Seminal Fluid and Cytokine Control of Regulatory T-Cells in Murine Pregnancy

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ABSTRACT

For successful pregnancy, the maternal immune system must tolerate the presence of a fetus that expresses alloantigens. The appropriate and timely acquisition of this state of tolerance is critical and emerging evidence suggests that it needs to be present from the time the embryo implants into the uterus. Recently it has been demonstrated that a subpopulation of lymphocytes termed $CD4^+CD25^+$ regulatory T cells (Treg cells) are required for immune tolerance of the fetus during pregnancy. Despite their importance the factors that control regulatory T cells during pregnancy, and in particular in the peri-implantation period, are poorly understood. Using mouse models we have assessed the role of the ejaculate and its components (sperm and seminal plasma) in coordinating Treg cells in the period prior to embryo implantation. We have also used mice with a null mutation in the interleukin 10 (IL-10) gene to assess the role of this cytokine in coordination of Treg cell populations in later pregnancy.

Experiments in the peri-implantation period just prior to implantation (day 3.5 post-coitum) showed that there was a significant increase (approximately 2-fold; $p<0.05$) in the total number of ($CD4^+Foxp3^+$) Treg cells in the iliac lymph nodes (LNs) that drain the uterus, but not in the distal inguinal LNs. This appeared not to be the result of a selective expansion in Treg cells but due to expansion of the entire $CD4^+$ cell pool, since the percent of $CD4^+$ cells expressing Foxp3 in any of the lymphoid tissues studied did not increase in response to mating. In addition, there was a similar increase in the density of these cells in the uterus just prior to implantation at day 3.5pc ($p<0.05$). By using males deficient in the sperm or seminal plasma components of the ejaculate we could show that the increase in both the lymph node and uterine Treg cell populations occurred in response to seminal plasma.

The role of seminal plasma in regulating expression of mRNAs encoding migratory molecules in the peri-implantation uterus, and the involvement of these genes in recruiting Treg cells following mating, was then assessed. We analysed the mRNAs for the chemokines *Ccl4, Ccl5, Ccl19*, *Ccl22*, the chemokine receptors *Ccr4, Ccr5, Ccr7* and the integrin *Cd103* using qRT-PCR. We showed a significant elevation in *Ccl19*

and *Ccr5* mRNA at day 3.5pc following mating to intact males. However the increase in mRNA was independent of factors associated with seminal fluid and might instead be regulated by ovarian steroid hormones.

Using *IL-10* null mutant (IL-10-/-) mice it was then shown that the cytokine IL-10 is involved in controlling Treg cell numbers in mid gestation. At gestational day (gd) 9.5, in IL-10-/- mice, there was an approximate 40% elevation in the proportion of $CD4^+$ cells expressing Foxp3 compared with wild-type control mice ($p<0.01$). This was seen in both the iliac LNs and inguinal LNs. In addition, there was a greater than 10-fold increase ($p<0.0001$) in the total number of Treg cells in the uterine-draining iliac LNs of IL-10-/- mice compared to wild-type mice. This was not seen in the inguinal LNs. Experiments comparing allogeneic and syngeneic mated mice showed that the proportional changes seen in the $CD4^+$ cell population was dependent on fetal alloantigens, although the elevation in total numbers still occurred in the absence of fetal alloantigens.

This study begins to unravel the process by which Treg cell populations are expanded and recruited into the uterus prior to embryo implantation and later in gestation. A greater understanding of this process may aid in the diagnosis and prevention of a range of pregnancy pathologies associated with immune dysregulation, such as pre-eclampsia and recurrent spontaneous abortion.

DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution to Leigh Guerin and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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ABBREVIATIONS

^{3}HTdR	Tritiated thymidine
ANOVA	analysis of variance
APC	Antigen presenting cell
B6	C57BL/6
cDNA	Complementary deoxyribonucleic acid
CG	Choriogonadotroptin
CSIF	Cytokine synthesis inhibitory factor
Ct	Cycle threshold
CTL	Cytotoxic T-lymphocyte
CTLA-4	Cytotoxic T-lymphocyte antigen 4
CV	Coefficient of variation
DC	Dendritic cell
E	Embryonic day
E2	Estradiol
Est	estrus
EtOH	Ethanol
FACS	Fluorescence-activated cell sorting
FasL	Fas lignad
FITC	Flurescein isothiocyanate
Foxp3	Forkhead box P3
gd	Gestational day
GFP	Green fluoresent protein
GITR	Glucocorticoid-induced tumor necrosis factor receptor
GM-CSF	Granulocyte-macrophage colony-stimulating factor

hCG	Human chronic gonadotropin
HLA	Human leukocyte antigen
HRP	Horseradish peroxidase
hrs	hours
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
IHC	Immunohistochemistry
IL	Interleukin
Int	intact
IPEX	immune dysregulation polyendocrinopathy, entropathy, X-linked
LAG-3	Lymphocyte-activation gene 3
LH	leutinizign hormone
LIF	Leukemia inhibitory factor
LN	Lymph node
LPS	Lipopolysaccharide
MHC	Major histocompatibility complexe
mins	minutes
mRNA	messenger ribonucleic acid
NK	Natural killer
Nrp1	Neuropilin-1
PBL	Peripheral blood leukocyte
PBS	Phosphate-buffered saline
PBST	PBS Tween-20
pc	Post-coitum
PD1	Programmed death-1
PE	Phycoerthrin

PGE2	Prostaglandin E2
qRT-PCR	Quantitative real-time polymerase chain reaction
RA	Retinoic acid
ROR	Retinoic acid-related orphan receptor
SEM	Standard error of mean
STAT5	Signal transducer and activator of transcription 5
SV-	vesiculectomised
TCR	T-cell receptors
TGF	Transforming growth factor
Th	T helper
TNF	Tumour necrosis factor
Tr1	T regulator 1
Treg	T regulatory Cell
uNK	Uterine natural killer
VAS-	vasectomised

CHAPTER 1

LITERATURE REVIEW

1.1 INTRODUCTION

Genetic diversity within mammalian species results in the generation of offspring that are genetically, and antigenically, disparate from their parents. Whilst this may have evolutionary benefits, it presents a problem for the immune system of eutherian animals. Immunological attack of 'non-self' antigens expressed by the conceptus should be incompatible with pregnancy success. To combat this, complex mechanisms that include adaptations of the innate and adaptive arms of the immune system have evolved to promote transient immune tolerance of fetal-antigens.

Since the 1950s, research into the mechanisms that facilitate this state of immune tolerance has revealed several overlapping pathways operating prior to conception and throughout gestation. These include modifications to the surface antigenic expression by the cells that form the maternal:fetal interface (Szekeres-Bartho 2002), expression of molecules that induce anergy or deletion of fetal reactive immune cells (Coumans*, et al.* 1999, Taglauer*, et al.* 2009, Vacchio*, et al.* 2005), as well as perturbations in the cytokine context in which fetal antigens are encountered by the immune system (Chaouat 2007). However importantly in opposition to original proposals that maternal tolerance of the fetus was mediated by immunological ignorance (Medawar 1953), we now have a body of evidence to suggest that immune tolerance in the adaptive arm of the immune system in pregnancy is in fact a result of functional recognition of fetal antigens (Aluvihare*, et al.* 2006, Jiang*, et al.* 1998, Mellor*, et al.* 2004, Trowsdale*, et al.* 2006). The exact mechanisms by which this recognition results in tolerance are not fully understood, however, we know that the network of cytokines both in the uterus and systemically is critical (Chaouat 2007, Saito*, et al.* 2003, Wilczynski 2005). One cytokine of importance is the Th2 cytokine, Interleukin (IL)-10 (Chaouat*, et al.* 1995, Robertson*, et al.* 2006, Sharma*, et al.* 2007).

Recently a specialized suppressive CD4 cellular subset known as $CD4^{+}CD25^{+}$ regulatory T (Treg) cells have been identified (Sakaguchi*, et al.* 1995). They have since been shown to be critical mediators of allogeneic immune tolerance (Aluvihare*, et al.* 2004) and are implicated as having an essential role in pregnancy success in both mice and humans (Aluvihare*, et al.* 2004, Heikkinen*, et al.* 2004, Saito*, et al.* 2005, Zenclussen 2005). Despite their apparent importance in the establishment and maintenance of immune

tolerance throughout pregnancy, the events and cytokines that regulate their abundance and function throughout pregnancy remain poorly understood.

This chapter will discuss current knowledge of the critical events that facilitate successful immune tolerance throughout pregnancy. It will describe the emerging role of Treg cells in immune suppression with a focus on their evident role in gestation. This will be discussed in regard to the factors and cytokines that regulate their abundance and function following mating and prior to embryo implantation in early pregnancy, and later gestation in both humans and mice, with an emphasis on the role of IL-10 in their regulation.

1.2 THE IMMUNOLOGY OF PREGNANCY

1.2.1 The immune paradox of pregnancy

The viviparous nature of mammalian reproduction combined with the antigenic disparities between conceiving partners results in an immunological paradox for the maternal immune system. Typically delegated to the role of recognition of, and attack upon, antigenically-foreign organisms and tissue, the immune system during pregnancy needs to develop a state of immune tolerance of the conceptus (Billington 2003). The recognition of the need for maternal tolerance of the developing fetus was first fostered in 1924 by C. Little, however the field of reproductive immunology research was introduced by Peter Medawar in 1953. Based on his knowledge of the role of tissue antigens in rejection of skin transplantation, he formulated the question of how the mother continues to nourish an antigenically foreign fetus within itself (Medawar 1953). He proposed 3 mechanisms by which this tolerance may be elicited; (a) the anatomical separation of fetus from mother; (b) the antigenic immaturity of the fetus; and (c) immunological indolence or inertness of the mother (Billington 2003). These 3 theories set the foundation for the field of reproductive immunology.

Since the proposal of these theories there have been significant advances in our understanding of the complex processes involved in maternal immune tolerance of the fetus. We now have a significant body of evidence to suggest that in opposition to the maternal immune system being ignorant and excluded from interacting with the fetus and its antigens, immune recognition of fetal antigens is instead critical for a successful

pregnancy (Chaouat, *et al.* 1985, James, *et al.* 2003, Kiger, *et al.* 1985, Tafuri, *et al.* 1995, Toder, *et al.* 1990, Zhou, *et al.* 1998). This is supported by work in other fields showing that cell mediated tolerance is most often a product of active recognition of, and responses to, antigens (Vacchio, *et al.* 2003, Vacchio, *et al.* 2005). Understanding the immune response and mechanisms of immune suppression directed toward the fetus involves a clear understanding of the nature, degree and limitations of the interactions between the maternal immune system and the conceptus.

1.2.1.1 The conceptus as an allograft

The analogy of a fetus as an allograft has limitations. Significant antigenic disparities exist between the mother and the conceptus, however the relationship between the mother's immune system and the conceptus differs significantly from that of a true allograft. Perhaps one of the most critical differentiating features is that the fetal and maternal circulatory systems are kept separate from each other (Billington 2003). In humans this separation is facilitated by fetal trophoblast cells that invade deep into the myometrium along the spiral arteries, and form the interface between the fetal compartment and the maternal immune system (Lightner, *et al.* 2008, Szekeres-Bartho 2002). Therefore the true fetal allograft is represented by the trophoblast cells and the extra-embryonic membrane (Billington 2003). As a result, the events of immune tolerance to the fetus must focus around the antigenicity and immune evasive properties of this interface.

1.2.1.1.1 Immunogenicity and antigenic properties of the fetal maternal interface

Medawar first hypothesised that there may be anatomical separation of the fetus from the mother (Medawar 1953) and indeed, albeit with restrictions, the placenta may be thought of as this barrier. However, unlike the initial intentions of the statement which implied that such a barrier would invoke ignorance of the fetus by the maternal immune system, the reality is that the mother's immune system can interact with the trophoblast cells on the placenta and is reactive to paternal and fetal antigens (Shao, *et al.* 2005, Tafuri, *et al.* 1995). However the antigenic properties of trophoblast cells are significantly modified in a manner that ultimately perturbs the ability of the immune system to recognise the alloantigenicity of trophoblast cells and consequently inhibits its

ability to elicit a directed response (Szekeres-Bartho 2002). This is predominately achieved by restricted expression of major histocompatibility complexes (MHC).

On human trophoblast cells, the expression of MHC class II antigens and classical highly polymorphic MHC class I antigens are significantly reduced or absent (Hutter*, et al.* 1996). On a transcriptional level, mRNA for human leukocyte antigen (HLA)-A, -B, -C, -E and –G are all detectable in trophoblast cell populations, however very few of these are translated and expressed as proteins (Guillaudeux*, et al.* 1995). HLA-A and HLA-B are completely absent from both cytotrophoblast cells and the syncytiotrophoblast cells that form the interface with the maternal immune system (Kawata*, et al.* 1984, Sunderland*, et al.* 1981). There is however limited expression of HLA-C, predominantly in the extravillous trophoblast cells of the cell columns (Hutter*, et al.* 1996, King*, et al.* 2000). In addition to this, placental cells express some non-classical MHC class I antigens, also known as MHC class Ib, specifically HLA-E and HLA-G (King*, et al.* 2000, King*, et al.* 2000, Kovats*, et al.* 1990).

The specific function or significance of the pattern of MHC regulation on the placenta is not fully understood, however several functions are evident and others are postulated. The low level or absent expression of the highly polymorphic HLA-A and -B acts to reduce the antigenicity of the fetal maternal interface, whilst the expression HLA-E and -G and even HLA-C may perform more complex functions (Szekeres-Bartho 2002). The reduced level of polymorphisms in these HLA subtypes, especially HLA-E and -G, ensure that there is little disparity between maternal and paternal HLA, thereby reducing the chances of inducing an alloantigen response (Szekeres-Bartho 2002). Furthermore, the expression of HLA-G may perform critical immunoregulatory roles (Carosella*, et al.* 2008, Rouas-Freiss*, et al.* 2007). Specifically, HLA-G can suppress immune responses by binding to inhibitory receptors and in particular inhibits the function of antigen presenting cells (APCs), natural killer (NK) cells and T-cell subsets including the cytotoxic activity of CD8 T-cells (Carosella*, et al.* 1999, Frédéric*, et al.* 2008, Kuroki*, et al.* 2007). Likewise it is postulated that HLA-C may have immunosuppressive functions (Hutter*, et al.* 1996). Evidence for such a role comes from pregnancies in which there is a disparity between maternal and fetal HLA-C, where it has been demonstrated that this results in an elevation in $CD4^{+}CD25^{bright}$ regulatory T cells (Tilburgs*, et al.* 2009).

The degree and pattern of expression of MHC on human placentas differs significantly from that of mice. Whilst classical MHC class I molecules aren't expressed on human placental tissue, their murine homologues are transcribed and expressed throughout gestation (Chatterjee-Hasrouni*, et al.* 1981, Hedley*, et al.* 1989, Philpott*, et al.* 1988). Specifically mRNA and protein of both the K and D haplotype of the H-2 antigens are expressed on extra embryonic tissues including trophoblast cells (Chatterjee-Hasrouni*, et al.* 1981, Philpott*, et al.* 1988). This suggests that the murine placenta is potentially more antigenic than the human equivalent, however this appears not to compromise immune tolerance. This is supported by experiments where over-expression of H-2 antigens or MHC class 1 antigens within trophoblast cell populations resulted in no impairment of pregnancy outcomes (Rogers*, et al.* 1998, Shomer*, et al.* 1998), indicating that despite the apparent antigenic nature of the murine placenta, a state of active suppression must exist to maintain immune tolerance of the fetus.

1.2.1.2 Mechanisms of immune tolerance during pregnancy

A large array of mechanisms have been proposed and demonstrated to be critical for the induction and maintenance of immune tolerance in pregnancy. These can target suppression of the immune system at both the induction and effector phase and can influence the actions of the humoral and innate arms of the immune system. The extensive and complex interactions of these different mechanisms of immune tolerance and their apparent levels of redundancy reflect the importance of appropriate immune tolerance in the success of reproduction. Some of the major mechanisms by which immune tolerance is elicited during gestation will be discussed.

Interaction with cells of the innate immune system is mediated by the expression of HLA-G on the human placenta (Carosella*, et al.* 1999, Kuroki*, et al.* 2007) acting to specifically suppress NK cell populations within the uterus via ligation of specific killer inhibitory receptors (Marchal-Bras-Goncalves*, et al.* 2001, Rouas-Freiss*, et al.* 1997). The presence of HLA-E and its homologue Qa-1 on the human and murine placenta respectively, further enhance this passive protection from NK cell attack as well as inducing tolerance within CD8 populations (Hu*, et al.* 2004, Jiang*, et al.* 2000, King*, et al.* 2000). There are further interactions between the innate immune system and the maternal-fetal interface, including interactions with uterine-NK (uNK) cells and macrophages, with uNK constituting about 70% of leukocytes in the early and mid-pregnant uterus (Bulmer*, et al.*

1991). However these leukocytes subsets appear to be more critical in vascular and tissue remodelling events in the uterus, rather than as mediators of maternal-fetal immune tolerance.

An additional local mechanism of immune tolerance may involve the expression of Fas ligand (FasL) at the fetal maternal interface (Hunt*, et al.* 1997). Interaction of FasL with its receptor Fas induces apoptosis in Fas^+ cells. Evidence suggests that FasL expression of trophoblast cells may induce apoptosis of maternal T-cell (Coumans*, et al.* 1999, Vacchio*, et al.* 2005). A similar role for the protein Programmed Death 1 (PD-1)/B7-H1 axis is hypothesized. Fetal-antigen specific T-cells that are deficient in PD-1 failed to undergo apoptosis and preferentially accumulative in lymph nodes (LNs) draining the uterus. This, however, does not result in detrimental effects on pregnancy (Taglauer*, et al.* 2009).

Tryptophan catabolism is implicated as having a critical role in the induction of immune tolerance at the fetal maternal interface. Indoleamine 2,3-dioxygenase (IDO) metabolizes tryptophan reducing its availability to T-cells and consequently inhibiting their proliferation (Munn*, et al.* 1999). IDO is known to be expressed by syncytiotrophoblast cells and DCs that reside in the uterus (Kamimura*, et al.* 1991, Terness*, et al.* 2007). Its critical role is indicated by experiments that show that the administration of the IDO blocking agent 1-methyl-tryptophan, leads to increased rates of fetal loss of allogeneic but not syngeneic fetuses (Munn*, et al.* 1998).

The systemic and local cytokine environment is implicated as having a critical role in the maintenance of immune tolerance during pregnancy. Pregnancy is associated with a deviation away from Th1 and toward Th2 cytokines. Studies in pregnant women show that peripheral blood leukocytes (PBLs) throughout all three trimesters show a cytokine expression profile that is dominated by Th2 cytokines such as IL-4, IL-6 and IL-10 and has low levels of Th1 cytokines such as IL-1β, IL-2 and interferon gamma (IFNγ) (Marzi*, et al.* 1996, Matthiesen*, et al.* 1998, Tranchot-Diallo*, et al.* 1997). Such a bias is equally present at the maternal fetal interface. Th2 cytokines including IL-4, IL-6, IL-10, IL-13 and transforming growth factor-β (TGFβ) are all produced by conceptus derived cells including trophoblast, decidua, chorionic and amniotic membranes (Bennett*, et al.* 1996, Dealtry*, et al.* 1998, Jones*, et al.* 1997, Roth*, et al.* 1996). This perturbation in the cytokine profile of the immune system is consistent with the remission seen in Th1

mediated autoimmune diseases such as rheumatoid arthritis and the exacerbation of Th2 mediated diseases such as lupus, that are noted during pregnancy (Del Prete*, et al.* 1994). Experiments in the CBA/J x DBA/2 abortion prone mouse model shows the fetal loss pathology is associated with a deficiency in Th2 cytokine, including IL-4 and L-10 (Chaouat*, et al.* 1995, Raghupathy 2001).

Together these multiple mechanisms mediate immune tolerance toward the fetus with redundancy and functional overlap between mechanisms providing back-up systems to ensure the integrity of tolerance for the period of gestation. The aforementioned systems are only some of those that are employed to maintain immune tolerance. Progressively we are discovering additional mechanisms that are important mediators of immune tolerance in pregnancy. Recently, regulatory T-cells have been implicated as having a critical role in the maintenance of allogeneic pregnancies. These will be discussed in sections 1.3 and 1.4.

1.2.2 The immune deviating properties of seminal fluid

Semen consists of the male gamete, the spermatozoa, and a fluid component that is a combination of secretions of the prostate gland, the seminal vesicle gland and the epididymis, with the seminal fluid component containing an array of proteins and bioactive moieties (Cooper 1990, Montagnon*, et al.* 1990). Traditionally, the seminal fluid has been seen as a supportive medium to facilitate spermatozoa transport into the female reproductive tract and to supply nutrients and protection to promote sperm health and consequently fertilization. However it is now believed that its role extends beyond this. Introduction of seminal fluid into the female reproductive tract induces a raft of changes within both local and distal tissues including the ovary, the uterus and systemic lymphoid tissues (Robertson 2005). These changes are induced by the diverse array of signalling moieties within semen including, estrogen, testosterone, prostaglandins, cytokines and growth factors (Aumuller*, et al.* 1992, Maegawa*, et al.* 2002). There is now extensive experimental and epidemiological evidence, that the consequence of seminal fluid being introduced into the female reproductive tract includes a range of physiological responses, one of which is to enhance paternal-antigen specific immune tolerance (Robertson 2007, Robertson*, et al.* 2003, Robertson*, et al.* 2002).

1.2.2.1 Antigenic properties of semen

The ability of semen to induce a state of paternal antigen specific immune tolerance is dependent on the presence of paternal antigens within the ejaculate. Sperm are known to be antigenic (Chamley, *et al.* 2007), with the presence of antisperm antibodies being present in both males and females (Ayvaliotis, *et al.* 1985). However the exact nature of the antigens expressed on sperm remains disputed. Human spermatozoa have been shown to express class II MHC and classical MHC class I antigens including HLA-A and HLA-B, at both the mRNA and protein levels (Arnaiz-Villena, *et al.* 1976, Bishara, *et al.* 1987, Martin-Villa, *et al.* 1996). However these findings remain controversial with other studies failing to identify the expression of MHC class I or II or β2-microglobulin (Anderson, *et al.* 1984, Ohashi, *et al.* 1990). In mice however, MHC class I, II and β2-microglobulin have all been confirmed to be expressed on ejaculated spermatozoa (Mori, *et al.* 1990, Wu, *et al.* 1990). Additionally, male-specific H-Y antigen is present on both murine and human spermatozoa (Sills, *et al.* 1998). Irrespective of the antigenic properties of spermatozoa, soluble antigens including MHC class I, as well as somatic cells, such as leukocytes and desquamated epithelial cells, are present within the ejaculate (Koelman, *et al.* 2000).

The ability of the maternal immune system to respond to antigens present within the semen remains controversial. Initial experiments within TCR transgenic mice show that maternal $CD8^+$ were incapable of responding to paternal MHC-I or minor (H-Y) antigens present within semen (Seavey, *et al.* 2006). However recent experiment using TCR transgenic mice who possess a CD8 T-cell population that is responsive a model minor histocompatibility molecule (β-actin) demonstrate that antigens within semen, and in particular in seminal plasma, are able to be processed and presented to T-cells within the uterine draining lymph nodes following insemination (Moldenhauer, *et al.* 2009). As such, the ejaculate may be the first and most common way that the maternal immune system is exposed to paternal antigen, some of which are later expressed on the developing conceptus. Consequently, the immune response that follows mating may be directive in any subsequent response in regard to paternal antigens expressed by the conceptus (Robertson, *et al.* 2001). Therefore the nature and type of immune response elicited following mating is thought to be a critical factor in the ongoing immune tolerance to the fetus generated throughout gestation.

1.2.2.2 The post-mating immune response

Following insemination, an inflammatory-like immune response is induced within the uterus in all mammalian species studied to date (Robertson 2005, Taylor 1982). This is characterised by the influx of a range of leukocytes including granulocytes such as neutrophils, leukocytes, macrophages and dendritic cells (DCs), which is transient in nature and is resolved by the time of embryo implantation (Bischof*, et al.* 1994, De*, et al.* 1991, Engelhardt*, et al.* 1997, McMaster*, et al.* 1992). This influx of immune cells mediates four categories of effector function: (1) clearance of excessive and obsolete sperm and protection from introduced pathogens; (2) activation of the female immune response to paternal antigens contained within the ejaculate; (3) tissue remodelling to facilitate uterine receptivity and embryo implantation; and (4) induction of expression of growth factors associated with embryo development (Robertson 2007). The post-mating immune response is mediated by proinflammatory cytokines up regulated in the female reproductive tract epithelial cells, including granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-6 and IL-8 (Robertson*, et al.* 1996, Sanford*, et al.* 1992, Sharkey*, et al.* 2007, Tremellen*, et al.* 1998). Additionally, there is an elevation in the expression of mRNA for a range of leukocyte chemo-attractant factors including *Ccl2, Ccl3, Ccl4, Ccl5, and Ccl11* (Pollard*, et al.* 1998, Robertson*, et al.* 1998, Sharkey*, et al.* 2007).

Induction of this immune response is primarily as a result of active signalling moieties within the seminal plasma of the ejaculate. Female mice mated with seminal vesicle deficient males displaying significantly decreased induction of GM-CSF and reduced leukocyte infiltration (Robertson*, et al.* 1996). Key signalling moieties within seminal plasma that have been identified to coordinate this response includes TGFβ and prostaglandin E2 (PGE2). TGFβ, once activated within the female reproductive tract, has been shown to stimulate the production of GM-CSF, and IL-6 (Robertson*, et al.* 1992, Robertson*, et al.* 1990, Sanford*, et al.* 1992, Tremellen*, et al.* 1998). Whilst PGE2 in humans has been shown to induce IL-8 and IL-10, and itself can induce a tolerogenic phenotype in DCs (Denison*, et al.* 1999, Kelly*, et al.* 1997). The net result of the function of the cytokines and chemokines is recruitment into the reproductive tract of abundant leukocyte populations following insemination.

1.2.2.3 Activation of maternal immune tolerance by seminal fluid

The influx into the uterus of professional APCs such as macrophages and DCs as a result of insemination leads to the uptake and trafficking of antigens from the female reproductive tract to the iliac (or para-aortic) lymph nodes that drain the uterus (Moldenhauer*, et al.* 2009). This is evidenced by lymph node hypertrophy and lymphocyte activation and proliferation (Johansson*, et al.* 2004, Moldenhauer*, et al.* 2009). Hostile responses to such antigens would be incompatible with pregnancy success. In opposition to immune activation leading to rejection, exposure to paternal seminal antigens has been shown to induce a state of immune tolerance in the female, with this tolerance being specific for paternal antigens present within the ejaculate (Beer*, et al.* 1974, Robertson*, et al.* 2009). Largely this state of immune tolerance is believed to be mediated by components that reside within the seminal plasma of the ejaculate, since sperm exposure in the absence of seminal plasma is unable to induce an equivalent state of tolerance compared to exposure to both, or seminal plasma alone (Beer*, et al.* 1974, Hancock*, et al.* 1986, Robertson*, et al.* 2009). The mechanisms by which this happens are still being elucidated, however the cytokine context induced following mating and in which the paternal antigens are presented within lymph nodes by APCs, is postulated to be key to facilitating this phenomenon. A recent study invokes exposure of the female to seminal components as having a role in expansion of $CD4^+CD25^+$ Treg cell populations, which consequently may contribute to the aforementioned immune tolerance (Robertson*, et al.* 2009).

1.3 REGULATORY T-CELLS AND IMMUNE SUPPRESSION

1.3.1 The discovery and classification of regulatory T-cells

The term “regulatory T-cells” refers to a family of T lymphocyte populations with suppressive/regulatory properties that are devoted to maintaining antigen-specific T-cell tolerance. The history of regulatory cells/suppressor cells in the immunology literature dates back to the 1970s when T lymphocytes that were capable of suppressing immune responses were first described and named “suppressor T-cells” (Gershon*, et al.* 1970). However with the lack of a definitive marker, questions regarding their authenticity were raised and attention shifted away from this cell population (Green*, et al.* 1993), until Treg

cells in their existing form were identified as a specialized T-cell subset in 1995 by Sakaguchi and colleagues (Sakaguchi, *et al.* 1995).

The current literature categorizes a wide variety of T-cell subsets under the umbrella term of regulatory T-cells (see Table 1.1), of these there are at least three subsets of $CD4^+$ regulatory T-cells that are perhaps the most clearly defined within the literature. These are categorized by their distinct suppressive mechanisms, phenotype, cytokine secretion and tissue of origin (Jonuleit, *et al.* 2003). These are type 1 regulatory T-cells (Tr1) cells, T-helper 3 (Th3) cells, and $CD4^+CD25^+$ T regulatory (Treg) cells. Each of these populations have the characteristic capacity to actively inhibit the proliferation and effector function of other T-cells (Fukaura, *et al.* 1996, Groux, *et al.* 1997, Suri-Payer, *et al.* 1998).

Table 1.1: Types of regulatory T-cells.

Type of regulatory T-cells	Origin	Phenotype	Mechanism of suppression
$CD4^+CD25^+$ T-cells[1]	Thymus/periphery	$CD4^+CD25^+Foxp3^+$	Contact dependent Secreted IL-10 Secreted or membrane TGFβ
Tr1 Cells[2]	Periphery	$CD4^+CD25^{+/-}$	Secreted IL-10
Th3 cells3	Periphery	$CD4^+CD25^{+/-}$	Secreted TGFβ
$CD8^+CD28^-$ T-cells[4]	periphery	CD8+CD28- $Foxp3^{+/-}$	Contact dependent
NKT cell[5]	Thymus?	$NK1.1^+TCR^+$ $CD4^{+/-}$	Induction of IL-10 from DCs
$CD4^+CD25^-Foxp3^+$ T-cell[6]	?	$CD4^+CD25^-Foxp3^+$	Contact dependent Secreted IL-10 Secreted or membrane TGFβ

[1](Askenasy, *et al.* 2008),[2](Roncarolo, *et al.* 2001),[3](Groux, *et al.* 1997),[4](Tulunay, *et al.* 2008),[5](Jiang, *et al.* 2007),[6](Nishioka, *et al.* 2006).

Tr1 cells were initially characterised in mouse models of inflammatory bowel disease as potent suppressors of antigen-specific immune responses mediated by IL-10 synthesis (Groux, *et al.* 1997). Th3 cells were discovered in mice as mediators of oral tolerance acting to inhibit induction of immunity through secretion of TGFβ (Weiner 2001). Of the three cell populations, Treg cells have fast become established as perhaps the most

potent and widespread suppressive cell lineage in the immune system. Unlike Th3 and Tr1 cells which appear to represent altered differentiation states of conventional $CD4^+CD25^-$ T-cells, Treg cells are believed to comprise a distinct and unique T-cell lineage (Weiner 2001). They have now been implicated in critical protective functions in autoimmune disease (Sakaguchi 2005), transplantation tolerance (Waldmann, *et al.* 2004), gastro-intestinal homeostasis (Coombes, *et al.* 2005) and inflammatory disease (Wahl, *et al.* 2004), and conversely, are a pivotal component of the patho-physiological immune tolerance induced by tumours (Munn, *et al.* 2006).

1.3.2 T regulatory 1 (Tr1) cells

The presence of Tr1 cells was initially described in mouse models where they were generated by TCR stimulation in the presence of IL-10 (Groux, *et al.* 1997). The distinction of Tr1 cells from Th1 and Th2 cells is primarily based on their cytokine secretion profile, with their surface phenotype resembling that of other Th cells. Predominantly Tr1 cells produce IL-10 and TGFβ along with lesser quantities of IL-5 and IFNγ (Roncarolo, *et al.* 2001).

The development of Tr1 cells, like Th1 and Th2 cells, is thought to arise from naïve Th0 precursors facilitated by the local cytokine milieu (McGuirk, *et al.* 2002). *In vitro* studies have demonstrated that human $CD4^+$ cells primed in the presence of IL-10 and tumour necrosis factor-α (TNFα) differentiate into Tr1 cells (Levings, *et al.* 2001). Like IL-12 and IL-4 that direct the development of Th1 and Th2 cells respectively, IL-10 is proposed to be the principle stimulating factor for the *in vivo* development of Tr1 cells (McGuirk, *et al.* 2002). This is believed to be mediated by the ability of IL-10 to inhibit the stimulatory capacity of DCs by obstructing their maturity through the down regulation of MHC class II expression as well as that of the costimulatory molecules CD80 and CD86 (McGuirk, *et al.* 2002).

The suppressive ability of Tr1 cells is mediated through their high level secretion of IL-10 and their moderate secretion of TGFβ (Beissert, *et al.* 2006), with most of the immunosuppressive effects of Tr1 cells being blocked by IL-10 neutralizing antibodies (Roncarolo, *et al.* 2001). The unique cytokine production of Tr1 cells appears to be regulated via antigen-specific signalling through the TCR complex, with activation stimuli that bypass the TCR (i.e. phorbol myristic acetate and calcium inophore) resulting in

production of a diverse range of cytokines, including IL-2 and IL-4, leading to the loss of the unique cytokine profile of Tr1 cells (Bacchetta*, et al.* 1994). Whilst IL-10 production is induced via TCR signaling, TGFβ production appears to be mediated via signalling through the Cytotoxic T-lymphocyte antigen-4 (CTLA-4) receptor, with blocking antibodies nullifying its production (Kitani*, et al.* 2000).

Tr1 cells appear to play an important role in the regulation of peripheral tolerance to both self and foreign antigens, suppressing self-reactive T-cells and limiting potentially pathogenic immune responses to foreign pathogens. IL-10 null mice are more susceptible to chronic colitis and conditions resembling rheumatoid arthritis (Kuhn*, et al.* 1993, Rennick*, et al.* 1997). However despite the importance of IL-10 in immune tolerance, any role for Tr1 cells in pregnancy still needs to be defined, but appears to have a degree of redundancy since allogenic pregnancy is not compromised in IL-10 null mice (White*, et al.* 2004).

1.3.3 T-helper 3 (Th3) cells

Th3 cells have been identified as actively promoting immune tolerance in a variety of mucosal tissues (Mosmann*, et al.* 1996). Th3 cells were first recognized as promoting active oral tolerance in mouse models whereby administration of low doses of oral antigens resulted in the amplification of TGFβ secreting $CD4^+$ T-cells (Chen*, et al.* 1994). Like Tr1 cells, Th3 cells are defined based on their distinctive cytokine profile, which is characterised by high levels of TGFβ secretion and low to no secretion of IL-2, IL-4 and IL-10 (Mosmann*, et al.* 1996).

Phenotypically Th3 cells resemble Th1 cells and indeed appear to be an extension of the Th1/Th2 spectrum. Sequence analysis of Th3 clone cDNA revealed that they utilized similar combinations of variable TCR genes to Th1 cells (Chen*, et al.* 1994). Due to similarity to Th1 cell clones it has been difficult to identify Th3 cells based on their phenotype. As such, like Tr1 cells, there have been no definitive surface markers identified for Th3 cells, making the study of these cells *in vivo* difficult. Indeed their identification is based solely on their cytokine profile.

The mechanism by which Th3 cells are generated largely remains unknown, however their induction is believed to be reliant on the cytokine environment and APC type in

their tissue of origin. It has been shown that the lymph node of origin is a critical determining factor in directing the developmental lineage of T-helper cells. Mucosal draining lymphoid tissues predominantly contain T-cells that produce IL-4 and TGFβ that in turn induce the differentiation of TGFβ producing cells from naïve T-cells (Daynes*, et al.* 1990, Seder*, et al.* 1998). Furthermore, the production of TGFβ by Th3 cells is proposed to positively feedback in order to promote further development of their cell lineage.

The suppressive mechanism employed by Th3 cells, like Tr1 cells, is dependent on their cytokine secretion, which is predominately TGFβ (Weiner 2001). TGFβ null mice have been shown to suffer from severe autoimmune disease (Gorelik*, et al.* 2000), and TGFβ has been shown to have suppressive effects on T-cells, B-cells and macrophages (Weiner 2001). However the TGFβ suppression pathway is not exclusively employed by Th3 cells and is known to be utilized by other suppressive cell lineages including $CD4^+CD25^+$ Treg cells. As such the contribution that the lack of Th3 cells has in the phenotype of TGFβ null mice is difficult to evaluate.

In the context of pregnancy the role of Th3 cells may be important in promoting tolerance of the alloantigens expressed on the developing fetus. Indeed many parallels can be drawn between tolerance induced in the mucosal reproductive tract and the gastro-intestinal tract, where Th3 cells are a major purveyor of tolerance. Furthermore the high levels of TGFβ reported in seminal plasma (Robertson*, et al.* 2002) may play a substantial role in promoting the early development of Th3 cells in the uterus and it's draining lymph nodes, which would aid in the acquisition of the optimal immunological environment prior to embryo implantation. However, the necessity of Th3 cells remains to be tested since immunocompetent TGFβ null mutant mice do not survive to reproductive age and suffer non-immune related reproductive disturbances (Ingman*, et al.* 2006).

1.3.4 $CD4^+CD25^+$ Treg cells are a distinct T lymphocyte subset

Treg cells are defined as a separate cellular subset on the basis of their surface phenotype as well as functional characteristics. Treg cells are a distinct subset amongst the four major classes of $CD4^+$ T-cells, together with Th1 cells, Th2 cells and Th17 cells (Zhu*, et al.* 2008), distinguished by their role in tolerance as opposed to immunity. They

comprise 1-3% of $CD4^+$ T-cells in humans and 5-10% of $CD4^+$ T-cells in rodents (Sakaguchi 2000, Shevach 2002). Their critical role in homeostasis is highlighted in mice that have a loss of function mutation in a gene critical for Treg development. As a result, these mice develop a fatal lympho-proliferative autoimmune disease at about 3 weeks of age (Brunkow*, et al.* 2001). Only identified as a specialised T-cell subset as recently as 1995 by Sakaguchi and colleagues (Sakaguchi*, et al.* 1995), Treg cells have fast established themselves as perhaps the most important suppressive cell lineage in the immune system and have become one of the most highly studied topics in immunity with an ever increasing citation rate in the literature (Shevach 2004). Treg cells appear to be a separate specialised cellular lineage with their critical function in many diseases reflecting this.

Unlike other regulatory T-cell subsets, Treg cells can be partially identified by their unique surface phenotype as well as by their unique expression of intracellular markers, primarily Forkhead box P3 (Foxp3) (see section 1.3.4.1). The mechanism by which Treg cells are generated still to some degree remains controversial, however it has become clear that there appear to be two distinctive pathways by which these cells are formed, either being generated in the thymus against self antigens or in the periphery against an array of antigens including foreign antigens (see section 1.3.4.2).

Unlike the aforementioned regulatory T-cells, Treg cells suppress in a contact dependent mechanism that appears to involve membrane bound TGFβ and CTLA-4, with blocking antibodies to these inhibiting suppression (Nakamura*, et al.* 2004, Nakamura*, et al.* 2001, Tang*, et al.* 2004)(see section 1.3.5). In addition they demonstrate an *in vivo* mechanism of suppression partially dependent on their paracrine production of IL-10 (see section 1.5.2). Furthermore Treg cells show the unique properties of 'linked suppression' and 'infectious tolerance' further defining them as distinct amongst T regulatory cells. Of the regulatory T-cell subsets, Treg cells are further defined by their apparent unique and essential role in reproductive processes.

1.3.4.1 Treg cell identification

Despite the unique suppressive properties of Treg cells compared with other lymphocytes, their cellular features are less distinct. Treg cells are generally identified on the basis of their constitutive expression of surface markers including the IL-2

receptor CD25 (Sakaguchi*, et al.* 1995), glucocorticoid-induced tumour necrosis factor receptor (GITR) (McHugh*, et al.* 2002, Shimizu*, et al.* 2002) CTLA-4 (Takahashi*, et al.* 2000), together with high expression of CD95 and in humans, low expression of CD45RB and CD127 (Liu*, et al.* 2006) (see Table 1.2). However each of these markers can also be dynamically expressed on the surface of other cell populations (Damoiseaux 2006) and there has been difficulty in identifying a definitive surface marker to distinguish Treg cells from related T-cells.

