Progesterone control of regulatory T cell abundance, phenotype and stability for pregnancy success

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A thesis submitted to the University of Adelaide in fulfilment of the requirements for admission to the degree Doctor of Philosophy

January 2020

“Nothing great is ever achieved without much enduring.”

St. Catherine of Siena

Table of contents

Abstract

Early pregnancy is characterised by adaptations in the maternal immune system to allow development of genetically disparate embryos. Maternal immune tolerance is mediated by a subset of anti-inflammatory T cells known as CD4+Foxp3+ regulatory T (Treg) cells. Absence or reduced function of Treg cells at embryo implantation causes infertility in mice and is implicated as a cause of reproductive disorders in women. The importance of adequate Treg cell responses during pregnancy is well recognised, however, the factors which control the strength and quality of this response are not well understood. One candidate regulator of Treg cells is the hormone progesterone (P4). We aimed to investigate how early pregnancy P4 exposure regulates Treg cell abundance, phenotype and stability, and the significance of this for pregnancy success.

The impact of a peri-implantation disruption in P4 bioavailability on the Treg cell response was investigated by administering the P4 antagonist RU486 to allogeneically mated females. At mid-gestation, a reduction in CD4+ T cells and Foxp3+ Treg cells was evident in the uterus-draining lymph nodes (udLNs) of females treated with low-dose RU486. These immune perturbations were evident at implantation, indicating a failure of normal Treg expansion following mating, suggesting that adequate P4 bioavailability from the outset of pregnancy is a requirement for robust Treg cell induction. Investigation of late-gestational outcomes revealed RU486-treated females had a reduction in pregnancy rate, and in females that retained their pregnancies, fetal growth restriction was evident. RU486-treated females additionally failed to show the normal P4 decline in late gestation, and had an extended gestation length compared to controls. As RU486 treatment perturbed the Treg cell response, we assessed the contribution of Treg cells to pregnancy success following P4 signalling disruption, by adoptively transferring Treg cells into RU486-treated females. Treg cell transfer, but not nonTreg cell transfer, improved both pregnancy rate and fetal weight in RU486 treated mice.

In vitro, P4 was found to suppress IFNγ expression in Treg and T effector cells cultured under inflammatory-polarising (Th1, Th17) and non-polarising (Th0) conditions, independent of the classical P4

receptor (PR). Using a membrane-impermeable form of P4 we demonstrated that T cells have the capacity to bind P4 at the membrane, and this capacity increases in early gestation in udLN Treg cells.

Finally, we investigated Treg cell stability during pregnancy using Treg cell fate-mapper Foxp3GFPCre.R26RFP mice. Preliminary results show that both Treg and exTreg cells increase in late-gestation. Whether exTreg cells are implicated in the inflammatory process of parturition and whether P4 bioavailability regulates Treg cell stability in pregnancy will be further investigated.

Overall, these studies demonstrate that P4 is a key regulator of Treg cells in early pregnancy and that Treg cells are an effector mechanism of P4 action for optimal fetal viability and growth. We show that P4 is a determinant of Treg cell abundance and phenotype and suggest this regulation occurs through both direct and indirect mechanisms of action. We propose that luteal-phase and early pregnancy P4 is essential to drive a robust Treg cell response necessary for pregnancy success.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution 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. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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I acknowledge the support I have received for my research through the provision of an Australian Government Research Training Program Scholarship.

Ella Green, BSc.

January, 2020

Acknowledgements

I would like to sincerely thank my supervisors Sarah Robertson, Lachlan Moldenhauer and Shaun McColl for their support during my PhD candidature. To Sarah, thank you for your mentorship, guidance and passion. Thank you for believing in me, allowing me to develop my own ideas and inspiring me to pursue research in the field of reproductive immunology. I truly value the countless thought-provoking discussions had on all things reproductive health and immune tolerance related. Lachie, thank you for being my first point of call for questions, advice and discussions, and for your excellent proofing skills.

I would like to thank past and present members of the Robertson laboratory - Lachie, John, Ali, Dexter, Kavita, David, Cam, Loretta, Tom, Holly, Ricky, Bridget, Jaz - who have helped me along the way with technical support and encouragement. To Dexter, Kavita and Holly, thank you for your wonderful company over the years. It's the end of an era and I have cherished your friendship, moral support and all the memorable moments we've shared. To John, thank you for your enthusiasm around the lab and thoughtful feedback. To Ali, thank you for inspiring me to take opportunities overseas and for your personal and professional insights.

Thank you to Becky and the Robker lab for the PRlacZ mice, and to Shaun and the McColl lab for all the reagents, technical advice and sorting support. To Ervin, I thank you sincerely for your mentorship and teaching me everything worth knowing about flow cytometry.

To my incredible family and friends; your presence and support in my life has been invaluable to my PhD completion. To Niko, Hayden, Kira, Adam, James and Clare - thank you for your love and understanding, for being my shoulders to lean on and supporting me in any way you can throughout this experience. I'm so lucky to have you all in my life. To Niko, thank you for giving me strength and being my biggest supporter, no matter what. To Kali, thanks for being the best listener of them all. And an extra special thanks goes out to Hayden for being my "IT support" and for his exceptional formatting skills.

To my salsa fam, thank you for the turning my bad days into good ones through laughs and our love of dancing. To the FLPs - Macarena, Bec and Renae - thank you for your friendship, laughs and "wine therapy". Brendan and Bettina, thank you for your friendship and encouragement – I'm lucky to have met you both. Thank you to everyone involved in Frontiers in Reproduction (FIR) 2018 for the truly unforgettable experience.

Finally, to Dad & Sue, and Mum, I sincerely thank you for everything. I am truly grateful for the opportunities you have given me in life and for your unconditional love, support and belief in me throughout these difficult years.

I am forever grateful for you all.

Publications arising from this thesis

1. Green ES, Moldenhauer LM, Groome HM, Kara EE, Robker RR, McColl SR, Robertson SA. (In Preparation) *Peri-implantation progesterone exposure programs Treg cells for pregnancy success*
2. Green ES, Moldenhauer LM, Robertson SA. (In Preparation) *Progesterone signalling at implantation controls timing of birth*
3. Green ES, Moldenhauer LM, Robertson SA. (In Preparation) Luteal-phase and early pregnancy progesterone regulation of maternal immune tolerance

Abstracts arising from this thesis

2019

Green ES, Moldenhauer LM, Groome, HM, McColl SR, Robertson SA. *Regulatory T Cells are a Key Agent of Progesterone Effects in Early Pregnancy.* (Oral Presentation, Invited Speaker) Immunology of Pregnancy Symposium, University Medical Center Groningen, Groningen, Netherlands

Green ES, Moldenhauer LM, McColl SR, Robertson SA. *Regulatory T Cells are a Key Agent of Progesterone Effects in Early Pregnancy.* (Oral Presentation) Society for Reproductive Investigation (SRI), Paris, France

2018

Green ES, Moldenhauer LM, McColl SR, Robertson SA. *Regulatory T cells protect against fetal loss in pregnancies compromised by peri-implantation disruption of progesterone signalling*. (Oral Presentation, 2nd prize) Adelaide Immunology Retreat (AIR) Meeting, Adelaide, South Australia

Green ES, Moldenhauer LM, McColl SR, Robertson SA. *Reduced progesterone bioavailability at implantation compromises regulatory T cell tolerance and impairs fetal growth and viability in later gestation.* (Oral Presentation) Frontiers in Reproduction (FIR) Symposium, Woods Hole, Massachusetts, USA.

2017

Green ES, Moldenhauer LM, McColl SR, Robertson SA. *Progesterone signalling at implantation is required for adequate Treg cell tolerance and fetal growth and viability in late-gestation.* (Poster Presentation) The Australasian Society for Immunology (ASI) Annual Meeting, Brisbane, Australia

Green ES, Moldenhauer LM, Kara EE, Chin PY, Robker RL, McColl SR, Robertson SA. *Disrupting progesterone signalling during implantation compromises Treg cell tolerance and is associated with poor fetal outcomes in late gestation.* (Poster Presentation) The American Society for Reproductive Immunology (ASRI) Annual Meeting, Chicago, USA

Green ES, Moldenhauer LM, McColl SR, Robertson SA. *Reduced progesterone signalling at implantation compromises Treg cell tolerance and impairs fetal growth and viability in later gestation*. (Oral Presentation) The Society for Reproductive Biology (SRB) Annual Meeting, Perth, Australia

2016

Robertson SA, Green ES, Moldenhauer LM, Kara EE, Robker RL, McColl SR. *Progesterone controls regulatory T cell proliferation and phenotype stability during early pregnancy in mice. (Poster Presentation) Society for the Study of Reproduction, San Diego, USA*

Green ES, Moldenhauer LM, Kara EE, Robker RL, McColl SR, Robertson SA. *Progesterone control of regulatory T cell abundance and phenotype.* (Oral Presentation, Invited Speaker, SRF-SRB Exchange Lecture) The Society for Reproduction and Fertility (SRF) Annual Meeting, Winchester, United Kingdom

Green ES, Moldenhauer LM, Kara EE, Robker RL, McColl SR, Robertson SA. *Progesterone control of regulatory T cell phenotype and abundance in pregnancy – a novel role for non-classical progesterone action*. (Oral Presentation, New Investigator Award Winner) The International Society for the Immunology of Reproduction (ISIR) Congress, Erfurt, Germany

Green ES, Moldenhauer LM, Kara EE, Robker RL, McColl SR, Robertson SA. *Regulatory T cell abundance and phenotype in pregnancy - a novel role for progesterone potentially independent of nuclear progesterone receptor.* (Poster Presentation) Inflammation in Reproduction, Pregnancy and Development (IRPD) Satellite Meeting, Palm Cove, Cairns, Australia

Green ES, Moldenhauer LM, Kara EE, Robker RL, McColl SR, Robertson SA. *Regulatory T cell abundance and phenotype in pregnancy - a novel role for progesterone potentially independent of nuclear progesterone receptor.* (Poster Presentation) International Congress of Immunology (ICI), Melbourne, Australia

2015

Green ES, Moldenhauer LM, Kara EE, Robker RL, McColl SR, Robertson SA. *Progesterone regulates regulatory T cell abundance and phenotype in pregnancy.* (Oral Presentation, David Healy New Investigator Award Winner) The Society for Reproductive Biology (SRB) Annual Meeting, Adelaide, Australia

List of Figures

List of Tables

Abbreviations

Ab	antibody
Ag	antigen
APC	antigen-presenting cell
ART	assisted reproductive technology
B6	C57Bl/6
BAC	bacterial artificial chromosome
BSA	bovine serum albumin
BSA-FITC	albumin-fluorescein isothiocyanate conjugate; bovine serum albumin-conjugated FITC
bp	base pair
CCL	chemokine (C-C) motif
CL	corpus luteum
CO_2	carbon dioxide
CXC	chemokine (C-X-C) motif
DC	dendritic cell
DNA	deoxyribonucleic acid
E2	estrogen; estradiol; 17β-estradiol
EAE	experimental autoimmune encephalomyelitis
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EtOH	absolute ethanol
exTreg	T cell previously classified as a $Foxp3^+$ Treg cell
FACS	fluorescence-activated cell sorting
FCS	fetal calf serum
FITC	fluorescein isothiocyanate

FMO	fluorescence minus one
Foxp3	forkhead box P3
geo. MFI	geometric mean fluorescence intensity
GFP	green fluorescent protein
GR	glucocorticoid receptor
Iono	ionomycin
i.p.	intraperitoneal
i.v.	intravenous
IFN	interferon
IL	interleukin
IMDM	Iscove's modified Dulbecco medium
IUGR	intrauterine growth restriction
IVF	in vitro fertilization
LN	lymph node
LPD	luteal phase deficiency
LPS	lipopolysaccharide
mAb	monoclonal antibody
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
mPR	membrane progesterone receptor
mRNA	messenger RNA
NK cell	natural killer cell
NK T cell	natural killer T cell
Nrp1	neuropillin 1
P4	progesterone; 4-pregnene-3, 20-dione

P4-BSA-FITC	progesterone 3-(O-carboxymethyl)oxime:BSA-fluorescein isothiocyanate conjugate; P4-FITC; progesterone-conjugated FITC
PALN	para-aortic lymph nodes; udLN
PBS	phosphate-buffered saline
pc	post-coitum
PCR	polymerase chain reaction
PE	preeclampsia
PGRMC1	progesterone receptor membrane component 1
PMA	phorbol 12-myristate 13-acetate
PR	progesterone receptor
PTB	preterm birth
pTreg	peripherally-induced Treg
qPCR	quantitative PCR
RA	recurrent abortion
RBC	red blood cell
RFP	red fluorescent protein
RNA	ribonucleic acid
RORγt	retinoic-acid receptor-related orphan receptor γt
RPMI	Roswell Park Memorial Institute medium
RT	reverse transcription
RU486	mifepristone
s.c.	subcutaneous
SEM	standard error of mean
TCR	T cell receptor
TdTomato	tandem dimer tomato red fluorescent protein variant; RFP
TEC	thymic epithelial cell

Teff	effector T cell
Tg	transgene
TGF	transforming growth factor
TLR	toll-like receptor
TNF	tumour necrosis factor
Treg	regulatory T cell
TSDR	Treg-specific demethylated region
tTreg	thymic-derived Treg cell
udLN	uterus-draining lymph nodes; PALN
WT	wildtype

Chapter 1

Review of literature

CHAPTER 1

1.1. INTRODUCTION

Pregnancy disorders including preterm birth, and preeclampsia, affect 10-12% of pregnancies and are the leading cause of perinatal morbidity and mortality, responsible for 16% of maternal deaths in developed countries (Sibai *et al.* 2005, Goldenberg *et al.* 2008, Steegers *et al.* 2010). These pregnancy complications are 10-30 times more prevalent within low socioeconomic status populations and in low income countries, where 99% of maternal deaths occur (Khan *et al.* 2006, Goldenberg *et al.* 2008). Many of these pathologies occur in conjunction with intrauterine growth restriction (IUGR), which can lead to both maternal and fetal complications and result in life-long impairment for the child (Longo *et al.* 2013).

Despite extensive research into these disorders over recent decades, their origins are still poorly understood. Recent work has highlighted that suboptimal maternal adaptation to pregnancy through poor uterine receptivity and placentation are central factors characterising these pathologies (Brosens *et al.* 2011, Roberts and Redman 2017). In addition to influence by environmental, hormonal and genetic factors, both of maternal and paternal origin, the immune system is clearly implicated in the pathology and cause of these conditions, which are often characterised by inflammation (Challis *et al.* 2009, Erlebacher 2013). It is well understood that the immune system plays an essential role in pregnancy. The semi-allogeneic fetus is in fact supported by the maternal immune system, which induces immune tolerance to protect the fetus from harmful immune responses. In particular, regulatory T cells (Treg cells), a specialised subset of $CD4^+$ T lymphocyte, are the key mediators of fetal-maternal tolerance. Through interaction with the maternal immune environment Treg cells promote an anti-inflammatory, tolerogenic immune response systemically and at the fetal-maternal interface. Importantly, the Treg cell response is perturbed during pregnancy complications in women, including preeclampsia (Santner-Nanan *et al.* 2009, Quinn *et al.* 2011) and preterm birth (Xiong *et al.* 2010, Schober *et al.* 2012, Steinborn *et al.* 2012, Koucky *et al.* 2014). Despite their relevance in these conditions, the details of their dynamics and regulation in pregnancy are not well understood. Regulation of T cells by hormones appears important for instructing cell phenotypes, and thus has relevance in pregnancy where maternal hormones, particularly progesterone, dominate. Notably, progesterone is a candidate regulator of Treg cells in pregnancy (Mao *et al.* 2010, Lee *et al.* 2012).

