgenic mice. *Nat Biotech,* 19**,** 746-750.

SATO, Y. 2008. VEGFR1 for Lymphangiogenesis: An Alternative Signaling Pathway? *Arterioscler Thromb Vasc Biol,* 28**,** 604-605.
SAVILL, J., DRANSFIELD, I., GREGORY, C. & HASLETT, C. 2002. A blast from the past: clearance of apoptotic cells regulates immune responses. *Nat Rev Immunol,* 2**,** 965-975.
SCHOFIELD, G. & KIMBER, S. J. 2005. Leukocyte Subpopulations in the Uteri of Leukemia Inhibitory Factor Knockout Mice During Early Pregnancy. *Biol Reprod,* 72**,** 872-878.
SCHOPPMANN, S. F., BIRNER, P., STOCKL, J., KALT, R., ULLRICH, R., CAUCIG, C., KRIEHUBER, E., NAGY, K., ALITALO, K. & KERJASCHKI, D. 2002. Tumor-associated macrophages express lymphatic endothelial growth factors and are related to peritumoral lymphangiogenesis. *Am J Pathol,* 161**,** 947-56.
SCHULTZ, G. A. & HEYNER, S. 1993. Growth factors in preimplantation mammalian embryos. *Oxf Rev Reprod Biol,* 15**,** 43-81.
SCHULZE-KOOPS, H. & KALDEN, J. R. 2001. The balance of Th1/Th2 cytokines in rheumatoid arthritis. *Best practice & research. Clinical rheumatology,* 15**,** 677-691.
SEGALOFF, D. L., WANG, H. & RICHARDS, J. S. 1990. Hormonal Regulation of Luteinizing Hormone/Chorionic Gonadotropin Receptor mRNA in Rat Ovarian Cells during Follicular Development and Luteinization. *Molecular Endocrinology,* 4**,** 1856-1865.
SENGER, D., GALLI, S., DVORAK, A., PERRUZZI, C., HARVEY, V. & DVORAK, H. 1983. Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. *Science,* 219**,** 983-985.
SENTURK, L. M., SELI, E., GUTIERREZ, L. S., MOR, G., ZEYNELOGLU, H. B. & ARICI, A. 1999. Monocyte chemotactic protein-1 expression in human corpus luteum. *Mol Hum Reprod,* 5**,** 697-702.
SERTA, R. T. & SEIBE, M. M. 1993. The influence of epidermal growth factor on progesterone production by human granulosa—luteal cells in culture. *Human Reproduction,* 8**,** 1005-1010.
SHAKIL, T. & WHITEHEAD, S. A. 1994. Inhibitory action of peritoneal macrophages on progesterone secretion from co-cultured rat granulosa cells. *Biol Reprod,* 50**,** 1183-9.
SHARIFI, B. G., ZENG, Z., WANG, L., SONG, L., CHEN, H., QIN, M., SIERRA-HONIGMANN, M. R., WACHSMANN-HOGIU, S. & SHAH, P. K. 2006. Pleiotrophin Induces Transdifferentiation of Monocytes Into Functional Endothelial Cells. *Arterioscler Thromb Vasc Biol,* 26**,** 1273-1280.
SHWEIKI, D., ITIN, A., NEUFELD, G., GITAY-GOREN, H. & KESHET, E. 1993. Patterns of expression of vascular endothelial growth factor (VEGF) and VEGF receptors in mice suggest a role in hormonally regulated angiogenesis. *J Clin Invest,* 91**,** 2235-43.
SIDHU, S. S. & KIMBER, S. J. 1999. Hormonal Control of H-Type α(1-2)Fucosyltransferase Messenger Ribonucleic Acid in the Mouse Uterus. *Biol Reprod,* 60**,** 147-157.
SIITERI, P. K., FEBRES, F., CLEMENS, L. E., CHANG, R. J., GONDOS, B. & STITES, D. 1977. PROGESTERONE AND MAINTENANCE OF PREGNANCY: IS PROGESTERONE NATURE'S IMMUNOSUPPRESSANT?*. *Annals of the New York Academy of Sciences,* 286**,** 384-397.
SILVA, M., DO VALE, A. & DOS SANTOS, N. 2008. Secondary necrosis in multicellular animals: an outcome of apoptosis with pathogenic implications. *Apoptosis,* 13**,** 463-482.