The search for a specific marker for Treg cells was aided by the identification of a mutation in a gene that resulted in the fatal human autoimmune disease 'immune dysregulation polyendocrinopathy, entropathy, X-linked' (IPEX) and the analogous disease in a spontaneous mutant mouse known as the 'scurfy' mouse. The responsible mutation was located in the gene that encodes a transcription factor of the forkhead/winged-helix family, known as Forkhead Box P3, (*FOXP3* or *scurfin*). Null mutation in this gene leads to a functional defect in the resultant protein and consequently the early onset of a severe lymphoproliferative/autoimmune disease (Brunkow*, et al.* 2001). The link between *Foxp3* expression and the development and function of the Treg cell was first made by Fontenot and colleagues who noted that *Foxp3* mRNA was elevated in the suppressive $CD4^{+}CD25^{+}$ lymphocyte population, and went on to demonstrate that *Foxp3* null mutant mice lacked Treg cells. Subsequently it was shown that transfer of $CD4^{+}CD25^{+}$ lymphocytes rescued *Foxp3* null mutant mice from autoimmune pathologies (Fontenot*, et al.* 2003). The critical role of Foxp3 in the development, function and origins of Treg cells has been further explored in recent publications utilising a $Foxp3^{gfp}$ knock-in allele, which validated this gene as the 'master-switch' for Treg development (Fontenot*, et al.* 2005, Fontenot*, et al.* 2005). Discovery of a definitive marker for Treg cells has allowed investigation of the origin and regulation of these cells, and has facilitated examination of their role in many disease states and physiological conditions.

Table 1.2: Markers for Treg cells.

Marker	Location	Level of expression by Treg cells	Specificity for Treg cells	Other expressing cell lineages
CD25	Surface	High	+++	Effector/memory T-cells
Foxp3	Intra cellular	High	++++	Giant cells
CD127	surface	low	+++ (humans only)	Effector/memory T-cells
CD95	Surface	High	++	Effector/memory T-cells
GITR	Surface	High	+++	Effector/memory T-cells
CTLA-4	Surface	High	+++	Effector/memory T-cells
CD45RB	Surface	Low	++	Effector/memory T-cells
Nrp1	Surface	High	++	Neurons
LAG-3	Surface	High	++	B-cells
PD-1	Surface	High	++	Activated T-cells

1.3.4.2 Ontogeny of Treg cells

The precise origins and pathways of generation of Treg cells is an active topic of research. It is becoming evident that there is heterogeneity in the ontogeny of Treg cells, with the processes through which Treg cells are generated, and the tissue in which they originate, being important factors in their suppressive function and target antigen repertoire as mature cells. At least two distinct pathways of Treg cell generation appear to exist. Firstly 'natural' Treg cells can originate via a selective process in the thymus based on the structure of their individual TCRs (Fontenot*, et al.* 2005, Itoh*, et al.* 1999). However the thymus appears not to be the sole source of Treg cells and this tissue cannot sufficiently account for the abundance and diversity of these cells. There is now compelling evidence to suggest that the majority of Treg cells are 'inducible' Treg cells generated within peripheral tissues, particularly in later life when the thymus undergoes extensive involution (reviewed in Akbar *et al.* 2007). As a result, the gross pool of Treg cells at any one time is the sum of the cells selected for survival in the thymus and those generated in the periphery.

1.3.4.2.1 Treg cell generation in the thymus

The fate of T-cells differentiated in the thymus is ultimately determined by the varying avidities for self-antigens of the TCRs expressed on their surface. T-cell survival in the thymus is the result of a sequential selection process based on the interaction between naïve T-cells and the thymic stromal epithelium. Depending on the avidity of the interaction between the TCR of an individual T-cell and the MHC/self-antigen peptide complex expressed by stromal cells, neglect, positive selection, or negative selection can ensue (von Boehmer*, et al.* 1989). Failure to interact with MHC molecules presenting self-antigen on the thymic epithelial cells leads to apoptosis of the T-cell through lack of TCR signalling (neglect). Positive selection is the result of a TCR signal of sufficient avidity to signify a T-cell's ability to recognise self MHC complexes, while a high avidity TCR/MHC interaction is indicative of excessive reactivity to self and hence a potentially pathological self-reactive T-cell, leading again to apoptosis of the T-cell (negative selection). In summary, "the thymus selects the useful, neglects the useless and destroys the harmful" (von Boehmer*, et al.* 1989).

The generation of natural Treg cells in the thymus is believed to occur as the result of an altered negative selection process. Commitment of naïve T-cells to Treg cell differentiation is thought to result when their TCR/MHC affinity is just weaker than that needed for negative selection, but at the extremity of the spectrum for positive selection (Jordan*, et al.* 2001) (Figure 1.1). In other words, Treg cells derived from the thymus are selected largely on the basis of their capacity to interact with self-antigens, and this interaction is implicit in their role for protection against pathological self-reactive immune responses. Thymic-generated Treg cells are thus thought to function primarily to protect from a range of autoimmune diseases (reviewed in Sakaguchi 2005).

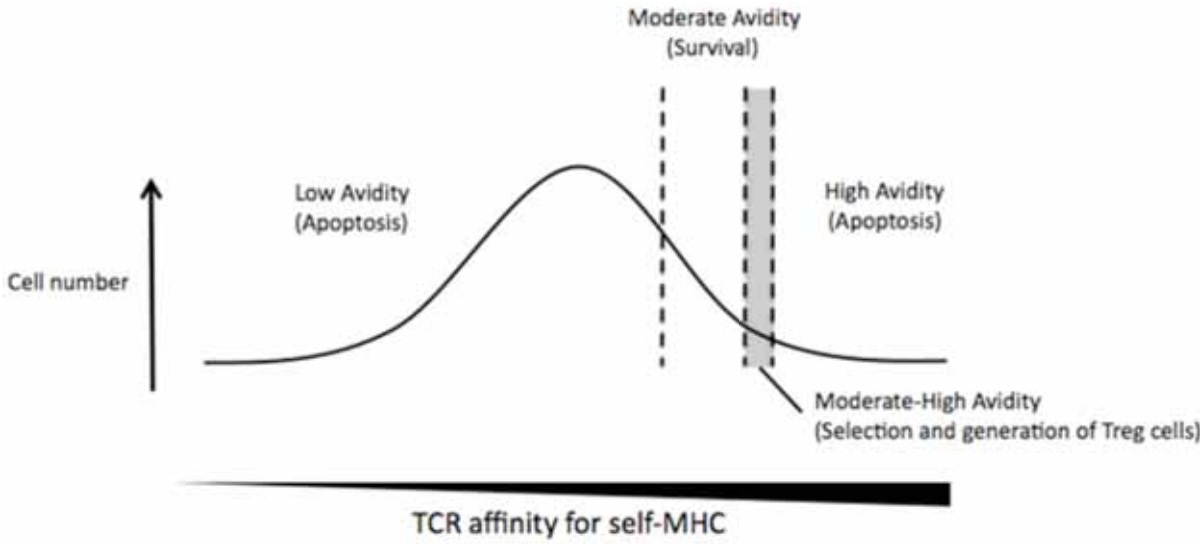


Figure 1.1: Thymic selection of 'natural' Treg cells. Engagement of the TCR of naïve T-cells by self-MHC in the thymus determines the fate of T-cells. TCR engagement by self-MHC on thymic stromal epithelial cells with an affinity at a level just below that needed to induce apoptosis induces the generation of Treg cells.

1.3.4.2.2 Treg cell generation in peripheral tissues

Maintenance of Treg cell numbers over the human life span, despite the extensive and early involution of the thymus, indicates an alternate source of Treg cell generation independent of the thymus. Peripherally there appears to be both *de novo* generation of inducible Treg cells, as well as maintenance and expansion of the existing Treg cell pool (Cozzo*, et al.* 2005, Seddon*, et al.* 1999).

The role of peripheral tissues in sustaining Treg cell populations was elegantly demonstrated by the observation that surgical excision of a tissue depletes the ability of donor Treg cells to prevent organ-specific autoimmune disease after adoptive transfer into intact recipients, compared with Treg cells from unmodified donors (Seddon*, et al.* 1999). This shows that the presence of peripheral tissue antigens is essential for the *de novo* generation of antigen-specific regulatory T-cells from naïve T-cell precursors (Seddon*, et al.* 1999). A similar conclusion is drawn from TCR transgenic mouse models, which show that with persistent low levels of antigenic stimulation, naïve cells can be induced to express the hallmark Treg cell marker Foxp3 and take on a suppressive phenotype (Abbas*, et al.* 2007).

A related explanation invokes a role for peripheral tissue antigens in providing a maintenance signal for existing Treg cells, promoting proliferation and survival after TCR engagement. Although it is widely reported that Treg cells are characteristically anergic following TCR stimulation *in vitro*, adoptive transfer experiments show that these cells can proliferate *in vivo* (Fisson*, et al.* 2003, Gavin*, et al.* 2002, Walker*, et al.* 2003), suggesting their proliferation contributes to maintaining and expanding the *in vivo* Treg cell pool. Critically, proliferation is dependent on the presence of cognate antigen for the Treg TCR (Walker*, et al.* 2003). The likely reality is that peripheral antigens are critical for both the generation of new Treg cells and maintenance of pre-existing Treg cells.

Whilst the role of peripheral tissues in Treg cell generation is established in rodent models, a new understanding of human peripheral Treg cell generation is evolving. Recently it has emerged that *FOXP3* expression is a constitutive product of human (but not mouse) T-cell activation, although typically expression is transient and rapidly down regulated (Wang*, et al.* 2007). This has led to speculation that *FOXP3* induction and subsequent suppression might be a default pathway in the activation of all human T-cells (Pillai*, et al.* 2007), however debate continues over the suppressive qualities of transiently $Foxp3^{+}$ T-cells (Allan*, et al.* 2007). Constitutive expression of *FOXP3* during T-cell activation supports the plausibility of peripheral Treg cell generation being the predominant pathway in humans, but raises the question of how and why *FOXP3* is extinguished in some cells but not others.

Importantly the pathway of peripheral Treg cell generation provides a mechanism by which these cells might be generated with affinities for antigens not expressed in the thymus. Instead peripherally generated Treg cells can express TCRs restricted to specific peripheral tissue antigens. This enables the immune system to regulate responses to exogenous antigens that are foreign to self, but against which aggressive responses are not appropriate, and equips the immune system with plasticity in the Treg cell repertoire facilitating dynamic change and adaptation to the environment. Importantly for reproductive events, this property allows the generation and expansion of Treg cell populations with affinities for reproductive antigens associated with the developing conceptus, which are absent from the thymus.

1.3.4.2.2.1 Treg cell control by dendritic cells, co-stimulatory signals and indoleamine 2,3-dioxygenase

The micro environmental context in which naïve $CD4^+$ cells encounter their cognate antigen is the principal determinant of their differentiation fate and development into Treg cells as opposed to Th1, Th2 or Th17 cells (Rutella*, et al.* 2004, Steinman*, et al.* 2003, Zhu*, et al.* 2008). The type and maturation of professional APCs, such as DCs, is a critical determinant in both the conversion of naïve T-cells into Treg cells, and the expansion and activation of pre-existing Treg cells (Rutella*, et al.* 2006).

Critically the functional phenotype of DCs, characterised by their expression of surface molecules such as costimulatory molecules (CD80/86) and their distinctive cytokine profile, is a determinant of the outcome of the DC/T-cell interaction. The interaction between either naïve $CD4^+$ T-cells or pre-existing Treg cells with a functional subpopulation of DCs, termed 'Tolerogenic DCs', is deterministic in the conversion of naïve $CD4^+$ T-cells into Treg cells or the expansion of pre-existing Treg cell populations (Rutella*, et al.* 2006, Steinman*, et al.* 2003). Importantly the interaction between a Treg cell and a DC is contingent on the presenting by the DC of the cognate antigen for the T-cell. As such, this pathway of Treg cell generation, activation or expansion is clonally specific and can induce and facilitate antigen-specific tolerance to a variety of exogenous antigens (Yamazaki*, et al.* 2006). These may include such antigens as fetal or trophoblast antigens.

Immature or semi-mature DCs, defined by their low expression of IL-12 and TNFα and their variable expression of MHC class II and CD80/86 in the absence of proinflammatory stimuli are capable of inducing the conversion of naïve T-cells into Treg cells (Rutella*, et al.* 2006) (see Figure 1.2). Conversely the expansion of pre-existing Treg cells is dependent on the presence of mature DCs characterised by their high expression of MHC class II, CD80/86 and IL-12 production (Rutella*, et al.* 2006). These DCs are most active when generated under inflammatory condition such as those generated in the presence of GM-CSF (Yamazaki*, et al.* 2003). This expansion is contingent on the expression of surface molecules, primarily the costimulatory molecules CD80 and CD86, with fixation inhibiting the ability of these DCs to expand Treg cells (Yamazaki*, et al.* 2003) (see Figure 1.2)

Another important characteristic of tolerogenic DCs is expression of IDO. In plasmacytoid DCs, IDO expression confers the ability to directly activate resting Treg cells for potent suppressor activity, and is implicated in converting $CD4^+CD25^-$ T-cells to $CD4^+CD25^+Foxp3^+$ Treg cells, and maintaining Treg cell suppressive function in both mice (Sharma, *et al.* 2007) and humans (Chen, *et al.* 2008). This is additional to the ability of IDO-expressing DCs to dominantly inhibit T-cell activation by other nonsuppressive DCs (Munn, *et al.* 2004). Bi-directional signalling between Treg cells and tolerogenic DCs may be one means of infectious tolerance, since Treg cells can condition DCs to express IDO and thereby exert a suppressive influence over neighbouring T-cells (Grohmann, *et al.* 2002, Munn, *et al.* 2002). Induction of IDO may occur via ligation of the costimulatory molecules CD80/CD86 by CTLA-4, a molecule constitutively expressed on Treg cells, ultimately interfering with the DCs capacity to activate effector T-cell function (Grohmann, *et al.* 2002).

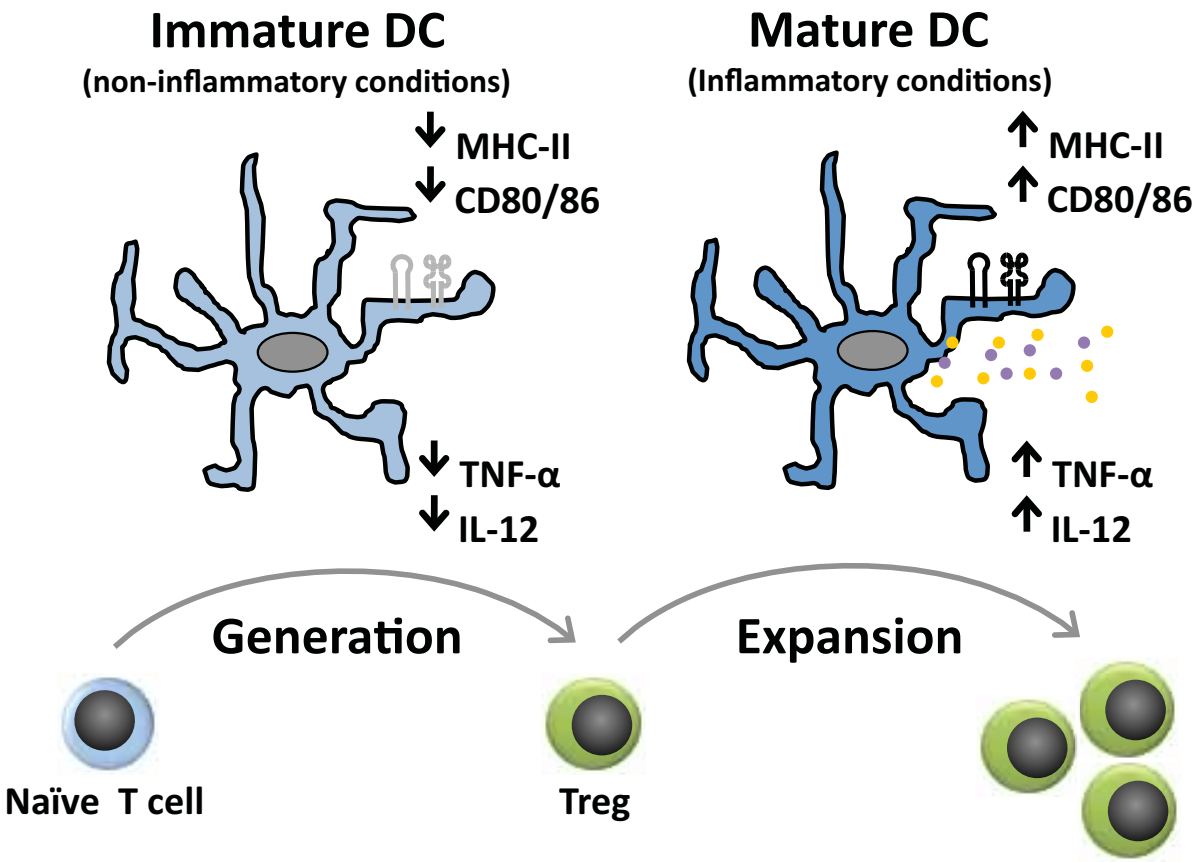


Figure 1.2: Dendritic cell maturation and Treg cell generation and expansion. Under non-inflammatory conditions immature DCs can direct the generation of naïve T-cells into Treg cells. These can then be further expanded by mature DCs that have typically matured under inflammatory conditions and the influence of cytokines such as GM-CSF.

1.3.4.2.2.2 Cytokines in Treg cell generation and lineage commitment

Like all T-cells, Treg cells require ligation of their TCR with cognate antigen and IL-2 in order to differentiate from naïve $CD4^+$ T-cell precursors. IL-2 exerts its effects by binding to a three-subunit membrane complex, which critically includes the high affinity α receptor CD25. Studies where IL-2 signalling is disrupted through the use of blocking antibodies, gene deletion of *IL-2, CD25*, or disruptions of the intracellular signalling of IL-2 via deletion of *STAT5,* reveal a critical role for IL-2 in Treg cell generation and maintenance in both the thymus and the periphery (Schorle*, et al.* 1991, Setoguchi*, et al.* 2005, Willerford*, et al.* 1995). IL-2 null mice display pathologies highly similar to those seen in *Foxp3* null 'scurfy' mice and eventually succumb to fatal lymphoprolipherative autoimmune disease (Kramer*, et al.* 1995, Sadlack*, et al.* 1993). The transfer of wild-type $CD4^+CD25^+$ cells can rescue mice containing IL-2 null T-cells, indicating that phenotype is a result of a deficiency of $CD4^+CD25^+$ cells and not an intrinsic defect in T-cells (Almeida*, et al.* 2002). The role of IL-2 in Treg cell generation and maintenance is further highlighted by experiments using bone marrow chimeras that shown the number of peripheral Treg cells are proportional to the number of IL-2 producing cells (Almeida*, et al.* 2006). Cumulatively these results implicate IL-2 as perhaps the most important growth and maintenance factor for Treg cells within the periphery.

Whilst IL-2 is critical in the maintenance of Treg cell fitness, the cytokine environment required for the fate commitment of naïve T-cells into Treg cells is dependent on factors additional to IL-2. One of the key factors in this lineage commitment is TGFβ. TGFβ has been shown to induce the expression of Foxp3 in both human and murine $CD4^+CD25^-$ *in vitro* (Chen*, et al.* 2003, Fantini*, et al.* 2004). Importantly, TGFβ alone is insufficient to mediate Treg induction, but must work in concert with IL-2 to induce suppressive function, with the two largely believed to play complementary roles in Treg cell generation (Horwitz*, et al.* 2003). The necessity and role of TGFβ in *in vivo* Treg development is more poorly defined. In murine models of diabetes, transient expression of TGFβ on islet T-cells in the priming phase of the disease was sufficient to prime the generation of Treg cells that were subsequently protective against future development of the disease (Peng*, et al.* 2004). However, in contrast to these findings are the observations that mice with $CD4^+$ cells that express a dominant-negative TGFβ receptor (dnTGFβR) that ultimately renders their $CD4^+$ cell population nonresponsive to TGFβ, still

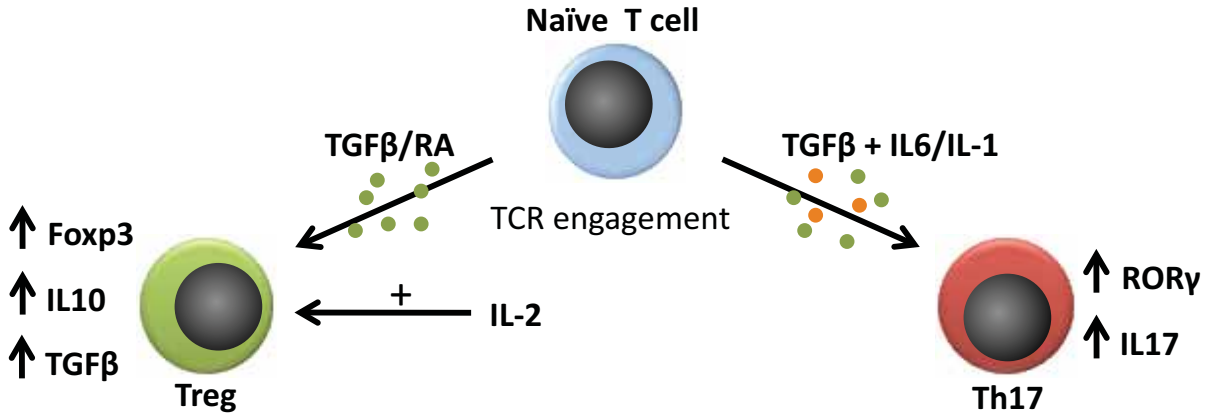
contain equivalent peripheral levels of Treg cells compared to wild-type mice (Fahlen, *et al.* 2005). This may reflect its redundant role in peripheral maintenance of Treg cells rather than the role of TGFβ in the *de novo* generation of Treg cells.

Recently the vitamin D derivative retinoic acid (RA) has been shown to be a potent mediator in facilitating the lineage commitment of $CD4^+CD25^-$ T-cells into Foxp3 expressing Treg cells in tandem with TGFβ. RA dramatically enhances the expression of Foxp3 in $CD4^+$ cells as well as the expansion and suppressive function of Treg cells (Benson, *et al.* 2007, Mucida, *et al.* 2007). Due to the ability of RA to induce gut homing, studies have predominantly focused on the role of RA in the induction of immune tolerance in the gastro-intestinal tract and subsequent oral tolerance (Pino-Lagos, *et al.* 2008). *In vitro* experiments show that RA greatly enhances the expression of Foxp3 in $CD4^+$ cells following antigen stimulation or anti-CD3 stimulation (Benson, *et al.* 2007). RA synergised with TGFβ to facilitate the conversion of over 90% of $CD4^+Foxp3^-$ cells into $CD4^+Foxp3^+$ cells, compared to less than 25% conversion in the absence of RA (Benson, *et al.* 2007). The role that RA has in Treg cell induction outside of the gut mucosa and the mesenteric lymph nodes remains to be investigated. However it is likely that a similar process of Treg cell generation and maintenance could be shared between mucosal sites, including the reproductive tract.

1.3.4.2.2.3 Dynamic Balance: The lineage commitment of Treg cells versus Th17 cells.

An emerging area of central interest is the relationship between Treg cells and pro-inflammatory IL-17-producing T (Th17) cells, which like Th1 cells are implicated in many autoimmune and inflammatory diseases (Zhu, *et al.* 2008). Treg cells and Th17 cells appear to share a common lineage with their relative abundance influenced dramatically by the cytokine environment in which T-cell priming occurs, particularly the ratio of IL-6 to TGFβ (Bettelli, *et al.* 2006). In the absence of IL-6, TGFβ1 simultaneously induces both Foxp3, and the Th17 cell master-switch, retinoic acid-related orphan receptor γt (RORγt). Foxp3 then directly interacts with RORγt to suppress the conversion of naïve T-cells to Th17 cells (Ichiyama, *et al.* 2008). However in the presence of IL-6, Foxp3 expression is down-regulated, allowing RORγt to induce IL-17 synthesis and the conversion of naïve T-cells to Th-17 cells. IL-1, like IL-6 can drive commitment to Th17 as opposed to Treg cells with this predominating over IL-6 in Th17 cell generation in humans (Yang, *et al.*

2008). Existing Treg cells, in the absence of sufficient TGFβ1, can function as inducers of Th17 cells and themselves convert to Th17 cells (Xu, *et al.* 2007). In addition to TGFβ, RA appears to be a critical negative regulator of Th17 cell induction and instead directs Treg cell development. This mutual antagonism and plasticity between Treg cells and IL-17 cells illustrates the fine line between a suppressive or pro-inflammatory immune outcome, and the major importance of the cytokine environment not only at the outset



but also for the duration of the response.

Figure 1.3: Cytokines and naïve T-cell commitment to Treg or Th17 cells. Depending on the cytokine environment, naïve T-cell can commit to Treg or Th17 cells following TCR engagement and activation. An environment containing TGFβ and IL-6 or IL-1 will drive Th17 cell commitment. In opposition to this an environment containing TGFβ alone or in combination with RA will drive naïve T-cell commitment toward Treg cells, which once formed are maintained by paracrine IL-2.

1.3.5 Mechanisms of immune suppression by Treg cells

The mechanisms by which Treg cells exert their suppressive action are still being defined. Treg cells have an extensive and diverse target cell repertoire, and heterogeneous pathways operate depending on the tissue and the identity of the target cell against which suppression is directed. Initially, suppression of activation and proliferation of $CD4^+$ T-cells was thought to comprise the principal function of Treg cells (Thornton, *et al.* 1998). However Treg cells are now known to inhibit proliferation and cytokine production in both $CD4^+$ and $CD8^+$ T-cells (Piccirillo, *et al.* 2001), to suppress B-cell proliferation and immunoglobulin production (Lim, *et al.* 2005), to inhibit cytotoxic function of NK cells (Ghiringhelli, *et al.* 2005) and inhibit maturation and function of APCs, including DCs and macrophages (Cederbom, *et al.* 2000, Misra, *et al.* 2004, Taams, *et al.* 2005). Thus Treg cells can target several stages of the adaptive immune response,

spanning the events of lymphocyte activation and proliferation, through to effector function.

Like all T-cells, Treg cells require engagement of their TCR with cognate antigen to activate their full effector function (Thornton*, et al.* 1998). In this regard Treg suppression is antigen-specific, and both pre-existing and newly-generated Treg cells must encounter antigens in order to exert their suppressive effects. However once activated, Treg cells are capable of targeting other cells in the vicinity and exerting suppression in an antigen non-specific manner (Karim*, et al.* 2004, Thornton*, et al.* 2000).

A major obstacle hindering the definition of Treg cell suppressive action is their apparent multiple and redundant effector mechanisms (Miyara*, et al.* 2007). However, the general consensus is that unlike other types of regulatory T-cells, Treg cells suppress largely in a contact-dependent manner (Annunziato*, et al.* 2002). Their *in vivo* and *in vitro* activities may differ, with *in vivo* experiments implicating TGFβ and IL-10 in paracrine actions that augment their contact-dependent mechanism of suppression (Wahl*, et al.* 2004).

Different suppressive mechanisms most likely operate synergistically, with each individual mechanism occupying a somewhat redundant role. It has recently been shown that Treg cells can act as an IL-2 'sponge', competitively depriving adjacent T-cells of autocrine IL-2, and thus preventing the IL-2-dependent processes of T-cell activation and proliferation (de la Rosa*, et al.* 2004, Scheffold*, et al.* 2005). Additional suppression is mediated by paracrine cytokine production or by cell–to-cell contact either directly between Treg cells and target/effector cells, or via third-party cells such as APCs (Taams*, et al.* 2005, Thornton*, et al.* 1998). Contact-mediated suppression results from ligation of a range of Treg cell surface molecules, namely CTLA-4, membrane-bound TGFβ and lymphocyte-activation gene 3 (LAG3) (Gorelik*, et al.* 2000, Huang*, et al.* 2004, Takahashi*, et al.* 2000, Tivol*, et al.* 1995).

A unique property of Treg cells is their ability to transfer suppressive capabilities to other cellular subsets in a phenomenon known as 'bystander suppression'. This can be achieved through Treg cells conferring upon target cells the ability to inhibit downstream steps in the immune cascade. For example, Treg cells can modify the function and phenotype of DCs or induce their production of suppressive cytokines such as IL-10

(Jonuleit*, et al.* 2002). This likely explains the notion of 'infectious immunity', a process by which suppression to a third- party antigen can be induced via physical association of that antigen with a previously tolerated antigen (reviewed in Waldmann *et al*. 2006). This is illustrated when animals of strain A, that are rendered tolerant to grafts from strain B, demonstrate prolonged tolerance of subsequent grafts from (B × C) F1 animals, and eventually tolerate grafts from strain C animals (Davies*, et al.* 1996, Waldmann*, et al.* 2006). Such a process allows for a limited repertoire of antigens to induce a dominant tolerant state to a much larger range of antigens that may subsequently be encountered in the same tissue site. This characteristic feature might be of special relevance in reproductive tissues, where a variety of antigens would be encountered in association with gametes and the developing gestational tissues.

1.3.6 Chemokines and Regulatory T-cell Migration.

The suppressive mechanisms utilised by Treg cells dictate that their spacial distribution is a critical determinant in their ability to provide specific suppression. In addition to this, the sequence and temporal migration of these cells is critical in their activation and subsequent suppressive potential. As such, the location of Treg cells throughout pregnancy, and their migration to lymphoid tissue or to tissues such as the decidua and the factors that influence this, are vital determinants of the balance between a suppressive or inflammatory response.

Like other T-cells, the migration and trafficking of Treg cells is largely dependent on the expression of chemokines within tissue and the presence of the cognate chemokine receptor on the surface of the Treg cell (Bono*, et al.* 2007, Curiel*, et al.* 2004, Mantovani 1999). As such, the differential expression of these factors is critical in determining the migration of Treg cells into their required site.

Insights into the specific chemokines and chemokine receptors that influence Treg cell migration can be gained from studies into the migration of Treg cells into inflammatory sites as well as their migration into allografts and their consequential influence on the resultant state or lack of tolerance. Additionally the factors that are responsible for the specific migration of Treg cells into the uterus throughout the estrus cycle and pregnancy have also been studied. These studies highlight some critical chemokines and chemokine receptors in the migration and function of Treg cells.

Studies in an islet allograft model showed that not only the site of migration of Treg cells was important, but the order in which the cells migrated determined their suppressive capabilities (Zhang*, et al.* 2009). The studies showed that for Treg cells to elicit their optimal suppression they first needed to be activated. This activation was dependent on their exposure to the allograft (Zhang*, et al.* 2009). Resultantly the study found that prolonged graft survival was dependent on the sequential migration of Treg cells from site of inflammation (the allograft) where they were activated, followed by subsequent migration into the draining lymph nodes.

The initial recruitment of Treg cells into the site of transplantation was dependent on CCR2, CCR4 and CCR5 as well as P- and E-selectin ligand. The subsequent migration to the draining lymph node was depended on the expression of the chemokine receptors CCR2, CCR5 and CCR7. The adoptive transfer of Treg cells deficient in these chemokine receptors failed to prolong graft survival, which was associated with a failure of these cells to migrate to the draining lymph nodes and a retention of these cells within the site of the allograft (Zhang*, et al.* 2009). The presence of the emigrated and activated Treg cells within the draining lymph nodes was needed to optimally prevent the infiltration of activated T-cells back into the graft (Zhang*, et al.* 2009).

1.4 TREG CELL ABUNDANCE AND FUNCTION IN PREGNANCY SUCCESS

1.4.1 Treg cells in rodent models of pregnancy

The first report implicating Treg cells as having a functional role in murine pregnancy appeared in 2004, when it was shown that $CD4^+CD25^+$ cells increase in number in the blood and lymph nodes of pregnant mice (Aluvihare*, et al.* 2004). An expansion in the Treg cell pool is detectable in lymph nodes draining the uterus from as early as 2 days after mating, while elevated blood levels do not become evident until after implantation (Aluvihare*, et al.* 2004). The increase in Treg cells is associated with accumulation of $Foxp3^+$ cells and *Foxp3* mRNA expression in the uterus, as well as elevated lymphocyte suppressive function in the spleen and lymph nodes. The systemic expansion in Treg cell populations occurs irrespective of fetal alloantigens, since Treg cell numbers are elevated over non-pregnant levels in both syngeneic and allogeneic pregnancies, suggesting involvement of factors such as pregnancy hormones or fetal-specific minor-

histocompatibility antigens (Aluvihare*, et al.* 2004, Zhao*, et al.* 2007). However, conceptus alloantigen leads to a greater increase in Treg cell numbers, and is associated with specific suppression of anti-paternal alloantigen reactivity (Zhao*, et al.* 2007). The early increase in Treg cell numbers, even prior to embryo implantation, was confirmed in another study (Thuere*, et al.* 2007). Interestingly, the increase in Treg cells is not sustained throughout pregnancy but progressively declines from mid-gestation to return to close to non-pregnant levels by fetal delivery at term (Zhao*, et al.* 2007).

The physiological necessity of Treg cells for pregnancy was elegantly demonstrated using an adoptive transfer model, wherein complete T-cell populations or populations depleted of $CD4^+CD25^+$ Treg cells were transferred into pregnant T-cell deficient mice (Aluvihare*, et al.* 2004). In the absence of Treg cells, allogeneic fetuses were uniformly rejected, while syngeneic fetuses were unaffected (Aluvihare*, et al.* 2004). These findings were confirmed utilizing another approach, using administration of the CD25-reactive PC61 monoclonal antibody to deplete $CD25^+$ cells *in vivo* (Darrasse-Jeze*, et al.* 2006). When mice mated with allogeneic males were administered PC61, activated $CD8^+$ and $CD4^+$ cell populations were expanded in the lymph nodes draining the uterus, indicating an activation of the local immune system. This resulted in fewer allogeneic fetuses surviving to term, while no effect on syngeneic pregnancies was observed (Darrasse-Jeze*, et al.* 2006). Collectively these studies support the notion that Treg cells are required to suppress maternal immune responses targeted against fetal alloantigens, rather than male-specific or trophoblast-specific minor histocompatibility antigens.

In a further demonstration of the importance of Treg cells for maternal fetal tolerance, fewer $CD4^+CD25^+$ cells were found in the decidual tissues of abortion-prone CBA/J mice mated with DBA/2 males. Adoptive transfer of Treg cells, purified from normal pregnant mice, elevated decidual *Foxp3* mRNA levels and prevented fetal loss (Zenclussen*, et al.* 2005). Interestingly, transfer of Treg cells from non-pregnant normal mice to the abortion prone mice was ineffective, and transfer of Treg cells on or after the day of embryo implantation (day 4 of pregnancy) did not prevent abortion (Zenclussen*, et al.* 2005). Consistent with this, depletion of Treg cells using PC61 during the first two days of pregnancy led to implantation failure (Zenclussen*, et al.* 2005). Together these findings show that Treg cells are essential during the first days of pregnancy, even prior to the

time of embryo implantation, and the factors that regulate Treg cell abundance and activity during this period may be critical.

1.4.2 Treg cells in human pregnancy

Several studies have examined the dynamics of lymphocyte subpopulations during pregnancy in women. An increased expression of CD25 on decidual lymphocytes compared to peripheral blood lymphocytes was described in 1992 (Saito*, et al.* 1992). However, due to the state of the art at this time no markers or attempts to define Treg cells were performed.

The first observations on $CD4^+CD25^+$ Treg cells in human pregnancy described an increase in this subset in decidual tissue early in pregnancy (Sasaki*, et al.* 2003). Subsequent reports demonstrated a systemic increase in peripheral blood $CD4^+CD25^+$ cells during early pregnancy with a peak in the second trimester and a subsequent decline to levels slightly higher than pre-pregnancy levels post partum (Heikkinen*, et al.* 2004, Somerset*, et al.* 2004). These early studies have later been confirmed in studies that more precisely identify Treg cells as $CD25^{high}$ cells rather than analysis of the heterogenic $CD25^+$ population (Saito*, et al.* 2005, Sasaki*, et al.* 2004, Tilburgs*, et al.* 2006, Zhao*, et al.* 2007). The function of the expanded $CD4^+CD25^+$ T-cell pool in pregnancy was confirmed to be suppressive *in vitro* and the population was highly enriched for Foxp3 (Somerset*, et al.* 2004). Several studies report Treg cells accumulation in decidual tissue at densities greater than in peripheral blood, indicating not only a role for peripheral expansion of Treg cell population but also a local recruitment to the site of action (Sasaki*, et al.* 2004, Tilburgs*, et al.* 2006). Evidence suggests that this expansion may be influenced by fetal alloantigens with Treg cells from peripheral blood of pregnant women suppressing anti-alloantigen responses *in vitro*, with enhanced capacity to suppress stimulation by paternal antigens as opposed to irrelevant third-party alloantigens (Mjosberg*, et al.* 2007 (e-pub ahead of print)).

It may be speculated that Treg cells have a greater role than simply promoting tolerance throughout gestation. The percentage of T lymphocytes that express CD4 and CD25 are significantly decreased in the deciduas of women who undergo a spontaneous vaginal delivery compared to an elective caesarean section (Sindram-Trujillo*, et al.* 2004). Furthermore, a sharp decrease in suppressive $CD4^+CD25^{high}$ Treg cells towards the end of

pregnancy and a concurrent rise in T-cells displaying an activated phenotype defined by a $CD4^{+}CD25^{low}$ phenotype was demonstrated late in pregnancy (Zhao*, et al.* 2007). This implies a potential role for Treg cells in the immunological changes preceding labor, and prompts speculation that their decline might be a causal factor in fetal expulsion from the maternal tissues. However, studies using more specific markers and animal models are needed to address the possibility of any active role of Treg cells in parturition.

1.4.3 Treg cells in infertility, miscarriage and pregnancy complications

Several studies report an association between Treg cell paucity and complications of pregnancy, particularly pre-eclampsia. There is abundant evidence that immunological factors are crucial for the development of this common disease, which in developed countries is responsible for about 15-20% of maternal mortality (Sargent*, et al.* 2006). In pre-eclampsia, Th1 dominance develops together with an increased inflammatory response towards the fetus (Saito*, et al.* 2003, Sargent*, et al.* 2006), raising the question of whether this might be secondary to impaired function or decreased numbers of Treg cells.

Initially studies exploring possible associations between Treg cell deficiencies and pre-eclampsia were conflicting. It was reported that the number of $CD3^{+}CD25^{+}$ lymphocytes is decreased in peripheral blood of women with gestational-induced hypertension, especially in the event of pre-eclampsia (Mahmoud*, et al.* 2003), however no Treg cell specific markers were used to confirm this. While a later study found no association between pre-eclampsia and changes in peripheral blood Treg cell levels (Paeschke*, et al.* 2005). Several subsequent studies that more specifically characterised Treg cells reported that $CD4^{+}CD25^{high}$ T-cells are significantly reduced in both the peripheral blood and decidual tissue of pre-eclamptic patients, compared to normal pregnant women (Darmochwal-Kolarz*, et al.* 2007, Sasaki*, et al.* 2007). A recent report demonstrates a decrease in $CD4^{+}Foxp3^{+}$ cells in preeclamptic women but showed no change in the level of $CD4^{+}CD25^{+}$ or $CD4^{+}CD25^{brighT\text{-}cells}$ (Prins*, et al.* 2009). Observations that the tryptophan catabolising enzyme IDO is decreased in pregnancies complicated by pre-eclampsia is consistent with diminished Treg cell activity in pregnancy (Nishizawa*, et al.* 2007, Santoso*, et al.* 2002), with decreased IDO potentially caused by, and contributing to, insufficient Treg cell activity (Grohmann*, et al.* 2002). The dynamic balance between Treg cells and Th17 cells may also be a causal factor in pre-eclampsia, with a preliminary

report suggesting a possible bias toward Th17 cells over Treg cells in women suffering third trimester pre-eclampsia (Santner-Nanan*, et al.* 2008).

Recurrent spontaneous abortion has also been associated with a mal-adaptation in the maternal immune system (Laird*, et al.* 2003). Several studies implicate an imbalance between suppressive and activated CD4 cells as being linked to pre-eclampsia. There was an increased number of $CD4^{+}CD25^{+}$ cells in the decidua at the time of spontaneous abortion compared to decidua recovered at therapeutic abortion (Quack*, et al.* 2001, Vassiliadou*, et al.* 1999). However the proportion of Treg cells amongst these cells was evidently reduced, since both decidual and peripheral blood $CD4^{+}CD25^{high}$ T-cells were lower in tissues recovered after spontaneous abortion, compared to therapeutic abortions or non-pregnant women (Sasaki*, et al.* 2004, Yang*, et al.* 2007, Yang*, et al.* 2008).