This review will summarise the literature on Treg cells, the maternal immune adaptation to pregnancy, and progesterone as an immunomodulator and discuss the potential role for progesterone in the regulation of Treg cells during pregnancy.

1.2. REGULATORY T CELLS

Regulatory T (Treg) cells are essential regulators of immune homeostasis through controlling and constraining excessive immune responses and inflammation. They also play key roles in mediating fetal-maternal tolerance, which is required for pregnancy success. An introduction to the history, types, functionality and plasticity of Treg cells Is detailed below.

1.2.1. Introduction

The adaptive immune system comprises a highly specialised set of cells and processes that coordinate with cells of the innate immune system to elicit effective responses against pathogenic microorganisms. These responses are largely mediated by T- and B-lymphocytes, adaptive immune cells that have the capacity to recognise a diverse range of antigens with high specificity and develop powerful effector functions and immunological memory. $CD4^+$ T cells are comprised of seven distinct subsets of T helper (Th) cells (Th1, Th2, Th17, Treg, Th22, Th9 and follicular helper T cells (Tfh)), each with distinct functions in adaptive immunity. The specific subsets are defined by their cellular function and cytokine expression, which is determined by specific transcriptional programs controlled by master transcriptional regulators (Zhu *et al.* 2010). Most of the Th subsets possess potent pro-inflammatory functions, which must be balanced by anti-inflammatory regulatory mechanisms to prevent excessive and undesirable immune responses that can lead to the development of autoimmunity or allergy. To serve this purpose, there exists a unique regulatory Th subset, regulatory T (Treg) cells, which are defined by their expression of the master transcription factor Forkhead Box P3 (Foxp3) of the forkhead/winged-helix family. Comprising approximately 1-3% of total Th cells in humans and 3-10% in mice, Treg cells are principal regulators of tolerance in the adaptive immune system. Treg cells maintain immune homeostasis by suppressing auto-reactive immune cells, inducing tolerance to self-antigens and non-pathogenic microorganisms and dampening immune responses to pathogens post clearance (Sakaguchi *et al.* 2008).

Suppressor cells were first identified as a functional subset distinct from T helper cells in the 1970s due to their ability to dampen adaptive immune responses (Gershon and Kondo 1971), however, a lack of specific biomarkers and cytokines profiles for these cells caused progress in the field of Treg cell biology to be delayed for many years. Sakaguchi *et al*. were the first to associate IL-2 receptor α-chain (CD25) surface molecule expression and $CD4^+$ T cells with suppressive phenotypes (Sakaguchi *et al.* 1995). They demonstrated that depletion of $CD25^+$ T cells caused various autoimmune diseases in mice including thyroiditis, arthritis and gastritis, and subsequent transfer of $CD4^+CD25^+$ T cells prevents disease

development. However, as $CD4^+$ effector Th subsets can also express CD25, it does not serve as a biomarker specific for only Treg cells. Foxp3 was subsequently identified as a unique biomarker for Treg cells due to the discovery of a spontaneous mutation in the *Foxp3* gene that caused severe autoimmune phenotypes in humans and mice (Brunkow *et al.* 2001). Fontenot *et al.* were the first to link Foxp3 expression and Treg cells. They demonstrated increased *Foxp3* mRNA levels could be found in $CD4^+CD25^+$ T cells, and that these cells were deficient in Foxp3 mutant mice. They subsequently demonstrated the transfer of $CD4^+CD25^+$ T cells into Foxp3 null mutant mice rescued them from autoimmune disease (Fontenot *et al.* 2003). This work not only identified Foxp3 as a distinctive Treg cell marker, but also demonstrated the striking necessity for Treg cells in the maintenance of peripheral tolerance. Foxp3 is now recognised as the master regulator of Treg cell development and function that not only regulates its own transcription, but directly or indirectly regulates the transcription of hundreds of genes that play essential roles in determining Treg phenotype, function and stability (Sakaguchi *et al.* 2008). In addition to Foxp3 expression, Treg cell lineage commitment also requires a Treg-specific pattern of CpG hypomethylation which is induced following T cell receptor (TCR) stimulation and leads to activation of Treg cell 'signature' genes including *Ctla4, Il2ra, Tnfrsf18* and *Ikzf2* which encode CTLA4, CD25, GITR and Eos, respectively (Zheng *et al.* 2010, Ohkura *et al.* 2012). In humans Treg cells exhibit much of the same characteristics and are typically defined as $CD25^+$ and $Cd127^{lo}$ and/or $FOXP3^+$.

1.2.2. Treg cell subtypes

Treg cells can be divided into two distinct subtypes, thymic-derived Treg cells (tTregs) and peripherally-induced Treg cells (pTregs) that differ in their developmental pathways and play different roles in the immune system. The classical paradigm of generation of the Treg cell subsets is as follows. In the thymus, where positive and negative selection of T cells based on the structure of their individual T cell receptors (TCRs) occurs, single positive $CD4^+$ thymocytes either differentiate into mature tTregs, if they have intermediate TCR/antigen affinity but high self-antigen affinity, or enter the periphery as naïve $CD4^+$ T cells if they have low affinity to self-antigen. In the periphery, clonal expansion of naïve $CD4^+$ T cells can result in differentiation into each of the $CD4^+$ T cell subsets, including pTregs (Jager and Kuchroo 2010). Differentiation into Th subsets is heavily influenced by the cytokine microenvironment present. Differentiation of naïve $CD4^+$ T cells into pTreg cells results from TCR-mediated activation by antigen presenting cells (APCs) in the presence of IL2 and TGFβ, which drives Foxp3 expression (Chen *et al.* 2003).

Generally, tTreg cells are through to mediate tolerance to self antigens and pTreg cells are thought to mediate tolerance to foreign antigens. tTreg cells generated in the thymus are predominantly specific for

tissue restricted antigens presented by medullary thymic epithelial cells (mTEC) (Lee and Lee 2018). These Treg cells initiate tolerance to antigen expressed throughout the body, including antigens of reproductive organs such are the ovary. Notably however, upon thymectomy of 3 day old mice, ovarian autoimmune disease is controlled by antigen-specific Treg cells in the ovarian-draining lymph nodes, demonstrating tolerance to some autoantigens such as the ovarian zona pellucida 3 (ZP3) are peripherally acquired (Samy *et al.* 2008, Tung *et al.* 2017). As well as pTreg cell generation to autoantigens, thymic Treg cells can respond to foreign antigens. Up to 10% of the T cell repertoire are thought to respond to alloantigens and this population of T cells that mediates responses in the context of organ rejection (Suchin *et al.* 2001). Thymic Treg cells can be activated to oral antigens, since the thymus was required for antigen-specific Treg cells capable of inducing oral tolerance in a model of experimental autoimmune encephalomyelitis (EAE) (Song *et al.* 2006). From these studies and others, it is clear that the simplistic, historical view of Treg cell development in some cases no longer applies and the many complexities of Treg cell activation and function in "self", "non-self", and "partly-self" responses are still being discovered (Lee and Lee 2018).

1.2.3. Treg cell function

In addition to expression of CD25, CD4 and Foxp3, Treg cells express many characteristic surface molecules, including cytotoxic T-lymphocyte protein 4 (CTLA-4), (glucocorticoid-induced tumour necrosis factor receptor (GITR), neuropillin 1 (Nrp1), $CD95^{high}$, and in humans, $CD45RB^{high}$ and $CD127^{low}$ (Gavin *et al.* 2007, Sakaguchi *et al.* 2008). Nrp1, which is regulated by TGFβ, is a cell surface molecule important for Treg cell stability. Importantly, it is recognised as a marker to distinguish between thymic and peripheral Treg cells as tTreg cells typically express Nrp1 at high levels whereas pTreg cells express Nrp1 at low levels (Weiss *et al.* 2012, Yadav *et al.* 2012). In humans, identification of tTreg and pTreg cells in humans is difficult as they cannot be identified by Nrp1, and the traditional tTreg marker, HELIOS, does not accurately distinguish between these subsets (Elkord 2016).

Treg cells are responsible for the maintenance of immune homeostasis throughout the body through their suppression of inflammatory immune responses (Rudensky 2011). Treg cell suppressive action importantly prevents adverse T effector cell immunity to non-dangerous self or foreign antigens (Bettelli *et al.* 2006, Sakaguchi *et al.* 2008). Once activated, Treg cells exert immune suppression by multiple non-specific, broad mechanisms, including through the secretion of anti-inflammatory cytokines IL10 and TGFβ. Importantly, Treg cell suppressive function inhibits the proliferation and cytokine production of the inflammatory T effector cells, Th1 and Th17 cells, which can be characterised by the production of the

inflammatory cytokines IFNγ and IL17, respectively. Effector T cell responses must be tightly controlled by regulatory T cells to ensure immune homeostasis and prevent excessive inflammation.

Treg cell suppressive effects can also be exerted in contact dependent ways, such as Treg expression of CTLA4, an effector molecule for Treg suppression. Activated Treg cell interaction with APCs causes down regulation of co-stimulatory molecules CD80/86, which are needed for naïve T cell activation (Sakaguchi *et al.* 2008). Furthermore, Treg cell interaction with dendritic cells (DCs) through CLTA4 causes indoleamine 2,3-dioxygenase (IDO) production by DCs, resulting in Th1 cell death (Fallarino *et al.* 2003). Treg cells also interact with macrophages, which adopt a regulatory, anti-inflammatory "M2" phenotpye in the presence IL10 and TGFβ (Tiemessen *et al.* 2007), and neutrophils, which Tregs recruit via CXCL8 (IL8) (Himmel *et al.* 2011).

In addition to general suppression of inflammatory responses, Treg cells are now appreciated to have a broad range of functions in organ homeostasis. The diverse functions of these cells are attributed to Treg cell populations in specific anatomical locations. Treg cells are known to localise to non-lymphoid sites including the intestines, skin, adipose tissue, tumours, and notably, the uterus and placenta. Treg cells are involved in processes of tissue remodelling and repair in various organ systems, for example, facilitating wound healing in the skin (Nosbaum *et al.* 2016, Lee and Lee 2018, Li *et al.* 2018). Treg cells are shown to interact with innate immune cells such as neutrophils and macrophages to control inflammation following tissue injury (Li *et al.* 2018).

1.2.4. Treg cell stability and plasticity

Foxp3 amplifies expression of genes encoding many classical surface and secreted molecules including CD25 and CTLA4, and represses expression of IL2 to induce a Treg-specific transcriptional program (Wu *et al.* 2006, Gavin *et al.* 2007). As part of facilitating Treg lineage commitment, Foxp3 also represses the master transcriptional regulators of other Th subsets, including T-Bet and RAR-related orphan receptor γt (RORγt), which control Th1 and Th17 activation and phenotype, respectively (Zheng and Rudensky 2007). Commitment to the Treg cell lineage also requires a specific CpG hypomethylation pattern which is induced following T cell receptor (TCR) stimulation and is independent of Foxp3 expression, leading to activation of Treg suppressive genes that encode CTLA4, CD25, GITR and Eos (Ohkura *et al.* 2012).

Treg cells were historically thought to represent a homogenous, stable lineage, however, it is now accepted that $CD4^+$ T helper (Th) cells, including $Foxp3^+$ Treg cells possess a high degree of phenotypic plasticity; the ability to express transcription factors and/or cytokines typically associated with other cell lineages (Voo *et al.* 2009, Levine *et al.* 2017). Treg cell phenotypical plasticity is dependent on

environmental conditions and leads to functional heterogeneity of Treg subsets. For example, various studies in mice and humans have demonstrated Foxp3$^+$ T cells can express the inflammatory cytokines IL17 and IFNγ that are characteristic of inflammatory T effector cells (Zhou *et al.* 2008, Voo *et al.* 2009, Feng *et al.* 2011, Zhao *et al.* 2012).

In addition to exhibiting phenotypic plasticity, numerous studies have shown that under some circumstances Treg cells can undergo lineage instability to lose Foxp3 expression. Lineage instability refers to the ability of Treg cells to lose their identity and be transcriptionally reprogrammed to various effector Th cells under certain environmental conditions (Sakaguchi *et al.* 2013). In mice Foxp3$^+$ T cells have been shown to switch lineage and become effector Th cells when appropriately stimulated in vitro and in vivo (Komatsu *et al.* 2009, Zhou *et al.* 2009). These converted Treg cells, known as exTregs, have been shown to drive disease pathology in a range of models of autoimmunity and inflammation (Zhou *et al.* 2009, Miyao *et al.* 2012, Hori 2014, Komatsu *et al.* 2014). This phenomenon was demonstrated in a model of autoimmune arthritis, where a fate-mapping mouse model to track past and present Foxp3 expression facilitated the identification of a unique population of pathogenic ex-Foxp3$^+$ IL-17-producing Th17 cells (Komatsu *et al.* 2014). Similarly, Treg fate-mapping experiments using transgenic mouse models revealed Foxp3$^+$ Treg cells can undergo transdifferentiation and convert to Th1-exTreg cells in type 1 diabetes (Zhou *et al.* 2009). These studies demonstrate that under polarising conditions in vivo, Treg cells have the potential to undergo genetic reprogramming. Further, they show the necessity for stable Treg cells in the maintenance of immune tolerance. In opposition of these studies, the Rudensky laboratory report Treg stability under homeostatic conditions with a tamoxifen-inducible Cre model which reports on the fate of Treg cells in a temporal fashion upon tamoxifen administration, suggesting that cells committed to the Treg fate will not undergo transdifferentiation. Notably, loss of Foxp3 expression could reflect instability of a minor population of Foxp3$^+$ T cells that had yet to commit fully to the Treg cell transcriptional program (Miyao *et al.* 2012). Regardless of the controversies over mouse models used in the literature, a prevailing conclusion is that Treg cell plasticity and instability have the potential to shape the outcome of immune responses in inflammatory environments.

Treg cell plasticity has also been observed in humans, such as the identification of FOXP3$^+$ Treg cells that are poised to express IL17 upon activation. These cells co-expressed FOXP3 and RORγt and maintained their suppressive function (Voo *et al.* 2009). Th17-like and Th1-like Tregs cells have been identified in patients with various autoimmune diseases including psoriasis, inflammatory bowel disease and rheumatoid arthritis and are in some cases are linked to the pathogenesis of these diseases (Jung *et al.* 2017, Di Giovangiulio *et al.* 2019) Investigation of how phenotypically plastic Treg cell subsets are

regulated in healthy subjects versus in pathologic disease states will be important for understanding the importance of these cells in human diseases.