SIMON, C., FRANCES, A., PIQUETTE, G. & POLAN, M. L. 1994a. Immunohistochemical localization of the interleukin-1 system in the mouse ovary during follicular growth, ovulation, and luteinization. *Biol Reprod,* 50**,** 449-57.

SIMON, C., FRANCES, A., PIQUETTE, G. N., EL DANASOURI, I., ZURAWSKI, G., DANG, W. & POLAN, M. L. 1994b. Embryonic implantation in mice is blocked by interleukin-1 receptor antagonist. *Endocrinology,* 134**,** 521-8.

SIMÓN, C., PIQUETTE, G. N., FRANCES, A. & POLAN, M. L. 1993. Localization of interleukin-1 type I receptor and interleukin-1 beta in human endometrium throughout the menstrual cycle. *Journal of Clinical Endocrinology & Metabolism,* 77**,** 549-55.

SIMON, C., VALBUENA, D., KRUSSEL, J., BERNAL, A., MURPHY, C. R., SHAW, T., PELLICER, A. & POLAN, M. L. 1998. Interleukin-1 receptor antagonist prevents embryonic implantation by a direct effect on the endometrial epithelium. *Fertil Steril,* 70**,** 896-906.

SJÖBLOM, C., WIKLAND, M. & ROBERTSON, S. A. 1999. Granulocyte–macrophage colony-stimulating factor promotes human blastocyst development in vitro. *Human Reproduction,* 14**,** 3069-3076.

SMITH, S., BAKER, P. N. & SYMONDS, E. M. 1997a. Placental apoptosis in normal human pregnancy. *American journal of obstetrics and gynecology,* 177**,** 57-61.

SMITH, S. C., BAKER, P. N. & SYMONDS, E. M. 1997b. Increased placental apoptosis in intrauterine growth restriction. *American journal of obstetrics and gynecology,* 177**,** 1395-1401.

SMITH, S. D., DUNK, C. E., APLIN, J. D., HARRIS, L. K. & JONES, R. L. 2009. Evidence for Immune Cell Involvement in Decidual Spiral Arteriole Remodeling in Early Human Pregnancy. *The American Journal of Pathology,* 174**,** 1959-1971.

SPENCER, T. E., HAYASHI, K., HU, J. & CARPENTER, K. D. 2005. Comparative Developmental Biology of the Mammalian Uterus. *In:* GERALD, P. S. (ed.) *Current Topics in Developmental Biology.* Academic Press.

STACKER, S. A., STENVERS, K., CAESAR, C., VITALI, A., DOMAGALA, T., NICE, E., ROUFAIL, S., SIMPSON, R. J., MORITZ, R., KARPANEN, T., ALITALO, K. & ACHEN, M. G. 1999. Biosynthesis of Vascular Endothelial Growth Factor-D Involves Proteolytic Processing Which Generates Non-covalent Homodimers. *Journal of Biological Chemistry,* 274**,** 32127-32136.

STANDAERT, F. E., CHEW, B. P. & WONG, T. S. 1990. Influence of blood monocytes and lymphocytes on progesterone production by granulosa cells from small and large follicles in the pig. *Am J Reprod Immunol,* 22**,** 49-55.

STANDAERT, F. E., ZAMORA, C. S. & CHEW, B. P. 1991. Quantitative and qualitative changes in blood leukocytes in the porcine ovary. *Am J Reprod Immunol,* 25**,** 163-8.

STANLEY, E. R., BERG, K. L., EINSTEIN, D. B., LEE, P. S. W., PIXLEY, F. J., WANG, Y. & YEUNG, Y.-G. 1997. Biology and action of colony-stimulating factor-1. *Molecular Reproduction and Development,* 46**,** 4-10.