Reduced responsiveness to pregnancy-associated expansion of Treg cell populations, evidenced by numerically fewer Treg cells as well as Treg functional deficiency, may underpin a reduced immunosuppressive capability and cause predisposition to miscarriage. In a comprehensive study, women experiencing repeated miscarriage were shown to have a reduced frequency of Treg cells within the peripheral blood $CD4^{+}$ pool, and reduced suppressive capacity, compared with normal fertile women (Arruvito*, et al.* 2007). Primary unexplained infertility has also been associated with reduced expression of *FOXP3* mRNA in endometrial tissue (Jasper*, et al.* 2006), suggesting that impaired differentiation and/or recruitment of uterine T-cells into Treg cells even prior to conception may affect the capacity to establish pregnancy in women.

1.4.4 Origin and antigen specificity of Treg cells in pregnancy

Defining the pathways of Treg generation and the consequences for specific functional roles is critical to understanding how Treg cells operate throughout gestation. We have incomplete knowledge of the tissue origins of decidual Treg cells and the roles of conceptus antigens and other factors including cytokines and hormones in driving their expansion in early pregnancy, however recent studies are starting to shed light on the role of hormones, predominantly estrogen, on Treg development and expansion. Both antigen-associated and antigen-independent mechanisms are likely to contribute to the further expansion in Treg cell populations observed in early pregnancy. The thymus

appears not to be the origin of the elevated numbers of Treg cells in pregnancy (Zhao*, et al.* 2007), so by default a peripheral tissue pathway of generation must occur. The relatively elevated numbers of Treg cells in tissues of mice bearing allogeneic pregnancies (Kallikourdis*, et al.* 2007, Zhao*, et al.* 2007) suggests fetal alloantigens act to drive Treg cell proliferation and migration. However, expansion of Treg cells in mice gestating syngeneic fetuses (Aluvihare*, et al.* 2004, Zhao*, et al.* 2007), albeit reduced, indicates alloantigen independent mechanisms for expansion throughout gestation.

In both mice and women, there is evidence of ovarian hormone-regulated fluctuations in uterine Treg cell populations. In mice, administration of 17-β-estradiol to ovariectomised mice causes elevation in Treg cell numbers and *Foxp3* mRNA expression. Additionally *in vitro* experiments using $CD4^+CD25^-$ cells show direct induction of Foxp3 in cells via CD3 and CD28 stimulation in the presence of E2 (Polanczyk*, et al.* 2004, Tai*, et al.* 2008). However Treg cells numbers in lymphoid tissues of pregnant mice poorly correlated with serum or uterine E2 levels *in vivio* (Tai*, et al.* 2008). Treg cells accumulate in the uterus during the estrus phase of the reproductive cycle as evidenced by elevated *Foxp3* mRNA expression, potentially in response to estrogen-induced expression of several chemokines that target chemokine receptors expressed by Treg cells (Kallikourdis*, et al.* 2007) (see section 1.4.5).

In women, a comparable expansion in $CD4^+CD25^+Foxp3^+$ Treg cells occurs in the peripheral blood during the late follicular phase of the menstrual cycle, when $CD4^+Foxp3^+$ cell abundance tightly correlates with serum E2 levels. This is followed by a dramatic decline in the luteal phase of the estrus cycle, which is consistent with levels in postmenopausal women (Arruvito*, et al.* 2007). Interestingly, the abundance of Treg cells detected in males was comparable to the level seen in women in their late follicular phase indicating an estrogen independent mechanism for Treg cell maintenance in males (Arruvito*, et al.* 2007). Direct actions of E2 on Treg proliferation and suppressive function may contribute to cycle-related fluctuations (Prieto*, et al.* 2006). Thus, each reproductive cycle elicits a relative expansion of Treg cell populations.

In addition to augmented Treg cell numbers as a result of E2 treatment, the suppressive potential of Treg cells may also be enhanced as a result of estrogen exposure. *In vivo* experiments show that E2 treatment over a period of 4 weeks in combination with TCR

stimulation via immunisation with antigens, increased the expression of the immunosuppressive molecule PD-1 exclusively in the Treg cell compartment (Polanczyk*, et al.* 2007, Wang*, et al.* 2009). Predominantly this augmented expression is localised within the intracellular compartments of Treg cells (Polanczyk*, et al.* 2007). PD-1 localisation has been suggested to be indicative of the activation status of Treg cells, with relocation from intracellular to surface expression relating to Treg cell activation (Raimondi*, et al.* 2006). However it is important to note that since Treg cells require exposure to antigen to proliferate and to exert their full suppressive function, estrogen alone would be insufficient to activate Treg cells for pregnancy, and instead can be viewed as potentiating Treg cells in preparation for stimulation by pregnancy-associated antigens. This may correlate with a relocation of PD-1 from intracellular to surface expression.

Whilst there is a growing case for estrogen being a critical factor in the increase in Treg cell numbers during pregnancy, its exact role is far from clear. There is insufficient *in vivo* evidence to support the suggestion that this is the primary driving factor that induces the changes observed during pregnancy. A recent report in humans that utilizes more specific Treg cell markers, analyzed the role of pregnancy hormones in Treg cell regulation during mid gestation. Contrary to other reports it showed a decrease in Treg cells in the peripheral blood of pregnant women and demonstrated *in vitro* that both estrogen and progesterone could replicate this decrease in phenotypic Treg cells (Mjosberg*, et al.* 2009). Additionally E2 and progesterone treatment of overectomized mice resulted in no change in the percentage of Treg cells despite significant elevation in their respective hormone levels. Hormones as a sole determinant of the augmentation of Treg cell number also fails to explain the very early changes in Treg cells noted prior to implantation (Aluvihare*, et al.* 2004), as estrogen levels during pregnancy fail to significantly change in the pre-implantation period (Schuurs*, et al.* 1990). The effect of progesterone on the formation of Treg cells also requires further evaluation. During pregnancy progesterone levels increase by up to 15-fold in mice and up to 1000-fold in humans (Schuurs*, et al.* 1990). However to date there is a paucity of studies on the effect of progesterone on Treg cell development.

Other pregnancy related hormones may also be critical in the control of Treg cell numbers. Recent work suggests a possible role for human chronic gonadotropin (hCG) in

regulatory T-cell localisation into the implantation site during pregnancy (Schumacher*, et al.* 2009). Treg cell exposure to hCG resulted in the expression of the hCG responsive receptor, the luteinizing hormone/choriogonadotroptin (LH/CG) receptor, which in *in vitro* assays resulted in the migration of Treg cells toward hCG (Schumacher*, et al.* 2009). Ultimately this may function to localize Treg cells to the implanting embryo in the first trimester of pregnancy when hCG is produced by the 6-8 day blastocyst and then subsequently by syncitiotrophoblast cells (Hoshina*, et al.* 1985), before hCG levels decline in the second and third trimesters of pregnancy.

Consistent with a role for normal embryonic development in regulating Treg numbers, $CD25^{+}CD3^{+}$ cells are decreased in peripheral blood of women with ectopic or anembryonic pregnancies compared to women with normal pregnancies (Ho*, et al.* 1996). Decidual $CD25^{+}CD3^{+}$ cells were also fewer in numbers, but interpretation of this study is confounded since no specific Treg cell marker was utilised. Conversely, Treg cells were more abundant in implantation sites in partial and complete molar pregnancies, where Treg cell numbers positively correlated with $CD8^{+}$ cytotoxic T-cells (Nagymanyoki*, et al.* 2007). An intact conceptus may therefore not be required for recruitment of Treg cells, but a role for trophoblast antigens cannot be excluded.

The temporal sequence of Treg cell expansion during gestation is critical for a proper understanding of the driving factors involved in the induction and maintenance of immune tolerance in pregnancy. Exposure to paternal antigen occurs in two waves in the reproductive process; initially during transmission of seminal fluid at coitus (Robertson*, et al.* 2001), and secondly when placental trophoblast cells invade maternal tissues after embryo implantation (Jaffe*, et al.* 1991, Redline*, et al.* 1989). This raises the possibility that male antigens present in seminal fluid may contribute to activating and expanding the antigen-specific Treg cell pool prior to conceptus antigen encounter. $CD4^{+}CD25^{+}$ Treg cell abundance increases within days after mating in mice (Aluvihare*, et al.* 2004, Tai*, et al.* 2008) and there is evidence of antigen specificity in the Treg cell response even before embryo implantation (Kallikourdis*, et al.* 2007, Zhao*, et al.* 2007). In both mice and women, seminal fluid elicits an inflammation-like response in the female reproductive tract associated with recruitment of DCs into the endometrial and cervical tissues (Robertson 2007, Robertson 2005, Robertson*, et al.* 2006, Sharkey*, et al.* 2007). These DCs are capable of processing male antigens in seminal fluid and activating T-cells

in draining lymph nodes (Johansson*, et al.* 2004, Moldenhauer*, et al.* 2009). Recent observations in mice exposed to seminal fluid in the absence of conception, support a role for seminal fluid in driving Treg cell activation and proliferation and mediating functional tolerance to paternal alloantigens at the time of embryo implantation (Robertson*, et al.* 2009). The high levels of TGFβ and prostaglandin E in seminal fluid (Robertson*, et al.* 2002) are likely to be important in skewing the T-cell response towards the Treg cell phenotype.

Such a process would explain epidemiological observations that exposure of the mother to the conceiving partners semen reduces the rate of pre-eclampsia (Marti*, et al.* 1977, Robillard*, et al.* 1994). Seminal fluid shares many of the same paternal antigens later expressed by the conceptus, however the nature of Treg cells would not necessitate that the full repertoire of conceptus antigens need be present at the site of initial Treg cell priming. When applied in the context of pregnancy, the notion of bystander tolerance conferred by Treg cells might explain why repeated exposure of the female reproductive tract to a limited panel of paternal antigens in seminal fluid, or alternatively prolonged exposure to a small number of trophoblast antigens early in pregnancy, may be sufficient to prime the maternal immune system to tolerate a wider repertoire of additional antigens, expressed later in gestational tissues.

1.4.5 Chemokines and Treg cell migration in pregnancy.

Studies specifically into the modulation of chemokines and chemokine receptors on Treg cells throughout the estrus cycle and during pregnancy highlight an important role for the chemokine receptor CCR5 and one of its ligands CCL4.

Real time quantitative PCR (qRT-PCR) studies in murine models show cyclical regulation of a variety of chemokines in uterine tissue throughout the estrus cycle, peaking at estrus. These included *Ccl3*, *Ccl4* and *Ccl22* (Kallikourdis*, et al.* 2007). However, of these only *Ccl4* showed an elevated expression during pregnancy. The temporal regulation of *Ccl4* mRNA was consistent with that of *Foxp3* mRNA with both showing similar patterns of regulation throughout the estrus cycle, and a comparative level of elevation at embryonic day (E)10.5 in pregnancy implicating CCL4 as possibly having a role in recruitment or retention of $Foxp3^{+}$ cells within the uterus (Kallikourdis*, et al.* 2007).

Despite this, no correlation between *Foxp3* mRNA and *Ccl4* mRNA was shown within individual samples by the authors.

Further supporting work for a role of CCL4 in Treg cell recruitment into the gravid uterus comes from studies that show an accumulation of T-cells expressing the CCL4 receptor, CCR5, within the uterus at mid-pregnancy (E10.5) (Kallikourdis*, et al.* 2007). These $CCR5^+$ T-cells were shown to phenotypically and functionally resemble Treg cells, with expression of CD25 as well as elevated levels of *Foxp3* mRNA. Additionally these cells were shown to be suppressive in mixed lymphocyte reactions, with $CD4^+CD25^+CCR5^+$ cells showing superior suppressive potential to $CD4^+CD25^+CC5^-$ cells (Kallikourdis*, et al.* 2007).

Interestingly CCR5 deficient mice and wild-type mice showed comparable levels of *Foxp3* mRNA in the gravid uterus at E10.5 (Kallikourdis*, et al.* 2007). This finding suggesting a level of redundancy in the recruitment of Treg cells via CCR5. An alternative explanation is that exposure to alloantigen induced an up regulation of CCR5 on antigen expressed cells. Consistent with this, allogeneic pregnancies showed a significantly higher level of $CD4^+CD25^+CCR5^+$ cells in the gravid uterus compared to syngeneic pregnancies (Kallikourdis*, et al.* 2007). This invokes two possible explanations, firstly that the presence of alloantigen induces Treg cell activation and the consequential up regulation of CCR5, or secondly that alloantigen is involved in the upregulation of chemokines that recruit $CCR5^+$ cells into the uterus. The ectopic marking of cells with a constitutively expressed green fluorescent protein (GFP) gene showed that CCR5 expressing cells preferentially accumulate in the gravid uterus, suggesting CCR5 mediated T-cell migration rather than an increase in the expression of CCR5 on cells following activation in the local tissue.

Inherently chemokine systems have a high level of redundancy that results in a relative insensitivity to the modulation of any individual chemokine or chemokine receptor. This high level of redundancy suggests that any recruitment of Treg cells into the uterus would likely be dependent on multiple chemokines and chemokine receptors on the Treg cells (Mantovani 1999). Additionally the necessary sequence and order of Treg cell migration for optimal activation and function would mean that the expression of specific chemokines within the uterus and iliac (uterine-draining) lymph nodes would be highly

variable throughout the course of pregnancy, depending on the stage of pregnancy and the corresponding physiological requirements.

1.5 THE ROLE OF INTERLEUKIN 10 IN IMMUNE TOLERANCE, REGULATORY T-CELL FUNCTION AND PREGNANCY OUTCOMES

1.5.1 Interleukin 10 function and immune regulation

IL-10, originally known as cytokine synthesis inhibitory factor (CSIF), is a pleitropic cytokine that has potent anti-inflammatory properties. IL-10 is produced by a diverse array of cellular subsets including T-cells, B-cells, monocytes, macrophages and some subsets of DCs. Of these sources it is perhaps T-cells that are the most potent source of IL-10, with Th2, Tr1, Th1 and recently Th17 cells representing the major subsets of T-cells that produce IL-10 (Mosser*, et al.* 2008). It has also been shown that $CD4^{+}CD25^{+}$ Treg cells are an additional source of IL-10 (Annacker*, et al.* 2001)

Like its production, IL-10 has effector functions on a diverse range of cellular subsets including DCs, macrophages, NK cells and Th1 cells, as well as being a potent co-stimulant of B-cell differentiation (Moore*, et al.* 1993). The major biological function of IL-10 appears to be directed at DCs and macrophages. It inhibits antigen presentation, the expression of MHC class II as well as that of the costimulatory molecules CD80/86, and ultimately inhibits DC maturation and as such antigen priming and activation of T-cells (Mosser*, et al.* 2008). IL-10 can also directly attenuate the effector function of T-cells (Annacker*, et al.* 2001).

IL-10 acts in a feedback loop to inhibit the production of proinflammatory cytokines including IL-2 and IL-12, IFNγ and TNFα primarily from APCs (Mosser*, et al.* 2008)(see Table 1.3). The function of such a mechanism was demonstrated in a model of the autoimmune disease experimental allergic encephalomyelitis (EAE), whereby it was shown that IL-10 subverted the onset of the disease by a mechanism that involved the inhibition of IL-12 release from APCs (Segal*, et al.* 1998).

Table 1.3: Influence of IL-10 on cytokine production.

Cytokine	Effector function of IL-10	Cytokine type/function
IL-2	inhibits	Type 1/Inflammatory
IFN-γ	inhibits	Type 1/Inflammatory
IL-4	inhibits	Type 2/Anti inflammatory
IL-5	inhibits	Type 2/Anti-inflammatory
IL-1α/β	inhibits	Inflammatory
IL-6	inhibits	Type 2/Inflammatory
IL-12	inhibits	Type 1/ Inflammatory
TNF-α	inhibits	Type 1/Inflammatory

The generation of IL-10 null mice has given many insights into the importance of IL-10 in homeostasis. The most apparent phenotype of these animals was the formation of severe spontaneous inflammation of the lower intestine (colitis) at approximately 2-3 months of age (Kuhn*, et al.* 1993, Rennick*, et al.* 1997, Sellon*, et al.* 1998). This was attributed to an over-exaggerated immune response to commensal microflora within the intestines (Kullberg*, et al.* 1998). In line with this, IL-10 has been shown to be critical in the control of early inflammatory responses in both systemic and local responses (Rennick*, et al.* 1997). As such it has been shown that IL-10 can inhibit the clearance of a range of infection organisms including *Klebsiella pneumonia, Candida albica, and Mycobacterium avium* (reviewed in Rennick *et al*.1997).

Converse to its deleterious role in the clearance of some infectious organisms, IL-10 has been shown to be critical in terminating and controlling inflammatory responses. Mice deficient in IL-10 succumb to typically avirulent strains of infectious pathogens such as *Toxoplasma gondii* (Gazzinelli*, et al.* 1996) as well as sub-lethal doses of bacterial mimetics such as lipopolysaccharide (LPS) (Berg*, et al.* 1995). The pathology was attributed to aberrant regulation of the $CD4^+$ T-cell population and overproduction of IL-12, IFNγ and TNFα (Gazzinelli*, et al.* 1996). The correct regulation of IL-10 is therefore critical for the coordination of an appropriate immune response to a range of infectious agents. The immune-regulatory properties of IL-10 also implicate this cytokine in the tolerance of tumours as well as influencing the susceptibility to autoimmune diseases (Segal*, et al.* 1998).

1.5.2 Interleukin 10 and regulatory T-cells

Whilst IL-10 has been shown to be produced by a range of T-cell subsets including CD8 T-cell as well as Th1 and Th2 CD4 T-cells, it has also been shown to have a considerable role in the regulatory T-cell subset, specifically $CD4^+CD25^+$ Treg cells and Tr1 (Maynard*, et al.* 2007). IL-10 has been shown to have functions in both the induction and effector phases of Treg cells. However IL-10 is not critical for the development of $CD4^+Foxp3^+$ Treg cells, with $Foxp3^+$ expressing cells with the ability to produce IL-10 still being present in IL-10 null animals (Maynard*, et al.* 2007). Primarily IL-10 is implemented in the suppressive function of Treg cells. Unlike other factors that are critical for Treg cell generation, maintenance and function, such as CTLA-4, IL-2 and TGFβ, animals deficient in IL-10 do not exhibit the multi-organ autoimmune disease that is indicative of Treg cell dysfunction. Consequently it is proposed that IL-10 possesses a somewhat reduced role in Treg cell function.

$CD4^+CD25^+$ Treg cells were first shown to produce IL-10 in 1996 (Asano*, et al.* 1996), with approximately 10-20% of $Foxp3^+$ Treg cells shown to secrete the cytokine (Maynard*, et al.* 2007). Investigation of the importance of IL-10 in Treg cell function has been performed extensively, utilising T-cell transgenic models and adoptive transfer of Treg cells from IL-10 null animals. Using an ovalbumin responsive T-cell transgenic model, it was shown that the importance of IL-10 in Treg cell function was specific to the stage of T-cell priming/proliferation or effector function (Dercamp*, et al.* 2005).

IL-10 deficiency resulted in increased proliferation and IFN-γ production of OT-I T-cells in response to ovalbumin expressing tumour cells. Depletion of Treg cells did not further facilitate the proliferation or IFN-γ production of these cells, suggesting a critical role of IL-10 in Treg cell control of CD8 T-cell proliferation and cytokine secretion (Dercamp*, et al.* 2005). However inhibition of cytotoxic T-lymphocyte (CTL) lytic function by Treg cells was shown to be independent of IL-10, as Treg depletion but not IL-10 deficiency enhanced antigen specific lytic function. Conversely it was shown that IL-10 could inhibit the production of the pro-inflammatory cytokine IL-12 by DCs in a mechanism independent of Treg cells. However the ability of DCs to prime an immune response *in vitro* was enhanced by IL-10 deficiency and Treg cell depletion independently and further facilitated in mice both depleted of Treg cells and deficient for IL-10, evoking a synergistic role for IL-10 and Treg cells (Dercamp*, et al.* 2005). Together these results

demonstrate that the utility of IL-10 by Treg cells to inhibit immune responses is strictly dependent on the stage of immune response and target cell of inhibition. With the role of IL-10 in Treg cell suppression being redundant, critical or synergistic depending on the site and stage of the immune response.

Consistent with the role of IL-10 being temporally defined, it has been shown that IL-10 may only be inhibitory in the early stages of infection (reviewed in Rennick *et al*. 1997). It was shown that the ability of Treg cells (defined as $CD25^{+}$ and $CD45RB^{low}$) to control the accumulation of effector and memory $CD4^{+}$ T-cells following adoptive transfer into lymphopenic hosts, was dependent on the presence of IL-10. However the ability of these cells to inhibit wasting disease within the host was still retained, further highlighting the complexity of IL-10 function in Treg cells (Annacker*, et al.* 2001).

Overall it is likely that IL-10 secretion by Treg cells is directed toward perturbing DC mediated responses rather than directly inhibiting T-cell effector functions. Through mechanisms such as altering the maturation state and cytokine profiles of DCs via IL-10, Treg cells can consequently inhibit T-cell proliferation.

1.5.3 Interleukin 10 in pregnancy

The cytokine milieu both locally in the uterus and systemically is critical to facilitating the appropriate state of immune-privilege as well as to allow correct embryo implantation and trophoblast invasion. One of the cytokines identified to be critical in this cytokine milieu is IL-10. IL-10 has been linked with preventing pregnancy pathologies such as preeclampsia, as well as influencing placental invasion and architecture, and providing protection from bacterial induced pregnancy loss.

Several researchers have addressed the role of cytokines, including IL-10, in association with pregnancy induced hypertension or preeclampsia. It has been shown that in women suffering from preeclampsia there is a significant perturbation of the cytokine balance away from type 2 cytokines and toward type 1 cytokines. This specifically included a significant decrease in the levels of systemic IL-10 (Azizieh*, et al.* 2005, Borekci*, et al.* 2007, Sharma*, et al.* 2007). Additionally polymorphisms in the IL-10 gene have been associated with an elevated likelihood of early pregnancy loss (Cochery-Nouvellon*, et al.* 2009). This may be as a result of a protective role for IL-10 against bacteria induced fetal loss.

Despite the evidence for IL-10 in the promotion of tolerance in pregnancy, studies in IL-10 null mice have shown that in the absence of IL-10 their ability to gestate allogeneic fetuses is not compromised (White*, et al.* 2004). Such a finding demonstrates a non-essential role for IL-10 in promoting maternal-fetal immune tolerance but does not discount a role for IL-10 in influencing the immune system in a manner conducive of heightened immune tolerance. Mice with a null mutation for IL-10 were shown to be 10-fold more sensitive to fetal loss induced by bacterial-mimetics such as LPS, with the surviving fetuses showing significant growth restriction in comparison to wild-type controls (Robertson*, et al.* 2007). This is primarily as a result of the deficiency of IL-10 leading to an inability to control the ensuing TNFα and IL-6 cytokine surge that followed the exposure to the bacterial-mimetic (Robertson*, et al.* 2007).

The cytokine profile in the uterine tissue is also critical to pregnancy success. Throughout murine gestation there is a significant change in the profile of DCs toward IL-10 production and away from IL-12 production. The level of IL-10 producing DCs peaked at gestational day (gd) 8.5 and subsided gradually toward delivery (Blois*, et al.* 2004). This predominance of IL-10 may both promote immune tolerance locally, and assist in trophoblast invasion and placental restructuring. IL-10 null mice have been shown to gestate fetuses with increased placental size, primarily due to an increase in the placental labyrinth (Roberts*, et al.* 2003). This ongoing immune tolerance in the face of IL-10 deficiency might be achieved by inducing tolerogenic phenotypes in other leukocyte subsets including T-cells, other DCs and importantly inhibiting macrophage activation and function which has been shown to promote trophoblast invasion (Renaud*, et al.* 2007).

1.6 SUMMARY AND RESEARCH QUESTIONS

There is a body of evidence that demonstrates a role for Treg cells in the maintenance of immune tolerance of the semi-allogeneic fetus during pregnancy. Experimental models and epidemiological evidence implicates inappropriate Treg cell function and numbers with immune pathologies such as pre-eclampsia and unexplained infertility. Whilst there is evidence for the expansion of the Treg cell populations in pregnancy in both humans and mice, the factors and nature of the temporal sequence of events that govern this

expansion remain poorly understood. Of particular interest is the identity and function of cytokines in pregnancy that may influence the numbers of Treg cells.

In mammalian species the introduction of semen into the female reproductive tract at the time of mating has a range of effects additional to fertilisation of the ovulated oocyte. One such effect is a coordinated response of the female immune system that involves an influx of leukocytes into the female reproductive tract and activation and expansion of local leukocyte populations in the regional lymph nodes. It has been demonstrated that the net consequence of this effect is a state of paternal antigen-specific immune tolerance that is postulated to be advantageous for the proceeding immune tolerance of the embryo following implantation. However the factors that are responsible for the induction of this immune-tolerant state following mating are not fully defined.

The experiments described in this thesis aim at addressing the involvement of semen and its constituents in regulating the expansion and localisation of Treg cells in the peri-implantation period. Additionally the role of the cytokine IL-10 in regulating Treg cell populations in pregnancy will be investigated.

1.7 HYPOTHESES

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

1. Seminal constituents increase the size of the Treg cell pool in the uterus and its draining lymph nodes during early pregnancy.
2. IL-10 influences the size and suppressive capacity of Treg cell populations during pregnancy.

1.8 RESEARCH AIMS

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

Aim 1.1 To analyse the influence of semen and its constituents (seminal plasma/sperm) in the expansion of Treg cells in lymph nodes and the uterus in the peri-implantation period.

Aim 1.2 To determine the influence of seminal constituents on the regulation of migratory molecules in uterine tissue, and the role these may have in Treg cell recruitment into the uterus in the peri-implantation period.

Aim 2.1 To investigate the physiological role of IL-10 in the number and suppressive function of Treg cells in pregnancy, using mice with a null mutation in the IL-10 gene (IL-10-/- mice).

CHAPTER 2

MATERIALS AND METHODS

2.1 ANIMALS AND SURGERIES

All mice used were between 6 and 12 weeks of age at the time of treatment and housed in specific pathogen-free conditions at the University of Adelaide Laboratory Animal Services (LAS) Medical School Animal House facility and maintained under the Australian Code of Practice for the care and use of animals for scientific purposes (NHMRC). Mice were housed on a 12:12 hr light-dark cycle, with food and water provided *ad libitum*. All experiments were approved by the University of Adelaide Animal Ethics Committee (ethics numbers M33-2004B and M72-2008).

IL-10 null mutant mice (IL-10-/-) were generated via targeted mutation of the *Il10* gene in 129/Ola embryonic stem cells, and propagated on a C57BL/6 (IL-10-/-) as per (Kuhn*, et al.* 1993) and were bred from homozygous breeding pairs in-house. C57BL/6 (B6) females and BALB/c male studs were purchased from LAS central animal facility.

Foxp3-GFP mice were kindly provided by Dr Alexander Rudensky and were generated a previously described (Fontenot*, et al.* 2005). Mice were back-crossed onto a B6 background for 8 generations, and were then on maintained by breeding homozygous breading pairs.

2.1.1 General procedures

IL-10-/- mice received broad-spectrum antibiotics (Oxymav 100, 100 g/kg oxytetracyclin hydrochloride; Mavlab, Slacks Creek, Australia) in autoclaved drinking water twice weekly at a concentration of 2 mg/ml to prevent the onset of colitis. In preliminary experiments a population of B6 wild-type mice were also treated with antibiotics for a period of 2 weeks prior to examination to ensure that there were no perturbation to any of the parameters studied as a result of antibiotic treatment.

All surgeries were performed on mice using autoclaved instruments, or instruments that had been submerged in 70% ethanol (ANALAR, Melbourne, Australia) prior to use. Mice were anaesthetised via intra-peritoneal (i.p.) injection of 0.5 mg/g of tribromoethanol (Avertin; Sigma-Aldrich, St. Louis, USA), with the exclusion of mice undergoing retro-orbital bleeding who were maintained under anaesthesia using fluothane (Veterinary Companies of Australia, Sydney, Australia). All surgical incisions were closed using 5/0 silk suture (Ethicon, Johnson and Johnson, Langhorne, USA).

Identification of coitus and ejaculation was done by the visual identification of a vaginal plug between 09:00 and 11:00 hrs for intact and VAS- mated females. The presence of sperm on vaginal smears confirmed mating in SV- mated females, while video recording with infra-red recording equipment was used to confirm mating with SV-/VAS- males. The morning of plug detection, detection of sperm on vaginal smears, or video confirmation of a mating event was termed day 0.5 post-coitum (pc).

Estrous mice were identified by cytological analysis of daily smears of vaginal epithelial cells, using phase contrast microscopy. Estrus was indicated by the presence of cornified epithelial cells with an absence of rounded "bright" epithelial cells and infiltrating leukocytes within the smear.

2.1.2 Vasectomies and vesiculectomies

Male BALB/c mice underwent vasectomy or the removal of the seminal vesicle glands (vesiculectomy), as previously described (Robertson*, et al.* 1996). Briefly, mice were anaesthetised and a small lateral incision was made to expose the abdominal cavity. To generate vasectomised males (VAS-) the vas deferens were ligated with 5/0 silk suture, then bisected using scissors. To generate seminal vesicle-deficient males (SV-), seminal vesicle glands were excised at the proximal tubule and coagulating glands were left intact. Wounds were then sutured using 5/0 silk suture. VAS- male mice were caged with normal cycling females two weeks post-surgery, to confirm their inability to sire pregnancy. To ensure male infertility, mated females were sacrificed at day 7.5 days pc to confirm the absence of implantation sites.

2.1.3 Retro-orbital bleeds

Retro-orbital bleeds were utilised to obtain blood from mice. Following anaesthesia a glass pipette containing heparin (Sigma-Aldrich) diluted in 1 x PBS was placed from the anterior side of the face into the orbital socket between the globe of the eye and the lower eye lid. Once the pipette had made contact with the medial orbital bone the pipette was rotated to rupture the orbital-venous sinus. Blood (200 µl) was then collected via capillary action into the pipette and transferred to a tube containing an equal volume of heparin in 1xPBS.

2.2 FACS AND REGULATORY T CELL IDENTIFICATION AND QUANTIFICATION

Lymphoid tissue including the medial iliac and inguinal lymph nodes (LNs) and the spleen, were excised from female mice. All lymphoid tissue nomenclature was consistent with published standards (Van den Broeck*, et al.* 2006). Anatomical sites were chosen to discern between events localised to the uterine-draining LNs (iliac LNs), or distal events such as those in the inguinal LN or spleen.

Single cells suspensions were made by homogenising lymphoid tissue by gently grinding the tissue between the frosted ends of two glass slides (Objektträger, HD Scientific, Melbourne, Australia). Liberated cells were suspended in 1 ml of FACS buffer (1xPBS/0.1% BSA/0.05% NaN_3). For quantification of total cell numbers 100 µl of this suspension was removed and transferred into a separate tube (see section 2.2.1).

The remaining proportion was washed with 2 ml of FACS buffer and centrifuged (300xg, 5 mins) to form a cell pellet and the supernatant was then removed. Cells were resuspended in 50 µl of FACS buffer containing 0.5 µg anti-Fc-γIIR antibody (FcBlock™, BD Pharmingen, BD Biosciences, San Diego, USA) for 10 mins at 4°C. Labelling of surface markers was performed via the addition of 100 µl of FACS buffer containing 0.5 µg each of fluorescein isothiocyanate (FITC) anti-CD8 (Ly-2), Phycoerthrin (PE) anti-CD4 (L3T4), (both from BD Pharmingen), and PE-Cy7 anti-CD25 (PC61; eBiosciences, San Diego, USA) for 30 mins at 4°C. Cells were then washed twice with 1 ml of FACS buffer followed each time by centrifugation.

Cells were subsequently fixed and permeabilized using a Foxp3 Staining Buffer Set (eBiosciences) according to the manufacturer's instructions. Briefly, cells were resuspended in 500 µl of 1xFix-Perm Buffer for 45 mins at 4°C. Cells were then washed twice with 1 ml of 1 x Permeabilization Buffer. Cells were subsequently resuspended in 50 µl of 1 x Permeabilization Buffer containing 0.5 µg of Fc Block™ and incubated for 10 mins at 4°C. This was followed by the addition of 50 µl of permeabilization buffer containing 0.25 µg of allophycocyanin (APC) anti-Foxp3 antibody (FJK-16s; eBiosciences) for 30 mins at 4°C. Cells were washed once more with 1 ml of permeabilization buffer, centrifuged and resuspended in 1 ml of FACS buffer for fluorescence-activated cell sorting (FACS).

Flow cytometry was performed on a FACSCanto (BD Biosciences, San Jose, USA) and data were collected using FACSDiva software (version 6.0, BD Biosciences). Gates were applied to the side scatter/forward scatter density plots to exclude debris and dead cells, and include lymphocytes. Additional gates were used to identify cells positive for CD4, CD8, CD25 and Foxp3 expression based on the unique fluorescence emission wavelength of the antibodies used.

2.2.1 Total cell number quantification

100 µl (1/10th) of the original lymphoid tissue cell suspension was incubated with 10 µl of FACS buffer containing 0.5 µg of FcBlock™ for 10 mins at 4°C. Following this cells were incubated with another 10 µl of FACS buffer containing 0.5 µg of PE anti-CD4 (L3T4) for 30 mins at 4°C. 860 µl of FACS buffer was then added, followed by 20 µl of CountBright™ Absolute Counting Beads (Molecular Probes, Invitrogen, Carlsbad, USA) to make a final volume of 1 ml. Flow cytometry was performed as above, and 1000 CountBright™ Absolute Counting Beads were counted based on their uniquely high fluorescence. The number of $CD4^+$ was assessed based on PE expression and the total number of cells was generated by the following equation:

Total number of $CD4^+$ cells = (Number of $CD4^+$ cells/1000)*total number of beads added*10.

The total number of any additional population (e.g. $CD8^+$ cells) was assessed based on their ratio relative to $CD4^+$ cells.

2.3 QUANTITATIVE REAL TIME PCR

2.3.1 RNA extraction

RNA extraction was performed as previously described (Bromfield 2006). Briefly estrus or day 3.5pc uterine tissue from mated mice was collected. Tissues were dissected under RNAse free conditions and freed from all connective tissue, then snap frozen in liquid nitrogen and stored at -80°C until processing. Tissues were homogenised in 1000 µl of RNA-Bee (Tel-Test, Friendswood, USA) using an Ulta-Turrax homogeniser until all tissue was disrupted, with special care taken to clean the homogeniser between samples. Following homogenisation 200 µl of chloroform (Unilab, Ajax Finechem, Taren Point, Australia) was added to the sample and gentle vortexed. Samples were incubated on ice and then centrifuged at 13000 rpm for 15 mins at 4°C. The aqueous RNA phase

was placed into a fresh tube and precipitated over night at -20°C using an equal volume of isopropanol (Sigma-Aldrich). The following morning samples were pelleted at 14000 rpm for 30 mins at 4°C. The RNA pellet was then washed twice by centrifugation in 500 µl of ice cold 75% ethanol at 13000 rpm for 30 mins at 4°C. The remaining pellet was air dried for approximately 45 mins before resuspending in 50 µl of RNAse free Milli-Q™ water. Contaminating DNA was removed by use of a commercial DNAse treatment, DNA-free™ (Ambion, Austin, USA), according to the manufacturer's instructions. Briefly, 5 µl of 10xDNAse I buffer and 2 µl of DNAse I were added to each sample and incubated for 30 mins at 37°C. DNAse activity was stopped by the addition of 10 µl of DNAse I inactivating reagent followed by a 2 min incubation at room temperature. Samples were again centrifuged at 13000 rpm for 1 min and the DNA-free RNA containing supernatant was collected for reverse transcription.

2.3.2 Reverse transcription and cDNA generation

Extracted RNA was quantified using spectrophotometry (DU-50, Beckman Coulter, Brea, USA) at a wavelength of 260 nm. The RNA concentration was determined according to the equation:

$$OD_{260}*40_{RNA}*\text{dilution factor} = \mu g/ml\ RNA$$

Samples were then resuspended at 2 µg/µl in RNAse free Milli-Q™ water. Samples were incubated with 2 µl of 500 µg/ml random hexamers (Geneworks, Adelaide, Australia) for 5 mins at 65°C and then chilled on ice for a further 5 mins. Reverse transcription (RT) master mix (8 µl 5 x RT buffer [Invitrogen, Melbourne, Australia], 2 µl DTT, 4 µl 10 mM dNTPs [AMRAD Pharmica Biotech, Melbourne, Australia], 2 µl Superscript III Enzyme [Invitrogen] per sample) was added (16 µl) to each sample, and incubated for 5 mins at 25°C, followed by 60 mins at 50°C. The RT reaction was stopped by heat inactivation of the sample for 10 mins at 70°C. RT samples were chilled on ice and stored at -20°C until real-time PCR application.

2.3.3 Oligonucleotide primer design

Oligonucleotide primers were designed utilising Primer Express software v2.0 (Applied Biosystems, Warrington, UK). RNA sequences were obtained through the Entrez Nucleotide database. Primers were designed to meet previously optimised criteria to suit

the Applied Biosystems real-time PCR analyser, and were targeted to sites that span introns to ensure absence of amplification of contaminating genomic DNA. All primers utilised for real-time PCR analysis were purchased from Geneworks. A list of primers along with corresponding nucleotide position, Genebank accession number and product size are shown in Table 2.1. Primer specificity was determined by gel electrophoresis 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.

Table 2.1: PCR Primers developed for qRT-PCR analysis.

Target mRNA	Primer sequence	Position	Genebank Accession#	Product size (bp)
Foxp3	5'-AGG AG AAG CTG GGA GCT ATG C-3' 3'-GTG GCT ACG ATG CAG CAA GA-5,	1004 1109	NM_054039	106
Ccl4	5'-CCA GGG TTC TCA GCA CCA AT-3' 3'-CAC AGC TGG CTT GGA GCA A-5'	137 268	NM_013652.2	132
Ccl5	5'-TGC CCA CGT CAA GGA GTA TTT-35 3'-TCT CTG GGT TGG CAC ACA CTT-5'	189 289	NM_013653.3	101
Ccl19	5'-GAC CTT CCC AGC CCC AAC T-3' 3'-CGG AAG GCT TTC ACG ATG TT-5'	239 340	NM_011888.2	102
Ccl22	5'-CAC CCT CTG CCA TCA CGT TT-3' 3'-CCT GGG ATC GGC ACA GAT AT-5'	157 270	NM_009137.2	114
Ccr4	5'-CCG TAC AAC GTG GTG CTT TTC-3' 3'-TTC TGT AGC CTG GAT GGC G-5'	876 977	NM_009916.2	102
Ccr5	5'-GCT CCT GCC CCC ACT CTA CT-3' 3'-TCA CGC TCT TCA GCT TTT TGC-5'	202 303	NM_009917.5	102
Ccr7	5'-GAA ACC CAG GAA AAA CGT GCT-3' 3'-CCG TGG TAT TCT CGC CGA T-5'	91 194	NM_007719.2	104
Cd103	5'-TGA TCT TCA AGA GAG CCG GG-3' 3'-TCT AGG ACA TGC TGC ATG GC-5'	764 871	NM_008399.2	108
β-actin	5'-CGT GGG CCG CCC TAG GCA CCA-3' 3'-ACA CGC AGC TCA TTG TA-5'	181 366	NM_007393.3	186

2.3.4 Quantitative Real-Time PCR

Using primers described in section 2.3.3, quantitative real-time PCR (qRT-PCR) was performed on cDNA samples using an ABI Prism 7000 Sequence Detection System (Applied Biosystem) and SYBR Green (Applied Biosystem) chemistry according to the manufacturer's instructions. Optimal primer concentration was determined by conducting PCR using serial dilutions of primer sets with a positive control sample of

cDNA. The optimal primer concentration for all primers was shown to be 0.5 µM in preliminary experiments. The efficiency of each primer set was determined by regression analysis across serial dilution of cDNA with only primers consistent with the housekeeper (β-actin) primer set were accepted. All primers were shown to be incapable of producing primer dimers as evidenced by no product amplification in non-template controls.

The optimal PCR conditions were as follows; 2 minutes at 50°C, 5 minutes at 95°C followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. Amplification plots for each sample were obtained and cycle threshold (Ct) limits determined using the ABI 7700 software package (Applied Biosystem).

Each sample was amplified in triplicate for each primer set. Quantity of cDNA for each sample was then normalised to the housekeeper gene β-actin using the $2^{-\Delta\Delta Ct}$ method, whereby the Ct difference between the housekeeper gene and the gene of interest was quantified (ΔCt). This difference was then subtracted from the average difference for the designated control group (ΔΔCt) and multiplied by the conversion constant -1 (-ΔΔCt). This allowed the data for all samples for each primer set to be expressed as a fold change relative to the mRNA content of control tissue.