1.3. IMMUNE RESPONSE TO PREGNANCY AND REGULATORY T CELLS

The implantation of the mammalian embryo in pregnancy presents a unique immunological challenge to the mother. The fetus and placenta, collectively called the conceptus, contains 50% paternally inherited genes and therefore expresses paternally derived major histocompatibility complex (MHC) molecules, making it antigenically distinct to the mother. Given this, the question of how the female immune system allows for the growth and development of the semi-allogeneic fetus, as opposed to exerting an immune response resulting in rejection, has fascinated both immunologists and reproductive biologists for many years and led to the development of the reproductive immunology field of research. Several key mechanisms mediate maternal tolerance to the fetus in pregnancy, including the induction of Treg cells as discussed below.

1.3.1. Immune recognition of the semi-allogeneic fetus

The maternal immune system is first, and most often, exposed to paternal antigens during transmission of seminal fluid at coitus, and later when the embryo makes intimate contact with the mother's uterine tissue when it penetrates the endometrium during implantation (Robertson and Sharkey 2001). The process of trophoblast invasion that follows leads to further antigen exposure, as placental trophoblast cells express fetal antigens (Holland *et al.* 2012). Furthermore, upon placental development, the maternal and fetal blood supply is shared, thereby allowing microchimeric fetal cells to exist in maternal circulation (Petroff 2011). Maternal exposure to both paternal and fetal antigens is a key indicator that recognition of fetal antigens is inevitable during pregnancy (Moldenhauer *et al.* 2009), and therefore makes the conceptus susceptible to rejection by the maternal immune system, which is tailored to respond to 'non-self' molecules in the form of rejection. Indeed, the implantation site and uterine stroma is heavily populated with both innate and adaptive leukocytes including natural killer (NK) cells, T ($CD4^+$ and $CD8^+$) lymphocytes, monocytes and granulocytes that have the capacity to drive an immune response given the appropriate cues (Mor *et al.* 2017).

In successful pregnancy, multiple mechanisms are induced to project the fetus from attack by the maternal immune system and to dampen inflammatory responses through the induction of immunological tolerance.

The existence of regulatory mechanisms that suppress the maternal immune system was first proposed by Sir Peter Medawar in 1953 (Medawar 1953). Fetal immune evasion strategies are known to exist, such as the expression of non-classical MHC molecules by placental trophoblast cells (Trowsdale and Betz 2006, Guleria and Sayegh 2007). Despite these mechanism of immune evasion, extensive research in mice shows that the maternal immune system has the capacity to recognise and respond to paternal antigens in seminal fluid (Moldenhauer *et al.* 2009, Shima *et al.* 2015). These studies demonstrate that the adaptive immune system is equipped to recognise paternal antigens during pregnancy, and is able to respond by eliciting either tolerance or rejection. Notably, the induction of immune tolerance that sustains pregnancy appears essential for implantation and placental development to occur. The key mediator of immune tolerance towards the antigenically distinct fetus is the induction of regulatory T (Treg) cells, which are proven essential for allogeneic pregnancy in mice (Aluvihare *et al.* 2004, Guerin *et al.* 2009). In addition to Treg cells, other immune cells including natural killer cells, macrophages and dendritic cells, play key roles in maternal tolerance and promote fetal development. The role of Treg cells and these immune subsets in pregnancy is discussed below.

1.3.2. Peri-conception immune adaptations to pregnancy

The immune response to pregnancy is first induced following coitus, in response to exposure to paternal antigens and immune-deviating chemokines and cytokines contained in semen. In addition to sperm, the ejaculate consists of seminal plasma which contains many signalling factors including TGFβ, PGE-type prostaglandins and miRNAs (Robertson 2007). Notably, seminal plasma is the greatest source of TGFβ in biological fluids (Robertson *et al.* 2002). In addition, semen contains numerous antigens, including MHC class Ia, class Ib and class II (Hutter and Dohr 1998). The presence of these antigens and potent signalling molecules in the female reproductive tract triggers a classical inflammatory cascade known as the "post-coital inflammatory response" characterised by induction of cytokines and chemokines in the uterine epithelium, leading to an influx of leukocytes, including neutrophils, macrophages and DCs into the uterus. Along with innate subsets, $CD4^+$, $CD8^+$ and γδ T cells are also recruited into the uterus in response to chemotactic signals (Heyborne *et al.* 1992, Kallikourdis *et al.* 2007, Mor *et al.* 2017).

The paternal antigens exposed to the uterus are processed by APCs such as uterine DCs, and are presented to naive T cells, in the lymph nodes (LNs) draining the uterus (udLNs). This leads to the activation and expansion of T cells that are specific for paternal antigens, including pTreg cells in the presence of appropriate environmental signals including the cytokines IL2 and TGFβ (Moldenhauer *et al.* 2009, Guerin *et al.* 2011). Treg cells are expanded locally in the uterus, but predominantly in the udLNs where they are found to subsequently traffic to the fetal-maternal interface following recirculation

(Aluvihare *et al.* 2004, Johansson *et al.* 2004). Treg cell accumulation in the uterus following mating is dependent on uterine expression of Treg-homing chemokines such as CCL4, which attracts $CCR5^+$ Treg cells in the pregnant uterus (Kallikourdis *et al.* 2007), and CCL19, a Treg chemokine upregulated in uterine epithelial cells upon seminal plasma and sperm exposure (Guerin *et al.* 2011). The activation of Treg cells prior to implantation appears key for resolving the post-coital inflammatory response to ensure embryo implantation is successful. Following Treg cell induction, an anti-inflammatory immune environment dominates throughout most of gestation, which is essential for preventing deleterious Th1 or Th17 expansion that can cause pregnancy failure (Rowe *et al.* 2011, Robertson *et al.* 2013). Notably, Treg cells are also recruited to the uterus prior to pregnancy periodically in response to hormone fluctuations throughout the menstrual cycle (Kallikourdis and Betz 2007).

1.3.3. Treg cells are mediators of maternal tolerance to the fetus in pregnancy

Treg cells are recognised as the key mediators of maternal tolerance to the fetus and are essential in mice for establishing the active immune tolerance required for embryo implantation (Aluvihare *et al.* 2004, Guerin *et al.* 2009). Aluvihare *et al*. were the first to demonstrate that Treg cell populations expand during pregnancy in mice and are required to prevent immunological rejection of the fetus by eliciting tolerance towards paternal antigens. They observed a high proportion of $CD4^+CD25^+$ lymphocytes in the uterus during pregnancy as early as 2 days after mating, which was reflected by a substantial increase in *foxp3* expression. Additionally $CD4^+CD25^+$ cell proportions were elevated in peripheral blood after implantation, and lymphocyte suppressive function increased in the spleen and lymph nodes (Aluvihare *et al.* 2004). The presence of Treg cells is most essential in the implantation period, as demonstrated by fetal rejection in their absence (Aluvihare *et al.* 2004, Zenclussen *et al.* 2005). Multiple in vivo models show that when Treg cells are depleted prior to mating or in the pre-implantation phase of pregnancy, then pregnancy progression is significantly compromised, with varying effects according to the extent of depletion (Aluvihare *et al.* 2004, Zenclussen *et al.* 2005, Shima *et al.* 2010). Transfer of CD25-depleted lymphocytes to T cell deficient mice caused gestational failure due to immunological rejection of allogenic fetuses. However, in syngeneic pregnancies whereby MHC was identical between mother and fetuses, Treg cells were not required (Aluvihare *et al.* 2004). Thus, Treg cells are essential mediators of maternal immune tolerance in allogenic pregnancies.

As described above, the immune response initiated at mating following exposure to seminal fluid is the crucial initiation step for Treg cell tolerance to be established and for successful embryo implantation to occur (Robertson *et al.* 2009). Zhao *et al.*, were the first to show that fetal alloantigen is a major factor driving the expansion of the Treg cell pool in early pregnancy, showing that $CD4^+CD25^+$ Treg cells

increased more in allogeneic pregnancy versus syngeneic pregnancy and contributed to a lower alloreactivity against paternal antigens (Zhao *et al.* 2007). Using ovalbumin (OVA) as a model paternal seminal fluid-derived antigen, Moldenhauer *et al.,* showed the expansion of OVA-antigen specific OT-II $CD4^+$ and OT-I $CD8^+$ T cells in the udLN following mating of females to Act-mOVA males (Moldenhauer *et al.* 2009). More recently, using tetramer-based enrichment to identify rare endogenous T cells specific for the model fetal antigen 2W1S, Rowe *et al.* elegantly demonstrated that pregnancy selectively stimulates the accumulation of maternal Treg cells with fetal specificity. These Treg cells expanded throughout pregnancy and persisted postpartum. These Treg cells retained protective memory to fetal antigen, rapidly re-accumulating during second pregnancies and mediating resistance to fetal resorption (Rowe *et al.* 2012). Recent evidence suggests that pTreg cells may have evolved with placentation in mammals, indicating the importance of this specific Treg cell subset in pregnancy (Samstein *et al.* 2012). In humans, Tilburgs *et al.,* showed a preferential recruitment of Treg cells from maternal peripheral blood to the decidua. These Treg cells were able to downregulate both fetal-specific and non-specific immune responses in vitro (Tilburgs *et al.* 2008).

Despite these studies, paternal/fetal antigen-specific pTreg cells cannot account for most of the Treg cells implicated in fetal maternal tolerance, raising the question of the involvement and antigen specificities of other Treg cells. There is evidence that a major population of Treg cells is expanded systemically prior to embryo implantation, independent of exposure to paternal antigens (Kallikourdis and Betz 2007). These Treg cells, found to be tTreg cells of thymic origin with assumed self-reactivity, accumulate in the uterus and udLNs during the estrus stage of the reproductive cycle in mice, just following ovulation. This occurs in response to increases in estradiol (E2) and P4 levels at ovulation (Arruvito *et al.* 2007, Kallikourdis and Betz 2007, Caligioni 2009, Teles *et al.* 2013). In women, Treg cells are expanded in the follicular phase of the menstrual cycle, which correlates with progressively elevating serum E2 levels (Arruvito *et al.* 2007). This selective trafficking of tTregs to the uterus is thought to take place in preparation for embryo implantation and pregnancy to occur. Chen *et al.,* demonstrated the importance of self-antigen specific Treg following embryo implantation in mice, showing that self-antigen specific Treg cells make up the majority of Treg cells in the udLN in the first half of pregnancy (Chen *et al.* 2013). Overall these studies indicate that both paternal antigen specific pTreg cells, and thymic-derived tTreg cells appear to play important roles in the maintenance of maternal tolerance during pregnancy. Elucidating the specific functions and regulation of distinct Treg cell subsets will be important for understanding the importance of each subset for pregnancy success in humans. The Treg cell response that occurs in women is similar to that seen in mice with a few key differences. In women, an increase in systemic and decidual Treg cell populations in the first trimester is observed. Circulating suppressive $CD4^+CD25^+$ cells increase in early

pregnancy, peaking at the second trimester, before declining at term (Somerset *et al.* 2004). Foxp3^{+} Treg cells with a unique activated memory phenotype are also present in the decidua in human pregnancy (Tilburgs *et al.* 2008, Feyaerts *et al.* 2017).

How Treg cells carry out their functions to mediate immune tolerance to the fetus has been widely investigated but is still not fully understood. Several general mechanisms of Treg cell suppression of immune responses apply to the situation of pregnancy. Treg cells are potent suppressors of inflammatory immune responses, influencing a diverse range of immune cells through their multiple suppressive actions including TGFβ and IL10 secretion. Importantly, Treg cells suppress Th1 and Th17 cell responses in pregnancy, as these inflammatory responses if uncontrolled can lead to immune rejection of the fetus (Guerin *et al.* 2009). To ensure the T cell response supports implantation and placental development but does not cause immunity and inflammation, the balance of T cells must be skewed towards Treg cells and away from Th1 and Th17 cells (Erlebacher 2013). Experiments in mice where Treg cells are depleted or overwhelmed by effector T cell phenotypes show the crucial importance of this T cell balance for pregnancy success (Samstein *et al.* 2012, Wegorzewska *et al.* 2014, Xin *et al.* 2014). Treg cell anti-inflammatory action in the udLN and uterus importantly prevents adverse T effector cell immunity to fetal antigens. Rowe *et al.*, showed sustained Treg cell expansion was required for maintaining pregnancy as even partial Treg cell ablation following administration of diphtheria toxin (DT) to susceptible Foxp3$^{DTR/WT}$ mice from mid-gestation caused fetal resorptions and allowed activation of paternal antigen-specific CD8^{+} T cells capable of producing IFNγ after stimulation (Rowe *et al.* 2012).

Treg cells interact with various immune cells in pregnancy including macrophages, uNK cells and DCs to promote anti-inflammatory and tolerogenic phenotypes and in turn, tolerance-promoting macrophages and DCs play a crucial role in expanding Treg cells (Steinman *et al.* 2003). The maternal uterine cytokine mileu includes TGFβ, Csf2 (formerly GM-CSF), IL4, IL10, Csf3 (formerly G-CSF) and prostaglandin E, which are identified as regulators of tolerogenic DCs (Guerin *et al.* 2009). Treg action on DCs causes them to produce indoleamine 2,3-dioxygenase (IDO) which mediates tryptophan catabolism causing toxic effects in T cells. IDO was demonstrated to play a role in successful tolerance in pregnancy through suppression of T cell activity against the allogeneic fetus (Munn *et al.* 1998, Fallarino *et al.* 2003). Treg cells also down-modulate expression of the costimulatory molecules CD80 and CD86 on APCs, impairing their ability to activate naïve T cells. Notably, these processes are dependent on Treg cell expression of CTLA4, which is indispensable for Treg cell function in vivo (Wing *et al.* 2008). Treg cell production of immunomodulatory cytokines TGFβ and IL10 modulate immune cell phenotypes in vivo. For example Treg cell derived TGFβ and IL10 suppresses uNK cell cytotoxic activity (Ghiringhelli *et al.* 2005), and controls DC release of IL15, a cytokine which modulates uNK cell function (Terme *et al.* 2008) and Treg

cells are known to localise with NK cells in the decidua basalis and may play a key role in regulating their phenotype during pregnancy (Zhang *et al.* 2016). Although IL10 specifically is dispensable for pregnancy success, it impaired host defence against prenatal *Listeria monocytogenes* infection (Rowe *et al.* 2011) and caused greater susceptibility to inflammation-induced pregnancy loss following lipopolysaccharide administration, potentially through altered Treg cell function, since the capacity of $IL10^{-/-}$ Treg cells was reduced in vitro (Prins *et al.* 2015). Treg cell involvement and interaction with other immune cells in early mouse pregnancy is summarised in Figure 1.