STANLEY, E. R., GUILBERT, L. J., TUSHINSKI, R. J. & BARTELMEZ, S. H. 1983. CSF-1--a mononuclear phagocyte lineage-specific hemopoietic growth factor. *J Cell Biochem,* 21**,** 151-9.

STAPLES, L. D., FLEET, I. R. & HEAP, R. B. 1982. Anatomy of the utero-ovarian lymphatic network and the composition of afferent lymph in relation to the establishment of pregnancy in the sheep and goat. *Journal of Reproduction and Fertility,* 64**,** 409-420.

STEFANCZYK-KRZYMOWSKA, S. & KRZYMOWSKI, T. 2002. Local adjustment of blood and lymph circulation in the hormonal regulation of reproduction in female pigs--facts, conclusions and suggestions for future research. *Reprod Biol,* 2**,** 115-32.
STEFANCZYK-KRZYMOWSKA, S., KRZYMOWSKI, T., WASOWSKA, B. & CHLOPEK, J. 2002. Retrograde transfer of ovarian steroid hormones to the ovary in the porcine periovarian vascular complex. *Experimental Physiology,* 87**,** 361-371.
STEWART, C. L., KASPAR, P., BRUNET, L. J., BHATT, H., GADI, I., KONTGEN, F. & ABBONDANZO, S. J. 1992. Blastocyst implantation depends on maternal expression of leukaemia inhibitory factor. *Nature,* 359**,** 76-79.
STOUT, R. D. 2010. Editorial: Macrophage functional phenotypes: no alternatives in dermal wound healing? *Journal of Leukocyte Biology,* 87**,** 19-21.
SUNDERKOTTER, C., STEINBRINK, K., GOEBELER, M., BHARDWAJ, R. & SORG, C. 1994. Macrophages and angiogenesis. *J Leukoc Biol,* 55**,** 410-22.
SZEKERES-BARTHO, J. & POLGAR, B. 2010. PIBF: The Double Edged Sword. Pregnancy and Tumor. *American Journal of Reproductive Immunology,* 64**,** 77-86.
SZEKERES-BARTHO, J. & WEGMANN, T. G. 1996. A progesterone-dependent immunomodulatory protein alters the Th1/Th2 balance. *J Reprod Immunol,* 31**,** 81-95.
SZEKERESBARTHO, J., CHAOUAT, G. & KINSKY, R. 1990a. A PROGESTERONE-INDUCED BLOCKING FACTOR CORRECTS HIGH RESORPTION RATES IN MICE TREATED WITH ANTIPROGESTERONE. *American journal of obstetrics and gynecology,* 163**,** 1320-1322.
SZEKERESBARTHO, J., KINSKY, R. & CHAOUAT, G. 1990b. THE EFFECT OF A PROGESTERONE-INDUCED IMMUNOLOGICAL BLOCKING FACTOR ON NK-MEDIATED RESORPTION. *American Journal of Reproductive Immunology,* 24**,** 105-107.
TABIBZADEH, S. 1994. Cytokines and the hypothalamic—pituitary—ovarian—endometrial axis. *Human Reproduction,* 9**,** 947-967.
TACHI, C., TACHI, S., KNYSZYNSKI, A. & LINDNER, H. R. 1981. Possible involvement of macrophages in embryo--maternal relationships during ovum implantation in the rat. *J Exp Zool,* 217**,** 81-92.
TAKAYA, R., FUKAYA, T., SASANO, H., SUZUKI, T., TAMURA, M. & YAJIMA, A. 1997. Macrophages in normal cycling human ovaries; immunohistochemical localization and characterization. *Hum Reprod,* 12**,** 1508-12.
TAMURA, K., TAMURA, H., KUMASAKA, K., MIYAJIMA, A., SUGA, T. & KOGO, H. 1998. Ovarian immune cells express granulocyte-macrophage colony-stimulating factor (GM-CSF) during follicular growth and luteinization in gonadotropin-primed immature rodents. *Mol Cell Endocrinol,* 142**,** 153-63.
THOMAS, M. L. 1989. The leukocyte common antigen family. *Annu Rev Immunol,* 7**,** 339-69.
THURSTON, G., RUDGE, J. S., IOFFE, E., ZHOU, H., ROSS, L., CROLL, S. D., GLAZER, N., HOLASH, J., MCDONALD, D. M. & YANCOPOULOS, G. D. 2000. Angiopoietin-1 protects the adult vasculature against plasma leakage. *Nat Med,* 6**,** 460-3.
THURSTON, G., SURI, C., SMITH, K., MCCLAIN, J., SATO, T. N., YANCOPOULOS, G. D. & MCDONALD, D. M. 1999. Leakage-resistant blood vessels in mice transgenically overexpressing angiopoietin-1. *Science,* 286**,** 2511-4.
TILLY, J. L., BILLIG, H., KOWALSKI, K. I. & HSUEH, A. J. 1992. Epidermal growth factor and basic fibroblast growth factor suppress the spontaneous onset of apoptosis