2.4 FOXP3 IMMUNOHISTOCHEMISTY OF UTERINE TISSUE

2.4.1 Tissue preperation

Estrus and day 3.5pc uterine tissues were collected from mice and placed directly into 4% paraformaldehyde fixative in phosphate-buffered saline (PBS; 80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 100 mM NaCl) for 24 hrs at 4°C. Following fixation, tissues were washed three times in 1 x PBS over 24 hrs before being transferred to 75% ethanol in preparation for processing and paraffin embedding. Tissues were processed and embedded in the Leica TP1020 Tissue Processor (Leica Microsystems, NSW, Australia) by the following dehydration and embedding protocol; 1 hr 75% ethanol (EtOH), 1 hr 85% EtOH, 1 hr 90% EtOH, 1 hr 95% EtOH, 2 x 1 hr 100% EtOH, 2 x 1 hr 100% Xylene, 2 x 1.5 hr paraffin wax under vacuum conditions. Tissues were then moulded into wax blocks and stored for sectioning on a Leica Rotary Microtome (Leica Microsystems, NSW, Australia). All tissue sections were cut longitudinally mid-sagittal in serial 7 µm sections and fixed to slides using a 45°C water bath. Tissue sections were then further fixed to slides and dehydrated over night at 37°C prior to histological processing.

2.4.2 Histological processing

Seven micron tissue sections were dewaxed in xylene (Unilab) and rehydrated through graduated dilutions of ethanol for 5 mins each (2 x 100% EtOH, 1 x 90% EtOH, 2 x 70% EtOh) followed by 1 x 5 mins in Milli-Q™ water and 1 x 5 mins PBS (pH 7.2). Antigen retrieval was performed by incubating slides (10 minutes at 120°C) in citrate buffer solution (10 mM sodium citrate, pH 6.0) in an autoclave (Athena MREC 916 DSL, Athertons, Thornbury, Australia). Slides were allowed to cool and then washed (2 x 5 mins) with PBS containing 0.025% Tween-20 (Sigma-Aldrich) (PBST, pH 7.4). Slides were then blocked with 10% rabbit serum (Sigma-Aldrich) and 10% mouse serum (Sigma-Aldrich) in PBST for 1 hr. Sections were then incubated with 100 µl of 1/400 of affinity purified anti-Foxp3 antibody (FJK-16s) over night in a humid chamber at 4°C. Primary antibody was washed of with 2 x 5 mins of PBST and then sections were incubated with 100 µl of 1/500 biotinylated secondary antibodies (Biotinylated rabbit anti-rat; Dako, Glostrup, Denmark) for 1 hr. Sections were treated with strepdavidin-conjugated horseradish peroxidase (HRP) (Vectastain ABC Kit; Vector Laboratories, Burlingame, CA) and detection was performed using diaminobenzadine (Vector Laboratories) according to the manufacturer's instructions. Negative controls were incubated with irrelevant isotype matched primary antibodies or no primary antibody.

2.4.3 Image capture and cell quantification

Slide images were captured using a NanoZoomer 1.0 (Hamamatsu, Shizuoka, Japan) at a zoom equivalent of a 20x objective lens and turned into a digital image. Cell density was calculated by manually counting Foxp3 positive cells and then dividing by the sectional area, which was calculated via manual tracing of the section outline using NDP.view (Hamamatsu).

2.5 *IN VITRO* SUPPRESSION ASSAY

2.5.1 Purification of $CD4^+CD25^+$ cells

$CD4^+CD25^+$ cells were isolated using a MACS $CD4^+CD25^+$ Regulatory T cell Isolation Kit for mice (Miltenyi Biotec, Bergisch Gladbach, Germany) as per the manufacturer's instructions with the exception of the composition of the MACS buffer (1 x PBS pH 7.2, 5.0% heat-inactivated fetal calf serum and 2 mM EDTA). Briefly, non-$CD4^+$ T cells were depleted by incubating 10^7 mesenteric leukocytes in 40 µl of MACS buffer and 10 µl of

biotin-antibody cocktail for 10 mins on ice. Thirty µl of MACS buffer, 20 µl of Anti-Biotin MicroBeads and 10 µl of Anti-CD25-PE antibody were then added and incubated for 15 mins on ice in the dark. Cells were washed with 2 ml of MACS buffer, centrifuged (300 x g, 5 mins) and the supernatant was removed. Cells were then resuspended in 500 µl of MACS buffer. The cell suspension was placed into a primed LD Column in a MidiMACS™ magnet (Miltenyi Biotech) and the column flow-through was collected. After washing the column twice with 1 ml of MACS buffer the flow through, which contains $CD4^+$ cells was, centrifuged and positive selection of $CD25^+$ cells was performed. This was achieved by resuspending the cells with 90 µl of MACS buffer and 10 µl of anti-PE MicroBeads, followed by 15 min incubation on ice in the dark. Cells were washed with 2 ml of MACS buffer, centrifuged and the supernatant was removed. The cell pellet was resuspended in 500 µl and placed into a primed MS Column in MiniMACS™ magnet. The column was washed with 2 x 1 ml of MACS buffer. The flow through was collected and this was the $CD4^+CD25^-$ cell population (T effector cells). The MS Column was removed from the magnet and 1 ml of MACS buffer was placed into the column and $CD4^+CD25^+$ (Treg) cells were flushed out by firm application of the plunger.

Following purification, $CD4^+CD25^-$ and $CD4^+CD25^+$ cells were checked for purity via FACS. $CD4^+CD25^-$ were routinely >95% pure and $CD4^+CD25^+$ cells were only used if >90% pure.

2.5.2 Suppression assay

Following isolation, $CD4^+CD25^+$ and $CD4^+CD25^-$ cells were resuspended in RPMI-1640 (Sigma-Aldrich) (cRPMI) containing 0.05 mM β-mecaptoethanol (BDH laboratory supplies, Poole, UK), 2 mM L-glutamine (BDH laboratory supplies, Poole, UK) and antibiotics (penicillin/streptomycin) (Sigma, Castle Hill, AU) and heat-inactivated FCS (CSL, Parkville, AU) at a concentration of 2×10^6 cells/ml.

In vitro suppression assays were performed by combining varying numbers of $CD4^+CD25^+$ cells (from B6 or IL-10-/- male mice) with 10^5 $CD4^+CD25^-$ cells (from B6 male mice) and 100 µl containing 10^6 stimulator cells (from BALB/c males) to give final $CD4^+CD25^-$:$CD4^+CD25^+$ (responder:suppressor) ratios of 1:1, 2:1, 3:1 and 4:1. The concentration of the $CD4^+CD25^+$ cells was adjusted so that the final volume of each reaction equalled 200 µl. Stimulator cells were generated via isolation of leukocytes from spleenocyte homogenates using Lympholyte® mammal (Cedarlane Laboratories, Burlington, Canada)

as per the manufacturer's instructions. These were then washed with 4 ml of cRPMI, centrifuged and resuspended in 3 ml of cRPMI. Stimulator cells were rendered anergic with 15 Gy of irradiation. Stimulator cells, $CD4^+CD25^-$ cells and $CD4^+CD25^+$ cells were incubated in 96 well U-bottom cell culture dishes (Nunc, Roskilde, Denmark) for 72 hrs (37°C, 5% CO_2).

Cell proliferation was assessed via the addition of 1.5 µCi of tritiated thymidine (^{3}HTdR) for the final 8 hrs of culture. Cells were then harvested onto glass filters (Millipore, Billerica, USA), using a Packard Filtermate 96 cell harvester (Packard, Meriden, USA). The ^{3}HTdR content of each well was assessed as counts per minute (taken as an average of 2 mins) using a TopCount NXT v2.53 Micro Scintillation Counter (Perkin Elmer, Massachusetts, USA). The degree of proliferation was assessed as a percentage relative to $CD4^+CD25^-$ cells combined with stimulator cells in the absence of $CD4^+CD25^+$ cells. All data points were the measured values from triplicate wells, and all experiments were performed 3 times.

2.6 PC61 TREATMENT

PC61 antibody was generated from the supernatant of PC 61 5.3 hybridoma cells (ATCC® number: TIB-222) by protein G affinity purification performed at Monoclonal Antibodies of South Australia Technologies (MABSA) facility.

Mice were administered 200 µl of 1 mg/ml PC61 antibody in 1xPBS via i.p. injection on day of plug detection (day 0.5pc), and another 200 µl of 1 mg/ml PC61 antibody 6 days later on gd6.5.

2.7 STATISTICS

Unless stated otherwise, data were analysed using a one-way ANOVA and Tukey post test or unpaired T-test using GraphPad Prism 5 for windows (GraphPad software Inc, San Diego, USA). Individual datum points were excluded as outliers if they were greater than 2 standard deviations from the mean. PCR data were log transformed to ensure normal distribution. Data are often displayed as mean±SEM (standard error of mean). Difference in groups were considered significant if $p<0.05$.

CHAPTER 3

SEMINAL FLUID COORDINATION OF REGULATORY T-CELLS

3.1 INTRODUCTION

Exposure of the female reproductive tract to semen at mating elicits an immune response in many mammalian species including both mice (Johansson*, et al.* 2004) and humans (Pandya*, et al.* 1985, Sharkey*, et al.* 2007). The physiological significance of this immune activation is still being elucidated, but considerable evidence supports the hypothesis that semen facilitates the induction of a state of immune tolerance (reviewed in Robertson 2005) and that this occurs in a paternal-antigen specific manner (Beer*, et al.* 1974, Lengerova*, et al.* 1963, Robertson*, et al.* 2009).

The mechanism by which semen leads to immune tolerance is believed to be mediated by its potent immune-deviating properties. Seminal plasma acts on uterine and cervical epithelial cells to induce the production and secretion of a range of cytokines including GM-CSF, LIF as well as IL-6 and IL-8 (Gutsche*, et al.* 2003, O'Leary*, et al.* 2004, Sharkey*, et al.* 2007). The consequence of this is the influx of a range of immune cells including neutrophils, macrophages and DCs into the female reproductive tract (McMaster*, et al.* 1992, Pandya*, et al.* 1985), this in turn leads to a state of regional lymph node hypertrophy as well as induction of characteristic markers of T-cell activation (Beer*, et al.* 1974, Johansson*, et al.* 2004), all classical signs that indicate an immune response. However the result of this post-mating immune response seems to be directed toward immune tolerance rather than rejection.

Evidence for the immune tolerance-promoting properties of semen arises from both animal and human experiments as well as epidemiological evidence. Murine models of allograft transplantation demonstrate the ability of semen to induce a state of paternal antigen-specific tolerance (Beer*, et al.* 1974, Robertson*, et al.* 2009). In humans, evidence from studies in IVF patients demonstrate that exposure to semen during the peri-transfer period resulted in an improvement in the viability of transferred embryos leading to increased pregnancy rates at 6-8 weeks of gestation (Tremellen*, et al.* 2000). Epidemiological evidence demonstrates that pregnancy pathologies are reduced in women chronically exposed to the semen of their conceiving partner, as opposed to those with limited exposure to semen due to use of barrier methods of contraception or limited periods of sexual cohabitation. Importantly this protection was lost upon a

change in conceiving partner suggesting an antigen- and partner-specific response (Klonoff-Cohen, *et al.* 1989, Robillard, *et al.* 1994).

The mechanism by which semen exposure results in a state of immune tolerance is not completely understood. It has been shown that the critical agents in semen that are responsible for immune activation and acquisition of immune tolerance are contained within the acellular fraction of the ejaculate (seminal plasma), with a state of tolerance even being induced in the absence of sperm or a conceptus (Robertson, *et al.* 2009, Sharkey, *et al.* 2007). Analyses of immunoactive moieties within the seminal plasma have identified several candidates that are present in high concentrations in seminal plasma and have been demonstrated to contribute to induction of the immune response. These include TGFβ (Tremellen, *et al.* 1998), 19-hydroxy-prostaglandin-E (in humans) (Templeton, *et al.* 1978), and IL-8 (Friebe, *et al.* 2003). It is postulated that these potent immune-deviating moieties can direct the immune response to paternal antigens present in semen in such a manner that a state of tolerance is achieved (Robertson, *et al.* 2003). Evidence from murine models showed that intra-vaginal administration of TGFβ lead to recruitment of leukocytes similar to that seen following mating and ultimately to an elevated level of cells expressing Foxp3 in the uterine lumen (Clark, *et al.* 2008).

The purpose of the experiments described in this chapter is to assess the influence of seminal components on the abundance of Treg cells in the local uterine-draining iliac LNs and distal secondary lymph tissues as well as in the reproductive tract. By using a mouse model whereby the mother is exposed to different components of the ejaculate, and then analysing the impact on Treg cell abundance in reproductive and lymphoid tissues, we investigated whether Treg cell abundance may in part be regulated by exposure of the female reproductive tract to semen in the peri-implantation period. This may provide insight into the mechanism by which semen exposure promotes a state of immune tolerance in the female after coitus.

3.2 VALIDATION OF REGULATORY T-CELL IDENTIFICATION AND QUANTIFICATION

Assessment of the accuracy, reliability and reproducibility of the identification and quantification of Treg cells is critical for experiments investigating these cells. The

co-expression of CD4 and CD25 was used to identify cells with a Treg phenotype, with this identification strategy being consistent with the classical and traditionally most common definition of these (Aluvihare*, et al.* 2004, Robertson*, et al.* 2009, Sakaguchi*, et al.* 1995). However in light of more recent literature that identifies Foxp3 as a definitive marker for $CD4^+$ Treg cells (Fontenot*, et al.* 2003, Fontenot*, et al.* 2005), the co-expression of CD4 and Foxp3 was additionally used. Therefore Treg cells will be defined as $CD4^+CD25^+$ and $CD4^+Foxp3^+$.

3.2.1 FJK-16s accurately identifies cells expressing Foxp3

Foxp3 staining was performed utilising flow cytometry analysis of cells stained with the anti-mouse/rat Foxp3 antibody FJK-16s, following fixation and permeabilization (see section 2.1). Cells positive for staining by FJK-16s were assessed for their expression of the additional markers CD4 and CD25. Overall 93.9% of cells labelled with FJK-16s were positive for CD4. Of the $CD4^+CD25^+$ cell subset, 84.5% were co-labelled with FJK-16s (Figure 3.1A), while 86.1% of cells stained by FJK-16s expressed both CD4 and CD25 (Figure 3.1B). Of $CD4^+CD25^-$ cells, 1.8% were co-labelled with FJK-16s, whilst 7.7% of cells stained with FJK-16s expressed CD4 but not CD25.

To confirm that FJK-16s was specifically labelling Foxp3 expressing cells, lymphocytes from mice containing a Foxp3-GFP fusion protein were fixed and permeabilized, and then labelled with FJK-16s. Of the cells that expressed the Foxp3-GFP fusion protein, 98.5% labelled by FJK-16s, whilst conversely 94.7% of cells labelled by FJK-16s expressed the Foxp3-GFP fusion protein (Figure 3.1C). Of the cells positive for either Foxp3-GFP expression or labelled by FJK-16s, 93.2% were positive for both markers.

3.2.2 Quantification of cells expressing Foxp3

To quantify the absolute number of cells expressing Foxp3 in lymphoid tissues, a proportion of the cell population was removed and stained for CD4 and 'spiked' with a known number of CountBright™ absolute counting beads. From this the number of total cells within any subpopulation could be quantified by comparison to CD4 cells. In order to ensure the reproducibility of cell quantification the intra- and inter-assay variation was assessed on a single mesenteric lymphoid cell sample from a male B6 mouse. Inter-assay variation was assessed by performing parallel runs using individual antibody

preparations on the same day. The assay was shown to be reproducible with coefficient of variations (CVs) shown in the table below.

Table 3.1: Absolute cells quantification intra and inter-assay variation

Count Beads – Variance	
Inter-assay	5.2%
Intra-assay	2.2%

3.2.3 $CD4^+CD25^+$ cells suppress effector T-cell proliferation

To confirm that cells expressing a Treg cell phenotype exerted an immune suppressive function, an *in vitro* mixed-lymphocyte proliferation assay was performed. $CD4^+CD25^+$ cells, which are highly enriched for $Foxp3^+$ cells (see Figure 3.1B), were combined in different ratios with $CD4^+CD25^-$ (T effector) cells and irradiated stimulator cells. $CD4^+CD25^+$ cells showed a dose-dependent ability to suppress the proliferation of stimulated T effector cells with approximately 85% suppression when equal numbers of T effector cells and Treg cells were present, and over 40% suppression when there were 4 times more T effector cells than Treg cells (Figure 3.3).

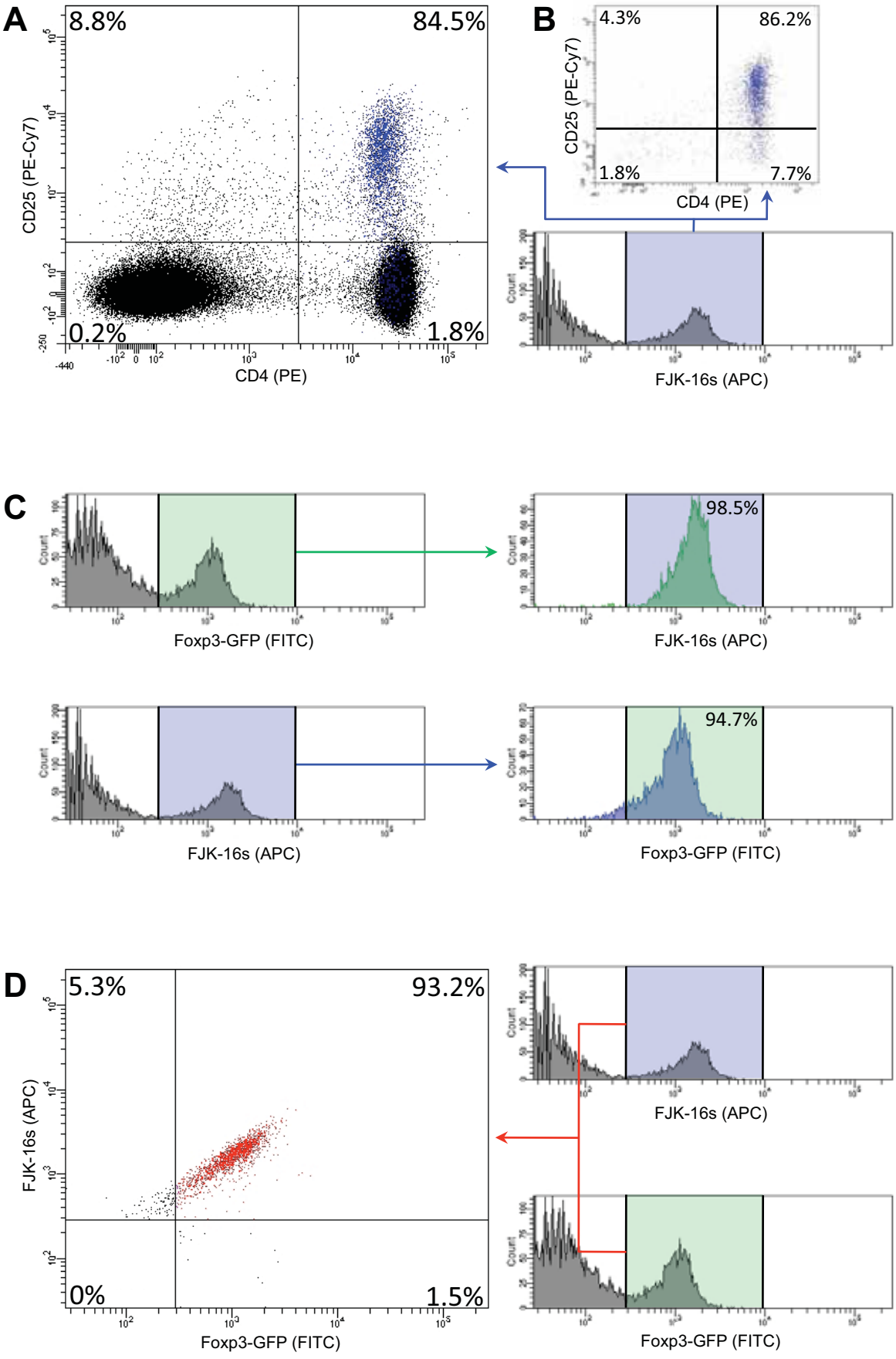

A
8.8%
84.5%
0.2%
1.8%
CD25 (PE-Cy7)
CD4 (PE)
B
4.3%
86.2%
1.8%
7.7%
CD25 (PE-Cy7)
CD4 (PE)
Count
FJK-16s (APC)
C
Count
Foxp3-GFP (FITC)
98.5%
FJK-16s (APC)
Count
FJK-16s (APC)
94.7%
Foxp3-GFP (FITC)
D
5.3%
93.2%
0%
1.5%
FJK-16s (APC)
Foxp3-GFP (FITC)
Count
FJK-16s (APC)
Count
Foxp3-GFP (FITC)

Figure 3.1: Localisation of Foxp3 protein in cells expressing both CD4 and CD25 and in cells expressing Foxp3-GFP. Lymphocytes from mice expressing a mutant Foxp3-GFP 'knock-in' gene were stained with PE-conjugated anti-CD4 and PE-CY7-conjugated anti-CD25 antibodies. Cells were then fixed and permeabilized followed by staining with an APC-conjugated anti-Foxp3 antibody (FJK-16s). **(A)** The distribution and percentage of cells with in the lymphocyte gate that are positive for Foxp3 as detected by FJK-16s. Percentages displayed in each quadrant indicate the percentage of all cells within that quadrant that are positive for FLK-16s staining. **(B)** The distribution and percentage of cells positive for Foxp3 expression as detected by FJK-16s (blue) and their respective level of expression of CD4 and CD25. Percentages within each quadrant indicate the proportion of the total FJK-16s positive population within that quadrant **(C)** The percentage of cells expressing Foxp3-GFP that stain positive for Foxp3 expression with FJK-16s antibody and the percentage of FJK-16s positive cells that express Foxp3-GFP. **(D)** Co-localisation of cells positive for FJK-16s antibody and expression of Foxp3-GFP protein.

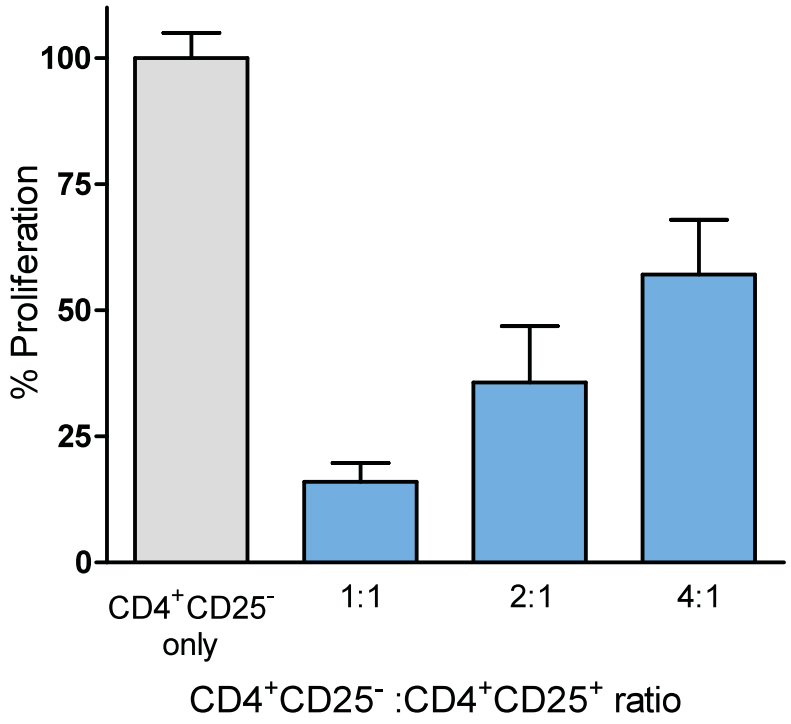


Figure 3.2: $CD4^+CD25^+$ cells suppress $CD4^+CD25^-$ cell proliferation in a dose-dependent manner. A constant number of $CD4^+CD25^-$ cells were co-cultured with isolated $CD4^+CD25^+$ cells at different ratios (from 1:1 to 4:1), and irradiated stimulator cells. Proliferation was assessed 72 hours later by tritiated thymidine (^{3}HTdR) incorporation. Data are means±SEM of 3 replicate wells, and are representative of n=3 experiments.

3.3 SEMINAL VESICLE-DERIVED FACTORS INDUCE EXPANSION OF THE REGULATORY T-CELL POOL

To investigate the role of different components of the ejaculate in regulating the abundance of Treg cells in lymphoid tissues in the post-coital/peri-implantation period, female (B6) mice were mated to either intact (BALB/c) males, males with their seminal vesicle glands removed (seminal vesiculectomy; denoted SV-), vasectomised males (VAS-), or males with both seminal vesiculectomy and vasectomy (SV-/VAS-). As a result females were exposed to sperm and seminal plasma, sperm alone, seminal plasma alone, or neither sperm or seminal plasma respectively (see Table 3.2). It is of note that formation of an embryo usually occurs in intact mating, occurs rarely in SV- mated animals, and never occurs in VAS- and SV-/VAS- matings (Bromfield 2006). Additionally the physical stimulation of the cervix at the time of mating leads to equivalent levels of progesterone in all mating groups irrespective of exposure to seminal components (Chambers*, et al.* 1979, Gangnuss*, et al.* 2004). As such, mice mated to SV-/VAS- males may be considered to be 'pseudo-pregnant' and therefore act as a control for changes in Treg cells that may occur due to the physical or neuroendocrine consequences of coitus. On day 3.5pc, female mice were sacrificed and the relative proportion and total abundance of $CD4^+Foxp3^+$ and $CD4^+CD25^+$ cells in lymphoid tissues, including the iliac LNs, inguinal LNs and the spleen, were analysed.

Table 3.2: Female exposure to seminal components at coitus.

[1]reduction in the number of oocytes to undergo first cleavage to 2 cell stage (Bromfield 2006)

In the uterine-draining iliac LNs, exposure to seminal factors did not result in an increase in the percentage of $CD4^+$ cells expressing Foxp3 or CD25. However surprisingly there was a modest (approximately 20%) increase in the percentage of $CD4^+$ cells expressing Foxp3 or CD25 in females mated with SV-/VAS- males (Figure 3.3 A,C; $p<0.001$ vs. estrus for both). A similar elevation of $CD4^+$ cells expressing Foxp3 or CD25 in females mated with SV-/VAS- males was also seen in the inguinal LNs (Figure 3.4 A,C; $p<0.01$ vs. estrus for both) and the spleen (Figure 3.5 A,C; $p<0.001$ vs. estrus for both).

Analysis of the total number of cells in each lymphoid tissue showed that there was an effect of male seminal factors on the total number of cells displaying a Treg phenotype ($CD4^+Foxp3^+$ and $CD4^+CD25^+$). In the iliac LNs there was an approximate 2-fold increase in the total number of $CD4^+Foxp3^+$ (Figure 3.3B) and $CD4^+CD25^+$ (Figure 3.3D) in females exposed to male seminal plasma at the time of mating (intact and VAS- mated animals) compared to virgin estrus mice ($p<0.001$ and $p<0.05$ respectively) and mice mated to SV-/VAS- males ($p<0.01$ and $p<0.05$). This however was not seen in the inguinal LNs (Figure 3.4 B,D) or spleen (Figure 3.5 B,D), where there was no significant effect of male seminal factors in comparison to estrus control animals. However there was a significant increase in the total number of cells expressing a Treg phenotype in the inguinal LNs of mice mated with VAS- males in comparison to the mice mated with SV-/VAS- males (Figure 3.4 B,D; $p<0.05$).

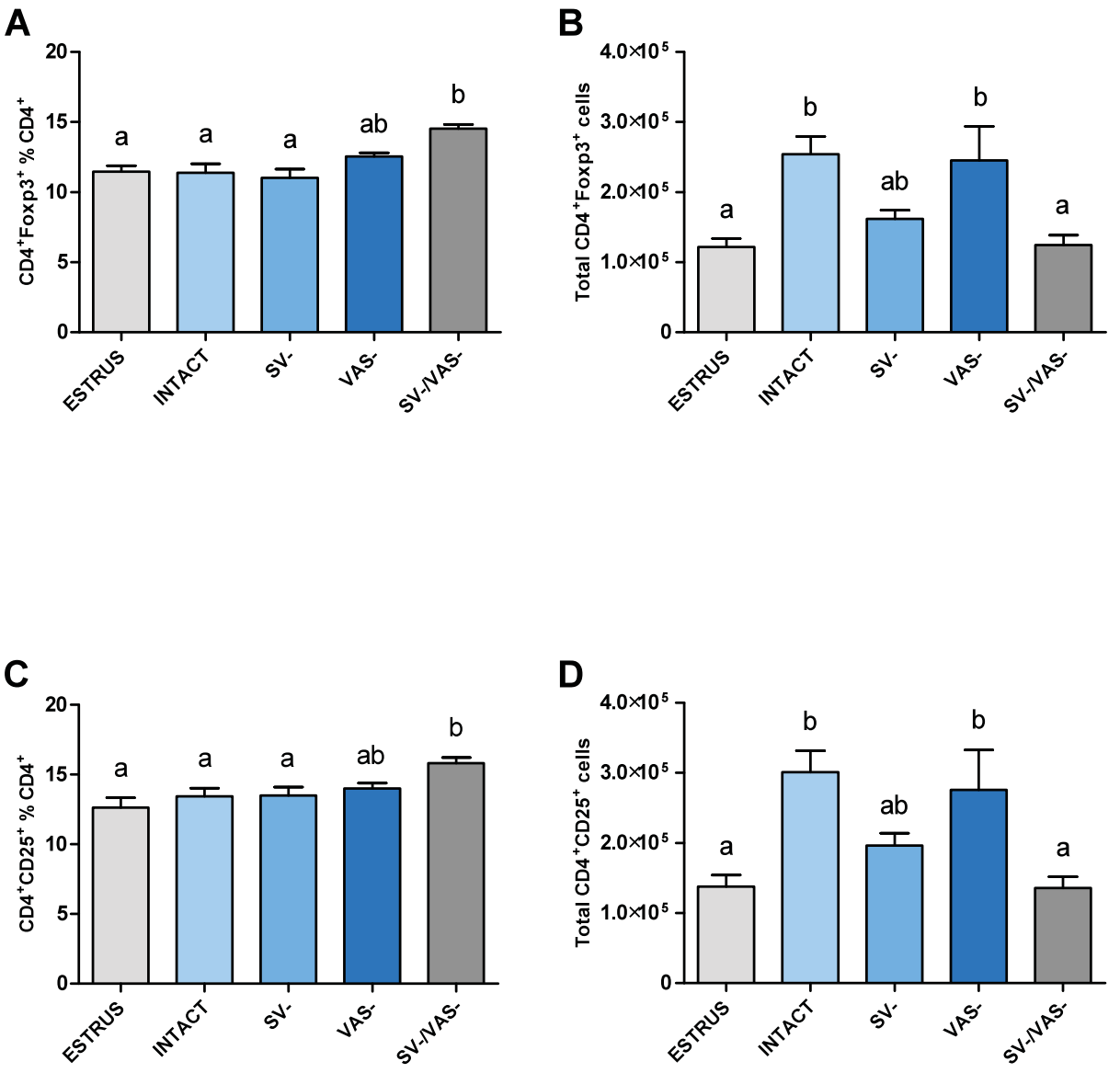


Figure 3.3: Effect of exposure to seminal factors on the proportion and total number of CD4 cells displaying a Treg cell phenotype in the iliac lymph nodes. B6 female mice were mated to either intact, seminal vesicle deficient (SV-), vasectomised (VAS-) or seminal vesicle deficient and vasectomised (SV-/VAS-) BALB/c males. Mice were then sacrificed on day 3.5pc and cells with a Treg phenotype were assessed. These were compared to mice that were in the estrus stage of the estrous cycle. **(A)** The percentage of $CD4^+$ cells expressing Foxp3. **(B)** The total number of cells positive for both CD4 and Foxp3. **(C)** The percentage of $CD4^+$ cells that express CD25. **(D)** The total number of cells positive for both CD4 and CD25. All data are expressed as means±SEM of n=10-24 females per group. Different letters indicate statistical significance between groups (p<0.05). Data were evaluated using a one-way ANOVA and a Tukey post test.

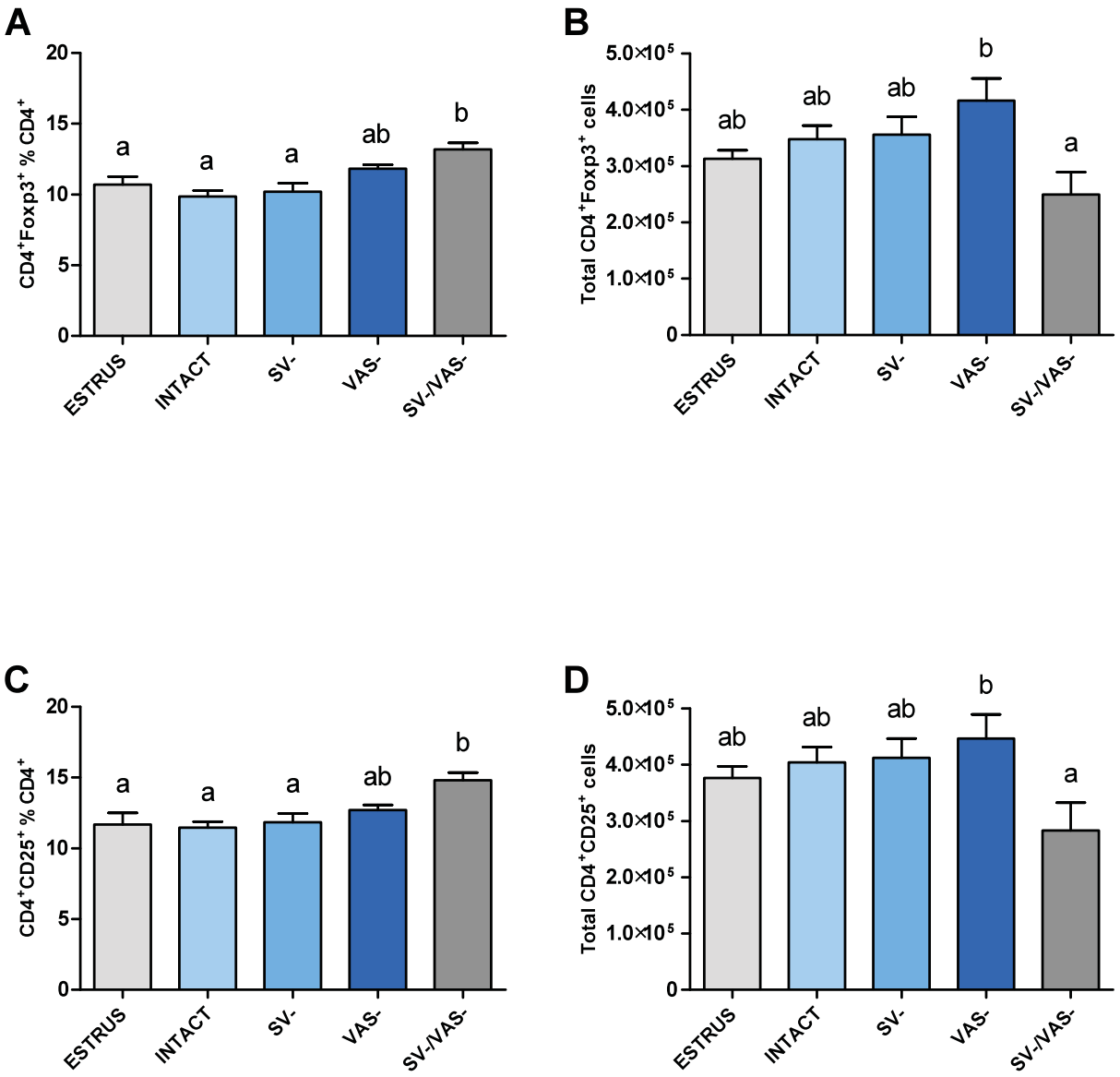


Figure 3.4: Effect of exposure to seminal factors on the proportion and total number of CD4 cells displaying a Treg cell phenotype in the inguinal lymph nodes. B6 female mice were mated to either intact, seminal vesicle deficient (SV-), vasectomised (VAS-) or seminal vesicle deficient and vasectomised (SV-/VAS-) BALB/c males. Mice were then sacrificed on day 3.5pc and cells with a Treg phenotype were assessed. These were compared to mice that were in the estrus stage of the estrous cycle. **(A)** The percentage of $CD4^+$ cells expressing Foxp3. **(B)** The total number of cells positive for both CD4 and Foxp3. **(C)** The percentage of $CD4^+$ cells that express CD25. **(D)** The total number of cells positive for both CD4 and CD25. All data are expressed as means±SEM of n=10-24 females per group. Different letters indicate statistical significance between groups (p<0.05). Data were evaluated using a one-way ANOVA and a Tukey post test

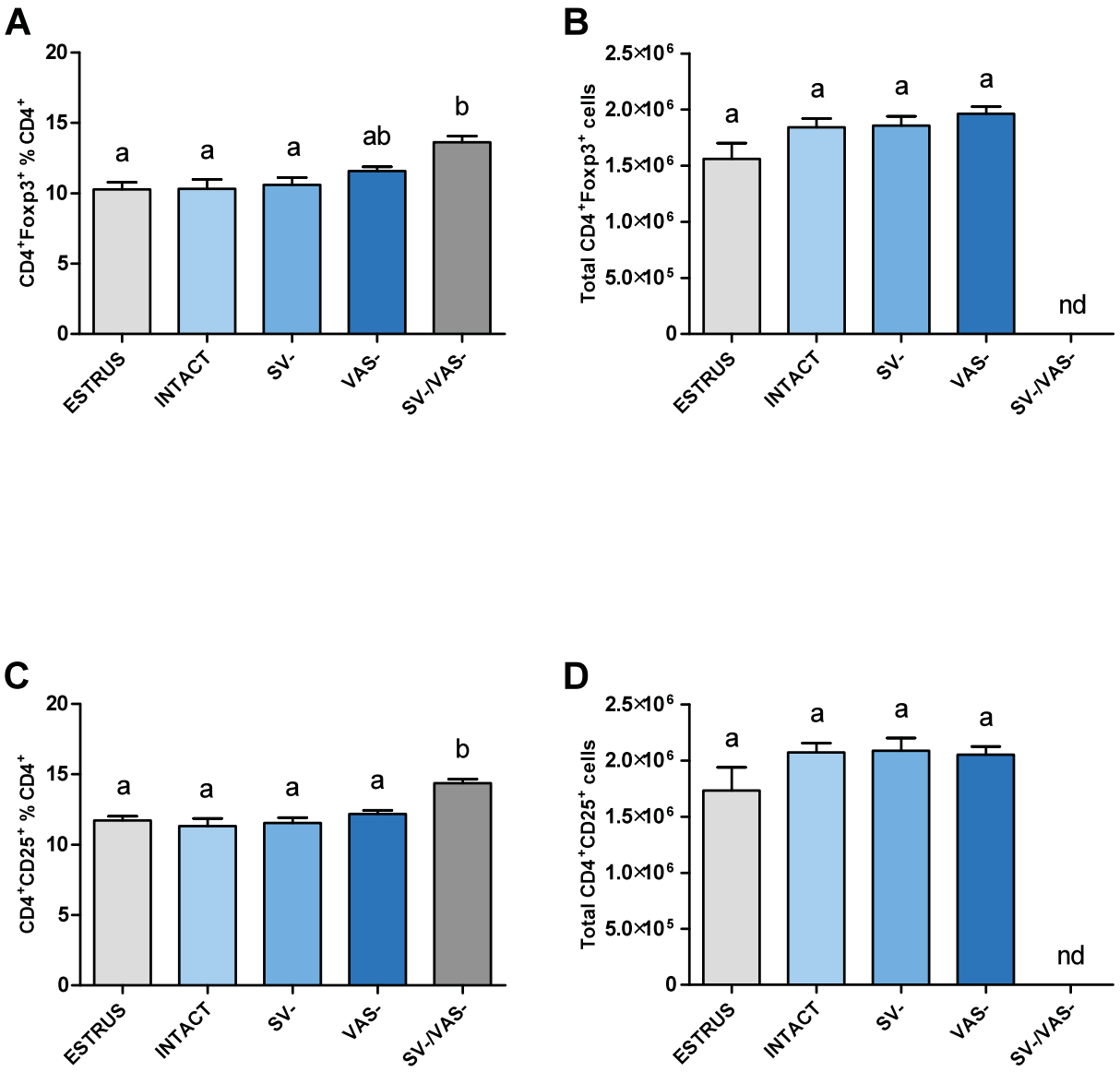


Figure 3.5: Effect of exposure to seminal factors on the proportion and total number of CD4 cells displaying a Treg cell phenotype in the spleen. B6 female mice were mated to either intact, seminal vesicle deficient (SV-), vasectomised (VAS-) or seminal vesicle deficient and vasectomised (SV-/VAS-) BALB/c males. Mice were then sacrificed on day 3.5pc and cells with a Treg phenotype were assessed. These were compared to mice that were in the estrus stage of the estrous cycle. **(A)** The percentage of $CD4^+$ cells expressing Foxp3. **(B)** The total number of cells positive for both CD4 and Foxp3. **(C)** The percentage of $CD4^+$ cells that express CD25. **(D)** The total number of cells positive for both CD4 and CD25. All data are expressed as means±SEM of n=10-24 females per group. Different letters indicate statistical significance between groups (p<0.05). Data were evaluated using a one-way ANOVA and a Tukey post test

3.4 SEMINAL VESICLE DERIVED FACTORS RESULT IN ELEVATED LEVELS OF REGULATORY T-CELLS IN THE UTERUS PRIOR TO EMBRYO IMPLANTATION

To investigate the role of different components of the ejaculate in regulating the abundance of cells expressing the Treg cell marker Foxp3 in uterine tissues prior to embryo implantation (day 3.5pc), female mice were mated to either intact males, SV- males, VAS- males or SV-/VAS- males thereby exposing them to different components of the male ejaculate (as shown in Table 3.1). On day 3.5pc, female mice were sacrificed and their uterine tissue was harvested and either processed for *Foxp3* mRNA analysis by qRT-PCR (see section 2.3) or fixed and embedded for immunohistochemisrty (IHC) analysis of Foxp3 protein (see section 2.4).