1.4. TREG CELLS IN PREGNANCY COMPLICATIONS

Although Treg cells have been proven to be irrefutably essential for successful allogeneic pregnancy in mice, their importance in women is not fully understood. As in mice, normal human pregnancy results in maternal immune activation and an increase in Treg cell proportions systemically and in the uterus (Saito *et al.* 2005, Sasaki *et al.* 2007, Tilburgs *et al.* 2008). Accumulating evidence suggests that Treg cells are of great importance for the progression of normal pregnancy in women, as shown by alterations in their number and functional capacity in many pregnancy complications. In addition, a skewing of the Treg/Teff ratio in favour of inflammatory T cell subsets is evident in these pregnancy complications.

1.4.1. Treg cells in preeclampsia

Preeclampsia complicates 2-8% of pregnancies, with sometimes life threatening impacts for maternal and fetal health (Duley 2009). It is well accepted that Treg cells are altered in subsets of women with preeclampsia (PE), a disorder affecting up to 10-15% of pregnancies. PE, which is characterised by gestational hypertension and is associated with poor remodelling of spiral arteries in early pregnancy, is a leading cause of maternal and fetal morbidity and mortality. Despite its prevalence, what causes the development of this disorder is not well understood, however, abundant evidence suggests that immune dysregulation plays a crucial role in PE development (Sargent *et al.* 2006, Ahn *et al.* 2011). Numerous studies have found that preeclamptic women have fewer Treg cells and a reduction in Treg suppressive capability. These Treg alterations are accompanied by increases in Th1 and Th17 cells as well as an increase in inflammatory mediators and inflammation towards the fetus (Saito and Sakai 2003, Sargent *et al.* 2006, Santner-Nanan *et al.* 2009, Quinn *et al.* 2011, Steinborn *et al.* 2012, Rahimzadeh *et al.* 2016). Collectively, this data has led to the hypothesis that Treg cell disturbances may cause the development of PE (Rahimzadeh *et al.* 2016). Rat models of preeclampsia demonstrate that Treg cells suppression of

inflammation reduces blood pressure and pathophysiology associated with preeclampsia (Cornelius *et al.* 2015, Cornelius *et al.* 2016), and a recent study showed that reduced Treg cells in early mouse pregnancy was causal to uterine artery dysfunction (Care *et al.* 2018).

1.4.2. Treg cells in term and preterm birth

Preterm birth (PTB) is defined as birth before 37 weeks' gestation and can result in severe infant morbidity and mortality. Spontaneous preterm birth accounts for the majority of preterm births and is associated with infection, inflammation, stress and a breakdown in fetal-maternal tolerance (Romero *et al.* 2014). Sterile inflammation in the absence of infection appears to be a key feature of many preterm births (Nadeau-Vallée *et al.* 2016). PTB often occurs in conjunction with intrauterine growth restriction (IUGR), which is linked to suboptimal placental development or function (Brosens *et al.* 2011).

Inflammation is a key part of normal on-time labour, with a pro-inflammatory signalling cascade mediated by the inflammatory cytokines IL1, IL6 and IL8 triggering gene associated with cervical ripening and uterine contractions (Romero *et al.* 2014). While the balance between immune subsets such as Treg cells to Teff cells, is key for maintaining pregnancy, a switch in innate and adaptive cell phenotypes in favour of cells with inflammatory potential appears important for the physiological process of labour in both term and preterm pregnancies (Gomez-Lopez *et al.* 2014). Term and PTB are characterised by an increase in responses from inflammatory macrophages and neutrophils (Gomez-Lopez *et al.* 2014). Preterm labour is associated with an inflammatory Th1 cytokine profile in the placenta, (El-Shazly *et al.* 2004) and decidual $CD4^+$ T cells were found to express IL1β, TNFα and the metalloproteinase MMP9 during spontaneous labour at term (Gomez-Lopez *et al.* 2013). IL17 likely derived from $CD4^+$ T cells, was found to promote inflammation in the chorioamniotic membranes of the fetal-maternal interface (Ito *et al.* 2010).

In contrast, to their essential role in mediating embryo implantation, Treg cells are thought to be nonessential for maintaining fetal development in late gestation in mice (Shima *et al.* 2010). In women, Treg cells are reported to decline at term (Somerset *et al.* 2004). It is suggested that alterations in Treg cells at the final stage of human pregnancy may be required to signal for parturition to occur (Schober *et al.* 2012, Steinborn *et al.* 2012). Preterm birth has been linked with further alterations in Treg cell number and function in a number of studies (Xiong *et al.* 2010, Schober *et al.* 2012, Steinborn *et al.* 2012, Koucky *et al.* 2014). Specifically, Treg cells with decreased expression of HLA-DR, a marker of differentiation and suppressive capacity, are reported in preterm labour (Schober *et al.* 2012).

1.4.3. Treg cells in abortion and infertility

There is an association between recurrent spontaneous abortion (RSA) and immune dysfunction, with a reduction in Treg cell numbers present in RSA (Laird *et al.* 2003, Sasaki *et al.* 2004, Rahimzadeh *et al.* 2016). Treg cells expressing Helios, a marker of Treg suppressive capacity, were reduced in the decidua of miscarriage patients compared to patients with normal progressing pregnancy (Inada *et al.* 2015). Increased Th1 cytokine responses have also been demonstrated in women with RSA and infertility (Kwak-Kim *et al.* 2003). In addition to complications of pregnancy, T cell imbalances are implicated in the pathophysiology of unexplained infertility. Primary unexplained infertility was associated with a decrease in *Foxp3* mRNA in endometrial tissue (Jasper *et al.* 2006). In addition, IL1A, TGFα and IFNγ were found to be elevated in uterine fluid of women with idiopathic infertility (Fitzgerald *et al.* 2016). These findings suggest that impairment in immune and Treg cell phenotypes is evident prior to conception in some women. Given the importance of Treg cells for pregnancy immune tolerance, Treg cell impairment in infertility may affect the capacity to establish pregnancy.

1.4.4. Summary and therapeutic potential for Treg cells in pregnancy complications

Studies of T cell responses in human pregnancy and infertility highlight the necessity for adequate Treg cells to constrain systemic and local inflammatory responses during pregnancy, which if uncontrolled can lead to adverse outcomes. The balance between regulatory subsets of T cells and inflammatory subsets is clearly important for successful pregnancy, however the dynamics and importance of specific T cell phenotypes during human pregnancy is still largely unknown. In addition to changes in Treg cell number, phenotype and function, many of these pregnancy complications are also linked with increased inflammation, and a change in the Treg:Teffector cell ratio. Therefore, impaired tolerance by Treg cells may be central in the environmental switch towards inflammation in these pathologies. Notably, at term there appears to be a switch in favour of immune activation and inflammation, which may be implicated in the timing of birth in normal and preterm pregnancies. Investigating the dynamic regulation of these T cell subsets throughout pregnancy will assist in developing a greater understanding of their role in pregnancy complications.

Given the involvement of suboptimal Treg cell responses in preeclampsia, infertility and other complications of pregnancy, there is clear potential for therapeutic interventions involving Treg cells to be developed in the future. Interventions to boost Treg cells are already under development to decrease pathology in a wide range of contexts including autoimmune diseases and transplantation (Allan *et al.* 2008). In the case of pregnancy complications, a clear understanding of how Treg cells are generated and regulated before and during pregnancy, their antigen specificity and the specific roles they play in

pregnancy, is not complete. Understanding the processes leading to Treg cell tolerance in pregnancy will help to understand the importance of Treg cells and how they can be therapeutically manipulated with the future goal of alleviating pregnancy disorders in which they are implicated.

1.5. TREG CELL PLASTICITY IN PREGNANCY

T cells are increasingly appreciated for their plasticity and adaptation to diverse environmental conditions, whereby they exhibit changes in gene expression and undergo cellular reprogramming to change their functions and phenotype. As described above Treg cell plasticity and instability has a role in driving inflammatory responses in a range of pathologies in mice, including inflammatory arthritis and autoimmune diabetes. It is well appreciated that Treg cells are important for pregnancy through constraining inflammatory Teff responses and assisting in optimal maternal vascular adaptations. While the balance between T cells subsets is important for overall pregnancy success, whether this balance in maintained and regulated through intrinsic changes in T cell phenotypes and fate is unknown. Pregnancy pathologies such as preterm birth are characterised by excessive local and/or systemic inflammation and a switch to Teff responses. In addition, in normal pregnancy there is a distinct switch towards an inflammatory state of immune activation that precedes parturition. Given this, the possibility that Treg cells exhibit plasticity in the context of both normal and/or pathological pregnancy is an important question. ***In this study, Treg cell stability during pregnancy will be investigated.***

1.6. PROGESTERONE

Progesterone (P4) is historically recognised as the major pregnancy hormone, as it is essential for successful reproduction in mice and humans due to its effects on ovulation, fertilisation and implantation (Graham and Clarke 1997). P4 is produced in high levels by the corpus luteum of the ovaries following ovulation and by the placenta in pregnancy. P4 regulates complex early reproductive events including decidualisation of the endometrium in preparation for embryo implantation (Franco *et al.* 2008). In women, administration of the progesterone receptor (PR) antagonist, Mifepristone (RU486), induces abortion in humans if given before 7 weeks gestation (Das and Catt 1987). The importance of P4 for the establishment of pregnancy is highlighted in women who experience luteal phase deficiency (LPD),

whereby a short or dysregulated luteal phase of the menstrual cycle causes insufficient P4 in early pregnancy resulting in impaired implantation and infertility (Mesen and Young 2015).

In mice, blocking P4 signalling in early pregnancy using RU486 causes implantation failure (Bagchi *et al.* 2005). When P4 signalling in the uterus was blocked with the PR antagonist, RU486, in the preimplantation period, many genes were dysregulated, including extracellular matrix/cell adhesion molecules, metabolic enzymes, immune modulators and cytoskeletal proteins (Cheon *et al.* 2002). PR-mediated P4 action was demonstrated to play an indispensable role in the normal development and function in the ovary, uterus, brain and mammary gland (Lydon *et al.* 1995). P4 has many targets on many different tissues and cells types. Most of P4 physiological actions are typically thought to be mediated through classical nuclear receptor pathway (PR) as evidenced by the aforementioned studies, however recent work suggests many of P4's rapid effects, non-genomic mechanisms on the cell surface and cytoplasm, mediated by membrane progesterone receptors (mPRs) and progesterone receptor membrane components (PGRMCs). The exact mechanisms of P4 action in many target tissues are unclear, as distinctions between many of the direct targets of P4 action and indirect effects of P4 regulation remain to be defined.

There are two receptor isoforms that arise from the PR gene (*Pgr)*, PR-A and PR-B, which function as ligand-activated transcription factors that differentially regulate the expression of a range of target genes (Giangrande and McDonnell 1999). In the absence of P4, PR is complexed to heat shock proteins (HSPs) in the cytoplasm. When ligands are present they diffuse across the cell membrane to the cytoplasm and bind PRs. Ligand-bound PRs then dissociate from HSPs, form homo- or heterodimers and translocate to the nucleus. They bind specific palindromic DNA binding sites known as progesterone response elements (PREs) in the promoters of PR target genes, leading to the initiation of transcription through transcription complex recruitment to the promoter (Jacobsen and Horwitz 2012).During pregnancy, PR is expressed in a wide range of tissues including the ovaries, placenta, uterus, mammary gland, thymus and brain where it regulates tissue-specific target genes (Graham and Clarke 1997, Brosens *et al.* 1999, Tibbetts *et al.* 1999). In many of these tissues, PR expression is regulated by 17β-estradiol (E2) and other estrogens bound to nuclear estrogen receptors, through their binding to estrogen-responsive elements in the promoter of the *pgr* gene (Graham and Clarke 1997).

Sufficient P4 is necessary for the establishment of pregnancy but P4 also functions to maintain myometrial quiescence until the end of pregnancy, when, in mice, luteal regression occurs causing P4 levels to decline and the events leading to labour to progress (Shynlova and Lye 2014). P4 is therefore a key

regulator of the timing of birth. Administration of RU486 later in gestation, at high doses, has been shown to cause PTB (Dudley *et al.* 1996).

Most of P4 actions have been determined from extensive studies in mice. However there are important differences in P4 dynamics across pregnancy between humans and mice. Firstly, the menstrual cycle in women and estrous cycle in mammals are acknowledged for their key differences in length and stages (Wang and Dey 2006). Secondly, the source of P4 production differs between human and mouse pregnancy. In many species including rodents, the corpus luteum of the ovary provides the main source of P4 throughout gestation. However, in humans, P4 is synthesised by placental trophoblast cells from mid-gestation, and secreted at high levels compared to all other mammals (Malassiné *et al.* 2003). The syncytiotrophoblast layer of the human placenta mediates various endocrine activities, whereas the placental labyrinth structure of the murine placenta completely lacks endocrine function. Finally, in humans, parturition is not characterised by a decline P4 levels like that seen in mice, but instead a functional withdrawal of P4 occurs, whereby PR-A and PR-B isoforms play distinct roles (Nadeem *et al.* 2016). While mouse models are essential to understanding the biology of pregnancy, these key differences must be appreciated when making inferences on human pregnancy from data generated in mice, and highlight the need for both animal and human studies to fully understand the role of P4 in normal and complicated pregnancy (Malassiné *et al.* 2003).

1.6.1. Progesterone regulation of immune cells and relevance to pregnancy immune adaptation

In addition to its well-documented physiological effects, P4 also possesses potent immunosuppressive properties, which aid in establishing appropriate immune adaptation to pregnancy (Druckmann and Druckmann 2005, Szekeres-Bartho 2009). Some of P4s effects on implantation success are mediated through anti-inflammatory action on immune cells. PR or its mRNA has been detected in various immune cells including T and B lymphocytes, macrophages and dendritic cells, confirming that P4 signalling through PR is important for normal immune cell regulation (Szekeres-Bartho *et al.* 1989, Pasanen *et al.* 1998, Butts *et al.* 2007).

Historically, P4 is known to influence human T cells by skewing their responses towards regulatory immune responses and away from inflammatory immune responses. Initial in vitro studies showed P4 could skew human peripheral blood $CD4^+$ T cell phenotypes from a Th1- to a Th2-type response and highlighted potential importance of this regulation during pregnancy (Piccinni *et al.* 1995, Piccinni *et al.* 2000). Exposure of maternal T cells to physiological doses of P4 decreases their proliferation and polyclonality, suppresses IFNγ, TNFα, IL10 and IL5 production and promotes IL4 production (Lissauer *et al.* 2015). Furthermore, P4 suppresses $CD4^+$ T effector responses, and promotes $Foxp3^+$ Treg cell

responses in cord blood T cells (Piccinni *et al.* 2000, Lee *et al.* 2011).Lymphocytes of pregnant women were shown to have increased PR expression compared to those of non-pregnant women (Szekeres-Bartho *et al.* 1989). Additionally, a link between PR expression and pregnancy pathologies associated with a lack of tolerance has been shown, with decreased peripheral blood lymphocyte expression of PR in observed in spontaneous abortion and in patients with clinical symptoms of threatened preterm delivery (Szekeres-Bartho *et al.* 1989).