in cultured rat ovarian granulosa cells and follicles by a tyrosine kinase-dependent mechanism. *Molecular Endocrinology,* 6**,** 1942-50.
TONELLO, A. & POLI, G. 2007. Tubal ectopic pregnancy: macrophages under the microscope.
TONG, W. & POLLARD, J. W. 1999. Progesterone inhibits estrogen-induced cyclin D1 and cdk4 nuclear translocation, cyclin E- and cyclin A-cdk2 kinase activation, and cell proliferation in uterine epithelial cells in mice. *Mol Cell Biol,* 19**,** 2251-64.
TONG, W. & POLLARD, J. W. 2002. Female sex steroid hormone regulation of cell proliferation in the endometrium. *In:* GLASSER, S. R., APLIN, J. D., GIUDICE, L. C. & TABIBZADEH, S. (eds.) *The Endometrium.* London: Taylor & Francis.
TOWNSON, D. H., WARREN, J. S., FLORY, C. M., NAFTALIN, D. M. & KEYES, P. L. 1996. Expression of monocyte chemoattractant protein-1 in the corpus luteum of the rat. *Biol Reprod,* 54**,** 513-520.
TREMELLEN, K. P., SEAMARK, R. F. & ROBERTSON, S. A. 1998. Seminal transforming growth factor beta1 stimulates granulocyte-macrophage colony-stimulating factor production and inflammatory cell recruitment in the murine uterus. *Biol Reprod,* 58**,** 1217-1225.
TREMELLEN, K. P., VALBUENA, D., LANDERAS, J., BALLESTEROS, A., MARTINEZ, J., MENDOZA, S., NORMAN, R. J., ROBERTSON, S. A. & SIMÓN, C. 2000. The effect of intercourse on pregnancy rates during assisted human reproduction. *Human Reproduction,* 15**,** 2653-2658.
TROWBRIDGE, I. S. 1978. Interspecies spleen-myeloma hybrid producing monoclonal antibodies against mouse lymphocyte surface glycoprotein, T200. *J Exp Med,* 148**,** 313-23.
TURNER, E. C., HUGHES, J., WILSON, H., CLAY, M., MYLONAS, K. J., KIPARI, T., DUNCAN, W. C. & FRASER, H. M. 2011. Conditional ablation of macrophages disrupts ovarian vasculature. *Reproduction*.
UCHINO, S., ICHIKAWA, S., OKUBO, M., NAKAMURA, Y. & IIMURA, A. 1987. Methods of detection of lymphatics and their changes with oestrous cycle. *Int Angiol,* 6**,** 271-8.
UEKI, M. 1991. Histologic study of endometriosis and examination of lymphatic drainage in and from the uterus. *Am J Obstet Gynecol,* 165**,** 201-9.
VAN DER HOEK, K. H., MADDOCKS, S., WOODHOUSE, C. M., VAN ROOIJEN, N., ROBERTSON, S. A. & NORMAN, R. J. 2000. Intrabursal Injection of Clodronate Liposomes Causes Macrophage Depletion and Inhibits Ovulation in the Mouse Ovary. *Biol Reprod,* 62**,** 1059-1066.
VOLL, R. E., HERRMANN, M., ROTH, E. A., STACH, C., KALDEN, J. R. & GIRKONTAITE, I. 1997. Immunosuppressive effects of apoptotic cells. *Nature,* 390**,** 350-351.
WANG, J., SNIDER, D. P., HEWLETT, B. R., LUKACS, N. W., GAULDIE, J., LIANG, H. & XING, Z. 2000. Transgenic expression of granulocyte-macrophage colony-stimulating factor induces the differentiation and activation of a novel dendritic cell population in the lung. *Blood,* 95**,** 2337-2345.
WANG, L. J., BRANNSTROM, M., ROBERTSON, S. A. & NORMAN, R. J. 1992. Tumor necrosis factor alpha in the human ovary: presence in follicular fluid and effects on cell proliferation and prostaglandin production. *Fertil Steril,* 58**,** 934-40.
WEGMANN, T. G., LIN, H., GUILBERT, L. & MOSMANN, T. R. 1993. Bidirectional cytokine interactions in the maternal-fetal relationship: is successful pregnancy a TH2 phenomenon? *Immunol Today,* 14**,** 353-6.