Analysis of relative *Foxp3* mRNA in day 3.5pc uterine tissue shows a significant elevation in females mated with intact and VAS- males compared to estrus controls (Figure 3.6A; $p<0.05$). This was confirmed following log transformation to adjust for skewness of data distribution (Figure 3.6B; $p<0.05$).

IHC analysis of uterine tissue from day 3.5pc mice shows that in females mated to intact or VAS- males, there was an elevation in the density of cells expressing Foxp3 protein (Figure 3.7). For intact and VAS- mated mice, the increase in density of $Foxp3^{+}$ cells was statistically significant compared to tissue from females mated to SV-/VAS- and SV- males ($p<0.05$). Additionally, VAS- but not intact mated females were significantly elevated compared to virgin estrus animals (Figure 3.7C; $p<0.05$).

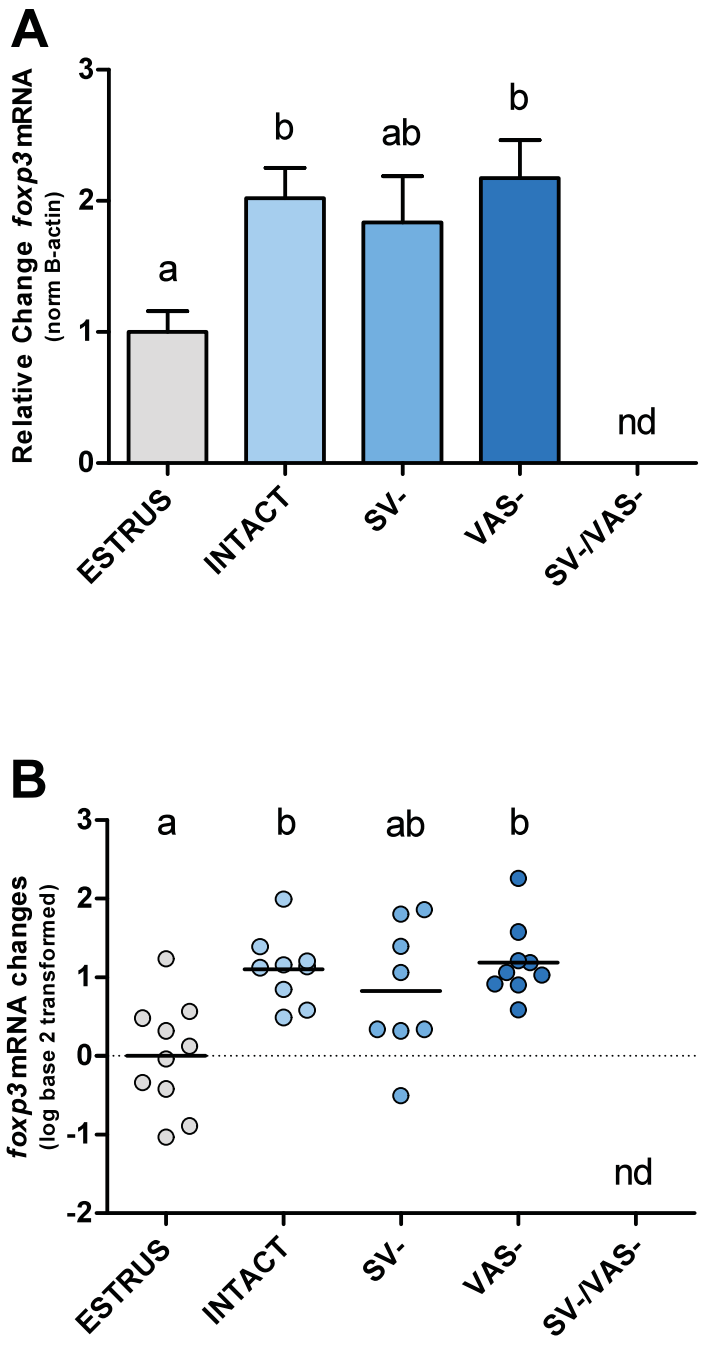
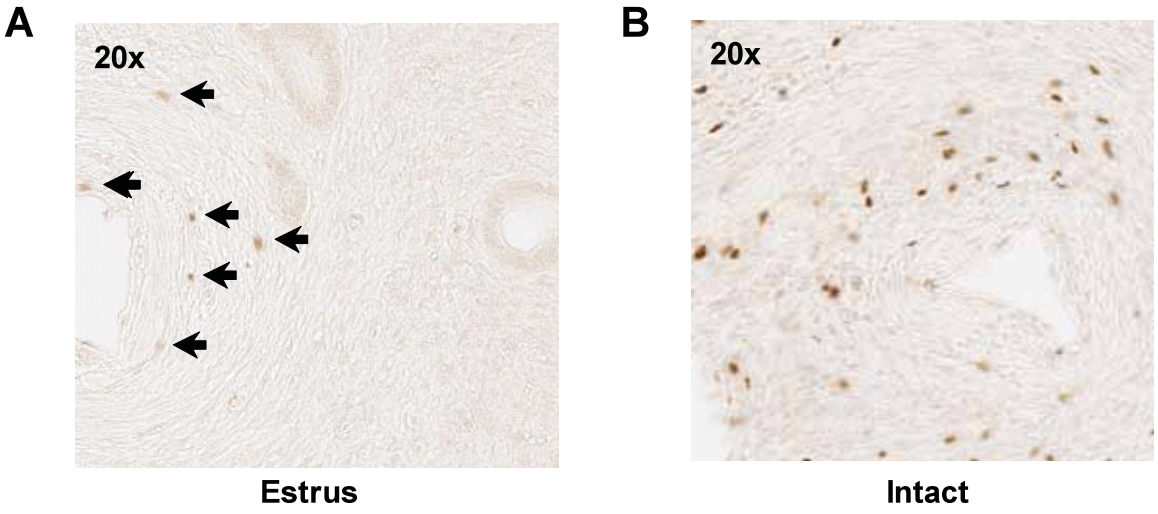


Figure 3.6: Exposure to seminal vesicle fluid increases *Foxp3* mRNA expression in the uterus of mice. B6 female mice were mated to either intact, seminal vesicle deficient (SV-) or vasectomised (VAS-) males. Mice were then sacrificed on day 3.5pc and the uterine tissue was collected. After mRNA isolation and reverse transcription *Foxp3* mRNA levels were assessed by qRT-PCR. These were compared to uterine tissue from mice in the estrus stage of the estrous cycle. **(A)** The mean±SEM of the fold difference of *Foxp3* mRNA for each mated group is shown. **(B)** The *Foxp3* mRNA expression following log transformation of data from uterine tissue from individual animals is shown (circles) along with the mean (straight line). Different letters indicate statistical significance between groups (p<0.05). Relative data were evaluated using a Kruskal-Wallis ANOVA and Dunn's multiple comparison post test. Following log transformation data were analysed using a one-way ANOVA and Tukey post test.



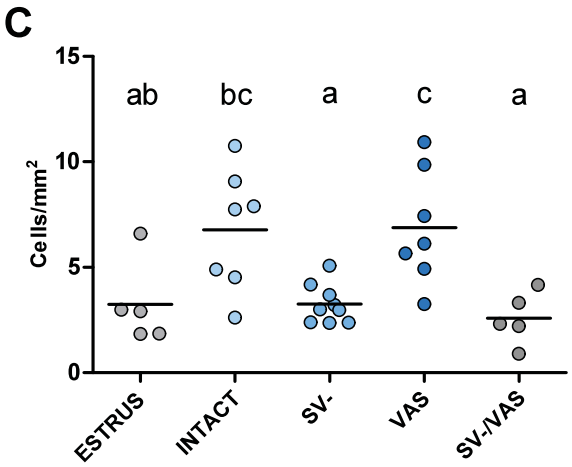


Figure 3.7: Exposure to seminal vesicle fluid increases the density of cells expressing Foxp3 in the uterus of mice. B6 female mice were mated to either intact, seminal vesicle deficient (SV-), vasectomised (VAS-) or seminal vesicle deficient and vasectomised (SV-/VAS-) BALB/c males. Mice were then sacrificed on day 3.5pc and the uterine tissue was collected, fixed and embedded, followed by incubation of longitudinal sections with anti-mouse/rat Foxp3 antibody FJK-16s. The number of cells in the uterus expressing Foxp3 was quantified via blinded manual counting, and areas were calculated using video image analysis. These were compared to uterine tissue from mice that were in the estrus stage of the estrous cycle. **(A)** 20x view of $Foxp3^+$ cells (indicated by arrows) in uterine tissue from a mouse in the estrus stage of its estrous cycle. **(B)** 20x view of $Foxp3^+$ cells in day 3.5 uterine tissue of an intact mated mouse. **(C)** The density of cells in the uterine tissue of individual animals is shown (circles) along with the mean (straight line). Different letters indicate statistical significance between groups ($p<0.05$). Statistical outliers have been excluded. Data were evaluated using a one-way ANOVA and a Tukey post test.

3.5 DISCUSSION

This study was undertaken to evaluate the influence of exposure of the female reproductive tract to semen and its constituent fractions, namely seminal plasma and sperm, on the abundance and distribution of Treg cells in uterine and lymphoid tissue during the peri-implantation period of pregnancy. Semen exposure did not result in an alteration in the proportion of $CD4^+$ cells that express a Treg phenotype ($CD4^+Foxp3^+$ or $CD4^+CD25^+$ cells) in comparison to control virgin estrus females. However semen, and specifically its seminal plasma component, resulted in an almost 2-fold increase in the number of Treg cells in the uterus and its associated lymph nodes (iliac LNs).

Previous studies analysing the abundance and regulation of Treg cells over gestation have relied on identifying Treg cells based on the presence of CD4 and CD25 (Aluvihare*, et al.* 2004, Heikkinen*, et al.* 2004, Robertson*, et al.* 2009, Somerset*, et al.* 2004, Zhao*, et al.* 2007), however CD25 is not an exclusive marker for Treg cells and also identifies activated T-cells (Caruso*, et al.* 1997). As a result there are significant limitations on the certainty of conclusions that can be made based on this identification strategy. Therefore, in this study the marker Foxp3 was also used to identify Treg cells amongst the $CD4^+$ T-cell subset. Foxp3 is a transcription factor that has been identified to be specific for Treg cells in mice, and has been shown to be the 'master switch' that induces commitment to a Treg cell lineage (Fontenot*, et al.* 2003, Fontenot*, et al.* 2005).

A population of $CD4^+Foxp3^+$ cells was identified that predominately express CD25, with more than 80% of the $CD4^+CD25^+$ cell population also expressing Foxp3 (Figure 3.1). However there was an additional population of $CD4^+CD25^-$ cells that also express Foxp3 (7.7% of $Foxp3^+$ cells; Figure 3.1B). Critically, these cells have also been shown to have suppressive function (Nishioka*, et al.* 2006). Therefore Treg cells were identified as either of two alternative phenotypes; $CD4^+Foxp3^+$ cells or $CD4^+CD25^+$cells, with the later being consistent with previously published identification strategies and so allowing comparison of the current data with that of previous findings. Importantly in this study, analysis of both populations gave consistent findings.

When the staining protocol was applied to cells from mice with an endogenously-expressed GFP protein under the promoter of the *Foxp3* gene, it could be demonstrated

that the FACS technique utilised was extremely efficient and accurate in identifying Foxp3$^+$ cells. However the intensity of the APC fluorophore conjugated to the anti-Foxp3 antibody appeared to provide superior discrimination of Foxp3$^+$ cells compared to the less bright GFP-tagged cells.

Alterations in the abundance of Treg cells in the peri-implantation period have previously been documented in mice, with an increase in the percentage of CD4$^+$ cells expressing CD25 as early as day 2.5pc in a range of lymphoid tissues (Aluvihare*, et al.* 2004). However the factors that may contribute to this regulation at such an early stage of pregnancy still remain to be investigated. It was proposed that maternal exposure to semen might explain such an effect. Unlike previous studies (Aluvihare*, et al.* 2004, Robertson*, et al.* 2009) no increase was seen in the percentage of CD4$^+$ cells expressing CD25 (or Foxp3) in any of the mated groups compared to estrus animals. However, consistent with previous findings (Robertson*, et al.* 2009) when total Treg cell numbers in lymphoid tissues were assessed, a significant increase in the total number of Treg cells in the uterine-draining iliac LNs was seen. This was shown to occur only in the iliac LNs with the inguinal LNs and spleen showing no alternation in total Treg cell numbers. The expansion seen in the iliac LNs following mating appears to be largely attributable to the seminal plasma component of the ejaculate, with VAS- mated animals showing comparable expansion in the Treg cell pool. This finding suggests a critical role for the immunoactive moieties in seminal plasma to be driving T-cell and Treg cell expansion prior to embryo implantation, with TGFβ being a leading candidate to mediate this effect (Robertson*, et al.* 2002).

In addition to the findings in the iliac LNs it was also found that seminal plasma has a role in regulating the abundance of Foxp3 expressing cells in the uterus in the peri-implantation period. Using *Foxp3* specific qRT-PCR it was demonstrated that uterine tissue from mice exposed to seminal plasma had significantly elevated *Foxp3* mRNA levels with a significant increase in Foxp3 mRNA in females mated to intact and VAS- males compared to virgin estrus females (Figure 3.6). These findings were supported at the protein level, with anti-Foxp3 IHC demonstrating an increase in Foxp3$^+$ cells in the uterus of females exposed to seminal plasma at the time of conception. This was evident in intact and VAS- mated animals which displayed elevated Foxp3$^+$ cell densities (Figure 3.7). However results at the mRNA levels weren't entirely consistent with those

seen at the protein level with SV- mated animals displaying a trend toward elevated Foxp3 mRNA levels but no difference at the protein level. This inconsistency in the findings may be explained by the differing parameters assessed by the two methods. Messenger RNA levels are quantified relative to the house keeper gene βactin, and as such can be interpreted as the amount of *Foxp*3 mRNA as a proportion of the total number of cells, whilst IHC assesses cell number in relation to tissue area. This means that any change in uterine size that wasn't as a result of increased cellularity, such as uterine oedema or alterations in connective tissue, would result in discordant results. With the mechanism of action of Treg cells being tightly associated with their spatial distribution, the elevated Foxp3 cell density in uterine tissue could suggest that Treg cells have a critical role in the uterus at the time just prior to embryo implantation.

Whilst no change was seen in the percentage of $CD4^+$ that express a Treg cell phenotype ($CD4^+Foxp3^+$ or $CD4^+CD25^+$), the approximate 2-fold expansion observed in total Treg cell numbers in the iliac LNs over such a short period of time (4 days) evokes the possibility of differential expansion of selective Treg cell clones that may possess specificity for paternal antigens in the ejaculate, some of which may later be expressed by the implanting conceptus. However the fact that the proportion of $CD4^+$ cells that express a Treg cell phenotype is unaltered suggests that any expansion within this population is equalled by a concurrent expansion with in the $CD4^+CD25^-$ (or $CD4^+Foxp3^-$) cell population. Whilst this does suggest that Treg cell are not specifically expanded, relative to non-regulatory $CD4^+$ T cells, it does not provide information regarding the ratio of antigen-specific Treg cells to antigen-specific CD4 effector cells. Analysis of the expansion of the entire Treg cell population may not be as critical for understanding the physiological role of these cells as looking at the role of specific antigens in their selective expansion or activation during pregnancy. In fact, one could argue that the alternative of a non-selective expansion of Treg cells could ultimately lead to a state of immune compromise. Thus it may be more informative to evaluate the level of fetal antigen-reactive effector cells as a ratio to fetal antigen-specific Treg cells, rather than simply looking at all CD4 cells as a ratio to all Treg cells. Whilst such an experiment may be technically challenging to perform, the development of TCR transgenic models specific for fetal antigens may help to provide insight (Erlebacher*, et al.* 2007, Moldenhauer*, et*

al. 2009). Additionally tetramers could be used to identify fetal specific T-cell clones, and hence discriminate between subsets of Treg cells and effector T-cells.

Whilst there is limited evidence for paternal antigen-specific responses in Treg cells in early pregnancy, it has been shown that prevention of abortion in the CBA/J x DBA mating combination can only be achieved if Treg cells are adoptively transferred from pregnant females, not virgin females, and only if transfer occurs in the peri-implantation period (Day 0.5 or Day 2.5). Additionally, "blocking" of Treg cells with anti-CD25 antibodies prior to embryo implantation resulted in diminished pregnancy rates (Zenclussen*, et al.* 2005). In later pregnancy it has been shown that the accumulation of Treg cells in the uterus is enhanced when fetal alloantigens are present (Kallikourdis*, et al.* 2007, Tilburgs*, et al.* 2008). Whilst in mid-gestation fetal alloantigen can result in enhanced Treg cell numbers, and paternal specific Treg mediated suppression (Zhao*, et al.* 2007). These findings are all suggestive of a role for antigens in driving the proliferation of Treg cell populations in pregnancy. However there is still a paucity of definitive evidence for a causal role of antigens of fetal or paternal origins in the expansion or activation of Treg cells in pregnancy.

The quantitative differences between the findings described here and those of others may result from variations in mating combinations, with different strains having been shown to have variations in the kinetics of Treg cell expansion over pregnancy (Thuere*, et al.* 2007). The mating combination of C57BL/6 females and BALB/c males used in this study differs from those in other experimental models (Aluvihare*, et al.* 2004, Thuere*, et al.* 2007). Additionally, the staining protocol varies from that used in papers that utilised the same mating combination (Robertson*, et al.* 2009). Specifically in regards to Robertson *et al.* (2009) the main point of difference is the use of the directly conjugated PE-Cy7 anti-CD25 antibody (PC61) in this study, as opposed to biotin-conjugated anti-CD25 antibody (7D5) that was labelled with a PE-CY5-streptavidin conjugate. These two different clones may have slightly different specificities or avidities for CD25 and the two different conjugates used may have significant differences in their brightness and compensation characteristics. The staining protocol and gating strategy employed in this study was extensively validated and was shown to be robust and reproducible. As such any variations in results will highly likely be due to variations in models used and perhaps variations in techniques employed to categorise Treg cells.

CHAPTER 4

MIGRATORY MOLECULES AND REGULATORY T-CELL ACCUMULATION IN THE PRE-IMPLANTATION UTERUS

4.1 INTRODUCTION

In the previous chapter it was shown that the seminal plasma component of semen is largely responsible for the elevation of Treg cell numbers seen in the lymph nodes draining the uterus and in the uterus itself during the peri-implantation period in mated mice. This raises the question of the mechanism by which seminal plasma influences Treg cells. There are several complimentary mechanisms by which exposure of the female reproductive tract to seminal plasma may result in an increase in Treg cells in the uterus. The increased availability of Treg cells, as a result of proliferation in the iliac LNs and subsequent entry into the circulation, may be assisted by elevated expression of migratory molecules within the uterus that facilitates enhanced recruitment of Treg cells from the systemic circulation and/or local expansion of recruited cell populations within the uterus itself.

Leukocyte migration within both lymphoid organs and peripheral tissues are coordinated by a complex network of migratory regulators known as chemokines (Viola*, et al.* 2008). Interactions between chemokines expressed in the target tissue and corresponding receptors on leukocytes (see Table 4.1 and Table 4.2) allows for selective migration of leukocyte subsets to their site of action. Conventionally such a process has been demonstrated to mediate effector T-cell responses in host defence processes including inflammation and viral clearance (Baggiolini 1998). However with the emergence of Treg cells, chemokine and chemokine receptor interactions have become implicated as critical factors in suppression of inappropriate immunity by mediating Treg cell recruitment to their site of action (Huehn*, et al.* 2005, Navarrete*, et al.* 2009). Due to the Treg cells having both a paracrine and contact-dependent mechanism of action, proper localisation of Treg cell is critical for their ability to effectively and appropriately suppress immune responses (Siegmund*, et al.* 2005, Zhang*, et al.* 2009).

A wide range of migratory molecules expressed on Treg cells have been identified as being involved in the recruitment of Treg cells to peripheral sites. These include chemokine (C-C motif) receptor 2 (CCR2), CCR4, CCR5 as well as the integrin $\alpha_E\beta_7$ (CD103) (Kallikourdis*, et al.* 2007, Siegmund*, et al.* 2005, Viola*, et al.* 2008, Zhang*, et al.* 2009). Additionally Treg cell migration and localisation within lymphoid tissues has been associated with CCR2, CCR5 and CCR7 (Debes*, et al.* 2005, Zhang*, et al.* 2009). Each of

these receptor molecules responds to corresponding chemoattractants typically in the form of chemokine (C-C motif) ligands (CCL), or E-cadherin in the case of CD103. In order to ensure robustness in the system, chemokine receptors are typically promiscuous and respond to multiple ligands (see Table 4.1) (Mantovani 1999). This means that a leukocyte expressing a specific chemokine receptor responds to the sum of all of the corresponding ligands for that receptor present within a tissue. As such the precise chemokine/chemokine receptor interaction that results in the recruitment of a leukocyte population into a tissue can be difficult to define.

In the cycling non-pregnant mouse uterus, the levels of chemokine mRNAs *Ccl3*, *Ccl4*, *Ccl5*, *Ccl22* and *Cx3cl1* have all been shown to fluctuate in a temporal pattern which correlates with that of *Foxp3* mRNA (Kallikourdis*, et al.* 2007) implying that these factors may have a role in accumulation of Treg cell in the uterus. Of these, however, only *Ccl4* stayed elevated in concert with *Foxp3* in mid pregnancy (gd10.5). This finding implicates *Ccl4* as possibly having a role in the accumulation of Treg cells in the pregnant uterus (Kallikourdis*, et al.* 2007). This is consistent with previous work that demonstrated the pregnant uterus was highly enriched for Treg cells that express the CCL4 receptor, CCR5, and that following adoptive transfer, $CCR5^+$ Treg cells accumulate in the pregnant uterus at almost twice the efficiency of $CCR5^-$ Treg cells (Kallikourdis*, et al.* 2007). The combination of these two findings suggests that CCL4 expression within the uterus during pregnancy may be critical in recruiting Treg cells that express CCR5. These cells may then function to suppress any local anti-fetal immune responses at the implantation site.

In the post-mating period the dynamic nature of chemokines has been demonstrated in the mouse uterus (Pollard*, et al.* 1998, Robertson*, et al.* 1998, Wood*, et al.* 1999). These studies predominately focused on the inflammation-like recruitment of leukocytes into the uterus following mating. Consistently it was shown that for the majority of chemokines studied, their amount of mRNAs peaked shortly following mating (day 0.5-1.5pc) and then subsided by day 3.5pc, with this pattern of regulation coinciding with leukocytes numbers in the uterus (Robertson*, et al.* 1998, Wood*, et al.* 1999).

Chemokines have been demonstrated to be critical for accumulation of Treg cells in the pregnant uterus. Whilst they have been shown to be dynamically regulated during the

estrus cycle and in the post-mating period, there have been no studies to date on the role of chemokine mediated accumulation of Treg cells in the peri-implantation period. This would seem to be pertinent given the importance of Treg cells in the maintenance of immune tolerance toward the conceptus. Based on this, we hypothesised that specific patterns of expression of mRNAs encoding migratory regulators, distinct from those in the estrous uterus, would be present in the peri-implantation uterus, and that seminal factors are important in controlling these.

Experiments performed in this chapter attempt to gain insight into the role of chemokines and their corresponding receptors in the recruitment of Treg cells into the peri-implantation uterus. Specifically the experiments addressed whether individual factors are elevated in the peri-implantation uterus, whether specific components of seminal fluid (sperm or seminal plasma) are required for their induction, and whether expression levels correlate with *Foxp3* mRNA levels.

4.2 MIGRATORY MOLECULE mRNA EXPRESSION IN THE PERI-IMPLANTATION UTERUS

To assess the possible role that chemokines and their receptors may have in inducing the migration of Treg cells into the uterus, a panel of primers were designed that were specific for a range of chemokines and chemokine receptors previously shown to be involved in Treg cell migration. These included the chemokines CCL4, CCL5, CCL19 and CCL22 and the receptors CCR4, CCR5, CCR7 and the integrin CD103 (see Table 4.1). The level of mRNAs for each chemokine and chemokine receptor present in uterine tissue samples recovered on day 3.5pc from B6 females (n=8-10 per group), were assessed by qRT-PCR. Uterine tissue from estrus females or females mated to intact, SV- or VAS-males was analysed.

Table 4.1: Migratory regulators of interest, their synonyms and molecules of interaction.

Migratory Regulator	Synonym	Ligand/Receptor
CCL4	Macrophage inflammatory protein 1-β (MIP-1β)	CCR5
CCL5	RANTES	CCR1 CCR3 CCR5
CCL19	CKb11 MIP-3β	CCR7 CCR11
CCL22	Macrophage derived chemokine (MDC)	CCR4
CCR4	CC CKR-4	CCL2 CCL3 CCL5 CCL22
CCR5	CC-CKR-5 CD195	CCL3 CCL4 CCL5
CCR7	CC-CKR-7 CD197 EBI1	CCL19 CCL21
CD103	integrin alpha E (itgae) $\alpha_E\beta_7$	E-cadherin[#]

(Modified from Viola *et al*, 2008;Uss *et al*. 2006)

Table 4.2: Expression of chemokine receptors by leukocyte populations.

Chemokine Receptor	Expressing Leukocyte Subsets (other than Treg cells)
CCR4	Th2[1] DCs[2] Macrophages[3] Granulocytes [4]
CCR5	Th1[1] DC[2] Macrophages[5] Granulocytes[6]
CCR7	B-cell (naïve and activate/memory)[1] T-cells (naïve and activate/memory)[1] DCs[2] Macrophages[5] Granulocytes[7]
CD103	T-cells (CD4 and CD8)[8] DCs[9]

[1](Kim, *et al.* 1999), [2](Sallusto, *et al.* 1998), [3](Katschke, *et al.* 2001), [4](Yousefi, *et al.* 2001), [5](Jeannin, *et al.* 2003), [6](Jan, *et al.* 2006), [7](Kobayashi, *et al.* 2003), [8](Cepek, *et al.* 1994), [9](Coombes, *et al.* 2007).

Comparison between mRNAs in tissue from estrus animals compared to that from animals mated to intact males revealed that only two of the migratory molecules analysed were significantly different on day 3.5pc following mating. These were *Ccl19* ($p<0.05$; Figure 4.1C) and *Ccr5* ($p<0.01$; Figure 4.2B). Both of these displayed elevated amounts, relative to estrus, following mating to intact males (approximately 1.9 and 1.8-fold respectively). Of the other migratory molecules analysed, *Ccl5* showed a trend ($p=0.062$; Figure 4.1B) toward elevated amounts following mating to intact males, whilst *Ccr7* showed a trend ($p=0.055$; Figure 4.2C) toward decreased amounts. All other mRNAs studied showed no notable changes following mating to intact males.

To determine the role of seminal plasma and sperm in regulating migratory molecule mRNA expression, additional groups of females mated with SV- and VAS- males were evaluated. Similar increases were seen in these groups as were seen in females mated with intact males, for *Ccr5* mRNA (Figure 4.2B). However the increase seen in *Ccl19* (Figure 4.1C) mRNA after mating with intact males was not seen after mating with SV- or VAS- males. Of note were *Ccl22* (Figure 4.1D) and *Ccr7* (Figure 4.2C) which both displayed significant lower mRNA expression when females were mated with SV- and VAS- males compared to tissue from estrous females (for *Ccl22,* $p<0.01$ and $p<0.001$ for SV- and VAS- mated animals respectively; for *Ccr7* $p<0.01$ for both groups).

Due to consistent changes seen in mRNA amounts of all three mating groups for *Ccl5*, the data from the individual mating groups were combined to assess possible changes as a result of mating. This revealed that there was a significant increase in the amount of *Ccl5* mRNA following mating ($p<0.05$).

By looking at the difference in Ct value between the migratory molecules of interest and that of β-actin (housekeeping gene) a semi-quantitative indication of the total amount of mRNAs present can be obtained (Table 4.3). Generally a lower Ct value indicates that the mRNA of interest was in greater abundance. Based on this it can be seen that at estrus the most abundant ligand is *Ccl5* followed by *Ccl4*, *Ccl22* and finally *Ccl19.* Of the leukocyte associated factors *Ccr5* mRNA was the most abundant, followed by *Cd103*, *Ccr4*, and *Ccr7*.

Table 4.3: Difference in Ct value relative to β-actin for migratory regulators of interest.

	Estrus		Intact		SV-		VAS-	
chemokine	**Mean**	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**
Ccl4	12.44	0.44	12.24	0.22	12.24	0.17	12.23	0.22
Ccl5	10.47	0.27	9.74	0.23	9.69	0.26	9.83	0.20
Ccl19	15.52	0.28	14.51	0.23	15.36	0.23	15.05	0.24
Ccl22	12.85	0.49	13.54	0.25	14.43	0.24	13.96	0.16
Ccr4	14.95	0.29	14.54	0.14	14.67	0.18	14.64	0.21
Ccr5	8.10	0.25	7.16	0.15	7.26	0.16	6.97	0.16
Ccr7	15.10	0.39	16.02	0.19	16.56	0.33	16.59	0.27
Cd103	10.08	0.24	9.70	0.14	9.87	0.18	9.77	0.28

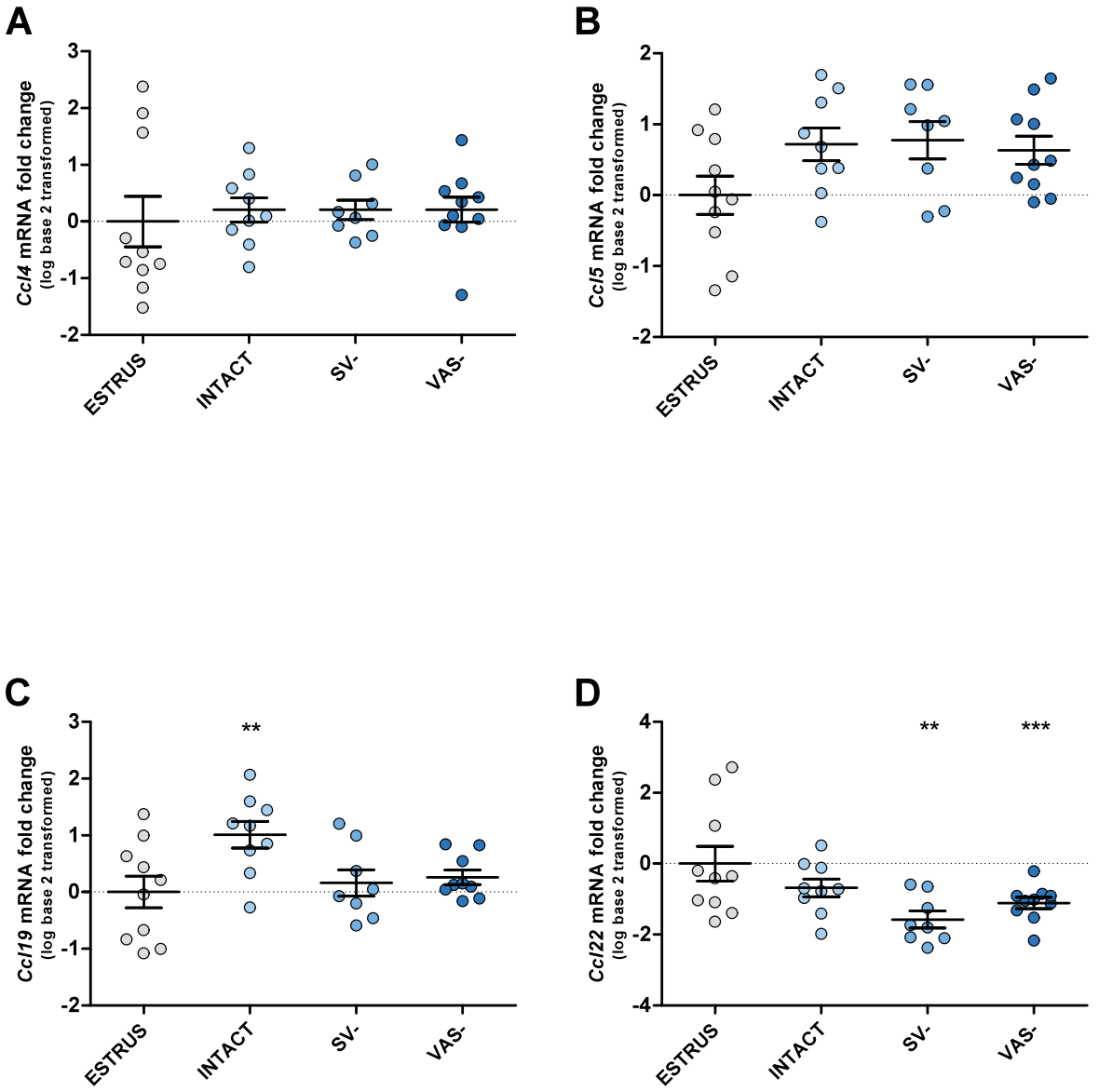


Figure 4.1: mRNA expression of chemokines *Ccl4, Ccl5, Ccl19* and *Ccl22* in estrous and day 3.5pc uterine tissue. B6 female mice were mated to either intact, seminal vesicle deficient (SV-) or vasectomised (VAS-) BALB/c males. Mice were then sacrificed on day 3.5pc and uterine tissue was collected. Tissue was processed for mRNA retrieval, and migratory molecules mRNAs were quantified by qRT-PCR. These were compared to uterine tissue from mice that were in the estrus stage of the estrous cycle. Data points are mRNA expression in individual mice, following log transformation (circles), along with the mean±SEM (straight line and error bars). **(A)** *Ccl4* mRNA in uterine tissue **(B)** *Ccl5* mRNA in uterine tissue **(C)** *Ccl19* mRNA in uterine tissue **(D)** *Ccl22* mRNA in uterine tissue. Statistical significance was assessed via a one-way ANOVA compared to estrus samples; *p<0.05 **p<0.01, ***p<0.001.

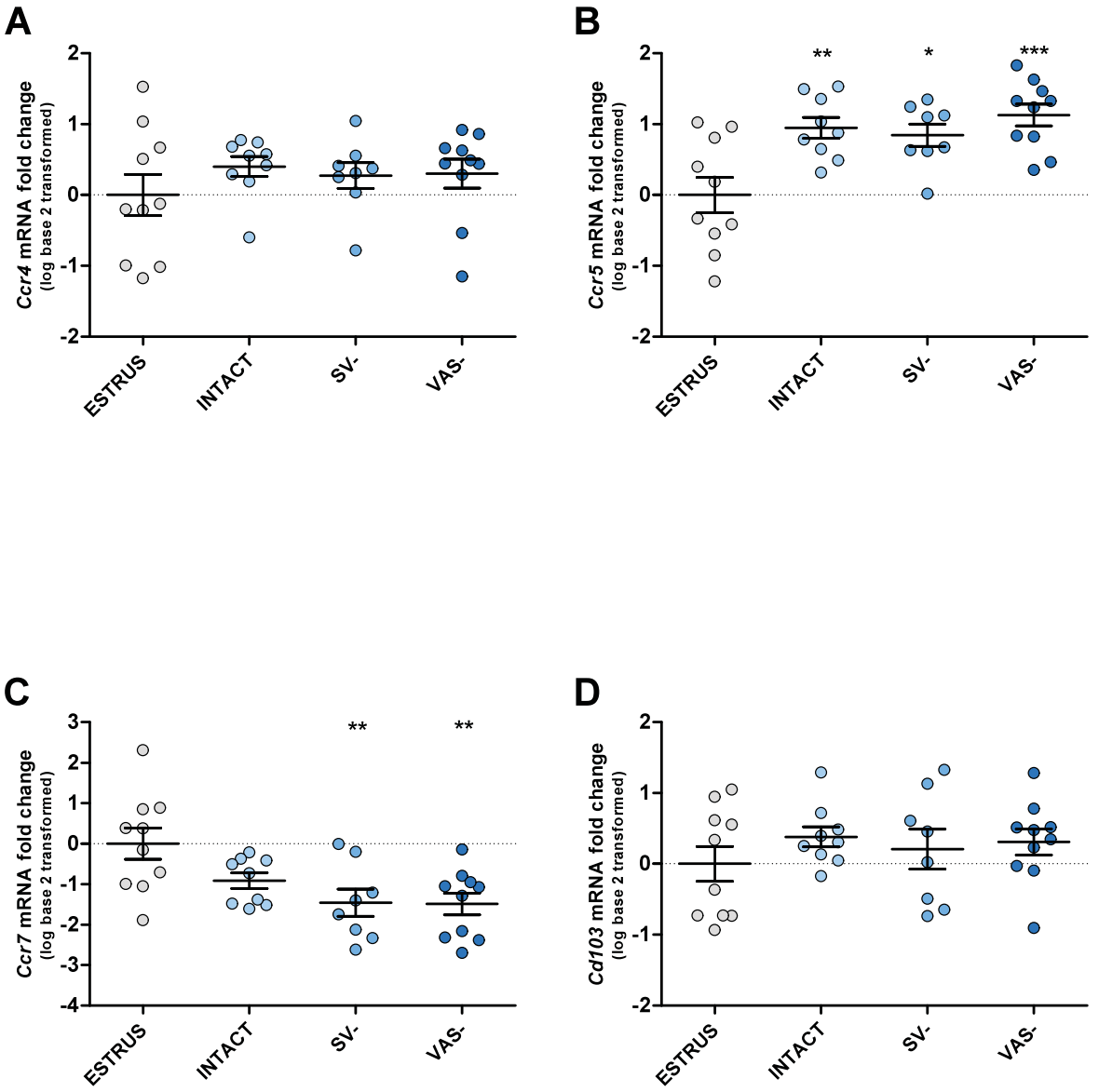


Figure 4.2: mRNA expression of chemokine receptors *Ccr4, Ccr5, Ccr7* and *Cd103* in estrous and day 3.5pc uterine tissue. B6 female mice were mated to either intact, seminal vesicle deficient (SV-) or vasectomised (VAS-) BALB/c males. Mice were then sacrificed on day 3.5pc and uterine tissue was collected. Tissue was processed for mRNA retrieval, and migratory molecules mRNAs were quantified by qRT-PCR. These were compared to uterine tissue from mice that were in the estrus stage of the estrous cycle. Data points are mRNA expression in individual mice, following log transformation (circles), along with the mean±SEM (straight line and error bars). **(A)** *Ccl4* mRNA in uterine tissue **(B)** *Ccr5* mRNA in uterine tissue **(C)** *Ccr7* mRNA in uterine tissue **(D)** *Cd103* mRNA in uterine tissue. Statistical significance was assessed via a one-way ANOVA compared to estrus animals; *p<0.05 **p<0.01, ***p<0.001.