In addition to $CD4^+$ and $CD8^+$ T cells, P4 also regulates γδ T cells, which express PRs (Szekeres-Bartho *et al.* 1999) plus innate immune cells discussed below. PR^+ γδ T cells increase in the peripheral blood of pregnant women and are thought to play an important role in pregnancy (Polgar *et al.* 1999).

1.6.2. Progesterone regulation of $CD4^+$ Treg cell responses

P4 mediates a major mechanism to suppress the $CD4^+$ T effector cell response and promote Treg cells. P4 effectively suppresses the generation Th1 cells and Th17 cells and induces Treg cell differentiation (Piccinni *et al.* 2000, Lee *et al.* 2011, Lee *et al.* 2012). Murine and human Treg cells induced by P4 have increased capacity to suppress the activation and expansion of effector T cells (Lee *et al.* 2011, Lee *et al.* 2012). P4 has also been shown to selectively repress *Ifng* gene expression via PR in $CD4^+$ T cells in mice (Hughes *et al.* 2013). This regulation is thought to be direct, as P4 was found to induce vitamin D receptor (VDR) gene expression in T cells, which is known to directly repress *Ifng* expression. Increased expression of VDR by P4 allowed highly sensitive regulation of T cell by vitamin D, allowing for enhance induction of Tregs and suppression of Th1 and Th17 differentiation (Xin *et al.* 2014, Thangamani *et al.* 2015).

There is additionally evidence of E2 and P4 regulated fluctuations in uterine Treg cell populations over the course of ovulatory cycles in preparation for pregnancy in women (Arruvito *et al.* 2007, Kallikourdis and Betz 2007) and mice (Caligioni 2009). P4 action on Treg cells has been investigated in the context of mouse pregnancy. Physiological pregnancy levels of P4 were found to increase the functional population of $CD4^+Foxp3^+$ cells in pseudopregnant mice (Mao *et al.* 2010). Moreover, in this study P4 increased splenic $CD4^+Foxp3^+$ cell proportions in mid gestation, and this was prevented by the addition the PR antagonist, RU486. In addition, PR was expressed by Treg cells and this expression was increased compared to PR expression in $CD4^+$ T cells (Mao *et al.* 2010).

Despite evidence for P4 regulation of Treg cells, a detailed understanding is lacking of the P4-Treg interplay during pregnancy and its importance for pregnancy success. In particular, the timing and mechanisms of P4 regulation of Treg cells is largely unknown, particularly in the context of early

pregnancy, when both P4 and Treg cells are known to play key roles in mediating embryo implantation and eliciting a tolerogenic environment for fetal growth. The potential role of P4 in the regulation of Treg cell responses in early mouse pregnancy is summarised in Figure 1. ***In this study, the P4 regulation of Treg cells in early pregnancy will be investigated.***

1.6.3. Progesterone regulation of innate immune cells

In addition to adaptive regulation, innate immune subsets are shown to be regulated by P4 in mice and women. P4 blocks the cytotoxic function of human peripheral blood NK cells, through suppression of NK cell degranulation (Laskarin *et al.* 2002). P4 inhibits DC maturation and promotes immature, tolerogenic DCs, which limit proliferation of activated T cells (Arck *et al.* 2007), but promote Treg cell expansion (Steinman *et al.* 2003).In rodents it was found that DCs express PRs and these receptors can mediate the effects of P4 (Butts *et al.* 2007, Butts *et al.* 2010). P4 was shown to regulate uterine DCs via PR, and this regulation was dependent on estrous cycle stage (Butts *et al.* 2010). P4 regulation of uterine DCs may be required for antigen presentation in early pregnancy leading to induction of paternal antigen-specific Treg cells, as one of the genes downregulated on day 3.5 pc in the uterus of mice administered RU486 in peri-implantation was MHC II (Cheon *et al.* 2002).

P4 suppresses activation of M1 and M2 murine macrophages in vitro (Menzies *et al.* 2011) and regulate macrophages via membrane-bound PRs (Lu *et al.* 2015). In the context of organ transplantation regulatory macrophages were shown to induce contact dependent differentiation of $Tigit^{+}Foxp3^{+}$ Treg cells capable of suppressing T cell immunity (Riquelme *et al.* 2018). Interestingly, this induction was dependent on progestogen associated endometrial protein (PAEP), which was found to be selectively upregulated in regulatory macrophages. In women, PAEP is found in both the decidua and seminal fluid, and plays an important role for embryo implantation and fetal maternal tolerance in early pregnancy (Lee *et al.* 2016). Expression of this key protein by uterine macrophages may therefore directly influence macrophage-T cell interactions necessary for Treg cell differentiation and tolerance to embryo implantation. Using a human monocyte cell line, the progestin Medroxyprogesterone acetate (MPA) was shown to cause differentiation of M2 macrophages of a decidual phenotype, which promote decidualisation of endometrial stromal cells and trophoblast invasion (Tsai *et al.* 2017).

1.6.4. Indirect mechanisms of P4 regulation of immune responses

P4 action on immune cells can occur through direct regulation of cells by P4 signalling, but it can also occur through indirect mechanisms, through effects in other cell subtypes. For example, stromal and epithelial cell regulation by P4 may affect T cells in pregnancy. P4 action represses NF-kB and MAPK

signalling in the uterus to suppress prostaglandin production and in turn inhibit pro-inflammatory cytokine production and leukocyte recruitment (Tan *et al.* 2012). PR regulates P4 action on uterine stromal and epithelial cells and is expressed temporally in these cell types in early pregnancy (Franco *et al.* 2012, Wetendorf and DeMayo 2012). One study showed that P4 promoted Treg cells and decreased Th17 cells via a mechanism partly dependent on thymic stromal lymphopoetin (TSLP) expressed by epithelial cells and keratinocytes (Xu *et al.* 2013). PRs are expressed additionally in the thymus, by thymic epithelial cells, and were shown to be essential for pregnancy success as they were required for the reversible thymic involution that occurs in pregnancy (Tibbetts *et al.* 1999). P4 is thought to limit thymic output of new $CD4^+$ and $CD8^+$ T cells, which is increased following ovariectomy in rats and reversed with P4 supplementation (Leposavic *et al.* 2014). There is some debate about the functional impact of hormonally-induced thymic involution during pregnancy, since recent-thymic emigrant (RTE) Treg cells are elevated in normal human pregnancy and perturbed in preeclampsia (Wagner *et al.* 2016, Hellberg *et al.* 2019). However, P4 regulation of thymocytes and thymic output likely has relevance for maternal immune tolerance.

1.7. SUMMARY, AIMS AND HYPOTHESES

Recent work suggests that events in the peri-implantation period are crucial for successful pregnancy in later gestation, and that immune dysregulation in this period may lead to complications in later pregnancy. As Treg cells are shown to be essential for early pregnancy in mice and are implicated in unexplained infertility and pregnancy complications, this suggests that an important component of Treg regulation occurs in peri-conception and early pregnancy. Therefore, investigation of Treg cell regulation in this period is warranted. Several studies show that paternal antigen-specificity of Treg cells can only partially explain their involvement in pregnancy. Maternal T cells are regulated by cytokines in seminal fluid as well as maternal hormones and cytokines. P4 is a candidate regulator of Treg cells in pregnancy but the specific role of maternal P4 in regulating early pregnancy Treg cells is not known. Several studies have shown a positive effect of P4 in promoting Treg cell number and function, however the physiological details of this regulation is not well understood, and no studies have investigated the regulation of Treg cells by peri-conceptional progesterone in vivo throughout pregnancy. Furthermore, the timing, mechanisms and importance of P4-Treg interplay for the overall pregnancy success in undefined. Here,

using various in vivo and in vitro models, we will investigate the role and importance of P4 in regulating Treg cells for pregnancy success,

Secondly, Treg cell generation and action is essential for pregnancy success, however, a firm understanding of the mechanisms of Treg cell phenotype commitment in pregnancy is lacking. Despite anti-inflammatory Treg cells predominating throughout gestation, the events occurring in late gestation prior to parturition, as well as in pregnancy pathologies, are characterised by an inflammatory state of immune activation. This includes increased activation of effector T cells that can produce inflammatory mediators. It is now appreciated that T cells exhibit a high degree of phenotypic plasticity and may possess lineage instability. The stability of Treg cells during pregnancy has not directly been addressed, but is relevant to our understanding how Treg cells are regulated in both normal and pathological pregnancies. To definitely measure Treg cell stability, the use of transgenic models allowing for the fate of Treg cells to be tracked in vivo can be employed. Various Treg fate-mapper models have been used to study Treg cell stability in the context of autoimmunity and inflammation (Zhou *et al.* 2009, Miyao *et al.* 2012). In this study, we will use a Treg fate-mapper model to investigate Treg cell stability during pregnancy.

The hypotheses of this project are:

1. P4 strengthens Treg cell phenotype and number in early pregnancy, which is crucial for the establishment and maintenance of maternal tolerance necessary for pregnancy success
2. The stability of Treg cells is crucial for the maintenance of fetal-maternal tolerance

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

1. Investigate the role of early pregnancy progesterone exposure in regulating Treg cells in vivo, and the significance of this for pregnancy success;
2. Investigate mechanisms of progesterone action in $CD4^+$ T cells and Treg cells;
3. Evaluate the stability of Treg cells throughout pregnancy, using a Treg fate-mapping mouse model

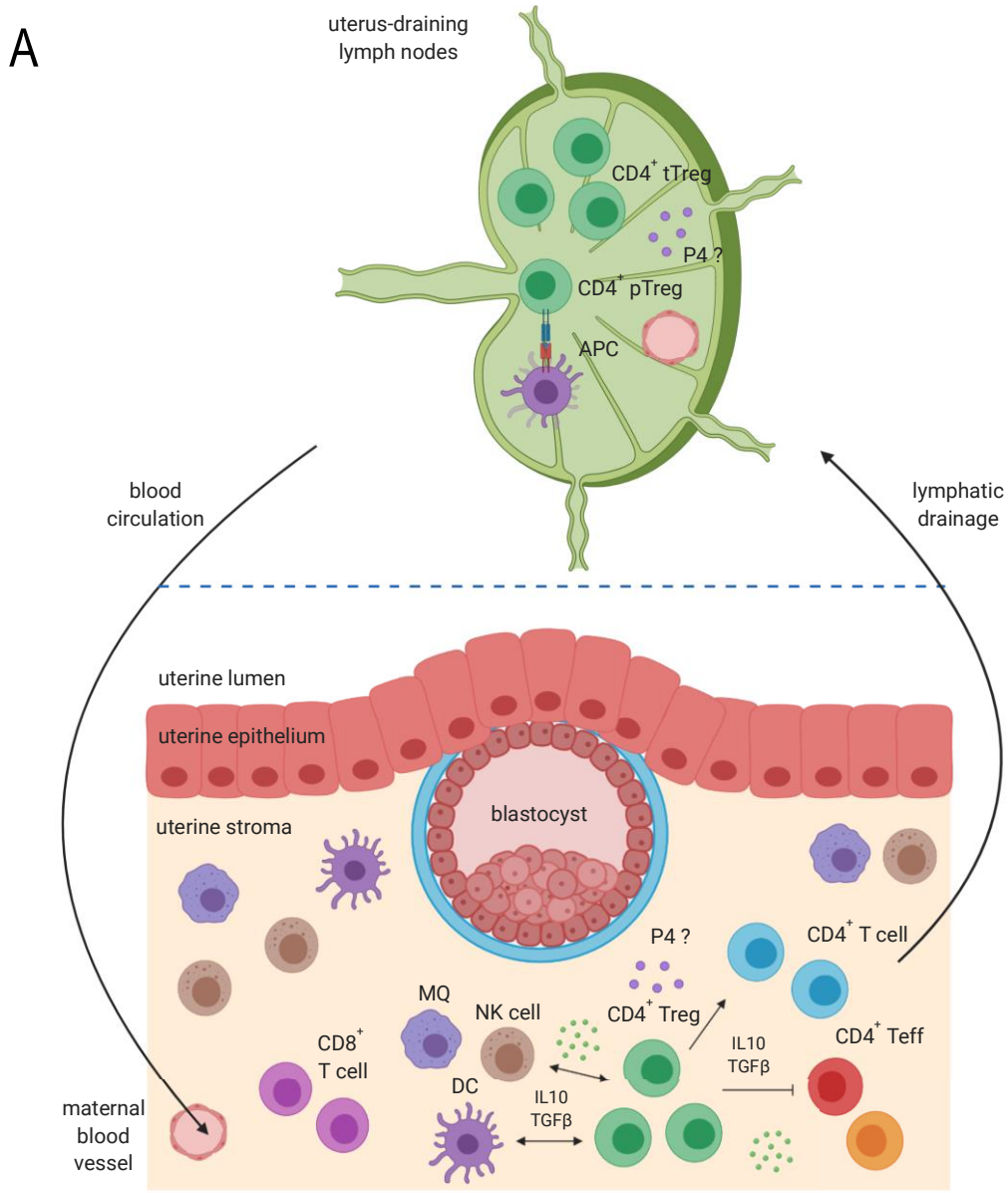

Figure 1.1 Current understanding of Treg cell involvement in early pregnancy maternal immune adaptations and possible role for progesterone in the regulation of Treg cells in the uterus and draining lymph-nodes.

(A) Treg cells are activated and expanded in the uterus-draining LNs (udLN) in early pregnancy. In the peri-conception period, thymic-derived Treg (tTreg) cells traffic to udLNs in response to hormones and cytokines. Following mating, paternal antigens delivered to the female reproductive tract are processed by maternal antigen-presenting cells (APCs) such has tolerogenic dendritic cells (DCs). In the udLN APCs present paternal antigen to naïve $CD4^+$ T cells and activate them to become paternal antigen-specific peripheral Treg (pTreg) in early pregnancy. Treg cells. Treg cells mediate maternal-fetal tolerance by suppressing inflammatory responses and promoting tolerogenic responses in a diverse range of target cells in the udLN and at the fetal maternal interface, where they can be recruited to following recirculation from the udLN. They secrete the immunosuppressive cytokines IL-10 and TGFβ that inhibit proliferation and cytokine production of pro-inflammatory Th1 and Th17 cells, cytotoxicity of NK cells and maturation and function of APCs including immunogenic DCs and M1 macrophages. They also interact with and promote tolerogenic cell phenotypes, for example tolerogenic DCs and M2 macrophages. The hormone progesterone (P4) promotes Treg cell expansion and is a candidate regulator of Treg cells in pregnancy, however the details of this regulation is largely unknown. P4 may function in the udLN and/or locally in the uterus to influence T cell phenotype, function and stability. Image concept adapted from Mor *et al.,* (Mor *et al.* 2017) and created with Biorender.com. Image not drawn to scale.

Chapter 2

Materials and methods

2.1. MICE

2.1.1. Housing and maintenance

All mice were housed in the specific pathogen-free conditions at the University of Adelaide Medical School Animal House (MSAH) or CSIRO barrier research facilities. All mice were kept on a 12h light/dark cycle and food and water were provided *ad libitum*. Mice were fed a diet of 10% fat chow (Envigo, Cambridgeshire, UK). All animal experiments were approved by the University of Adelaide Animal Ethics Committee (ethics numbers #M-2014-22, #M-2015-115 and #M-2016-185) and conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes 8th edition 2013. All experiments utilised females between 8 and 16 weeks of age at the time of mating or treatment. Females were virgin before the onset of experiments.