WIKTOR-JEDRZEJCZAK, W., BARTOCCI, A., FERRANTE, A. W., AHMED-ANSARI, A., SELL, K. W., POLLARD, J. W. & STANLEY, E. R. 1990. Total absence of colony-stimulating factor 1 in the macrophage-deficient osteopetrotic (op/op) mouse. *Proceedings of the National Academy of Sciences,* 87**,** 4828-4832.

WIKTOR-JEDRZEJCZAK, W., URBANOWSKA, E., AUKERMAN, S. L., POLLARD, J. W., STANLEY, E. R., RALPH, P., ANSARI, A. A., SELL, K. W. & SZPERL, M. 1991. Correction by CSF-1 of defects in the osteopetrotic op/op mouse suggests local, developmental, and humoral requirements for this growth factor. *Exp Hematol,* 19**,** 1049-54.

WIRA, C. R., GRANT-TSCHUDY, K. S. & CRANE-GODREAU, M. A. 2005. Epithelial Cells in the Female Reproductive Tract: a Central Role as Sentinels of Immune Protection. *American Journal of Reproductive Immunology,* 53**,** 65-76.

WOOD, G. W., HAUSMANN, E. & CHOUDHURI, R. 1997. Relative role of CSF-1, MCP-1/JE, and RANTES in macrophage recruitment during successful pregnancy. *Molecular Reproduction and Development,* 46**,** 62-70.

WOOD, G. W., HAUSMANN, E. H. S. & KANAKARAJ, K. 1999. Expression and regulation of chemokine genes in the mouse uterus during pregnancy. *Cytokine,* 11**,** 1038-1045.

WOOD, G. W., MAMATA, D., SANFORD, T. & CHOUDHURI, R. 1992. Macrophage colony stimulating factor controls macrophage recruitment to the cycling mouse uterus. *Developmental Biology,* 152**,** 336-343.

WU, R., VAN DER HOEK, K. H., RYAN, N. K., NORMAN, R. J. & ROBKER, R. L. 2004. Macrophage contributions to ovarian function. *Hum Reprod Update,* 10**,** 119-33.

WULFF, C., WILSON, H., LARGUE, P., DUNCAN, W. C., ARMSTRONG, D. G. & FRASER, H. M. 2000. Angiogenesis in the Human Corpus Luteum: Localization and Changes in Angiopoietins, Tie-2, and Vascular Endothelial Growth Factor Messenger Ribonucleic Acid. *Journal of Clinical Endocrinology & Metabolism,* 85**,** 4302-4309.

WULFF, C., WILSON, H., RUDGE, J. S., WIEGAND, S. J., LUNN, S. F. & FRASER, H. M. 2001. Luteal Angiogenesis: Prevention and Intervention by Treatment with Vascular Endothelial Growth Factor TrapA40. *Journal of Clinical Endocrinology & Metabolism,* 86**,** 3377-3386.