4.3 CORRELATIONS BETWEEN MIGRATORY MOLECULE mRNAS AND *FOXP3* mRNA EXPRESSION IN THE UTERUS

Analysis of the level of migratory molecule mRNAs in the uterine tissue of female mice that had been exposed to varying components of the ejaculate highlighted some candidate molecules that may be associated with the elevation of Treg cells seen in the peri-implantation uterus. To further investigate the possible association between these migratory molecules and attraction of $Foxp3^+$ Treg cells into the peri-implantation uterus, the amounts of migratory molecule mRNAs in mated mice (intact, SV- and VAS-mated groups) were plotted against values for *Foxp3* mRNA within individual tissue samples. The combination of all three mated groups allowed for greater statistical power when analysing correlations.

Of the migratory molecules analysed, the mRNAs of only 3 were positively correlated with *Foxp3* mRNA levels in mated animals. These were *Ccl19* (Figure 4.3C; p=0.0018; r^2=0.3275), *Ccr4* (Figure 4.4A; p=0.0006; r^2=0.3790) and *Cd103* (Figure 4.4D; p<0.0001; r^2=0.6294). Comparisons of the relative distribution of tissue samples from intact mated mice (light blue dots), compared to SV- mated mice (blue dots) and VAS- mated mice (dark blue dots) revealed no overt differences in distribution between groups.

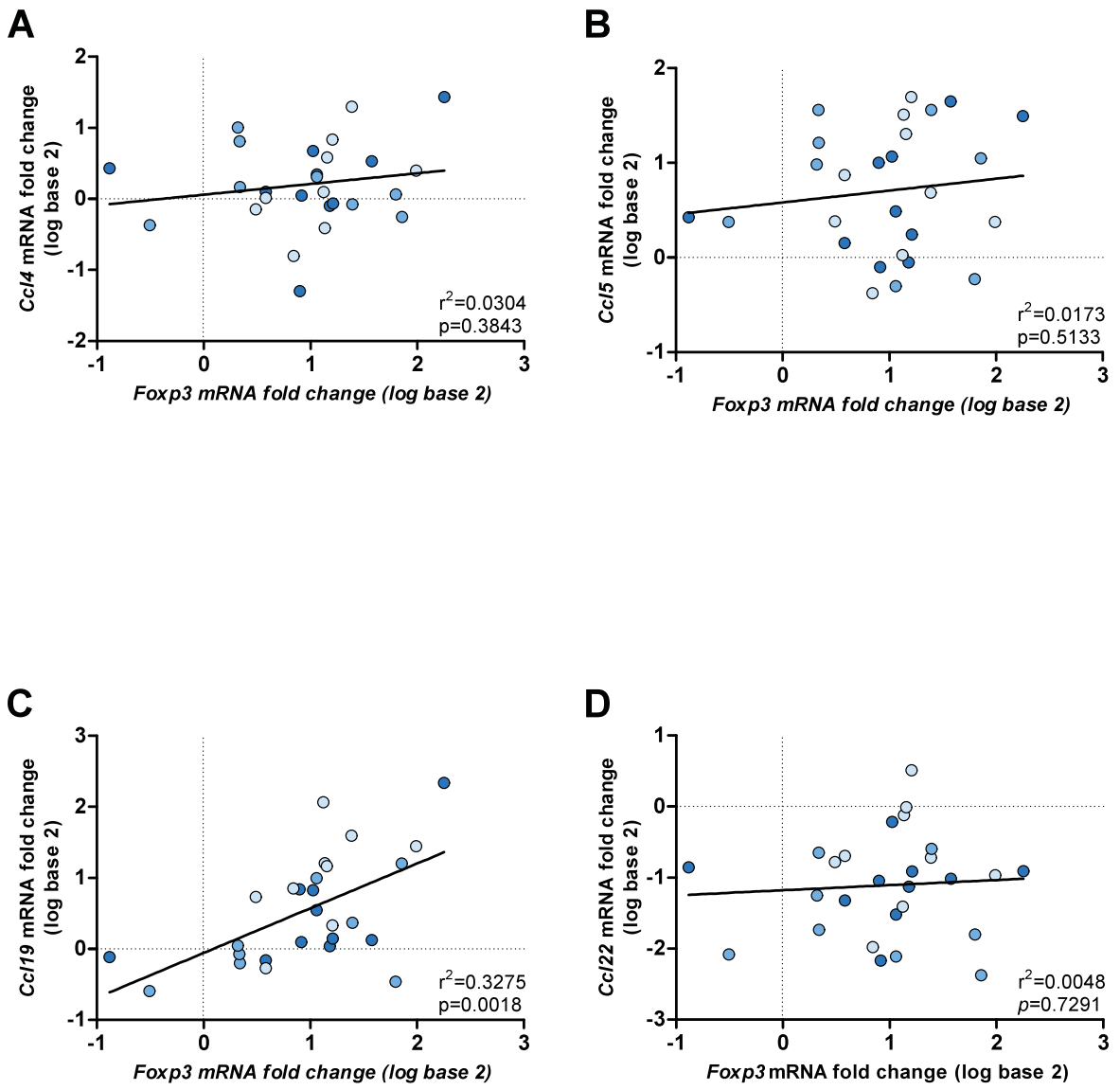


Figure 4.3: Correlations between *Ccl4, Ccl5, Ccl19* and *Ccl22* mRNAs and *Foxp3* mRNA in day 3.5pc uterine tissue. Uterine tissue was collected on day 3.5pc from B6 female mice mated with BALB/c males. Tissue was processed for mRNA retrieval, and migratory molecule mRNAs and *Foxp3* mRNA were quantified by qRT-PCR. Linear regression analysis was performed to assess correlations between mRNAs of migratory molecule and *Foxp3* following log-transformation. Individual points represent the co-expression of *Foxp3* mRNA (x axis) against the migratory molecule of interest (y axis) within the same sample. Intact mated mice are indicated by light blue dots, SV- by blue dots and VAS- by dark blue dots **(A)** Correlation between *Foxp3* mRNA and *Ccl4* mRNA. **(B)** Correlation between *Foxp3* mRNA and *Ccl5* mRNA. **(C)** Correlation between *Foxp3* mRNA and *Ccl19* mRNA. **(D)** Correlation between *Foxp3* mRNA and *Ccl22* mRNA. Correlations were assessed using linear regression analysis with statistical significance being inferred if $p<0.05$. The coefficient of determination is denoted as the r^2 value.

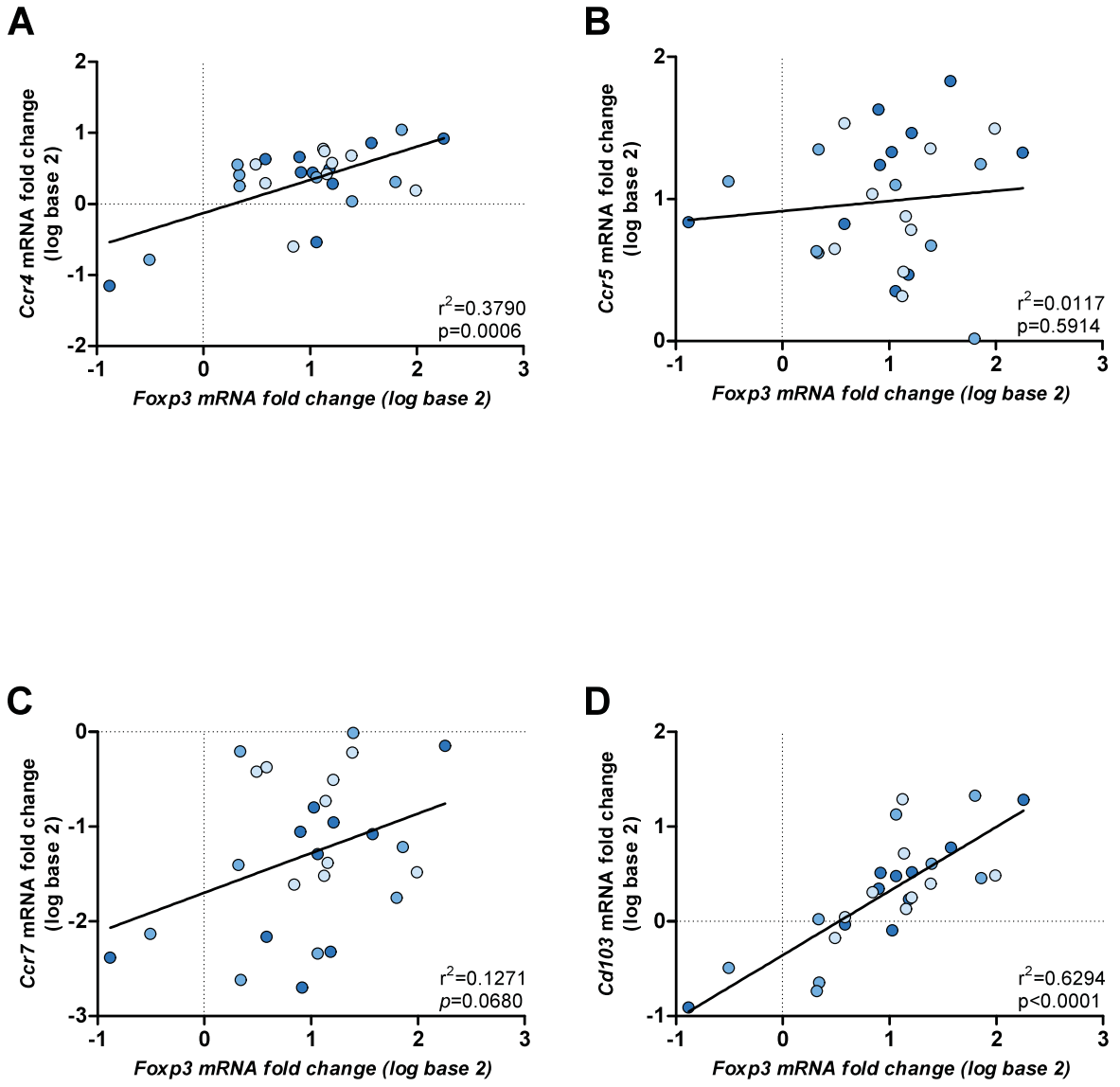


Figure 4.4: Correlations between *Ccr4, Ccr5, Ccr7* and *Cd103* mRNAs and *Foxp3* mRNA in day 3.5pc uterine tissue. Uterine tissue was collected on day 3.5pc from B6 female mice mated with BALB/c males. Tissue was processed for mRNA retrieval, and migratory molecule mRNAs and *Foxp3* mRNA were quantified by qRT-PCR. Linear regression analysis was performed to assess correlation between mRNAs of migratory molecule and *Foxp3* following log-transformation. Individual points represent the co expression of *Foxp3* mRNA (x axis) against the migratory molecule of interest (y axis) within the same sample. Intact mated mice are indicated by light blue dots, SV- by blue dots and VAS- by dark blue dots **(A)** Correlation between *Foxp3* mRNA and *Ccr4* mRNA. **(B)** Correlation between *Foxp3* mRNA and *Ccr5* mRNA. **(C)** Correlation between *Foxp3* mRNA and *Ccr7* mRNA. **(D)** Correlation between *Foxp3* mRNA and *Cd103* mRNA. Correlations were assessed using linear regression analysis with statistical significance being inferred if $p<0.05$. The coefficient of determination is denoted as the r^2 value.

4.4 DISCUSSION

This study was performed to evaluate a possible role of chemokines in recruiting Treg cells into the peri-implantation uterus and the role of seminal fluid in controlling these mechanisms. Like other lymphocyte subsets, Treg cells have been shown to migrate along chemoattractant gradients, with these chemoattractants taking the form of chemokines (Bono*, et al.* 2007). Using qRT-PCR the abundance of mRNA was analysed for a range of migratory molecules in uterine tissue from mated and estrous females. To evaluate if exposure of the female reproductive tract to seminal fluid influenced the expression of migratory molecule mRNA in the peri-implantation period, uterine tissue from day 3.5pc B6 mice was recovered from females mated with VAS- and SV- males where the sperm and seminal plasma components are deficient respectively. This allowed discernment of those factors that may be regulated by seminal fluid in a manner similar to *Foxp3* mRNA.

Coordinated localisation of Treg cells in lymphoid organs or their target peripheral tissue is a determinant of their suppressive function (reviewed in Huehn *et al.* 2005). Based on this we decided to assess the level of mRNAs for a range of migratory regulators at day 3.5pc as this represents the time just prior to embryo implantation into the uterus. As such if a tolerogenic micro-environment within the uterus is evoked to facilitate optimal embryo implantation, then it should be established by this time point.

A range of migratory molecules are associated with Treg cell localisation, with specific chemokine/chemokine-receptor interaction dictating tissue specificity (Huehn*, et al.* 2005, Viola*, et al.* 2008). To assess how migratory molecules may be involved in the accumulation of Treg cells in the uterus in the peri-implantation period, a panel of chemokines and chemokine receptors known to be involved in Treg cell localisation were selected for investigation, including the chemokines CCL4, CCL5, CCL19 and CCL22, the chemokine receptors CCR4, CCR5, CCR7 and the integrin CD103 (see Table 4.1) (Curiel*, et al.* 2004, Forster*, et al.* 2008, Kallikourdis*, et al.* 2007, Suffia*, et al.* 2005, Yurchenko*, et al.* 2006).

Of the 4 ligands analysed, only *Ccl19* showed a significant increase in the amount of mRNA expressed in any of the mating groups assessed (Figure 4.1C). There was an

approximately 1.9-fold increase in the amount of *Ccl19* mRNAs in the uterine tissue of mice mated to intact males. This was not seen when either the sperm or seminal plasma component of the ejaculate was absent. This result is indicative of a requirement for seminal plasma in combination with sperm and/or the conceptus to elevate *Ccl19* expression. Correlations between the amounts of *Ccl1*9 mRNA and *Foxp3* mRNA within mated animals revealed a strong positive correlation (p=0.0018; r^2=0.3275) between the two. This further supports a role for *Ccl19* in the recruitment of $Foxp3^+$ cells into the mated uterus.

CCL19 is a chemokine that under non-inflammatory conditions is almost exclusively expressed in secondary lymphoid organs (Gunn*, et al.* 1998, Link*, et al.* 2007). As such CCL19 is primarily implicated in the recruitment of lymphocytes, including Treg cells, to lymphoid tissues rather than peripheral sites. This migration is primarily facilitated by CCL19 interacting with one of its receptors, CCR7 (reviewed in Forster *et al*. 2008). However in our samples no correlation could be seen between *Ccl19* and *Ccr7* mRNA (data not shown). Alternatively elevated CCL19 may act as a chemoattractant for $CD62L^+$ cells, with $CD62L^+$ Treg cells displaying enhanced suppressive capabilities and preferential migration toward CCL19 (Fu*, et al.* 2004). Unfortunately *Cd62l* mRNA was not assessed in this study. The expression of *Ccl19* in non-lymphoid tissue is surprising but not unprecedented, with CCL19 documented in other peripheral tissues in a variety of species, including the intestinal tract (Shang*, et al.* 2009), pancreas (Bouma*, et al.* 2005) as well as the uterus (Daikoku*, et al.* 2004). However analysis of Ct values showed *Ccl19* mRNA was one of the lowest in regard to total amount of all the chemokines analysed. Therefore *Ccl19* mRNA likely represents only a small proportion of the total chemokine mRNA amounts, suggesting perhaps that only a low proportion of uterine cells express it. Whether this is reflected in low levels of protein or perhaps very location-restricted synthesis, requires experiments to quantify and localise CCL19 protein. However, it does not discount this chemokine as being important in the recruitment of Treg cells into the uterus.

Of the remaining chemokine ligands the only other to show altered expression due to male factors was *Ccl22*, which showed a significant decrease in the SV- and VAS- mated groups compared to virgin estrus animals. Surprisingly there was not a significant

change compared to the estrous group in the amount of *Ccl22* mRNA when these two seminal fluid components were both present in the intact mated group.

Of the chemokine receptors analysed, only *Ccr5* showed a significant elevation in mRNA expression following mating (Figure 4.2B). *Ccr5* showed consistent elevations in the amount of mRNA in all of the three mated groups studied, suggesting that its regulation may be independent of the ejaculate and is more likely related to hormone regulation or other events resulting from neuro-endocrine stimulation at coitus. Elevated progesterone, which has been shown to be comparable at day 3.5pc in both pseudo-pregnant mice and pregnant mice (Chambers*, et al.* 1979), may be a factor regulating *Ccr5* mRNA expression.

The magnitude of *Ccr5* mRNA elevation for each of the mated group was similar to that seen for *Foxp3* mRNA levels (see section 3.4). This indicates that it may have an involvement in the recruitment of $Foxp3^+$ cells into the peri-implantation uterus. This is consistent with published reports which implicate CCR5 as a key chemokine receptor for the accumulation of Treg cells into the pregnant uterus (Kallikourdis*, et al.* 2007). The accumulation of $CCR5^+$ cells into the uterus in mid gestation (gd10.5) has been suggested to occur in response to expression of the chemokine CCL4 (Kallikourdis*, et al.* 2007, Kallikourdis*, et al.* 2007). However this factor may not be of paramount importance in early gestation as the current results do not show a significant increase in *Ccl4* mRNA in any of the mated groups analysed in this study. CCR5 has also been demonstrated to represent a marker for antigen experienced "effector" Treg cells, with $CCR5^+$ Treg cells exhibiting enhanced suppressive function compared to $CCR5^-$ Treg cells (Kallikourdis*, et al.* 2007, Wysocki*, et al.* 2005). As such, the elevation noted in *Ccr5* mRNA levels might demonstrate a mechanism of Treg cell recruitment following mating, but could also reflect an increase in the relative proportion of activated/experienced Treg cells.

While the amounts of *Ccr5* mRNAs were significantly elevated in a manner implicating its involvement in the elevation of *Foxp3* mRNA following mating, when the amount of *Ccr5* mRNA was correlated to the amount of *Foxp3* mRNA within individual samples there was no correlation between the two (Figure 4.4B). Although previous studies have shown consistent patterns of regulation between CCR5 ligands (namely CCL4) and *Foxp3* mRNA in uterine tissue (Kallikourdis*, et al.* 2007), none have correlated levels in individual

samples. If the pattern of regulation was the only factor assessed in this study, then the conclusion could be drawn that *Ccr5* was a principle factor in Treg recruitment into the peri-implantation uterus. However given the poor correlation between *Ccr5* levels and *Foxp3* mRNA it can be concluded that *Ccr5* may be positively regulated in the uterus following mating, but it may not be a principal factor controlling the accumulation of Treg cells. CCR5 is also expressed on other lymphocyte subsets such as macrophages and granulocytes, with both of these being in greater abundance in the uterus compared to T-cells, and as such are likely responsible for the increase in *Ccr5* mRNA seen. Also of note is that there is no association between *Ccl4* and *Foxp3* (Figure 4.3A) or *Ccl4* and its receptor *Ccr5* (data not shown) which is the primary ligand/receptor axis proposed to be employed in the recruitment of Treg cells into the non-pregnant cycling and mid-gestation uterus (Kallikourdis*, et al.* 2007, Kallikourdis*, et al.* 2007). This does not however dismiss the possibility that an increase in *Ccr5* mRNA following mating is partially due to an enhanced proportion of activated/experienced Treg cells.

Analysis of correlations revealed that *Ccr4* was positively associated with *Foxp3* mRNA levels. However it showed no significant elevation over levels at estrus in any of the mated groups analysed. CCR4 has been implicated in the accumulation of Treg cells in tolerated allografts (Lee*, et al.* 2005), and their presence in ovarian carcinomas is linked to a poor prognosis (Curiel*, et al.* 2004), suggesting CCR4 expression on Treg cells is linked to enhanced peripheral tolerance. Thus *Ccr4* may not be a principal factor involved in increased recruitment of Treg cells into the peri-implantation uterus, but it may be consistently expressed on a subset of Treg cells in the uterus. This would provide a possible explanation for the correlation seen between *Ccr4* and *Foxp3* mRNAs.

Like *Ccr4*, *Cd103* (integrin α_E) mRNA showed a very significant correlation with Foxp3 mRNA (Figure 4.4D; $p<0.0001$; $r^2=0.6294$). CD103 defines a unique subpopulation of highly suppressive and tissue specific Treg cells (Banz*, et al.* 2003, Huehn*, et al.* 2004) with it being expressed on approximately 30% of $CD4^+CD25^+$ cells (Lehmann*, et al.* 2002). $CD103^+$ Treg cells have been demonstrated to have enhanced suppressive properties as well as elevated responsiveness to chemokines (Huehn*, et al.* 2004, Lehmann*, et al.* 2002). Importantly $CD103^+$ Treg cells display specific affinity for epithelial sites due to their ability to bind to E-cadherin expressed at high levels on epithelial cells (Cepek*, et al.* 1994). As a result $CD103^+$ Treg cells accumulate in mucosal sites such as the gut

(Lehmann*, et al.* 2002), however there are no previous studies reporting evidence for a role in Treg cell recruitment into the uterus. With a role for CD103 in Treg cell homing to peripheral tissue, especially mucosal tissue, it is not surprising that we found a strong correlation between *Cd103* and *Foxp3* mRNA levels in the uterus. It is perhaps somewhat surprising that *Cd103* was not positively regulated by seminal plasma given that its expression is induced by TGFβ, which is in abundance in seminal plasma (Robertson*, et al.* 2002). However the regulation of its ligand E-selecin would be of interest and given the role of CD103 in epithelial and mucosal tissue, its potential for the regulation of Treg cell density in the uterus throughout pregnancy warrants further investigation.

The degree to which causal links can be concluded from the experiments within this study are limited. The data presented here are only associations between patterns of regulation and correlations between the level of mRNA of migratory molecules and *Foxp3* mRNA. Significant differences can arise between mRNA levels and resulting protein levels, meaning that mRNA levels documented here may not be reflective of final protein levels. Additionally none of the migratory molecules analysed in this study are exclusive for Treg cells. This study does however provide insight into possible candidates for further investigation and the use of chemokine or chemokine receptor knock-out mice would reveal further information about the relative contributions of individual chemokine/chemokine receptor interactions.

One line of reasoning supported by the work presented in this chapter is that the increase in Treg cell density in the uterus following exposure to seminal plasma may not be solely a result of cell accumulation and recruitment. It is possible that Treg cell proliferation in the uterus contributes to the elevation in cell density. Such a mechanism would result in an increase in Treg cell numbers without the need for notable changes in chemokines.

The findings presented in this chapter build on those presented in chapter 3 and further dissect the mechanisms that result in the accumulation of Treg cells in the uterus in the peri-implantation period and the contribution of seminal factors and potentially additional factors such as the hormonal changes that follow mating. The elevated availability of Treg cells in the peripheral blood after generation in the iliac LNs combined

with the altered expression of chemokines in the peri-implantation uterus may both contribute to an increase in uterine Treg cells. An outstanding question relates to the proliferation of Treg cells in the uterus and additional analysis of proliferation indicators such as Ki67 would help to elucidate the relative contribution of this. Ultimately the gross pool of Treg cells in the peri-implantation uterus may be attributable to all 3 of these mechanisms. As such only the study of all of these mechanisms in combination will fully elucidate the relative contribution of each of these factors.

CHAPTER 5

INTERLUKIN-10 INFLUENCES THE ABUNDANCE OF REGULATORY T-CELLS DURING GESTATION

5.1 INTRODUCTION

Interleukin 10 is a cytokine with a wide range of immune-regulating functions and distinct cellular origins (Mosser, *et al.* 2008). It is produced by a large array of immune cells with its predominant lymphocytic source being Th2 cells (Mosser, *et al.* 2008). IL-10 has a diverse range of target cells, however it appears to elicit its most significant effector functions on DCs (Mosser, *et al.* 2008).

The most potent effect of IL-10 on DCs is inhibition of the production of IL-12 and the expression of MHC class II as well as costimulatory molecules such as CD80 and CD86 (Buelens, *et al.* 1995, Segal, *et al.* 1998). Consequently this results in inhibition of DC priming and inhibition of alloantigen-specific responses (Caux, *et al.* 1994). The effect of IL-10 on T-cells is mediated via this mechanism in addition to directly affecting the T-cells themselves. These include inhibition of $CD4^+$ T-cell proliferation and a decrease in IL-2, IL-12 and TNFα production (de Waal Malefyt, *et al.* 1993, Schandene, *et al.* 1994). The net result of this is a general immune suppression (Annacker, *et al.* 2001, Moore, *et al.* 2001).

The involvement of IL-10 in immune suppression is further augmented by its role in the generation and function of suppressive T-cells subsets such as Tr1 cells and Treg cells. In relation to Treg cells, IL-10 has been shown not to be essential for their generation (Maynard, *et al.* 2007), but is however implicated as one of the factors utilised by Treg cells to elicit their suppressive effects, and may be relevant in formation of an environment conducive to Treg cell generation (Annacker, *et al.* 2001, Dercamp, *et al.* 2005).

The involvement of IL-10 in a wide-range of suppressive actions in the immune system and its expression in gestational tissues implies this cytokine plays a leading role in mediating tolerance in pregnancy. Indirect evidence for a role of IL-10 in reproductive success can be gained from experiments in humans. Polymorphisms in the gene encoding IL-10, and reduced levels of systemic IL-10, have both been related to pregnancy complications (Cochery-Nouvellon, *et al.* 2009, Sharma, *et al.* 2007). Treg cells are shown to be potent sources of IL-10 during pregnancy. Treg cells exhibit a significant increase in IL-10 production in normal pregnant women compared to non-

pregnant controls (Forger, *et al.* 2008, Forger, *et al.* 2009). However, in opposition to an essential role of IL-10 in reproductive success, reproductive outcomes in mice with a null mutation in the IL-10 gene are not compromised, irrespective of the antigenicity of their gestating offspring (White, *et al.* 2004). However IL-10 is critical in the prevention of inflammatory induced fetal loss and preterm delivery, with mice deficient in IL-10 being 10-fold more sensitive to fetal loss induced by the bacterial-mimetic LPS (Robertson, *et al.* 2006).

The ability for mice to maintain pregnancy in an IL-10 deficient environment could be due to the employment of additional mechanisms of suppressing immune responses toward fetal alloantigens, a role which Treg cells may fulfil. The experiments described in this chapter endeavour to define the role of IL-10 in controlling the generation and function of Treg cells in pregnancy. To address this, the effect of IL-10 null mutation on Treg cell numbers and function in pregnant mice was analysed.

5.2 INTERLEUKIN-10 INFLUENCES TREG CELLS ABUNDANCE DURING PREGNANCY

To analyse the influence that IL-10 may have on the abundance of Treg cells during gestation, IL-10-/- mice on a B6 background (termed IL-10-/- mice) were mated to BALB/c males and the proportion and numbers of Foxp3 expressing $CD4^+$ cells within the iliac LNs draining the uterus, and the distal inguinal LNs, were analysed over the course of pregnancy (days 3.5pc, gd 6.5, 9.5, 12.5 and 17.5) using FACS (see section 3.1). This was compared to data obtained from BALB/c mated wild-type B6 mice (termed wild-type mice).

5.2.1 Kinetics of Treg cell expansion during pregnancy in WILD-TYPE mice

In wild-type mice, both the iliac LNs and the inguinal LNs showed a progressive increase in the proportion of $CD4^+$ cells that expressed Foxp3 over the period of gestation (Figure 5.1A). A significant elevation in the percentage of Treg cells, relative to the proportion of the $CD4^+$ T-cell population, could first be seen in the iliac LNs at gd6.5 (mean value=13.4% compared to 11.5% in estrus mice; $p<0.05$) with the highest percentage at gd16.5 (17.1%; $p<0.001$; Figure 5.1A). The pattern of regulation in the inguinal LNs was similar to that seen in the iliac LNs, albeit with a consistently lower percentage of $CD4^+$ cells expressing Foxp3 (Figure 5.1B). However unlike the iliac LNs the inguinal LNs did

not show a significantly elevated percentage of $CD4^+Foxp3^+$ cells, compared to estrus until gd16.5 (10.5% and 13.8% respectively; $p<0.001$).

Assessment of the total number of $CD4^+Foxp3^+$ cells showed no significant elevations over the course of gestation in either of the LNs analysed, with the exception of day 3.5pc in the iliac LNs (Figure 5.2A). With the exception of this, both the iliac and inguinal LNs displayed similar patterns of Treg cell numbers throughout gestation.

5.2.2 Kinetics of Treg cell expansion during pregnancy in IL-10-/- mice

Unlike the wild-type mice which showed a progressive increase in the proportion of $CD4^+$ cells expressing Foxp3 over the duration of pregnancy, IL-10-/- mice had comparable proportions of Treg cells at all time points measured excluding gd9.5 (Figure 5.1). Like wild-type mice however, there was a similar relationship in the pattern of change over the course of pregnancy between the iliac and inguinal LNs. On gd9.5 there was a significant elevation in the percentage of $CD4^+$ cells that express Foxp3 in both the iliac and inguinal LNs (approximately 35% and 29% respectively compared to females at estrus; Figure 5.1A and B; $p=0.001$ and $p<0.05$ respectively). This elevation was transient and was not present at the previous time point (gd6.5) or following time point (gd12.5). Interestingly, in both LNs analysed the proportion of Treg cells in IL-10-/- mice was significantly elevated over wild-type mice at all time points analysed, except for gd6.5 and gd16.5.

Analysis of the total Treg cell abundance in IL-10-/- mice showed significant differences from wild-type mice. In both lymph nodes there was a trend toward elevated numbers of Treg cells across all time points, with the exception of day 3.5pc in the inguinal LNs (Figure 5.2B). In contrast, the pattern of change in total number of Treg cells was different between the iliac and inguinal LNs. The pattern of regulation in the abundance of Treg cells in the inguinal LNs of IL-10-/- mice was similar to that of wild-type mice, albeit at an elevated level (Figure 5.2B). On the other hand the pattern of regulation in the iliac LNs of IL-10-/- mice showed no similarities to wild-type mice (Figure 5.2A). Unlike in the iliac LNs of wild-type mice there was no significant elevation in the abundance Treg cells at day 3.5pc in IL-10-/- mice. Instead there was a trend toward a consistently higher abundance of Treg cells at all time points, with a dramatic elevation at gd9.5 (approximately 11-fold compared to wild-type; $p<0.001$, and approximately 2.7-

fold compared to gd6.5; $p<0.001$; Figure 5.2A). This elevation was transient not being seen at the time points either side of gd9.5. This was reflective of extensive lymphadenopathy seen at this time point (Figure 5.3).

5.2.3 Density of Foxp3$^+$ cells at gd9.5 in implantation sites of IL-10-/- and wild-type mice

The significant elevations in the proportion and total abundance of Foxp3$^+$ cells in the iliac LN of IL-10-/- mice at gd9.5 was not reflected in increased numbers of Foxp3 expressing cells in implantation sites at gd9.5, with the implantation sites of both wild-type and IL-10-/- mice displaying similar densities of Foxp3$^+$ cells (Figure 5.4A). Foxp3$^+$ cells were evenly distributed throughout the maternal decidual tissue in implantation sites of both IL-10-/- and wild-type mice (Figure 5.4B and C).

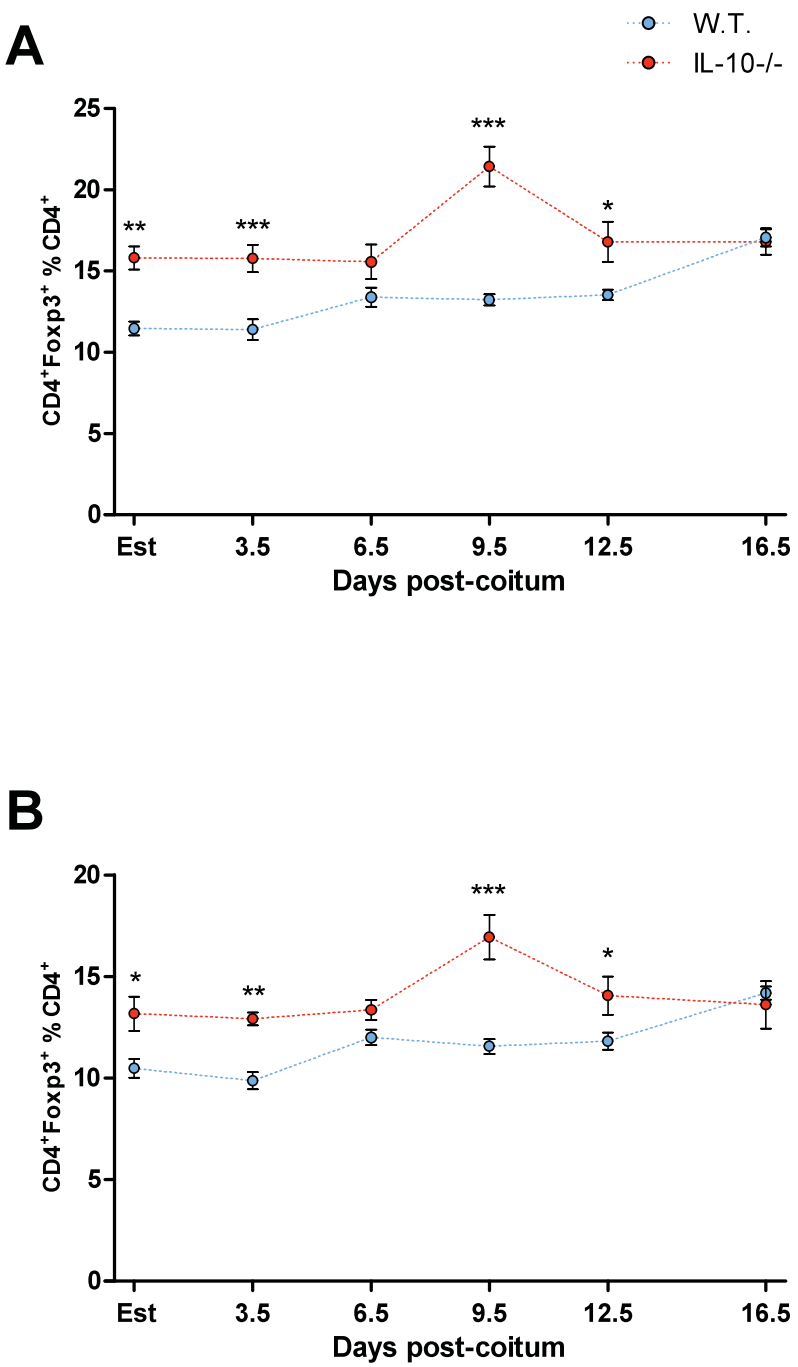


Figure 5.1: Effect of IL-10 deficiency on the size of the Treg cell population as a percentage of $CD4^+$ cells in iliac and inguinal LNs over the course of pregnancy. IL-10-/- or wild-type female mice were mated to BALB/c males. Mice were sacrificed at varying time points of gestation and the proportion of $CD4^+$ cells with a Treg phenotype was assessed. **(A)** The percentage of $CD4^+$ cells expressing Foxp3 in the iliac lymph nodes of IL-10-/- mice (red) and wild-type mice (blue circles). **(B)** The percentage of $CD4^+$ cells expressing Foxp3 in the inguinal lymph nodes of IL-10-/- mice (red) and wild-type mice (blue). All data are expressed as means (circles) ±SEM of n=6-18 females per group.

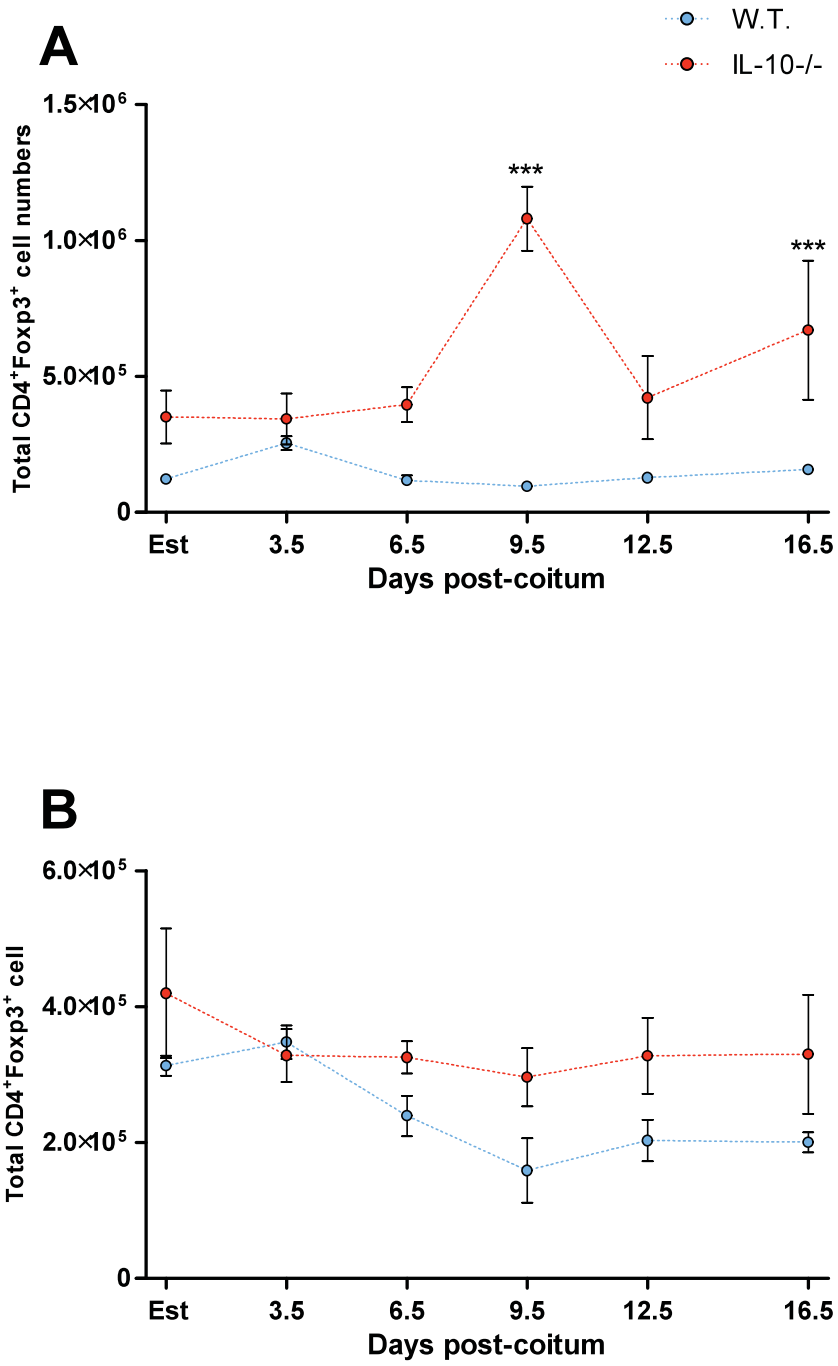


Figure 5.2: Effect of IL-10 deficiency on the total number of Treg cells in iliac and inguinal LNs over the course of pregnancy. IL-10-/- or wild-type female mice were mated to BALB/c males (allogeneic) or B6 males (syngeneic). Mice were sacrificed at varying time points of gestation and the level of cells with a Treg phenotype was assessed. **(A)** The total number of $CD4^+Foxp3^+$ cells expressing Foxp3 in the iliac lymph nodes of allogeneic mated IL-10-/- mice (red) and wild-type mice (blue). **(B)** The total number of $CD4^+Foxp3^+$ cells expressing Foxp3 in the inguinal lymph nodes of allogeneic mated IL-10-/- mice (red) and wild-type mice (blue). All data are expressed as means (circles) ±SEM of n=6-18 females per group

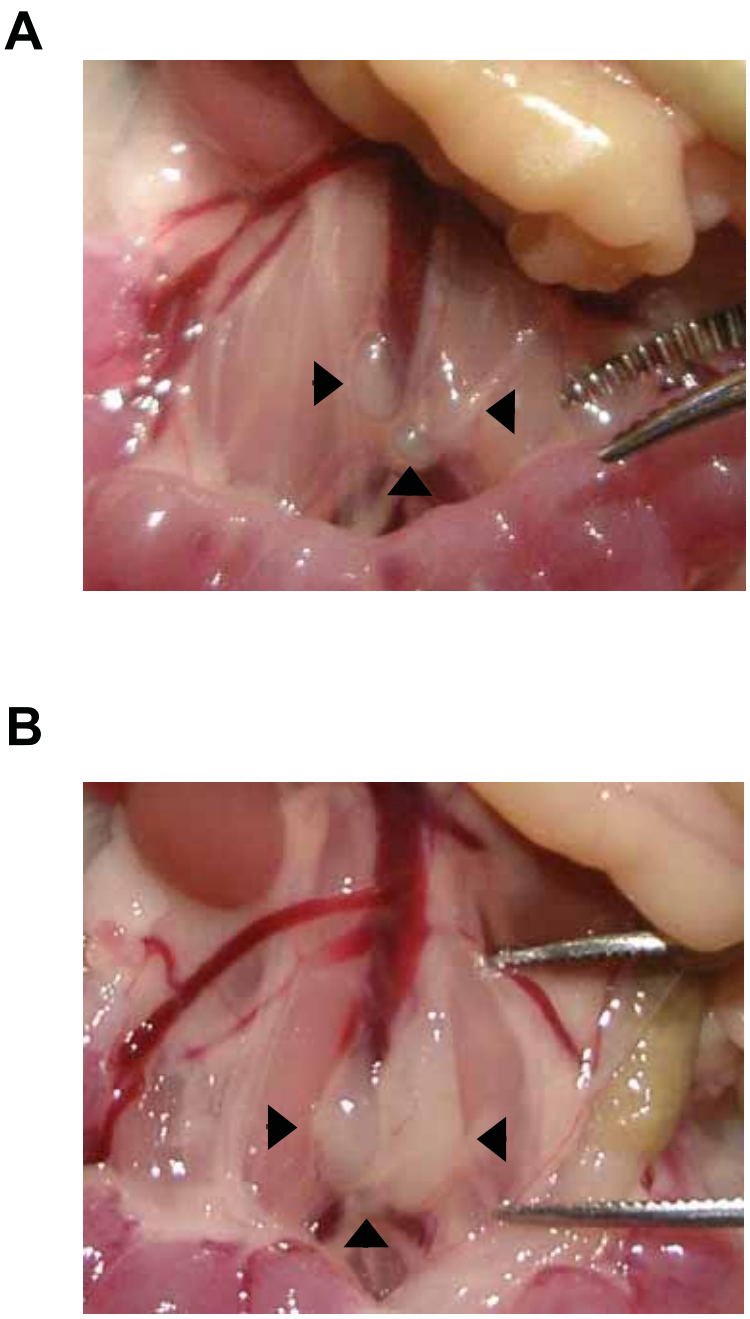


Figure 5.3: The macroscopic appearance of the iliac lymph nodes in wild-type and IL-10-/- female mice at gd9.5. Representative images of lymph nodes from wild-type and IL-10-/- female mice at gd9.5. The medial Iliac LNs (arrows) can be seen ventral to the aorta located slightly anterior of the aortic bifurcation into the common iliac veins. **(A)** The iliac LNs of wild-type female mice. **(B)** The iliac LNs of IL-10-/- female mice at gd9.5, displaying extensive lymphadenopathy.