2.1.2. Strains

2.1.2.1. C57Bl/6

C57Bl/6 (B6) female mice (B6/h; Harlan sub-strain) were purchased from the University of Adelaide Laboratory Animal Services (LAS) and were used to establish the model of RU486 administration in early pregnancy (Chapter 3). After it was established that the B6/h strain was a sub-strain, B6 (C57Bl/6J; Jackson sub-strain) mice were purchased from Animal Resource Centre (ARC, Perth, Western Australia), and used for all experiments. Where possible, experiments performed using B6/h mice were replicated with the B6/J strain, with no major differences between strains observed for the immune and pregnancy parameters measured in this study.

2.1.2.2. Ly5.1

Mice harbouring the naturally occurring congenic CD45.1 (Ly5.1, *Ptprca*) allele, B6.SJL-Ptprca Pepcb/BoyJ (Ly5.1), were purchased from ARC. These mice are on B6 background but express a different isoform of CD45 (Ly5), compared with standard Ly5.2-expressing C57Bl/6 mice. This strain was utilised in adoptive T cell transfer experiments to enable the in vivo tracking and visualisation of donor T cells in congenic recipient mice (Chapter 4).

2.1.2.3. Rosa26RFP

B6.Cg-Gt(ROSA)26Sor$^{tm(CAG-tgTomato)Hze}$/J (Rosa26RFP) mice, originally generated by Madisen *et al.,* (Madisen *et al.* 2010), were purchased from The Jackson Laboratory (Bar Harbor, ME, USA; ID#007914).

These mice harbour a targeted mutation of the Gt(ROSA)26Sor (Rosa26 or R26) locus with a loxP-flanked STOP cassette preventing transcription of a CAG promoter-driven red fluorescent protein variant, tdTomato (referred to herein as RFP). Under the control of the Rosa26 constitutive promoter, RFP is expressed following Cre-mediated recombination to excise the STOP cassette. Thus, when crossed to a cre-expressing strain, these mice provide conditional and constitutive RFP expression and are therefore useful for tracking cell fate in specific cre-expressing transgenic lines. A colony of homozygous mutant Rosa26RFP mice (RFP/RFP) was established at the University of Adelaide Medical School Animal House (MSAH). The colony was maintained by crossing homozygous RFP/RFP breeders to create offspring of the same genotype.

2.1.2.4. Foxp3GFPCre

Mice expressing the fluorescent marker green fluorescent protein (GFP) and the recombinase Cre under the control of the Foxp3 promoter, B6129S-Tg(Foxp3-EGFP/cre)1aJbs/J (Foxp3GFPCre), were purchased from The Jackson Laboratory (ID#023161). Originally generated by Professor Jeffrey Bluestone (University of California, San Francisco), these mice enable reliable detection of Foxp3 expressing cells using GFP as a reporter (Zhou *et al.* 2008). Due to the presence of the Cre recombinase, these mice also enable Treg-specific modification of conditional genes when crossed to mice expressing loxP-flanked genomic DNA. Chapter 6 describes the Foxp3GFPCre mouse model in detail. Mice were generated by bacterial artificial chromosome (BAC) transgenesis, whereby a BAC containing the entire Foxp3 gene was modified by inserting an Enhanced GFP (eGFP) fused to a humanised Cre recombinase coding sequence. The BAC transgene was inserted into NOD/ShiLtJ fertilized oocytes and maintained on the same background. Foxp3GFPCre mice were then backcrossed to the B6 strain for 8 generations, before being maintained by crossing to B6126SF1/J.

A breeding colony was established and maintained at University of Adelaide MSAH initially by crossing mice hemizygous for the Foxp3GFPCre transgene (GFP^+). The Jackson Laboratory report this strain is difficult to breed on the B6 background and some males carrying the GFP transgene are infertile. We observed the strain breeds poorly even on the mixed B6126SF1/J background. Also consistent with reports from Jackson Laboratory on this strain, we observed hemizygous pups to be smaller in size than their wild-type (WT) littermates, with females being notably smaller even post weaning. Hemizygous females took longer to reach reproductive age and hemizygous x hemizygous breeding was often unsuccessful. To enable reproductive success, the colony was maintained by crossing hemizygous mice with wild-type littermates. Better breeding was observed when the male breeder was hemizygous as opposed to the female breeder. Breeding trios of 2 females and 1 male were set up to maximise breeding

efficiency and provide co-parenting for offspring. Increased breeding efficiency was observed when cages were provided with enhancement such as crinkle nest bedding. Additionally, wet food was provided to GFP^+ offspring to support their growth.

2.1.2.5. Foxp3GFPCre.R26RFP

The Treg cell fate-mapping strain, Foxp3GFPCre.R26RFP, allows the fate of Treg cells to be tracked in vivo. This strain was created by crossing Rosa26RFP mice to Foxp3GFPCre mice. The Cre recombinase driven by the *Foxp3* promoter causes constitutive RFP expression, marking cells that currently and previously expressed Foxp3. This, combined with the GFP reporter for Foxp3 expression, enables past and present Treg cells to be detected, whereby current Treg cells are GFP^+RFP^+, and cells previously expressing Foxp3 (exTregs) are GFP^-RFP^+. Chapter 6 describes the Treg fate mapping model in detail. Similar Treg cell fate-mapping models have been generated using a combination of the particular Cre/lox reporter strains used in this study and other strains (Zhou *et al.* 2009, Rubtsov *et al.* 2010, Komatsu *et al.* 2014). A breeding colony of Foxp3GFPCreR26RFP mice was established and maintained at University of Adelaide MSAH. The strain was initially established by crossing Rosa26RFP homozygous females with Foxp3GFPCre hemizygous males. The resultant offspring were screened for the GFP transgene and used for breeders. The GFP^+ x non-carrier breeding strategy was used as above (2.1.1.4). Due to the poor breeding observed in the Foxp3GFPCre strain as described above (2.1.1.4), the Foxp3GFPCre.R26RFP strain also exhibited poor breeding. All breeders in the colony were RFP hemizygous (RFP/+) or homozygous (RFP/RFP) and ongoing breeding was performed to achieve complete RFP homozygosity within the colony. Validation of the model by flow cytometry to measure GFP and RFP transgene expression was performed prior to experiments (Chapter 6). Experimental females were GFP^+ and RFP/RFP.

2.1.2.6. PRLacZ

Mice with a targeted insertion of the LacZ Reporter in the murine progesterone receptor (*Pgr*; PR) gene (PRLacZ) were originally provided by Prof. John Lydon (Baylor College of Medicine, Houston, TX, USA) (Ismail *et al.* 2002). The insertion of LacZ disrupts the transcription of both isoforms of PR (PR-A and PR-B). Thus, mice homozygous for LacZ insertion are a phenocopy of the well described PR deficient (PRKO) strain (Lydon *et al.* 1995) and are referred to herein as $PR^{-/-}$. Mice heterozygous for the lacZ insertion ($PR^{+/-}$) are a phenocopy of WT mice. $PR^{-/-}$ mice are infertile, but importantly, $PR^{+/-}$ exhibit normal fertility. A breeding colony of PRLacZ mice was established and maintained at the University of Adelaide MSAH by Assoc. Prof. Rebecca Robker and members of the Ovarian Biology Laboratory. Mice were maintained

on a mixed B6126SF1/J background. WT, $PR^{+/-}$ and $PR^{-/-}$ mice were generated by crossing $PR^{+/-}$ mice at every generation

2.1.2.7. BALB/c

BALB/c male mice, purchased from ARC, were used as stud males in all mating experiments. BALB/c males were separated from cage mates for at least one week prior to set up as stud males. Stud males were proven to be fertile before mating experiments were conducted and each male was used for multiple studies in the Robertson Laboratory. Stud males were aged between 10 weeks and 12 months.

2.1.3. Genotyping

Genotyping was performed for Rosa26RFP, Foxp3GFPCre, Foxp3CreR26RFP and PRLacZ mice, either to establish the colony, for ongoing colony maintenance, or to screen for experimental mice. The genotype of each mouse in the colony was determined by diagnostic PCR of ear tissue DNA. Ear notches from individual colony mice were taken at the time of weaning and stored at -20°C until DNA extraction.

Either traditional DNA extraction and purification methods, or quick extraction methods were used. For traditional DNA extraction, genomic DNA was isolated by digesting tissue in 250 µL digestion solution (20 nM EDTA, 50 mM Tris, 120 mM NaCl, 1% [w/v] SDS, pH 8.0) and 5 µL proteinase K (10 mg/mL; Promega, Fitchberg, WI, USA) at 55°C for 4h or overnight on a shaking incubator (180 rpm). Cellular debris and proteins were precipitated out using 250 µL 4 M ammonium acetate (Sigma-Aldrich, Sigma; St. Louis, MO, USA) and absolute ethanol was used to precipitate the DNA. The resulting pellet containing the isolated DNA was washed in 70% ethanol, air-dried and resuspended in 300 µL Milli Q (MQ) water at 37°C with gentle shaking for 1 hr.

For quick extraction, the QuickExtract™ DNA Extraction Solution (Lucigen, Middleton, WI USA) was used. Briefly, 150 µL of QuickExtract™ was added to tubes containing ear notch samples. Samples were vortexed and incubated in a heat block at 65°C for 10 min. Following this, samples were vortexed and transferred to a heat block at 98°C for 2 min to complete the extraction procedure.

Genotyping PCR was performed in 0.6 thin-walled PCR tubes (Axygen, Union City, CA, USA) using an Applied Biosystems GeneAmp® PCR system 9700 Thermal Cycler (Applied Biosystems, Foster City, CA, USA). All primers used for PCR amplification are listed in Table 2.1. All primers were purchased from GeneWorks (Adelaide, South Australia). Primers were diluted to 20 µM in sterile MQ upon arrival and stored at -20°C. For Rosa26RFP and Foxp3GFPCre genotyping, PCR was performed according to Jackson Laboratory protocols for each strain. For Foxp3Cre.R26RFP genotyping, both protocols were run to determine the genotype for both GFP and RFP. Reactions were prepared using the KAPA 2G Robust

HotStart PCR Kit (Sigma) using the follow components; KAPA 2G buffer (1.0 x), MgCl2 (2 mM final), dNTPs (0.2 mM final), primers (Table 2.1; 0.5 µM), KAPA 2G Taq polymerase (0.01U/µl) and MQ water. 10 µL of buffer and 2 µL genomic DNA was used for a total volume of 12 µL per reaction. PCR cycling conditions were as follows: 1 x 94°C for 2 min; 10 x [94°C for 20 sec, 65°C for 15 sec with a -0.5°C decrease per cycle, 68°C for 10 sec]; 28 x [94°C for 15 sec, 60°C for 15 sec, 72°C for 10 sec]; 72°C for 2 min; and hold at 10°C. Following the PCR, 6 x Gel loading dye (Thermo Fisher Scientific, Waltham, MA, USA) was added to samples (1 x final) for visualisation of products by gel electrophoresis.

For PRlacZ genotyping, PCR reactions were prepared with GoTaq® Flexi DNA polymerase and associated buffers (Promega). Reactions contained the following components; 5 x Green GoTaq Flexi buffer (1.0 x), MgCl2 (2 mM final), dNTPs (0.25 mM final), primers (Table 2.1; 1.2 µM for PR1a and PR3 and 0.6 µM for PR2), Go Taq polymerase (0.025 U/µl) and MQ water. 18.4 µL of buffer and 1.6 µL genomic DNA was used for a total volume of 20 L per reaction. PCR cycling conditions were as follows: 1 x 95°C for 2 min; 35 x 95°C for 1 min, 60°C for 30 sec and 72°C for 1 min hold 72°C for 10 min; and hold at 15°C.

Genotypes were determined by separating PCR products on a 2% (w/v) agarose (Promega) gel containing 1 x Gel Red® nucleic acid stain (Biotium, Hayward, CA, USA) in 1 x Tris/Borate/EDTA (TBE) buffer at 80 -100 V. Samples were run alongside a DNA ladder; Benchtop 100 bp DNA ladder (Promega) or pUC19 DNA/Msp1 Marker (Thermo Fisher Scientific) to confirm the size of resolved DNA fragments. Gels were visualized and photographed using the Bio-Rad Gel Doc™ EZ system (Bio-Rad, Hercules, CA, USA) and Image Lab software (Bio-Rad). The genotype of each mouse was determined using controls of known genotype. Table 2.1 depicts expected products for each strain, and Figures 2.1 - 2.3 show representative gels with detectable genotypes for Rosa26RFP, Foxp3CreGFP and PRLacZ mutations, respectively.

2.2. IN VIVO STUDIES

2.2.1. Mating

For all mating and pregnancy experiments, female mice were mated to MHC-disparate (allogeneic), proven fertile BALB/c stud males. Naturally cycling virgin females were housed with BALB/c studs overnight for mating to occur (maximum 2 females per stud). Female mice were checked between 0800-1100 hrs for the presence of a copulatory plug at the vaginal opening which is indicative of a mating event.

The presence of a plug was designated as day 0.5 post-coitus (pc). “Day pc” is used hereafter to indicate the number of days following the mating event and therefore in pregnant females also corresponds to day of gestation. Mated or “plugged” females were separated from the stud male and placed in a new cage. Females that mated on the same night were housed together, with a maximum of 3 females per cage.

2.2.2. Estrous cycle tracking

In all experiments with non-pregnant females, and in most mating experiments, the estrous cycle (reproductive cycle) of each female was tracked by daily vaginal smearing. To determine the stage of estrous cycle, the cellular composition of wet mounts of vaginal lavage fluid (‘vaginal smear’) was analysed (Byers et al., 2012). Vaginal smears were performed by lavage (gently using a pipette) of the vagina with 20 µl phosphate-buffered saline (PBS; Gibco, Thermo Fisher Scientific). Smears were deposited on SuperFrost glass slides (HD Scientific Supplies Pty Ltd, Sydney, New South Wales) and coverslipped. Slides containing vaginal smears were analysed under a phase contrast microscope (Olympus, Adelaide, South Australia) to determine the stage of estrous. Estrous cycles of were tracked for at least 1 full cycle (4-5 days) before females were utilised in experiments.

For mating studies, mice in the ‘proestrus’ stage of their cycle (the stage prior to ovulation) are known to be receptive to mating and were placed with a stud male when determined to be in this stage. For studies involving non-pregnant mice, females were used for experiments at specific stages of their estrous cycle as specified in text, to control for hormonal influences.

To maximise the number of females at particular stages of pregnancy for experiments containing flow cytometry analyses, a large number of animals were required to be mated on the same night. To achieve this, estrous cycles were tracked until enough females were in proestrus and then females were placed with stud males on the same night to yield a sufficient number of mated females. To increase the likelihood of females being in the same stage of their estrous cycle on the same day, soiled bedding from genetically distinct (BALB/c) males was added to female cages daily, for at least three days. Known as the “Whitten Effect”, this technique synchronises estrous cycles of females housed together and encourages ovulation (Whitten 1958).