WUU, Y. D., PAMPFER, S., BECQUET, P., VANDERHEYDEN, I., LEE, K. H. & DE HERTOGH, R. 1999. Tumor necrosis factor alpha decreases the viability of mouse blastocysts in vitro and in vivo. *Biol Reprod,* 60**,** 479-83.

XU, F. & STOUFFER, R. L. 2009. Existence of the lymphatic system in the primate corpus luteum. *Lymphat Res Biol,* 7**,** 159-68.

YAGEL, S., HURWITZ, A., ROSENN, B. & KEIZER, N. 1987. Progesterone enhancement of prostaglandin E2 production by fetal placental macrophages. *Am J Reprod Immunol Microbiol,* 14**,** 45-8.

YAN, D., WANG, X., LI, D., QU, Z. & RUAN, Q. 2011. Macrophages overexpressing VEGF, transdifferentiate into endothelial-like cells in vitro and in vivo. *Biotechnology Letters***,** 1-8.

YAN, Z., HUNTER, V., WEED, J., HUTCHISON, S., LYLES, R. & TERRANOVA, P. 1993. Tumor necrosis factor-alpha alters steroidogenesis and stimulates proliferation of human ovarian granulosal cells in vitro. *Fertil Steril,* 59**,** 332-8.

YEE, G. M. & KENNEDY, T. G. 1988. Stimulatory effects of prostaglandins upon endometrial alkaline phosphatase activity during the decidual cell reaction in the rat. *Biol Reprod,* 38**,** 1129-36.

YOSHIDA, S., ONO, M., SHONO, T., IZUMI, H., ISHIBASHI, T., SUZUKI, H. & KUWANO, M. 1997. Involvement of interleukin-8, vascular endothelial growth factor, and basic fibroblast growth factor in tumor necrosis factor alpha-dependent angiogenesis. *Mol Cell Biol,* 17**,** 4015-23.

YOUN, J. I., NAGARAJ, S., COLLAZO, M. & GABRILOVICH, D. I. 2008. Subsets of myeloid-derived suppressor cells in tumor-bearing mice. *J Immunol,* 181**,** 5791-802.

ZAREIE, M., MCKAY, D. M., KOVARIK, G. G. & PERDUE, M. H. 1998. Monocyte/macrophages evoke epithelial dysfunction: indirect role of tumor necrosis factor-α. *American Journal of Physiology - Cell Physiology,* 275**,** C932-C939.

ZHANG, F., TANG, Z., HOU, X., LENNARTSSON, J., LI, Y., KOCH, A. W., SCOTNEY, P., LEE, C., ARJUNAN, P., DONG, L., KUMAR, A., RISSANEN, T. T., WANG, B., NAGAI, N., FONS, P., FARISS, R., ZHANG, Y., WAWROUSEK, E., TANSEY, G., RABER, J., FONG, G.-H., DING, H., GREENBERG, D. A., BECKER, K. G., HERBERT, J.-M., NASH, A., YLA-HERTTUALA, S., CAO, Y., WATTS, R. J. & LI, X. 2009. VEGF-B is dispensable for blood vessel growth but critical for their survival, and VEGF-B targeting inhibits pathological angiogenesis. *Proceedings of the National Academy of Sciences,* 106**,** 6152-6157.

ZHANG, H., MCELRATH, T., TONG, W. & POLLARD, J. W. 2005. The molecular basis of tamoxifen induction of mouse uterine epithelial cell proliferation. *J Endocrinol,* 184**,** 129-40.

ZHAO, Y.-G., XIAO, A.-Z., CAO, X.-M. & ZHU, C. 2002. Expression of matrix metalloproteinase -2, -9 and tissue inhibitors of metalloproteinase -1, -2, -3 mRNAs in rat uterus during early pregnancy. *Molecular Reproduction and Development,* 62**,** 149-158.