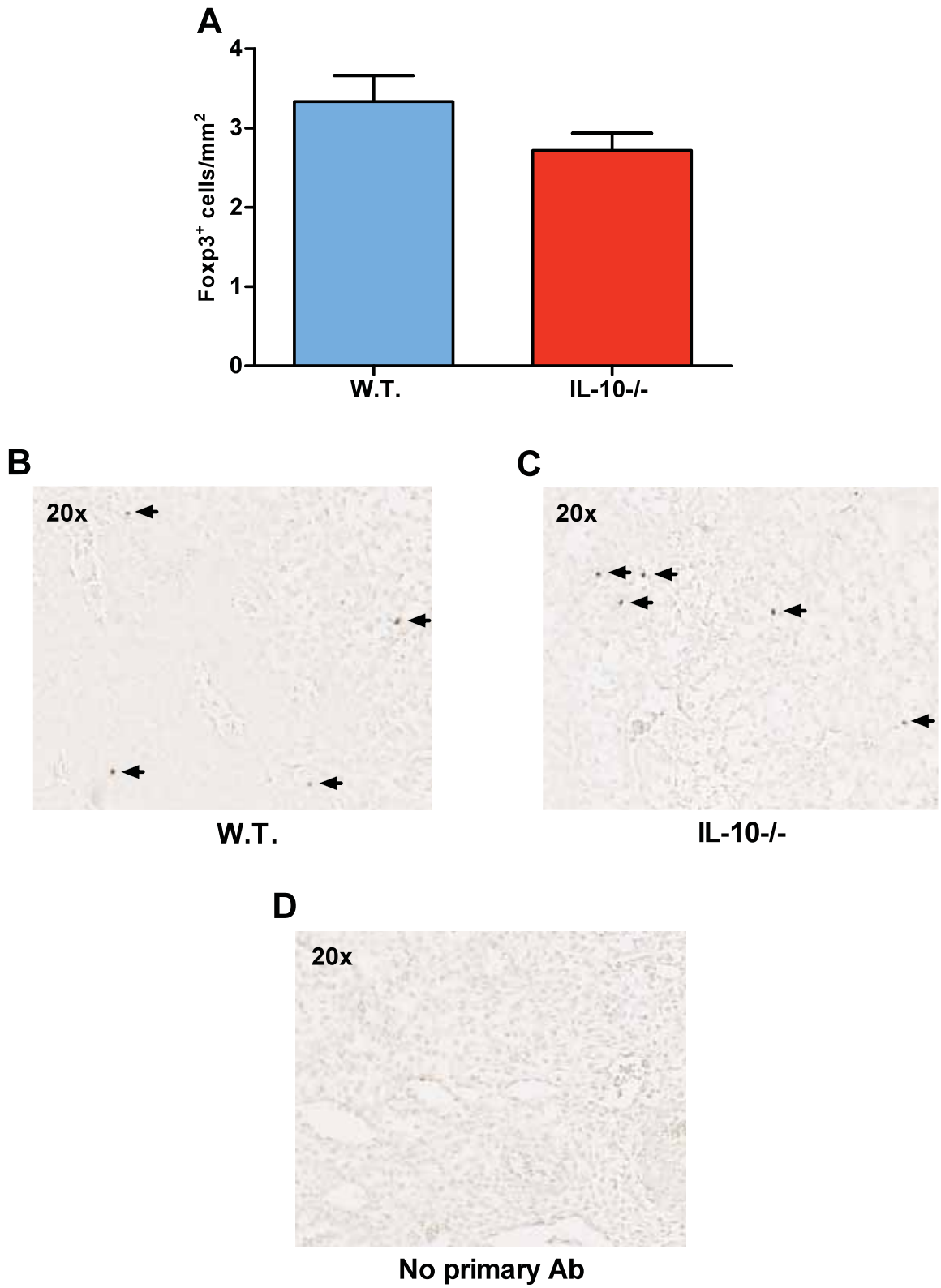


Figure 5.4: Effect of IL-10 deficiency on the density of Foxp3$^+$ cells in the implantation sites of gd9.5 wild-type and IL-10-/- mice. Implantation sites from wild-type and IL-10-/- mice were dissected on gd9.5 and fixed and processed. **(A)** Data are the mean±SEM of the density of cells expressing Foxp3 in decidual tissue at the implantation site. **(B)** Foxp3$^+$ cells (brown) in the decidual tissue of gd9.5 implantations of wild-type female mice. **(C)** Foxp3 cells (brown) in the decidual tissue of gd9.5 implantations of IL-10-/- female mice. **(D)** IHC of decidual tissue with no primary anti-Foxp3 antibody.

5.3 THE INFLUENCE OF FETAL ALLOANTIGENS ON REGULATORY T-CELLS AT MID GESTATION IN IL-10-/- FEMALE MICE

To explore the role that fetal MHC alloantigens may have in interaction with IL-10 deficiency to influence the Treg cell population on gd9.5, IL10-/- female mice were mated with syngeneic wild-type B6 males. The females were then sacrificed on gd9.5. The proportion and total abundance of Treg cells in the iliac and inguinal LNs was analysed and compared to the data obtained for IL-10-/- female mice that had been mated with BALB/c males.

It was demonstrated that the absence of fetal MHC alloantigen altered some of the Treg cell parameters measured. In the absence of fetal MHC alloantigens there was no significant increase in the proportion of $CD4^+$ cells expressing Foxp3 at gd9.5 compared to virgin estrus controls (Figure 5.5A,C). This was significantly different to the elevation seen in the presence of MHC alloantigens ($p<0.01$ and $p<0.05$ for iliac and inguinal LNs respectively). In relation to total $CD4^+Foxp3^+$ cell numbers, in the iliac LNs there was a significant elevation in the total number of $CD4^+Foxp3^+$ cells relative to estrus, irrespective of the presence of fetal MHC alloantigens (Figure 5.5B; $p=0.001$ allo-mated and $p<0.05$ syn-mated). In the inguinal LNs there was no change in their numbers relative to estrus in the absence of fetal MHC alloantigens (Figure 5.5D). This was in contrast to the decrease seen in the presence of fetal MHC alloantigens.

When total cell numbers of $CD4^+$ cells were analysed, disregarding changes in the proportion of Foxp3 expressing cells, key differences could be seen between the behaviour of $CD4^+$ cells in the iliac and inguinal LNs. Analysis of the total $CD4^+$ cell numbers in the iliac LNs show that in IL-10-/- female mice, in the absence of fetal MHC alloantigens, there was a significant elevation in the abundance of $CD4^+$ cells at gd9.5 relative to estrus, with this increase being equal to that seen in the presence of alloantigen (Figure 5.6A). However, in the inguinal LNs of IL-10-/- mice the total number of $CD4^+$ cells in syngeneic pregnant females on gd9.5 was equal to that seen at estrus. This was in contrast to allogeneic mated IL-10-/- female mice where a trend toward decreased total $CD4^+$ cells relative to estrus was evident, which was similar to that seen in wild-type pregnancies (Figure 5.6B; $p=0.081$ and $p<0.05$ respectively).

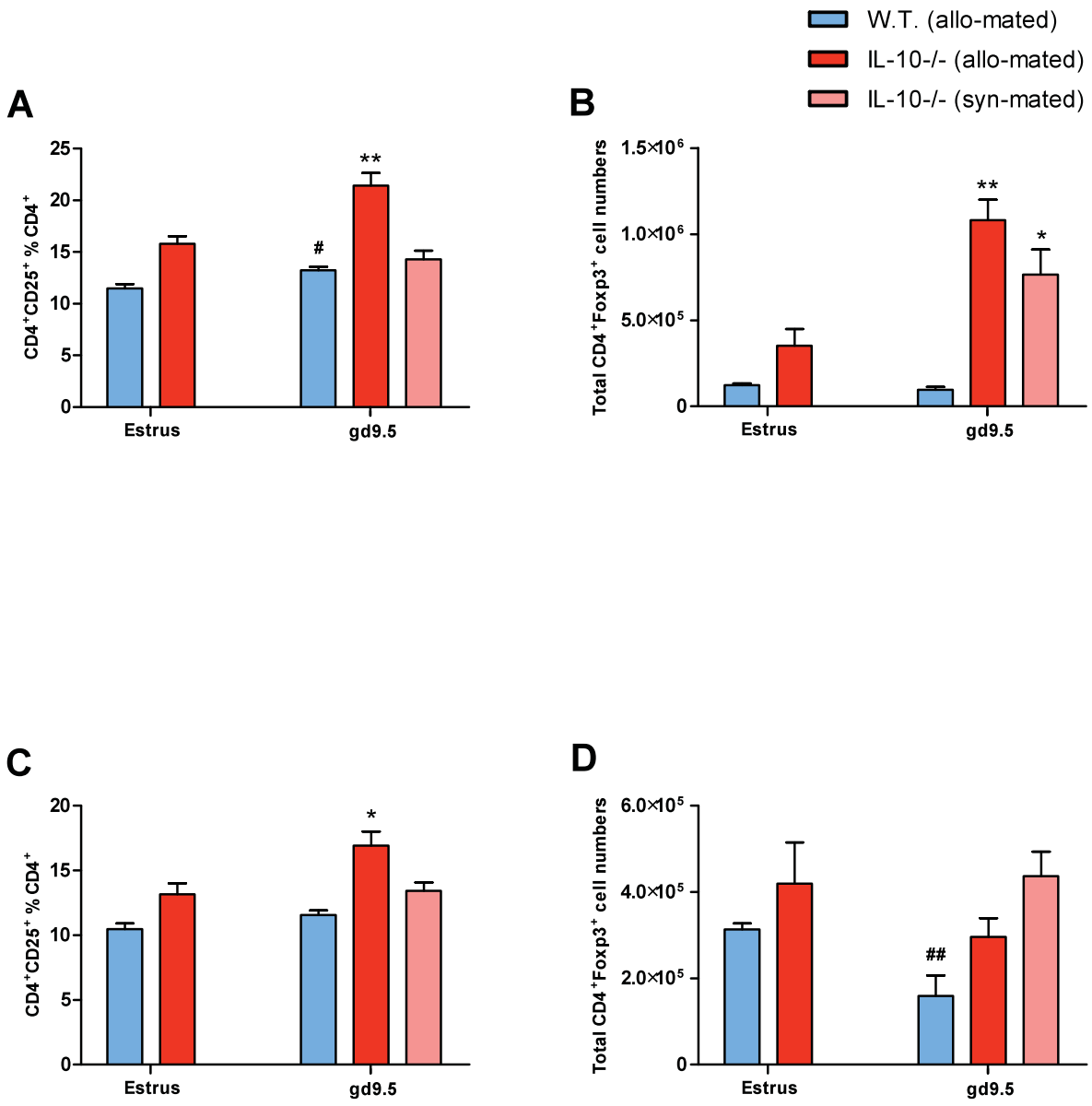


Figure 5.5: The effect of fetal alloantigens on the proportion of $CD4^+$ expressing Foxp3 and the total size the $CD4^+Foxp3^+$ cell population in iliac and inguinal LNs in IL-10-/- and wild-type mice at gd9.5. Wild-type females were mated to BALB/c males and IL-10-/- female mice were mated to BALB/c males (allogeneic) or wild-type B6 males (syngeneic). Mice were sacrificed at day 9.5 of gestation. **(A)** The percentage of $CD4^+$ cells expressing Foxp3 in the iliac LNs. **(B)** The total number of $CD4^+Foxp3^+$cells in the iliac LNs. **(C)** The percentage of $CD4^+$ cells expressing Foxp3 in the inguinal LNs. **(D)** The total number of $CD4^+Foxp3^+$cells in the inguinal LNs. All data are expressed as means±SEM of n=5-18 females per group. Statistical evaluation between estrus and gd9.5 mated groups was performed using a one way ANOVA compared to the respective estrus groups, *p<0.05, **p<0.01 relative to IL-10-/- estrus mice, $^{\#}p$<0.05, $^{\#\#}p$<0.01 relative to wild-type estrus mice.

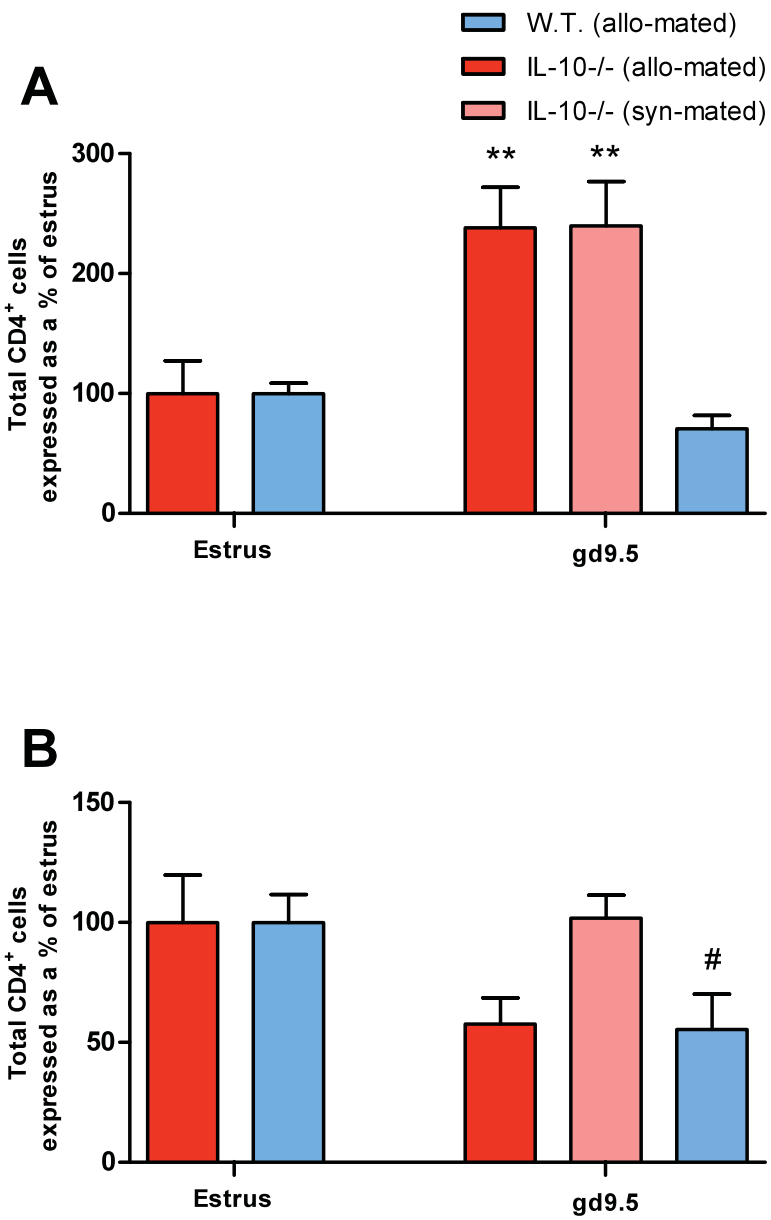


Figure 5.6: The effect of fetal alloantigen in expanding the total size of the CD4$^+$ cell population in IL-10-/- mice at gd9.5 relative to estrus. IL-10-/- female mice were mated to BALB/c males (allogeneic) or B6 males (syngeneic). Mice were sacrificed at day 9.5 of gestation. **(A)** The total number of CD4$^+$ cells (expressed as a percentage relative to the respective estrus mice for each group) in the iliac LNs of syngeneic (IL-10-/-) and allogeneic mated (IL-10-/- and wild-type) mice. **(B)** The total number of CD4$^+$ cells (expressed as a percentage relative to the respective estrus mice for each group) in the inguinal LNs of syngeneic (IL-10-/-) and allogeneic mated (IL-10-/- and wild-type) mice. All data are expressed as means±SEM of n=5-15 females per group. Statistical evaluation between estrus and gd9.5 mated groups was performed using a one way ANOVA compared to the respective estrus groups, *p<0.05, **p<0.01 relative to IL-10-/- estrus mice, $^{\#}p$<0.05 relative to wild-type estrus mice.

5.4 IL-10 ALTERS THE PROPORTION AND ABUNDANCE OF LYMPHOCYTE POPULATIONS IN MID-GESTATION

Since IL-10 deficiency was found to lead to an elevation in the size of the $CD4^+$ T-cell population, its influence on other lymphocyte subsets was investigated (Figure 5.7). $CD4^+$ and $CD8^+$ cells were analysed as a proportion of all the cells that fell within the forward-scatter/side-scatter gate set up to identify lymphocytes, as well as their proportion relative to each other. This was done to allow for analysis of the relative expansion of subsets within the LNs. Comparisons were made between cell proportions in the corresponding LNs of allogeneic mated wild-type and IL-10-/- mice at gd9.5 (Table 5.1,Table 5.2 and Figure 5.7), and due to the differences noted between the pattern of regulation of $CD4^+$ cells in the iliac LNs versus the inguinal LNs in IL-10-/- mice, these two sites were compared within genotype groups to compare the local response with the distal response.

The proportion of lymphocytes expressing CD4 or CD8 amongst cells that displayed a forward-scatter and side-scatter profile characteristic of lymphocytes, were significantly different in lymph nodes from gd9.5 wild-type or IL-10-/- female mice (Table 5.1). The proportion of cells that displayed neither T-cell marker ($CD4^-CD8^-$ cells) was increased in both the iliac and inguinal LNs from IL-10-/- mice (Table 5.1 and Figure 5.7; $p<0.001$ and $p<0.05$), and there were also significantly lower proportions of $CD4^+$ cells ($p<0.001$ and $p=0.001$). In the iliac LNs there was a significantly lower proportion of $CD8^+$ cells ($p<0.001$) when IL-10 was absent. When the proportion of $CD4^+$ cells to $CD8^+$ cells were compared, there was a significant decrease in proportion of $CD4^+$ cells and an increase in $CD8^+$ cells in both lymph node sites studied in IL-10-/- mice compared to wild-type females (Table 5.2 and Figure 5.7).

Comparison of LN sites from mice with the same genotype demonstrated that the cell populations within the iliac and inguinal LN from wild-type mice were comparable, with the exception of the $CD4^+CD8^+$ cell population, which was higher in the inguinal LNs (Table 5.1). There were however significant differences between the iliac and inguinal LNs of IL-10-/- mice, with the iliac LNs displaying a decrease in both $CD4^+$ and $CD8^+$ cells and a resultant increase in cells expressing neither marker (Table 5.1 and Figure 5.7;

p<0.01 for all). However comparisons between $CD4^+$ and $CD8^+$ cells showed that they existed in the same ratio in both LN sites (Table 5.2 and Figure 5.7).

Table 5.1: Proportion of $CD4^+$, $CD8^+$ and $CD4^-CD8^-$ subsets in the lymph nodes of IL-10-/- mice at gd9.5.

	Iliac		Inguinal	
	Wild-type	IL10-/-	Wild-type	IL10-/-
Cell type	Mean±SEM (%)	Mean±SEM (%)	Mean±SEM (%)	Mean±SEM (%)
$CD4^+$	32.1±1.1	17.4±1.0***	36.6±0.5	25.4±1.8** ##
$CD8^+$	23.2±0.52	15.4±1.2***	25.8±0.6	23.4±1.5##
$CD4^+CD8^+$	0.21±0.02	0.30±0.03	0.34±0.03#	0.40±0.04
$CD4^-CD8^-$	44.4±1.6	66.9±2.2***	37.3±0.7	50.9±2.9* ##

*p<0.05, **p<0.01, ***p<0.001 compared to wild-type mice for the same lymph node. #p<0.05, ##p<0.01, ###p<0.001 compared to iliac LNs for the same genotype.

Table 5.2: Comparisons of $CD4^+$ to $CD8^+$ cells in the lymph nodes of IL-10-/- mice at gd9.5.

	Iliac		Inguinal	
	Wild-type	IL10-/-	Wild-type	IL10-/-
Cell type	Mean±SEM (%)	Mean±SEM (%)	Mean±SEM (%)	Mean±SEM (%)
$CD4^+$	58.23±0.4	52.65±0.8***	58.35±0.5	51.63±1.0***
$CD8^+$	41.40±0.4	46.45±0.8**	41.12±0.5	47.57±0.9***
$CD4^+CD8^+$	0.37±0.04	0.91±0.06***	0.54±0.04	0.80±0.05**

*p<0.05, **p<0.01, ***p<0.001 compared to wild-type mice for the same lymph node. #p<0.05, ##p<0.01, ###p<0.001 compared to iliac LNs for the same genotype.

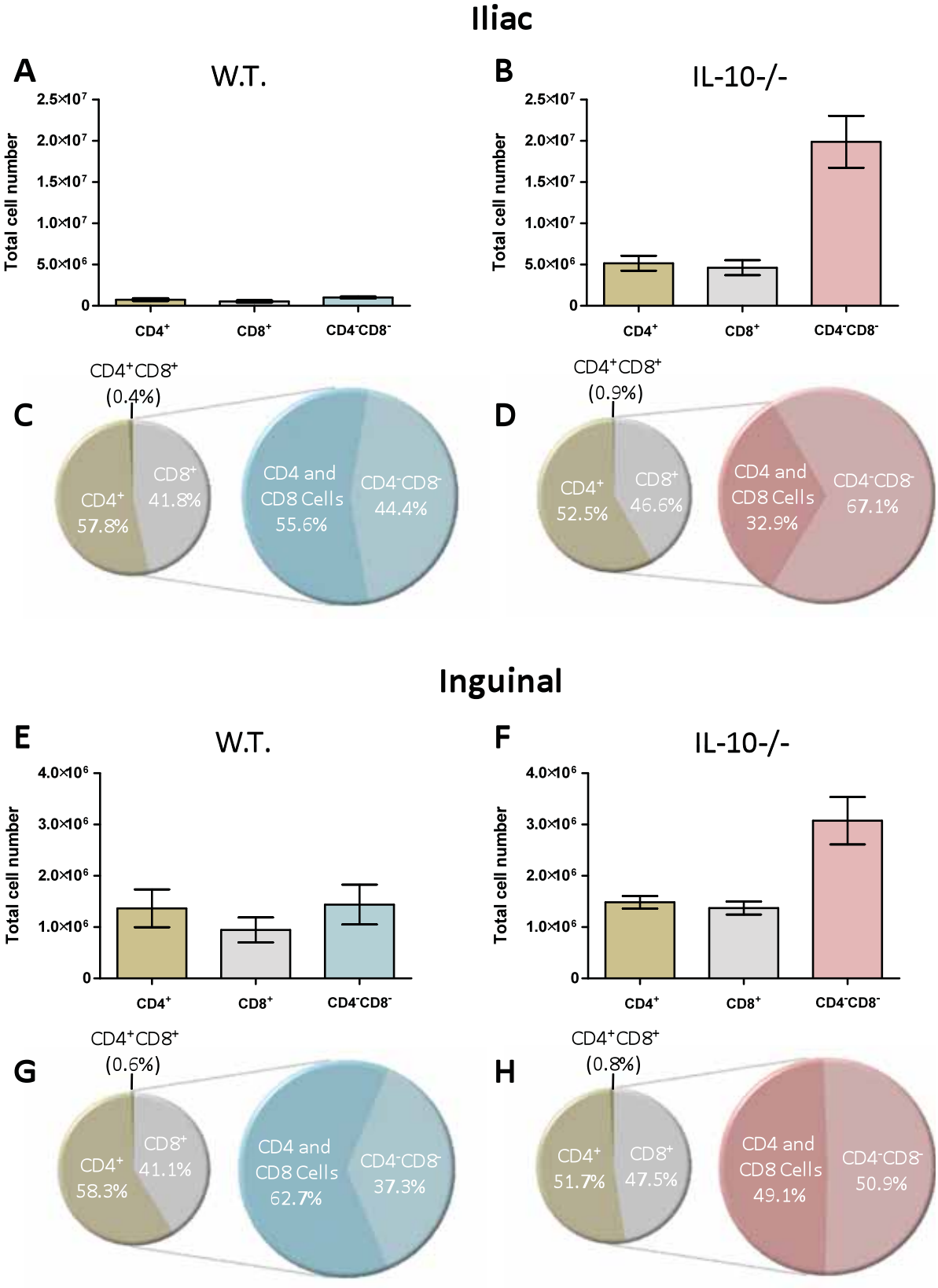

Iliac
A
W.T.
Total cell number
2.5×10⁷
2.0×10⁷
1.5×10⁷
1.0×10⁷
5.0×10⁶
0
CD4⁺
CD8⁺
CD4⁻CD8⁻
B
IL-10-/-
C
CD4⁺CD8⁺ (0.4%)
CD4⁺ 57.8%
CD8⁺ 41.8%
CD4 and CD8 Cells 55.6%
CD4⁻CD8⁻ 44.4%
D
CD4⁺CD8⁺ (0.9%)
CD4⁺ 52.5%
CD8⁺ 46.6%
CD4 and CD8 Cells 32.9%
CD4⁻CD8⁻ 67.1%
Inguinal
E
W.T.
4.0×10⁶
3.0×10⁶
2.0×10⁶
1.0×10⁶
F
IL-10-/-
G
CD4⁺CD8⁺ (0.6%)
CD4⁺ 58.3%
CD8⁺ 41.1%
CD4 and CD8 Cells 62.7%
CD4⁻CD8⁻ 37.3%
H
CD4⁺CD8⁺ (0.8%)
CD4⁺ 51.7%
CD8⁺ 47.5%
CD4 and CD8 Cells 49.1%
CD4⁻CD8⁻ 50.9%

Figure 5.7: Absolute number and proportions of CD4 and CD8 lymphocyte populations in the iliac and inguinal LNs at gd9.5 in wild-type and IL-10-/- mice. (A) The absolute cell numbers of lymphocyte subsets in the iliac LNs of wild-type mice. **(B)** The absolute cell numbers of lymphocyte subsets in the iliac LNs of IL-10-/- mice. **(C)** The proportion of cells expressing CD4, CD8 or neither marker in the iliac LNs of wild-type mice. **(D)** The proportion of cells expressing CD4, CD8 or neither maker in the iliac LNs of IL-10-/- mice. **(E)** The absolute cell numbers of lymphocyte subsets in the inguinal LNs of wild-type mice. **(F)** The absolute cell numbers of lymphocyte subsets in the inguinal LNs of IL-10-/- mice. **(G)** The proportion of cells expressing CD4, CD8 or neither marker in the inguinal LNs of wild-type mice. **(H)** The proportion of cells expressing CD4, CD8 or neither marker in the inguinal LNs of IL-10-/- mice. Bar graphs represent the mean±SEM of n=5-6 for each group. Pie charts represent the mean of n=5-6 for each group.

5.5 $CD4^+CD25^+$ CELLS FROM WILD-TYPE AND IL-10-/- MICE ARE EQUALLY SUPPRESSIVE *IN VITRO*

Due to the documented involvement of IL-10 in the suppressive mechanism of Treg cells (see section 1.3.5 and 1.5.2), the functional capability of Treg cells from IL-10-/- mice was assessed to ascertain if IL-10 deficiency altered their function. To do this *in vitro* suppression assays were performed (n=3; see section 2.5).

Treg cells from IL-10-/- mice showed comparable suppressive function to those from wild-type mice. This was seen across the three ratios of T-effector cells to Treg cells that were tested, with Treg cells from the two genotypes exhibiting approximately 51% and 43% suppression respectively at a ratio of 4 T-effector cells to 1 Treg cell.

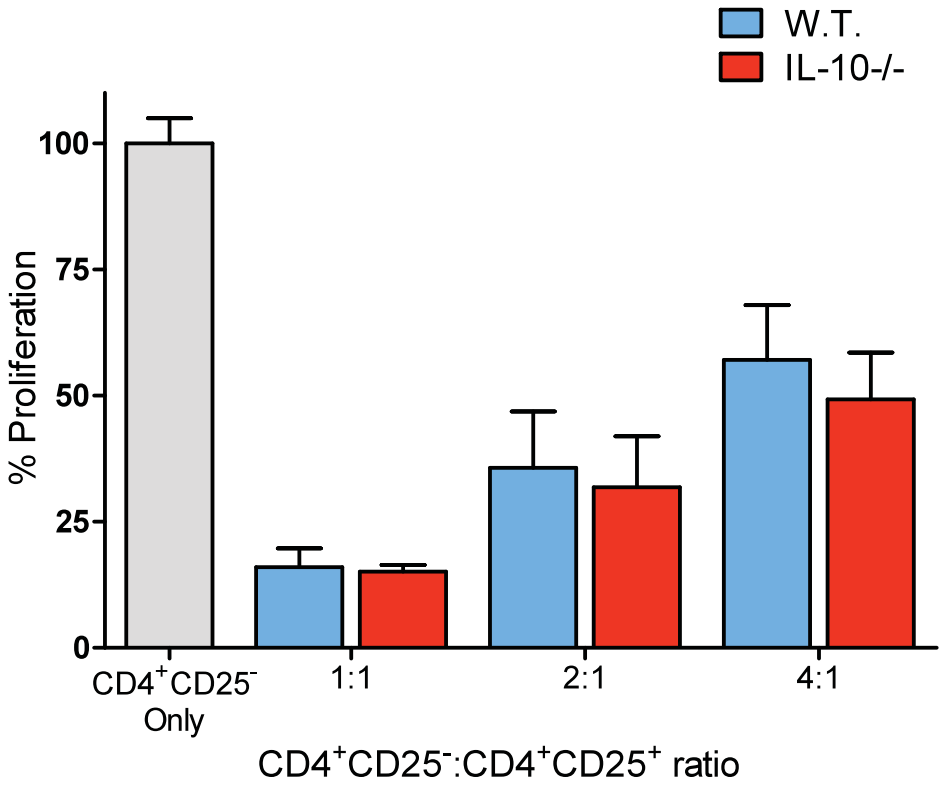


Figure 5.8: Effect of IL-10 null mutation on *In vitro* suppression function of $CD4^+CD25^+$cells. A constant number of $CD4^+CD25^-$ (Teffector) cells were co-cultured with isolated $CD4^+CD25^+$ (Treg) cells from wild-type mice (blue bars) and IL-10-/- mice (red bars) at different ratios (from 1:1 to 4:1), and irradiated stimulator cells. Proliferation was assessed 72 hours later based on tritiated thymidine (^{3}HTdR) incorporation. Data are means±SEM of 3 replicate wells, and is representative of n=3 experiments.

5.6 PARTIAL TREG CELL DEPLETION DOES NOT ADVERSELY AFFECT PREGNANCY IN WILD-TYPE OR IL-10-/- MICE

Due to the increased abundance of Treg cells in pregnant IL-10-/- mice (section 5.2) and the lack of any notable adverse effects on pregnancy in these mice (section 1.5.3) it was hypothesised that the increase in Treg cell numbers may compensate for the loss of IL-10, and as such play a more critical role in the maintenance of fetal tolerance in IL-10-/- mice than in wild-type mice. The necessity for Treg cells in the maintenance of pregnancy in IL-10-/- mice compared to wild-type mice was tested by partial depletion of Treg cells from both genotype groups and comparing pregnancy parameters at gd17.5. Mice were administered anti-CD25 antibody (PC61) which depletes CD25 expressing cells. This was done using 2 x 200 µg doses, the first at day 0.5pc (the day of plug detection) and the second at gd6.5. This was sufficient to maintain $CD4^{+}Foxp3^{+}$ cells below 50% of their pre-treatment levels for a period of 17 days (Figure 5.9). Mice were then sacrificed at gd17.5 and the number of implantation sites, fetal resorptions and individual fetal and placental weights for all viable fetuses were assessed. These values were compared to control data obtained from mice treated with PBS carrier at the same time points.

Comparison between PC61 treatment and PBS treatment for wild-type mice revealed that administration of 2 x 200 µg doses of PC61 did not negatively affect pregnancy outcome, with no detrimental effect on the number or viability of implantation sites, or fetal weights. IL-10-/- female mice showed results similar to wild-type mice, with PC61 administration having no detrimental effect on the parameters of pregnancy measured (Table 5.3). In agreement with pervious findings (White*, et al.* 2004), there was a significant increase in both the fetal and placental weight in IL-10-/- mice treated with PBS compared to PBS treated wild-type mice ($p<0.001$ and $p<0.05$). There was also a significant increase in fetal weights in PC61 treated IL-10-/- mice compared to PC61 treated wild-type mice ($p<0.05$).

Table 5.3: Pregnancy parameter from wild-type and IL10-/- deficient mice treated with PBS or PC61 mAb.

	Wild-type		IL-10-/-	
	PBS	PC61	PBS	PC61
	Mean±SEM	Mean±SEM	Mean±SEM	Mean±SEM
Females pregnant/mated	6/7	7/7	6/6	5/6
Implantation sites	6.7±1.2	8.5±0.4	5.6±0.2	6.5±0.9
Viable fetuses (%)	97.6±2.4	91.6±2.7	93.4±4.1	92.7±4.3
Fetal weight (mg)	867.2±16	908.3±11	967.2±23***	979.5±21*
Placental weight (mg)	99.5±2	102.2±2	108.8±15*	103.6±11
Fetal : Placental ration	8.79±0.2	8.98±0.2	8.94±0.2	9.50±0.2

*p<0.05, ***p<0.001 compared to wild-type mice of the same treatment group. There was no effect of PC61 treatment on any parameter in either wild-type or IL-10-/- mice compared to PBS. Data were evaluated using a one-way ANOVA and a Tukey post test.

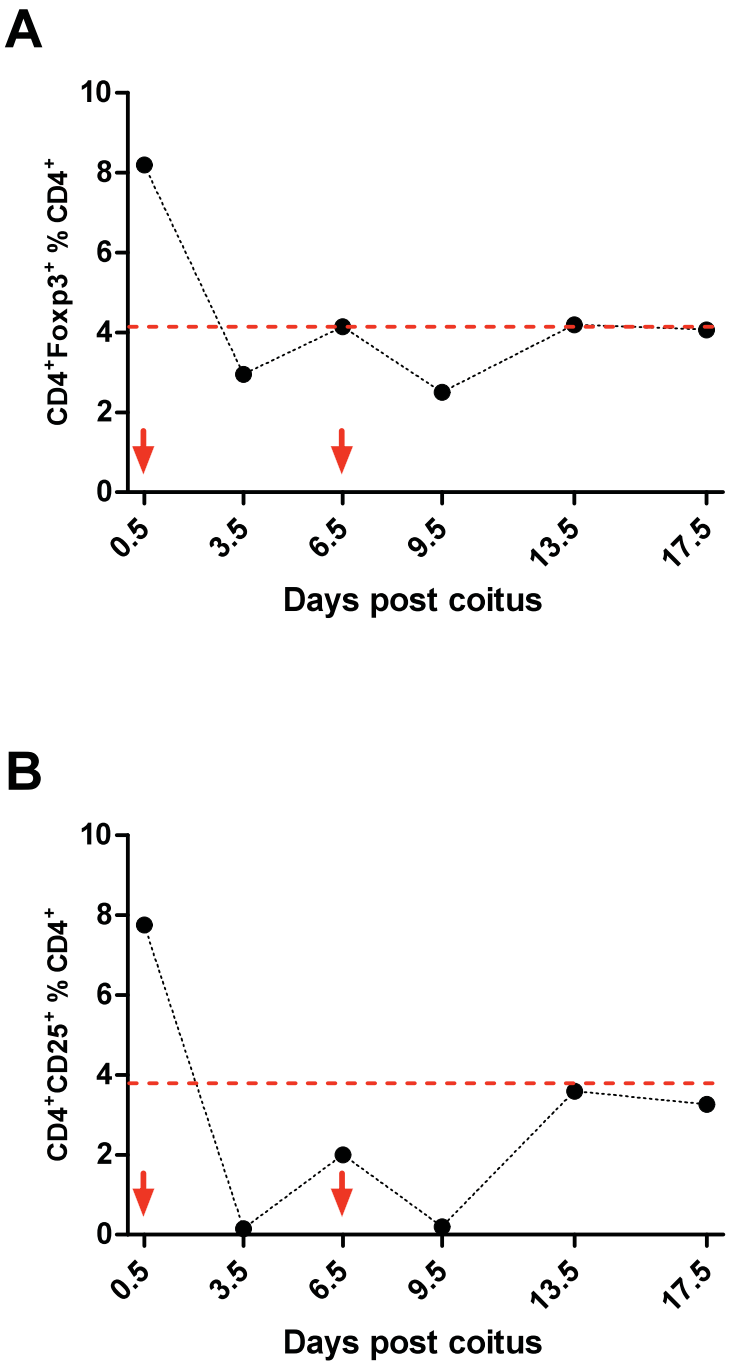


Figure 5.9: PC61 depletion of Treg cells *in vivo*. B6 mice expressing a mutant Foxp3-GFP knock-in allele were administered 2 x 200 µg doses of PC61 antibody (red arrows) and the levels of Treg cells in the blood were analysed over time. (A) The percent of $CD4^+$ cells expressing Foxp3 in blood over time. (B) The percent of $CD4^+$ cells with detectable CD25 expression in blood over time. All data are expressed as means (n=4) with 50% of original levels indicated with red dashed line.