2.2.3. Treatments

2.2.3.1. RU486 treatment

To reduce progesterone (4-pregnene-3, 20-dione; P4) signalling in early pregnancy the P4 antagonist, RU486 (Mifepristone; Sigma), was administered to mated B6 females on days 1.5 and 3.5 pc. A 20 mg/mL

RU486 stock was prepared by dissolving 20 mg RU486 in 1 mL absolute ethanol (EtOH). The solution was vortexed until completely dissolved and stored at -20°C until use.

On the day of injection, the RU486 stock was diluted in sesame oil (Sigma) to a range of concentrations. In initial experiments to determine an optimal dose (Chapter 3), RU486 was prepared at the following concentrations: 0.1 mg/ml (final dose 0.5 mg/kg); 0.2 mg/mL (1 mg/kg); 0.4 mg/mL (2 mg/kg); 0.8 mg/mL (4 mg/kg) and 1.6 mg/mL (8 mg/kg). In all subsequent studies (Chapters 3 and 4), the 1 mg/kg dose was used. A control group of 1% absolute EtOH diluted in sesame oil was used in all studies.

Injections were performed at between 1000 h and 1400 h. RU486 suspensions were vortexed for 10 sec prior to loading of suspensions into a 1 mL syringe. Air bubbles were removed and a 26 gauge needle was applied to the syringe. Mice were injected with RU486 subcutaneously in the neck scruff. Mice were weighed prior to injection and 5 µL/g body weight (i.e. 100 µL for a 20 g mouse) was injected to yield the mg/kg doses specified. Care was taken to avoid excessive oil from leaking out of the injection site. Animals treated with RU486 were killed at various days of pregnancy for analysis (days 4.5, 6.5, 9.5, 16.5, 18.5 pc) or allowed to give birth. In some experiments, RU486-treated mice were administered with either T cells via adoptive transfer or with lipopolysaccharide (LPS) in late pregnancy, as described below.

2.2.3.2. Lipopolysaccharide treatment

To induce an inflammatory challenge in late-gestation, LPS (Escherichia coli 0111B4 serotype; Sigma) a component of gram negative bacteria, was administered to pregnant B6 females on day 16.5 pc. A protocol for administration of low-dose LPS treatment to induce inflammatory stress during pregnancy is well established within the Robertson Laboratory. LPS stock was prepared by diluting to 1 mg/mL in PBS + 0.1% BSA (Bovine Serum Albumin, Sigma). Aliquots were frozen at -20°C until use. On the day of injection, LPS was diluted to 10 µg/mL in 200 µl PBS containing 0.1% BSA. Thawed LPS aliquots were discarded after use. At 1200 to 1300 hrs, pregnant mice were weighed and 2 µg (200 µL) was injected intraperitoneally. Control mice were injected with PBS containing 0.1% BSA. Dams administered LPS were analysed in late gestation (d18.5 pc) or at birth as described in 2.2.5 and 2.2.6, respectively.

2.2.3.3. T cell adoptive transfer

Treg ($CD4^+CD25^+$) non-Treg ($CD4^+CD25^-$) cells isolated from pregnant mice (described in 2.3.3) were adoptively transferred to RU486-treated B6 mice, 8-10 h following the final RU486 injection on day 3.5 pc. Treg or non-Treg cells were suspended in sterile 1 x PBS containing 1% fetal bovine serum (FBS, Gibco) at 2 x 10^6 cells/mL. Cell suspensions were kept on ice until injection. Suspensions were loaded into 0.5 ml 29 gauge Ultra-Fine™ insulin syringes (BD) and any air bubbles were carefully removed. Mice

were prepared for injection by brief warming using a heat lamp or advanced animal warming system (Datesand, Manchester, UK) followed by placement in a homemade restraint exposing the tail of the mouse. The tail was wiped with 70% EtOH to enable better visualization of the tail vein. 2 x 10^5 cells (100 µL) were administered intravenously via tail vein injection. Previous studies demonstrate this dose of $CD4^+CD25^+$ Treg cells is sufficient to improve pregnancy outcomes in the abortion prone CBA/2 x DBA/J mouse model (Zenclussen *et al.* 2005, Zenclussen *et al.* 2006). Vehicle control mice were injected with 100 µL sterile PBS containing 1% FBS. Following the procedure, pressure was applied briefly to the site of injection, and mice were monitored for signs of distress. Mice transferred with T cells were analysed (pregnancy outcomes and/or flow cytometry) either on day 6.5 pc (Chapter 4), day 18.5 pc (Chapter 4) or at birth.

2.2.4. Blood and tissue collection

2.2.4.1. Blood and serum isolation

For terminal procedures involving blood collection, cardiac bleeds were performed. Mice were first anaesthetised by intraperitoneal injection of 15 µL/g body weight of 2% Avertin (2.2.2-tribromoethanol; Sigma-Aldrich). Once under deep anaesthesia, blood was harvested by cardiac puncture using a 20 gauge needle and 1 mL syringe. Following the procedure mice were euthanized by cervical dislocation. For flow cytometry experiments, blood was immediately added to 50 µL heparin sulphate (Sigma; prepared according to manufacturer's instruction) to prevent clotting and kept on ice for further processing (2.3.1.1.3). For experiments requiring serum for progesterone measurements, non-heparinised blood was left at room temperature (RT) for a minimum of 10 min before centrifugation at 2000 rpm for 10 min in a benchtop centrifuge. The resulting serum contained in the supernatant was aliquoted and frozen at -80°C for ELISA (2.3.4).

2.2.4.2. Spleen and lymph nodes

In cases where blood was not harvested, mice were euthanized via cervical dislocation prior to tissue collection. Spleen, uterus draining para-aortic lymph nodes (udLN) and other lymph nodes (inguinal, brachial, axillary, mesenteric) were carefully excised using scissors and forceps. udLN were visualised by their location between the uterine horns, and positioning surrounding the aorta. Three to four individual udLNs were isolated per mouse. Spleen and LNs were collected in petri dishes containing PBS or media for further processing, as described in 2.3.1.1.

2.2.4.3. Uterus

The uterus was isolated from non-pregnant and pregnant mice mouse at different stages of pregnancy. Uteri were removed by excision from the ovaries and cervix and fat and mesentery was trimmed from each uterine horn. In some cases outlined in text, uteri were collected, fixed in formalin (Sigma) using standard procedures and stored in 70% ethanol for future immunohistochemical processing and analysis.

For experiments where mated females were euthanized in early pregnancy prior to embryo implantation (d3.5 pc), blastocyst flushing was performed to confirm the presence of viable blastocysts, an indicator of mating and fertilisation. Briefly, uteri were collected in a petri dish containing PBS at incubated at 37°C. PBS was flushed though each uterine horn and the presence of blastocysts/unfertilized eggs was assessed using a dissecting microscope.

2.2.4.4. Decidua

Decidua was isolated from mice on day 16.5 and 18.5 of pregnancy according to 'The Guide to Investigation of Mouse Pregnancy: Implant Site Dissections' (Pang *et al.* 2014). Briefly, uteri was removed and each implantation site was isolated. The uterine muscle was removed with scissors, then the decidual tissue was removed from the placenta using forceps. The decidua was placed in PBS for further processing as described in 2.3.1.1

2.2.5. Assessment of gestational outcomes

2.2.5.1. Pregnancy rate, implantations and resorptions in early-mid gestation

Mice were weighed on day 0.5 pc following the presence of a copulatory plug, and at various stages following mating. If no weight gain was evident, mice were sacrificed on their prospective experimental day and the uterus was examined for evidence of pregnancy and resorptions.

For experiments prior to day 5.5 pc, implantations sites were not able to be visualised by unaided inspection of the uterus. On day 6.5, implantations sites were able to be visualised by inspection of the uterus. Implantations were visualised as small, rounded "bumps" in the uterus. For experiments from day 9.5 pc, the number of implantations and resorbing/failing implantation sites were visualised and quantified. Implantations were classified as either viable or resorbing/non-viable depending on characteristics including size and appearance. Resorbing or failing implantation sites were classified as implantation sites that were significantly smaller in size and/or paler in colour in comparison to the other implantation sites. As described by Pang *et al.* in 'The Guide to Investigation of Mouse Pregnancy' (Pang *et al.* 2014) these implantation sites have a very high chance of fetal resorption prior to birth.

2.2.5.2. Maternal, fetal and placental late-gestational outcomes

On day 16.5 or 18.5 pc pregnant mice were euthanised for experimental analyses. The uterus from each female was removed, and the number of viable, dead and resorbed implants was recorded. A pregnant dam was defined by the presence of at least one viable pup. However, data from females with complete resorptions or non-viable implants was also recorded. Resorptions were implantation sites characterised by their discoloured appearance (haemorrhagic or black) and failed development.

Each implantation was dissected and the uterine wall removed with scissors. The placenta was removed and the fetus was dissected from the fetal membranes and umbilical cord. Viable fetal and placental weights were recorded and expressed as absolute numbers and a ratio (fetal:placental weight ratio). In some studies, the placenta was fixed in formalin, using standard procedures for future histochemical and immunohistochemical analyses such as Masson's Trichrome staining.

2.2.6. Monitoring of birth and tracking of fetal growth trajectories

Gestation length of pregnant B6 mice was measured using video recording with a Sony HandyCam Video Camera with nightshot mode capability (Sony, Tokyo, Japan). Animal housing IVC bottoms were placed on the floor with Perspex sheets containing perforations placed on top. Dams were given adequate food and hydration via a NECTA H20 gel pack (Able Scientific, Adelaide, South Australia) for hydration for the duration of filming. The tripod and video camera was set up above the mice. Late pregnant dams were monitored for the time of parturition and the number of pups born. Timing of birth was designated after delivery of the first pup in the litter. The labour process generally lasted for 30-60 min. The viability of pups was recorded and pup birth weight was measured at 12–24 hrs after delivery. Pup viability and weight was also assessed at 7 days postpartum, and at 21 days postpartum, where the number, sex, and weight of surviving pups was recorded. On 21 days postpartum mother and pups were humanely euthanised.

2.3. EX VIVO AND IN VITRO STUDIES

2.3.1. Flow cytometry

2.3.1.1. Tissue processing and sample preparation

Mice in estrus/proestrus and on various days post coitus were killed and immune and reproductive tissues were collected as described in (2.5). Tissues were processed and single cell suspensions were prepared

as described below for different tissues. Cells were prepared and washed in either PBS or media depending on the experiment to follow. For experiments examining cytokine production by ex vivo stimulation, complete RPMI-1640 media (cRPMI) media or complete Iscove's Modified Dulbecco Media (cIMDM) media was used to prepare single cell suspensions. cRPMI consisted of RPMI (Gibco) supplemented with 10% FBS and 2% Penicillin/Streptomycin (Gibco). For studies with a focus on Th17 cells (e.g. T cell differentiation assays, Treg fate-mapping experiments) cIMDM was used to prepare cells. cIMDM was made by supplementing IMDM with 10% FBS (Gibco), 100 U/mL Penicillin/Streptomycin and 0.002 M L-Glutamine (Gibco).

Spleen

Spleens were homogenized by gentle crushing through a 70 μm cell strainer (BD Biosciences, San Diego, CA, USA) using the plunger end of a 5 mL syringe (BD) into a petri dish containing ≥ 2 ml PBS/media. The suspension was transferred to a 15 mL tube and resuspended to a total of 10 mL PBS/media before centrifugation at 300 g for 6 min to pellet the splenocytes. Supernatant was discarded and erythrocytes were lysed from by incubating suspensions in 5 mL of pre-warmed 1 x mouse red blood cell lysis buffer (0.155 M NH_4Cl, 10 mM $KHCO_3$, 99.2 μM EDTA disodium salt in RO water) at 37°C in for 5 min. 5 mL of PBS was added to stop the reaction and samples were washed and resuspended to 10 mL for cell counting.

Lymph nodes

Lymph nodes (LNs) were gently crushed between the frosted ends of two SuperFrost glass slides (HD Scientific Supplies Pty Ltd, NSW, Australia) moistened with PBS or media, into a petri dish containing >2 mL PBS or media. Suspensions were transferred to 15 mL tubes and resuspended to 10 mL for centrifugation as described above. The supernatant was discarded and suspensions were resuspended to 1-2 mL for cell counting.

Blood

Lymphocytes were immediately isolated from the blood using Lympholyte™ (Cedarlane, Hornby, Canada), according to the manufacturer's instructions. Briefly, Lympholyte was layered underneath the blood using a glass Pasteur pipette, the sample was centrifuged for 20 min at 600 g at 25ºC and the interface layer containing lymphocytes was collected and transferred to a separate tube and washed in cRPMI.

Uterus and decidua

Uterus and decidua samples were digested enzymatically prior to further processing. Samples were mechanically disrupted using scissors before addition to 1-2 mL 2% FBS in RPMI supplemented with 0.1% DNAse I (Sigma) and 10% of 10 mg/mL collagenase type I (Sigma). Samples were incubated at 37°C with constant shaking for 1 hr. 50 µL of 0.1 M EDTA was added to each sample before incubation for a further 10 mins. Samples were placed on ice and chunks of tissue and viscous tissue debris were removed from the samples by straining through a 70 µM strainer. Samples were underlayed with 500 µL of FBS supplemented with 10% EDTA and centrifuged at 1000 g for 10 min to pellet viable cells. Following this, in late gestation uteri and decidua samples, erythrocyte lysis was performed (as described for spleen) prior to washing again and cell counting.

In late pregnancy experiments, this protocol was further optimised to improve the elimination of contaminants for FACS staining (such as auto-fluorescent uterine cells) and enrich specifically for leukocytes by using Lympholyte™ as described in 2.3.1.1.2, following the cell filtration step. Following lymphocyte isolation, cells were then resuspended for cell counting in 1-2 mL media.

2.3.1.2. Cell counting

Viable cells were enumerated by dilution of cell suspensions in 0.8% Trypan Blue (Sigma) and counting on a haemocytometer (Hawksley, London, England). For spleen samples a 1/10 dilution of cell suspension (10 µL) in Trypan Blue (90 µL) was used. For lymph nodes, blood, decidua and uterus, a 1/5 dilution of cell suspension (10 µL) in 40 µL Trypan Blue was used. Concentration of viable cells was calculated using the formula: *Total cell count = average cell count x dilution factor (DF) x 10^4 x volume of cell suspension (mL).* Suspensions were resuspended to final concentration of 5 x 10^6 - 1 x 10^7 cells per mL in media or PBS and typically 1 x 10^6 cells were added to 96-well U bottom plate (Corning; Amsterdam, Netherlands; #9377) for cytokine stimulation or staining as follows.