5.7 DISCUSSION

The experiments described in this chapter show significant changes in the composition of the lymphocyte pool in the secondary lymphoid tissues of IL-10-/- mice compared to wild-type mice. Specifically in relation to Treg cells there were increases in both the proportion of $CD4^+$ cells expressing Foxp3, and in the total abundance of $CD4^+Foxp3^+$ cells in IL-10-/- mice compared to wild-type mice. In IL-10-/- mice there was a similar proportion of Treg cells at both the beginning and end of gestation, with a transient elevation in the proportion of Foxp3 expressing $CD4^+$ cells in mid-pregnancy (gd9.5). Analysis of the total abundance of Treg cells showed a similar shift in IL-10-/- mice compared to wild-type mice. Again at gd9.5 there was a significant and transient elevation in Treg cell numbers, however in this case it was only evident in the uterine-draining iliac LNs. Further analysis of the proportion of lymphocyte types represented in each of the LNs showed that IL-10 deficiency significantly altered the balance of lymphocyte subsets and this was more dramatically altered in the iliac LNs than the inguinal LNs. Despite the alteration in Treg cell numbers caused by IL-10 deficiency, no additional reliance on these cells in pregnancy could be demonstrated in IL-10-/- mice, since partial depletion did not compromise pregnancy outcomes in either wild-type or IL-10-/- mice.

In this study the kinetics of expansion of the Treg cell populations in B6 females mated to BALB/c males was assessed (Figure 5.1). The degree of increase seen in these experiments (51% and 30% over estrus levels in the iliac and inguinal LNs respectively) was less than that reported by others (Aluvihare*, et al.* 2004, Thuere*, et al.* 2007, Zhao*, et al.* 2007). The difference in proportional increase between this and previous studies was mainly attributable to a greater proportion of Treg cells measured in virgin animals (11.46% in iliac LNs) compared to other studies (approximately 8% Zhao *et al*. 2007 and 4% Aluvihare *et al*. 2004). Such differences are likely to result from the use in this study of virgin animals only in the estrus stage of their estrous cycle, compared to the use of virgin animals in random stages of the cycle in other studies (Aluvihare*, et al.* 2004, Zhao*, et al.* 2007). It has been demonstrated that the number of Treg cells significantly varies depending on the stage of the estrus cycle in both uterine-draining LNs (Robertson*, et al.* 2009) as well as in the uterus (Kallikourdis*, et al.* 2007) with highest numbers at estrus

and lowest at diestrus. This combined with the use differing identification protocols and FACS categorisation strategies (see section 3.2) may explain the elevated numbers in virgin mice reported herein.

An additional reason for the difference between the results in this study and those of previous studies may be the B6 x BALB/c mating combination used. Previous studies have used a variety of mating combinations with variable haplotypes, including B6 (females, $H2^b$ haplotype) x CBA (males, $H2^k$), CBA x DBA ($H2^d$), CBA x BALB/c ($H2^d$) and BALB/c x B6 (Aluvihare*, et al.* 2004, Thuere*, et al.* 2007, Zhao*, et al.* 2007). The influence of different mating combinations in the coordination of Treg cells in pregnancy was demonstrated by mating CBA females to males with the same haplotype, but who were of different strains. This resulted in different Treg cell responses in the pregnant females (Thuere*, et al.* 2007), suggesting that genes additional to major MHC antigens can influence Treg cell populations, and emphasises the importance of mating combinations when analysing Treg cell responses over pregnancy in mice.

When the same kinetic study performed in wild-type mice was performed using mothers with a null mutation in the IL-10 gene, significant differences were seen in the pattern of regulation of Treg cells as well as the magnitude of regulation. This was true for both the proportion of $CD4^+$ cells expressing Foxp3 in the iliac and inguinal LNs (Figure 5.1), and for the total number of cells within the iliac LNs, but not in the inguinal LNs (Figure 5.2A,B). Most strikingly at gd9.5 there was an increase in the proportion of $CD4^+$ cells expressing Foxp3 in the iliac and inguinal LNs, and in the iliac LNs an increase in the total number of $CD4^+Foxp3^+$ cells of over 270% with respect to gd6.5. Therefore there is substantially greater activation and/or proliferation of lymphocytes in the absence of IL-10, and this change appears to have a selectively greater impact on Treg cell than other lymphocytes.

Macroscopic analysis of the iliac LNs of IL-10-/- mice at gd9.5 showed significant lymphadenopathy (Figure 5.3) indicating extensive cellular hyperplasia. No such phenomenon was seen in the inguinal LNs indicating that the noted lymphadenopathy was related to local (most likely uterine-associated) factors. Localised increases in the size and cellularity of LNs draining the uterus during mouse pregnancy are well documented, and have been largely related to the presence of fetal alloantigens (Ansell,

et al. 1978, Maroni*, et al.* 1973). With this in mind, the role of fetal alloantigen in the noted changes in the proportion and total number of Treg cells and other cellular subsets at gd9.5 was investigated by mating IL-10-/- B6 female mice with B6 male mice.

The role of fetal MHC alloantigen is evidently variable, depending on the parameter and lymph nodes being assessed. In IL-10-/- mice, fetal MHC alloantigen appears to be important in regard to the proportion of $CD4^+$ cells expressing Foxp3, but appears to be less important in regard to the changes seen in the total cellularity of the iliac LNs, with the response in IL-10-/- mice being equivalent, irrespective of the MHC antigen disparity. Instead the change in cellularity appeared to be more dependent on the IL-10-/- genotype. Interestingly the IL-10-/- genotype resulted in an increase in the relative proportion of $CD4^-CD8^-$ cells compared to wild-type animals in both lymph nodes analysed, with these cells likely to be B-cell or NK cells. However a greater increase in the $CD4^-CD8^-$ cellular subset was seen in the iliac LNs as opposed to the inguinal LNs and indicates that it is highly likely that local factors, possibly related to the developing conceptus, are interacting with the immune system to generate this response.

The above-presented results pose several questions; firstly what are the factors driving the lymphoproliferative response seen at gd9.5 in IL-10-/- mice and secondly, what are the implications of the perturbations seen in Treg and lymphocyte abundance in the iliac LNs?

Fetal MHC alloantigen appears to not be critical in regard to the changes seen in the cellularity of the iliac LNs. However, this does not preclude a role for minor histocompatibility antigens, such as tissue specific antigen deriving from the placenta or fetus, or H-Y antigens, as these are still present in syngeneic pregnancies. An IL-10 deficient environment may lead to altered presentation of these minor histocompatibility antigens that may produce the noted lymph node hyperplasia. IL-10 can inhibit maturation of DCs and as such decrease their ability to process and present antigen and secrete proinflammatory cytokines (Buelens*, et al.* 1995, Moore*, et al.* 2001). Therefore a deficiency of IL-10 allows for greater DC maturation, increased antigen presentation, and an enhanced inflammatory environment with increased IL-12 (Segal*, et al.* 1998). This change in DC function could result in altered processing and presentation of minor histocompatibility antigens to induce lymphocyte proliferation.

The timing of such a response fits with the kinetics demonstrated for maternal exposure to model minor histocompatibility conceptus antigens (Erlebacher*, et al.* 2007, Moldenhauer*, et al.* 2009).

Alternatively, expansion with in the cellular subsets, including Treg cells, may be in an antigen-nonspecific manner that may be related to an altered cytokine environment present during pregnancy in combination with the absence of IL-10. Such an environment may induce non-specific proliferation of cellular subsets. However, the factors and alterations in the complex cytokine networks as a result of a deficiency in IL-10 in combination with pregnancy induced factors that would provide a stimulating factor across a diverse array of cell populations would be difficult to attribute to one specific cytokine and would likely be a result of a combination of cytokines and additional factors.

The explicit localisation of the lymphadenopathy to the iliac LNs would suggest factors related to the local immune environment. These would be additional to minor histocompatibility antigens that are not localised to the uterine draining LNs, but can be found systemically throughout the mother (Erlebacher*, et al.* 2007, Moldenhauer*, et al.* 2009). The noted lymphadenopathy coincides with the peak of cells expressing the DC marker CD11c+ in the uterus, and is just preceded by a period when there is normally a significant drop in the maturation state of uterine DCs and a dominance of IL-10 production over IL-12 production within these cells (Blois*, et al.* 2004). In an IL-10 deficient environment the convergence of these factors may result in significantly enhanced antigen presentation within the uterine-draining lymph nodes.

While there are some obvious factors that may explain the timing of the induction of cellular expansion in the iliac LNs in IL-10-/- mice, it is more difficult to explain its short-lived, transient nature. One possible explanation is that the antigenic factors or cytokine environment that induces this response is only present over a short period of time in mid gestation. However for the resolution of this degree of lymphadenopathy a large number of cells would have to undergo apoptosis, which seems unlikely. A related hypothesis is that the large degree of expansion in the Treg cell pool is sufficient to induce suppression of the ongoing immune response and ultimately forges a dominant-suppressive environment and resolution of the lymphadenopathy. Alternatively,

following the extensive cellular expansion there is likely to be migration of the cells into the peripheral blood and recruitment into the tissues, which would account for the rapid diminution in cellularity seen between gd9.5 and gd13.5. It is also of note that the timing of these changes coincides closely with the establishment of maternal blood flow into the fetal labyrinth compartment of the placenta. Maternal blood flow into the fetal labyrinth has shown to be temporally located between gd9.5 and gd10.5 (Muntener*, et al.* 1977), whilst the observed lymphadenopathy is prior to this (strictly at gd9.5), the transient nature and resolution of this phenomena may be related to the changes in maternal blood flow in the labyrinth.

Secondary lymphoid organs, and specifically site draining lymph nodes, have been proposed to be key locations for the establishment of maternal tolerance toward the fetus (Taglauer*, et al.* 2009). As such the perturbations described in this chapter should logically impact on the degree and integrity of this maternal-fetal tolerance. However previous studies (White*, et al.* 2004) and the work presented above (Table 5.3) demonstrates that this is in fact not the case, with fetuses from IL-10-/- animals having higher birth weights than those from wild-type animals. The extensive and dramatic increase seen in the number of Treg cells (predominantly in the iliac LNs) may provide some possible insight into this phenomenon. Speculatively it may be that the absence of IL-10 would necessitate an enhancement in additional suppressive mechanism such as Treg cells. As such, the elevation seen in the Treg cell compartment during pregnancy in IL-10-/- mice may be a response to maintain the required level of immune tolerance toward the fetus. It should then stand to reason that the reliance on Treg cells for the maintenance of immune tolerance of the fetus would be elevated, and that any depletion or loss of function of these cells should lead to enhanced fetal loss compared to the wild-type situation.

The importance of Treg cells in the maintenance of pregnancy in IL-10-/- mice was evaluated by treating both wild-type and IL-10-/- pregnant female mice with the anti-CD25 antibody PC61. In this study PC61 treatment had no significant effect on pregnancy outcome compared to PBS treatment on either wild-type or IL-10-/- mice (Table 5.3). This was surprising considering the fetal loss reported previously with PC61 treatment at a dose of 1 x 100 µg (Darrasse-Jeze*, et al.* 2006), which is lower than the dose used in the current study. That PC61-mediated Treg cell depletion at such a low

level can manifest in pregnancy loss is surprising considering the extensive documentation that PC61 is in fact poor at depleting $Foxp3^+$ cells. Characterisation of the effect of PC61 treatment on Treg cell populations *in vivo* has shown that treatment fails to eliminate a significant proportion of $Foxp3^+$ cells (Couper*, et al.* 2007). Treatment of mice with PC61 doses as high as 1 mg every second day for a fortnight was insufficient to induce organ-specific autoimmune disease that is characteristic of Treg cell deficiency (McHugh*, et al.* 2002).

In the current experiment we chose a level of treatment (2 x 200 μg doses) that was estimated to be on the threshold for pregnancy loss in wild-type animals. If Treg cells are more important in the absence of IL-10, it was hypothesised that dosing at this level may have resulted in fetal loss in IL-10-/- mice. Unfortunately the dosing protocol used failed to induce fetal loss in either genotype. To properly explore the importance of Treg cells in

IL-10-/- pregnant mice compared to wild-type pregnant mice, depletion of Treg cells to a level where some fetal loss was seen in wild-type mice would allow for more sensitive interrogation of the function of Treg cells in the absence of IL-10. One possible solution to this would be to increase the dose of PC61 given by approximately 2.5-fold. Doses of 2 x 500 μg given during the pre- or peri-implantation period have recently been used to induce fetal loss in wild-type mice (Saito, S; personal communication). Unfortunately attempts using higher doses of PC61 could not be achieved during the term of this thesis, due to a lack of availability of sufficient quantities of PC61 antibody.

The results presented in this chapter show a significant perturbation in the immune response to pregnancy in the absence of IL-10. This is manifested in a significant degree of lymphadenopathy in the uterine-draining iliac LNs which appears to be independent of fetal MHC antigens, and is specific for mid gestation (gd9.5). Interestingly, there is a significant increase in the proportion of $CD4^+$ cells expressing Foxp3 in both local and systemic LNs in allogeneic pregnancies in IL-10-/- mice, resulting in a large (approximately 11-fold) increase in the total number of $CD4^+Foxp3^+$ cells, which is confined to the Iliac LNs. The functional significance of these changes and mechanism by which they take place require further investigation before conclusions on their significance can be made.

CHAPTER 6

GENERAL DISCUSSION

The evolutionary drive for genetic diversity in eutherian animals has prompted the generation of complex mechanisms of immunological tolerance to negate adverse immune responses against the semi-allogeneic fetus. The importance of the reproductive process has ensured that mechanisms that promote maternal immune tolerance of the conceptus are robust and display many levels of redundancy. Despite this it is evident that failure to achieve an appropriate level of tolerance can manifest as pathologies of pregnancy.

The events and temporal sequence that ultimately result in the formation of this tolerant state are still ill-defined. However, there is a growing body of evidence that supports the hypothesis that the act of mating itself has a role in the induction of maternal-fetal immune tolerance (Robertson*, et al.* 2001). The introduction of the ejaculate into the female reproductive tract has a range of effects on the local leukocyte population, with these mainly being attributable to the acellular seminal plasma component of the ejaculate (Robertson 2007). Factors within the seminal plasma coordinate changes that induce a state of functional tolerance toward antigens within the ejaculate (Robertson*, et al.* 2009). Despite these observations the exact mechanisms that promote paternal-specific immune tolerance following insemination are ambiguous.

One mechanism by which maternal-fetal tolerance is enacted is via the suppressive lymphocyte subset termed $CD4^{+}CD25^{+}$ regulatory T cells. It has been shown that Treg cells are indispensible for the maintenance of allogeneic pregnancies in mice (Aluvihare*, et al.* 2004). In humans it has been shown that the proportion of the T-helper cell population that express a Treg cell phenotype is increased during gestation (Heikkinen*, et al.* 2004, Saito*, et al.* 2005), and that women who suffer primary unexplained miscarriage have diminished levels of the Treg cell marker Foxp3 in their endometrial tissue. With Treg cells implicated as being critical in pregnancy success and the noted ability of semen in generating paternal-specific tolerance, the factors that influence the level and function of Treg cells during pregnancy and in the peri-implantation period warrants further investigation. To date few studies have addressed the role of Treg cells in the pre and peri-implantation period.

The studies in this thesis provide evidence for the ability of the ejaculate to regulate Treg cell levels in the female reproductive tract prior to embryo implantation. This provides

insight into a possible mechanism by which the ejaculate promotes maternal-fetal tolerance. Additionally this thesis shows that in the absence of IL-10 there are dramatic alterations in the kinetics and magnitude of changes in the Treg cell population in mid-gestation.

The studies presented in this thesis are some of the first to analyse Treg cells in pregnancy using the hallmark Treg cell marker Foxp3. Previous studies have relied on categorising Treg cells based on their co-expression of CD4 and CD25 (Aluvihare*, et al.* 2006, Robertson*, et al.* 2009, Zenclussen 2005, Zhao*, et al.* 2007). Categorisation using this combination of markers presents a limitation when interpreting data, as CD25 is shown to be an activation marker for all $CD4^+$ cells. As such the $CD25^+$ population represent a heterogeneous population of cells composed of both activated T-cells (Caruso*, et al.* 1997) and Treg cells (Sakaguchi*, et al.* 1995). Also, it has been demonstrated that there is a population of $CD25^-$ cells that express Foxp3 that display suppressive function (Nishioka*, et al.* 2006). The categorisation of T-cells as $CD4^+Foxp3^+$ has been shown to be specific for Treg cells in mice (Fontenot*, et al.* 2003). The use of Foxp3 to define Treg cells in this study therefore allows for more accurate classification than has been used in the majority of previous studies analysing Treg cells in pregnancy. However for the purposes of comparison with previous studies, the experiments herein also measure $CD4^+CD25^+$ cells.

In chapter 3 the role of the ejaculate in influencing Treg cell numbers in the uterus, and secondary lymphoid organs in the peri-implantation period was investigated. Surgery was performed on stud males who underwent vasectomy, or seminal vesiculectomy, or both and as such had only the seminal plasma component, or the sperm component of the ejaculate or neither. Consequentially females were exposed to differing components of the ejaculate at mating. The influence of this on ensuing Treg cell numbers in uterine tissue and secondary lymphoid organs was assessed in comparison to estrous females and those mated with intact males.

In contrast to other studies (Aluvihare*, et al.* 2004, Robertson*, et al.* 2009) no changes were found in the proportion of $CD4^+$ cells that expressed Foxp3 in the uterine-draining iliac LNs or the distal inguinal LNs at day 3.5pc. However, consistent with previous studies (Robertson*, et al.* 2009) it was demonstrated that there was a greater than 2-fold

increase in the total number of Treg cells in iliac LNs. This was attributable to the seminal plasma component of the ejaculate as it was not seen in SV- or SV-/VAS- mated mice, and was not evident in the inguinal LNs. Treg cell numbers in the uterus were also quantified. Like in LNs, exposure of the female reproductive tract to seminal plasma resulted in an elevation in the number of $Foxp3^+$ cells and the level of *Foxp3* mRNA in uterine tissue.

In order to elucidate a possible mechanism by which Treg cells could be recruited into the reproductive tract in response to seminal plasma exposure, regulators of leukocyte migration were assessed in chapter 4. The level of mRNAs of migratory regulators, including chemokines, chemokine receptors and the integrin *Cd103* was assessed in uterine tissue exposed to varying components of the ejaculate. A similarity between the regulation of *Foxp3* mRNA and that of *Ccr5* mRNA was observed. This suggests a possible role for CCR5 in Treg cell recruitment in the peri-implantation period, consistent with previous published reports that propose CCR5 expression on Treg cells as being critical for their recruitment into the uterus both throughout the estrous cycle and in the pregnant uterus (Kallikourdis*, et al.* 2007). However *Ccr5* mRNA expression did not correlate with *Foxp3* mRNA expression. Three other migratory regulators studied (*Ccl19*, *Ccr4* and *Cd103*) did show significant correlations with *Foxp3*. All these three showed tendencies toward elevated expression following mating, but only *Ccl19* showed a significant elevation in at least one of the mated groups, with its abundance being significantly higher following mating to intact males. Whilst the results suggested that none of the migratory regulators studied were solely responsible for the accumulation of Treg cells into the peri-implantation uterus, it is likely that their regulation works in concert with additional factors to culminate in elevated Treg cells. The increased numbers of Treg cells as a result of proliferation in the iliac LNs and subsequent efflux into the peripheral blood would mean that there is increased availability of Treg cells to be recruited into the uterus. This action may be supplemented by expansion of the local Treg cell population in the uterus due to proliferation. These three mechanisms in combination may result in the noted increase in $Foxp3^+$ cell density (see Figure 6.1).

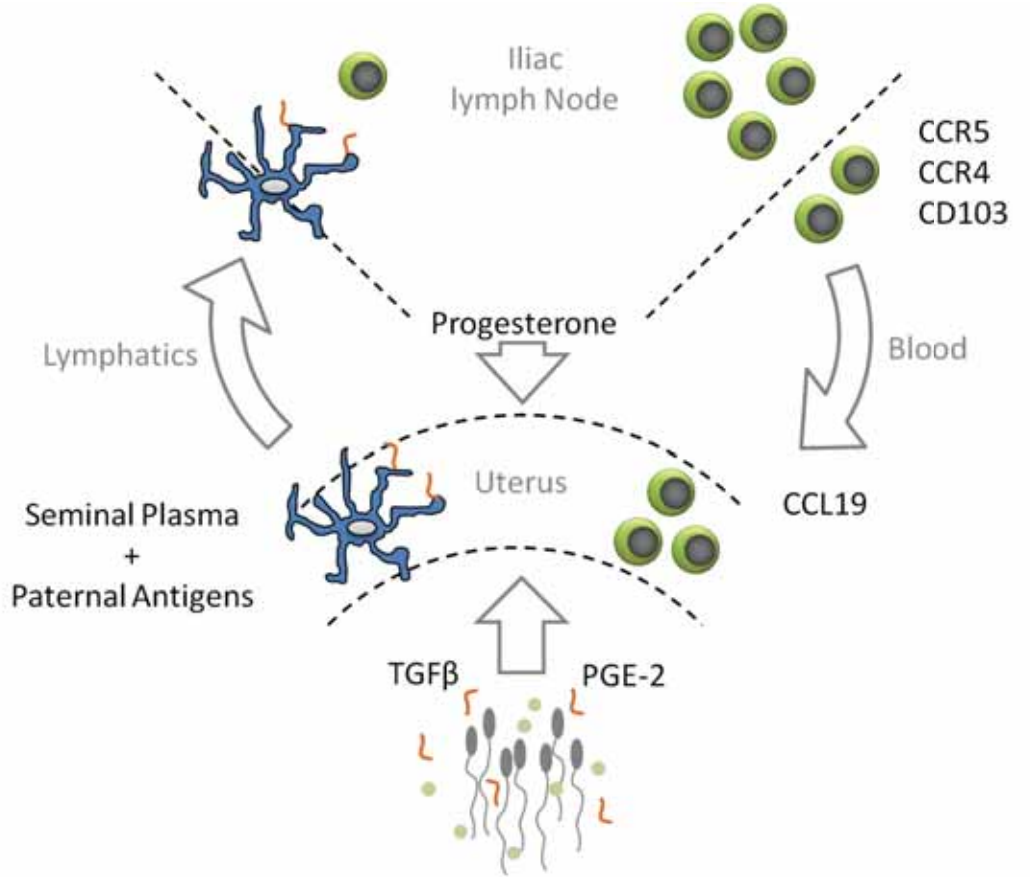


Figure 6.1: Seminal plasma-derived factors induce the proliferation of Treg cells in the iliac LNs which can subsequently migrate via the blood into the peri-implantation uterus to promote tolerance of the implanting embryo. The immune-active moieties in the seminal plasma component of the ejaculate along with paternal antigens can drive the proliferation of Treg cells in the uterine-draining iliac LN. These then efflux into the peripheral blood from where they can be recruited into the uterus under chemokine regulation and may subsequently proliferate further.

In chapter 5 we examined at the kinetics of Treg cell expansion over the length of gestation. It was shown that the proportion of $CD4^+$ cells expressing Foxp3 significantly increase from estrus to late pregnancy (gd17.5), and that this expansion was systemic, being seen in both the iliac LNs and the inguinal LNs. Both of the sites showed consistent patterns of Treg expansion. These findings are supportive of previous findings that demonstrated an elevated proportion of $CD4^+$ cells with a Treg phenotype over the course of murine pregnancy (Zhao*, et al.* 2007). Interestingly while the proportion of $CD4^+$ cells expressing Foxp3 increased in both sites analysed, the total number of Treg cells were not significantly altered during pregnancy. This was with the exception of day 3.5pc which, as previously discussed, displayed a greater than 2-fold increase in total Treg cell numbers in the iliac LNs only. It needs to be taken into account however that the number of Treg cells in a lymph node at any one time is a reflection not only of *de*

novo generation of cells, but also efflux of cells into the blood from where they would be available for migration into the pregnant uterus.

The influence of IL-10 on Treg cell numbers was investigated in chapter 5. The kinetics of Treg cell expansion was assessed in IL-10-/- females. In the absence of IL-10 there were significant alterations in the pattern of regulation of Treg cells in regard to the proportion of $CD4^+$ cells expressing Foxp3, with the most notable time point being gd9.5. In both the iliac and inguinal LNs there was a significant and transient elevation in the proportion of $CD4^+$ cells expressing Foxp3. Experiments using syngeneic mating partners demonstrated that this elevation was due to the presence of fetal alloantigens. Additional to this was a greater than 11-fold elevation in the total number of Treg cells in the iliac LNs at gd9.5. This was accompanied by a significant degree of lymphadenopathy. Interestingly this was seen only in the iliac LNs, and was shown to be independent of alloantigens.

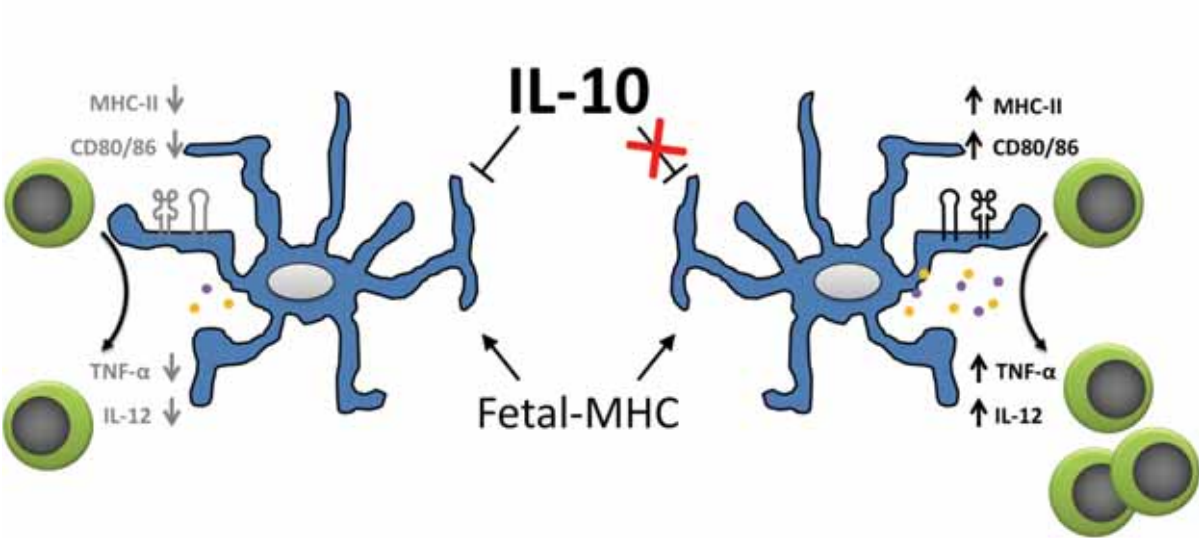


Figure 6.2: In the absence of IL-10, fetal MHC antigens drive an increase of the proportion of $CD4^+$ cells expressing Foxp3 at gd9.5. In the absence of IL-10 DCs may develop a phenotype characterised by elevated MHC class II expression and an increase in co-stimulation (CD80/86). This combined with an increase in the production of pro-inflammatory cytokines may allow for increased presentation of fetal-derived MHC antigens and may drive an elevation in the proportion of Foxp3 expressing cells amongst the $CD4^+$ population.

In the absence of IL-10 we documented a significant change in the relative phenotypes of lymphocyte cells within the LNs. This was altered more substantially in the iliac LNs than in the iliac LNs, presumably as a result of pregnancy and/or conceptus-associated factors that might include minor-histocompatibility antigens draining to this site. This in combination with an expected alteration DC phenotype, as a result of the absence of

IL-10, may allow for enhanced presentation of the fetal minor-histocompatibility antigens and result in the extensive iliac LN hyperplasia (see Figure 6.3). However, despite the expansion of lymph cell populations in the absence of IL-10, in the absence of fetal-MHC alloantigens there was no selective expansion in Treg cells. In spite of these changes in the lymphocyte populations there was no compromise in pregnancy outcomes in the absence of IL-10, which is consistent with previous studies in IL-10-/- mice (White*, et al.* 2004).

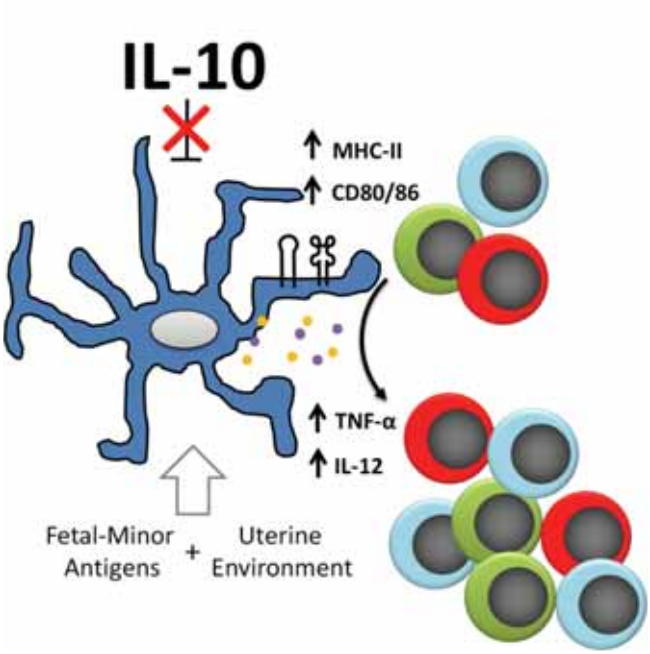


Figure 6.3: IL-10 deficiency alters DC phenotype and induces lymph-cell expansion. In the absence of IL-10 DCs may develop a more phenotype characterised by elevated MHC class II expression and an increase in co-stimulation (CD80/86). This combined with an increase in the production of pro-inflammatory cytokines and an altered uterine cytokine environment may allow for altered presentation of fetal-derived minor-histocompatibility antigens such that lymph cell proliferation is enhanced.

Depletion of Treg cells using the antibody PC61 in IL-10-/- mice demonstrated that in the absence of IL-10, mice were no more prone to fetal loss induced by moderate Treg cell depletion. Interestingly this study revealed that 50% Treg cell depletion was insufficient to induce overt pregnancy loss or fetal growth restriction in allogeneic-mated wild-type B6 mice. This finding was in opposition to previously reported studies that demonstrated fetal loss with doses of PC61 antibody lower than those used in this study (Darrasse-Jeze*, et al.* 2006). However it is consistent with reports that suggest that higher doses of PC61 are needed before fetal loss can be induced (Saito, S; personal communications).

Experiments within this thesis explore the kinetics and distribution of Treg cells during pregnancy, with a specific focus on the period following mating and just prior to embryo implantation. Evidence suggests that there is a requirement for Treg cells prior to embryo implantation (Zenclussen 2005). However to date no extensive studies have been published on the temporal importance of Treg cells in pregnancy. The paucity of such studies is likely a result of the difficulties in tracking these low abundance cells, and in acutely depleting Treg cell populations. The advent of mice with a highly sensitive diphtheria toxin receptor conjugated to the Foxp3 promoter (Foxp3-DTR) (Kim*, et al.* 2007, Lahl*, et al.* 2007) that allows for selective depletion of Treg cells, will enable the thorough investigation of the degree to which Treg cells are important in pregnancy and at which stages of pregnancy they are important. We have made attempts to establish a colony of such mice in Adelaide but were unable to achieve pregnancies in founder mice due to male-related infertility. Currently attempts are underway to generate this mouse line from a heterozygous background. The establishment of this colony will allow for a greater ability to interrogate the importance of Treg cells in both wild-type and IL-10-/- pregnancies.

Additional to the importance of Treg cells in pregnancy, the pathways of generation that account for the well-documented increase in the proportion of $CD4^+$ cells expressing Foxp3 in pregnancy warrants investigation. Expansion in the proportion of $CD4^+Foxp3^+$ cells may originate from either the clonal expansion of pre-existing Treg cells or the *de novo* generation from $Foxp3^-$ cells. The use of T-cell receptor transgenic (TCR-Tg) mice on a recombination activating gene (RAG) deficient background will be informative in relation to the role that *de novo* generation may play in Treg cell expansion in pregnancy. Due to failure in T cell exposure to their cognate antigen within the thymus, TCR-Tg x RAG-2-/- mice lack Treg cells (Itoh*, et al.* 1999, Thorstenson*, et al.* 2001). As a result any $Foxp3^+$ cells can only be generated by the *de novo* pathway. The combination of this model with those used for studying the maternal immune response to model paternal-antigens (Erlebacher*, et al.* 2007, Moldenhauer*, et al.* 2009) could be used to study the origins of Treg cells in pregnancy. Such a model would take the form of adoptively transferring ovalbumin (OVA) reactive cells from RAG-2 deficient mice, such as those from DO11.10 x RAG-2-/- mice (Thorstenson*, et al.* 2001), into mothers gestating fetuses expressing OVA. If Treg cells were generated *de novo* during gestation then this would

be evidenced by the presence of Foxp3+ cells in the transferred population following exposure to fetal antigens. Similar experiments could be done in wild-type TCR-Tg mice. However limited conclusions could be made due to the existence of secondary TCRs generated by the endogenous rearrangement of the TCR-α chain. This ultimately results in the presence of thymic generated $Foxp3^+$ cells. As such, any Treg cell expansion seen in response to pregnancy could not be solely attributed to either clonal expansion or *de novo* pathways of generation.

The models outlined above could also be used to answer another pertinent question regarding Treg cell generation in pregnancy. That is the question of the involvement of fetal antigens in driving Treg generation/expansion. An initial study showed equivalent expansion of Treg cells during pregnancy irrespective of the alloantigen status of the gestation fetus (Aluvihare*, et al.* 2004). However these results have come into question after a more recent study showed that in the presence of fetal alloantigen there was enhanced expansion of Treg cells (Zhao*, et al.* 2007). By utilising the specific nature of TCR-Tg T cells responsive to specific fetal antigens it can be investigated whether fetal MHC-antigens, minor trophoblast antigens, or the hormonal changes in combination with pregnancy or pregnancy alone are sufficient to generate or expand Treg cell populations.

In addition to analysing the levels of Treg cells (proportion of $CD4^+$ cells and total numbers), the specific function of the Treg cell population needs to be analysed in pregnancy. The function of Treg cells has previously been suggested to be antigen non-specific once the cells have been activated via TCR engagement (Karim*, et al.* 2004, Thornton*, et al.* 2000). It would therefore stand to reason that even in the absence of Treg cell expansion, enhanced activation of the pre-existing Treg cell pool during pregnancy could lead to an elevated suppressive state. To this effect expansion of specific clones of Treg cells that are reactive to fetal alloantigens, which would also be activated as a result of TCR engagement, could produce potent suppression without displaying significant expansion relative to the total pool of Treg cells. As such it may be fallible to analyse Treg cells by looking at the Treg cell population as a whole. Instead analysis of the expansion of specific Treg cell clones may prove more accurate. Such experiments may be performed by adoptive transfer of labelled TCR-Tg Treg cells or by

the use of TCR specific tetramers that would allow labelling of cells reactive to model fetal antigens.

In light of recent finding that show the "Jekyll and Hyde" nature of Treg cell precursors, which can form suppressive Treg cells or pro-inflammatory Th17 cells depending on the cytokine environment (Bettelli*, et al.* 2006), quantification of Treg cell function and abundance may alone not suffice. Instead the ratio of Treg cells to Th17 cells may be more indicative of the overall balance of "tolerance". The regulation of Th17 cells throughout pregnancy and their involvement in pathologies of pregnancy remains to be reported on, however emerging evidence suggests a role for Th17 cells in pre-eclampsia, whilst in normal pregnancy their systemic levels appear not to be altered (Nakashima*, et al.* 2009, Santner-Nanan*, et al.* 2008). Future studies may look at not only the levels of Treg cells but analyse this with respect to the level of Th17 cells.

With the spatial distribution of Treg cells being implicit in their function, the factors that account for their accumulation within the uterus are important to understand in assessing their role in pregnancy. On this premise we assessed the level of mRNA for a range of migratory regulators in the peri-implantation uterus. Day 3.5pc was specifically chosen as it just precedes the implantation of the embryo and as such, if a tolerogenic micro-environment within the uterus was required, then it should be established by this time point. This assumption however fails to account for transient up-regulation of chemokines that result in the recruitment of Treg cells prior to day 3.5pc but are then reduced back to baseline levels by the time of embryo implantation. The result of this coordinated response could be the noted elevation of Treg cell density in the peri-implantation uterus. More detailed analysis of chemokine levels in the days following mating but prior to implantation could provide more insight into which chemokines may be involved in recruitment of Treg cells into the uterus. This would however be complicated by the large influx of leukocytes into the uterus following mating, which would make it difficult to attribute Treg cell recruitment to the involvement of any one dynamically-regulated chemokine. The use of chemokine receptor null mutant mice, such as those deficient in CCR4 or CD103 would be a valuable tool for addressing this. In particular the identification of the role of CCL19 in Treg cell migration in the peri-implantation period would warrant the use of a CCL19 null mutant mouse to address the relative contribution of this cytokine.

With the establishment of pregnancy following implantation the Treg cell population undergoes dynamic changes as shown in chapter 5 and previous studies (Aluvihare*, et al.* 2004, Thuere*, et al.* 2007, Zhao*, et al.* 2007). The influencing factors that shape these changes are poorly understood and are likely due to a multitude of factors including fetal antigens, hormonal changes and perturbations in the cytokine environment during pregnancy. It is well documented that during pregnancy the balance of cytokines are biased towards a predominance of type 2 cytokines over type 1 cytokines (Wilczynski 2005). In chapter 5 we studied the consequence that deficiency in the type 2 cytokine IL-10 had on Treg cell populations during pregnancy. We noted a significant perturbation in Treg cell numbers on gd9.5. The factors that lead to, and the consequences of this remain unclear.

Analysis of DCs during pregnancy in an IL-10 deficient environment might provide insights into the influence of IL-10 on their maturation status and their ability to influence lymphocytic cell populations. Changes in DC maturation, which leads to alterations in MHC II expression and costimulatory molecules would be expected to have significant effects on lymphocyte activation. The consequence of this may be the documented lymphadenopathy and the significant elevation seen in Treg cells and other lymphocyte populations. Particular focus would need to be placed on the microenvironment of the uterus and the draining iliac LNs. Of interest may be the uterine and systemic levels of IL-12 which has been shown to be negatively regulated by IL-10 (Segal*, et al.* 1998), and can induce DC maturation (Rutella*, et al.* 2006). Any knowledge regarding the cytokine network that influence Treg cells will be instrumental in understanding the complex mechanisms that ultimately dictate Treg numbers in pregnancy.

It is likely that the absence of IL-10 compromises the function of other suppressive cellular subsets such as Tr1 cells or Th3 cells, which whilst they haven't been directly implicated in maternal/fetal immune tolerance are likely to be important. However, despite such significant alterations in the immune system during pregnancy, reproductive outcomes remain uncompromised. This is testament to the robust nature of the immune tolerance in pregnancy. The resultant elevation in Treg cells in the absence of IL-10 may be a mechanism by which this robustness is maintained. In other words, the elevation in Treg cells may act to maintain an immunologically tolerant state

that might not otherwise occur in the absence of IL-10. It may be that in an IL-10 deficient environment Treg cells become more important in the maintenance of pregnancy. We were unable to provide evidence to support such a theory, however this is most likely due to insufficiency in our approach with a failure to more completely deplete Treg cells. The advent of the aforementioned Foxp3-DTR transgenic mice may provide an additional tool to address this question. Backcrossing these animals with IL-10-/- mice will allow for greater control over the depletion of $Foxp3^+$ cells in IL-10 deficient animals compared to the use of PC61 utilised in this thesis. This will then provide data to demonstrate if indeed IL-10-/- animals are more sensitive to Treg cell loss than wild-type mice, and hence are more dependent on Treg cells for maintenance of immune tolerance. Alternatively it is possible that IL-10 deficiency does not compromise maternal-fetal immune-tolerance. To this end, the noted elevation of Treg cells in IL-10-/- animals might not have any compensatory function but might simply manifest as enhanced tolerance of the fetus.

The results presented in this thesis increase our understanding of the factors involved in coordination of Treg cell responses during murine pregnancy. The role of Treg cells in the control of the immune system implicate them in an ever-increasing range of immune pathologies including allergies, autoimmune disease, cancer and pregnancy pathologies. Any fundamental knowledge pertaining to factors that result in the alteration of Treg cell numbers or function aids in our understanding of the origins and the factors that influence this important T cell population. We have used murine models to help elucidate the spatial and temporal sequences that govern Treg cells during gestation, with a specific focus on pre-implantation events. We have also looked at how alterations in the systemic cytokine environment, specifically a deficiency in IL-10, can alter the Treg response during gestation. Whilst we remain some distance from decisively identifying the complex networks that influence Treg cell numbers, functions and migration, the data presented in this thesis are a step forward. An understanding of murine Treg cell biology will inform studies to investigate possible influential factors in human Treg cell biology. With the involvement of these cells in an ever-growing range of pathologies of pregnancies in women, their manipulation presents an exciting new therapeutic target.

CHAPTER 7

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