2.3.1.3. Cytokine Stimulation

For experiments requiring quantification of intracellular cytokines, following cell count, samples were resuspended in media (cRPMI or cIMDM) and seeded in 96-well plate at 1 x 10^6 cells per well in 50 µL (actual conc/mL). Cells were washed once with media, supernatant was removed and cell were resuspended in 50 µL media supplemented with 20 ng/mL phorbol 12-myristate 13-acetate (PMA; Sigma), 1 nM ionomycin (Invitrogen) and 1/1500 dilution of Golgi Stop (as per manufacturers instruction, BD). Aliquots of PMA (1 mg/ml) and ionomycin (1 mg/ml) were stored at -80°C until use. Cells were incubated for 4-5 hrs at 37°C in 5% CO_2. Following stimulation, cells were washed twice with 1 x PBS for FACS

staining. Unstimulated controls were included in each experiment to assist with gating the cytokine expression levels.

2.3.1.4. FACS staining and data acquisition

FACS staining was performed in 96-well plate following cell counts and cytokine stimulation if required. 1 x 10^6 cells were stained per well. Cells were first washed in 1 x PBS and incubated with a 50 µL of a 1/1000 dilution of fixable viability dye (BD; FVS620 or FVS700) for 20 min in dark to discriminate between live and dead cells. Cells were then washed twice in FACS buffer and resuspended in 50 µL of a 1/100 dilution of α-Fc-cIIR antibody (Fc block; BD Biosciences) in FACS buffer at RT for 15 min to block Fc receptors. Fluorophore-conjugated surface antibodies (Table 2.2) were diluted in FACS buffer to a total volume of 10 µL per well. The antibody cocktail was added to cells and samples were incubated for 25 min in the dark at 4°C. For stains containing multiple violet dyes, antibodies were first diluted in Brilliant Stain Buffer (BD; 10 µL per well) before adding the antibody cocktail to samples. Following cell surface staining, samples were washed twice in ice cold FACS buffer. Samples were then fixed and permeabilised using the Foxp3 Staining Buffer Set (eBiosciences, Thermo Fisher Scientific) according to the manufacturer's instructions. Cells were typically fixed overnight and intracellular staining performed the following morning. Cells were washed in ice cold 1 x permeabilisation wash (1:10 dilution of buffer solution in MQ water) before being resuspended in 50 µL of fluorophore-conjugated intracellular antibodies (Table 2.3) diluted in permeabilisation wash. For stains containing multiple violet dyes, antibodies were first diluted in Brilliant Stain Buffer (BD; 10 µL per well) before addition to permeabilisation wash. Samples were incubated in the dark at 4˚C for 30 min. Following staining, cells were washed twice with ice cold FACS buffer and resuspended in PBS or FACS buffer for analysis. In most experiments (and all using fixation sensitive dyes such as APC-Cy7), cells were acquired on the day of intracellular staining. However, for some experiments, cells were resuspended in 200 µL of 1% methanol-free formaldehyde (16% stock diluted in PBS; Thermo Fisher Scientific). In this case samples were analysed up to 5 days post-staining.

Data was acquired on a BD Canto II, BD Aria III or BD LSR Fortessa X-20, using FACS Diva Software. Flow cytometers were calibrated daily using CS&T to track performance of machine and ensure consistency of results particularly for continual studies. Where possible, at least 5 x 10^5 events were acquired per sample to maximise the number of viable cells for analysis. In some cases, multiple wells of the same sample were stained and pooled for acquisition to maximise the number of cells in populations of interest (e.g. Treg cells). In each experiment single-colour controls were run to enable compensation, and "fluorescence minus one" (FMO) controls were run to assist with gating positive/negative populations.

All flow cytometry data was analysed using FlowJo software (Treestar, Ashland, OR, USA). Figure 2.4 depicts the general FACS gating strategy used for lymph nodes (e.g. udLNs), spleen and blood samples to identify CD4 and CD8 T cell subsets including Treg cells, and to measure cytokine production in T cells following stimulation. Figure 2.5 depicts the general FACS gating strategy used for uterus and decidua samples to identify CD4 and CD8 T cell subsets including Treg cells. These strategies form the basis of most FACS analysis in this thesis, specifically with relation to single cell gating, live/dead cell discrimination and general T cell gating. Cases where the gating differs significantly from this will be demonstrated in text where results are presented.

2.3.2. P4-FITC T cell assays

For experiments investigating the capacity of T cells to bind to a membrane-impermeable form of P4 (P4-FITC) (Chapter 4), spleen and LN single cell suspensions from estrus or day 3.5 pc mated mice (with the presence of blastocysts confirmed) were prepared in warmed media as described in 2.3.1.1 and 2.3.1.2. Progesterone 3-(O-carboxymethyl)oxime:BSA-fluorescein isothiocyanate conjugate (P4-BSA-FITC; P4-FITC) and albumin-fluorescein isothiocyanate conjugate (BSA-FITC) were purchased from Sigma. BSA-FITC was used as a control for background FITC fluorescence in P4-FITC. P4-FITC (8.68 mol. P4 /mol. BSA; 3.97 mol., FITC /mol. P4-BSA) was made up by dissolving to 5 mg/mL in MQ and further diluted to 1 mg/mL in FACS buffer. BSA-FITC (12 mol. FITC /mol. albumin) was made up by dissolving to 1 mg/mL in MQ and was further diluted 1/3.02 in FACS buffer, to an equimolar concentration of FITC in P4-FITC. Both solutions were further diluted 1/18.05 in FACS buffer to reach a final P4 concentration of 10 µg/mL in P4-FITC.

Splenocytes were resuspended to 1 x 10^7 cells/mL in warm cRPMI media and rested for 3 hrs at 37°C in 5% CO_2. Cells were then washed 3 x in ice cold PBS to remove media, re-counted and plated at 1-2 x 10^6 cells/well. For each sample, 2 wells were plated (one for P4-FITC and one for BSA-FITC). All of the following steps were performed on ice and at 4°C in the dark. Cells were incubated with viability dye, Fc block and surface stained for flow cytometry, as described in 2.3.1.4. Following surface staining, cells were washed and incubated with 50 µL of a 1/36.1 dilution of P4-FITC (final concentration 0.5 µg/ 50 µL P4) or BSA-FITC in FACS buffer for 45 min. Cells were washed, fixed and intracellular stained as described in 2.3.1.4 before data was acquired. To calculate true FITC fluorescence as a reflection of the P4 membrane binding capacity and control for background staining of the BSA-FITC component, FITC positive staining in BSA-FITC stained samples was subtracted from FITC positive staining in respective P4-FITC samples. An FMO control was used to set the $FITC^+$ gates in all experiments.

2.3.3. T cell in vitro polarization assays

For experiments examining Treg and T effector (Teff) cell polarisation in the presence or absence of P4, splenocyte suspensions from estrus B6, $PR^{+/+}$ or $PR^{-/-}$ mice were prepared as described in 2.3.1.1 and 2.3.1.2. Following cell counting, splenocytes were resuspended to a final concentration of 2 x 10^6 cells/mL in cIMDM media and placed on ice until ready for use.

A stock solution of 50 mg/ml progesterone (Sigma; P4) was prepared by dissolving P4 in absolute EtOH. The solution was vortexed for 1 min, heated 56°C for 10 min, followed by further vortexing until particulates were completely dissolved. The stock was further diluted in EtOH to 10 mg/mL, before dilution to 1 mg/mL in cIMDM. To control for the addition of P4, absolute EtOH was diluted to the same concentration in media.

α-mouse-CD3 (clone 2C11) Ab was diluted to a final concentration of 10 µg/mL in 1 x PBS and 50 µL was added to each well of a 96-well U-bottom cell culture plate (Corning). Plates were incubated at 37°C for 90 min and subsequently then kept at 4°C until ready for use. T helper (Th) cell polarising media for non-polarising (Th0-), Th1- and Th17-polarising conditions (Kara *et al.* 2013) were prepared at 2 x the final concentration in cIMDM. Cytokines and neutralising antibodies used for each polarising condition is described in Table 2.4. α-mouse CD28 was added to each Th-polarising media at 2 µg/mL. P4 (or control) was added to Th-polarising media at 2 x the final concentration (final concentration 0.5 µg/mL). When α-mouse-CD3 plates were removed from incubations they were washed twice with 200 µL 1 x PBS. 100 µL of 2 x Th-polarising media containing 2 x P4 or vehicle were added to wells, followed by the addition of 100 µL splenocytes (2 x 10^5 cells/well). Cells were cultured for 2 days at 37°C in 5% CO_2. Cells were subsequently washed twice with 1 x PBS and restimulated for 4 hrs before FACS staining (as described in 2.3.13 and 2.3.1.4).

2.3.4. T cell isolation for in vivo adoptive transfer

For Treg cell transfer experiments, Treg and non-Treg cells were isolated from mid-pregnant (day 11.5-14.5 pc) females. Spleen and LNs (udLN, inguinal, brachial, axillary and mesenteric) were collected and processed as described in 2.3.1.1 and cells were pooled (from different tissues and from multiple mice where possible) following spleen erythrocyte lysis and prior to cell counting. From each donor, approximately 1 - 2 x 10^8 starting cells were yielded for cell isolation. Cells were resuspended to 1 x 10^8 cells/mL and processed using the StemCell $CD4^+CD25^+$ cell isolation kit (StemCell Technologies Inc., Vancouver, BC, Canada) as per manufacturer's instructions to isolate $CD4^+CD25^-$ (non-Treg) and $CD4^+CD25^+$ (Treg) cells. Briefly, $CD4^+$ cells were first isolated by a negative selection cocktail using a

magnet separation technique. Then, $CD25^+$ cells among $CD4^+$ cells were isolated by positive selection using a CD25-PE-Streptavidin labelling method. Additional steps of two extra 10 sec incubations in the magnet and collection of the 'flow through' suspension were necessary to eliminate excess/loose magnetic streptavidin particles from $CD25^+$ isolate samples. Following T cell isolation the purity of isolated $CD4^+CD25^-$ and $CD4^+CD25^+$ cells was assessed using flow cytometry, staining for live cells along with surface antibodies for CD4 and CD25. The purity of isolated cells was routinely determined to be >85%. Final concentration was adjusted to purity. 2×10^5 cells were transferred to each recipient female via tail vein injection as described in 2.2.3.3. This protocol enabled $0.8 - 1.2 \times 10^6$ Treg and $1 - 1.4 \times 10^7$ non-Treg cells to be isolated per donor.

2.3.5. Progesterone ELISA

Blood serum isolated from mated female mice at various stages of gestation (as described in 2.2.4.1) was frozen at -80°C. Aliquots were defrosted and processed using the ALPCO Mouse/Rat Progesterone ELISA kit (ALPCO, Salem, NH, USA) according to the manufacturers' instructions. This kit has a detection range of 0.4-100 ng/mL and has minimal cross-reactivity with other steroid hormones tested. Serum from mice in later stages of pregnancy was diluted with kit diluent to ensure a quantifiable reading was achieved. Absorbance of samples was measured at 450 nm. A standard curve was plotted using mean absorbance readings from the kit calibrators and this was used to calculate the concentration of each sample (ng/mL), accounting for dilution factor.

2.4. STATISTICAL ANALYSES

Most data analysis was performed using GraphPad Prism 8 for Windows (GraphPad software, San Diego, CA, USA). Parametric tests were run for all experiments and were chosen as part of the experimental design. As small data sets were often used, normality tests were not run on data (Motulsky 2017). For data comparing two groups, paired or unpaired T-tests were performed as appropriate. Data comparing three or more groups was analysed by one-way ANOVA with Fisher's LSD test. Comparisons were made between the control group and treatment groups, or between every group, as appropriate. For Chapters 3 and 4, ROUT's test was used to determine outliers greater than two standard deviations from the mean, which were excluded from analysis. For other chapters, outliers were not removed from analysis. To analyse categorical data (e.g. pregnant vs non-pregnant) χ^2-tests were performed.

Late-pregnancy fetal and placental data was analysed using IBM SPSS Statistics Version 25 (SPSS Inc., Chicago, IL, USA) using Linear Mixed Model ANOVA, with mother as the subject. To account for differences in fetal and placental weight due to litter size, viable litter size was accounted for as a covariate in the model and data are represented as estimated marginal means.

Statistical tests are specified in figure legends. All data is presented as mean ± SEM (standard error of mean) with scatter plots using symbols to depict data from individual mice where appropriate. The number of mice used per experiment is reported in each figure legend throughout the thesis. Statistical significance is indicated by; * $p \geq 0.05$, ** $p \geq 0.01$, *** $p \geq 0.001$. # is used to indicate trends in data (p values of <0.1 and >0.05).

For experiments involving pregnant mice, due to constraints with getting all mice pregnant on a single day, it was necessary to analyse animals over several experimental days. Data was validated for reproducibility and preliminary analysis of data on different experimental days showed no consistent effect of day of experiment on the results, with control mice showing no more than -/+ 20% variation. Therefore, the scale of the impact of the treatment was comparable between different experimental days. Unless otherwise stated in the Figure legend (e.g. Figure 5.1) the data presented is from at least four independent experiments each with similar results, with each symbol representing data from an individual mouse.

Table 2.1 Primers used for genotyping PCR

Strain	Allele	Primer	Sequence	Product Size	Detected for genotype
Foxp3GFPCre; **Foxp3GFPCre.R26 RFP**	GFP: Tg (Foxp3-EGFP/cre) 1aJbs	12444	FWD 5' – CGG GTC AGA AAG AAT GGT GT – 3'	300 bp	Hemizygote
		12445	REV 5' – CAG TTT CAG TCC CCA TCC TC – 3'		
	Internal Positive Control	oIMR8744	FWD 5' – CAG TTT CAG TCC CCA TCC TC – 3'	200 bp	Hemizygote WT
		oIMR8745	REV 5' – GTC AGT CGA GTG CAC AGT TT – 3'		
Rosa26RFP; **Foxp3GFPCre.R26 RFP**	RFP: Gt(ROSA)26Sor(tdTomato-WPRE)	tdTomato (RFP) / oIMR9105	FWD 5' – CTG TTC CTG TAC GGC ATG G – 3'	196 bp	Heterozgyote Mutant
		WPRE / oIMR9103	REV 5' – GGC ATT AAA GCA GCG TAT CC – 3'		
	WT	oIMR9020	FWD 5' – AAG GGA GCT GCA GTG GAG TA – 3'	297 bp	Heterozgyote WT
		oIMR9021	REV 5' – CCG AAA ATC TGT GGG AAG TC – 3'		
PRLacZ	Pgr	Pgr Fwd	FWD 5' – TAG ACA GTG TCT TAG ACT CGT TGT TG – 3'	590 bp	Heterozygous WT
		Pgr Rev	REV 5' – GAT GGG CAC ATG GAT GAA ATC – 3'		
	Neo Mutant	Pgr Fwd	FWD 5' – TAG ACA GTG TCT TAG ACT CGT TGT TG – 3'	110 bp	Mutant Heterozygous
		Neo	REV 5' – CTT CAC CCA CCG GTA CCT TAC GCT TC – 3'		

Table 2.2 Monoclonal surface antibodies used in flow cytometric analyses

anti-	Conjugate	Clone	Source	Stock conc. (mg/ml)	Used conc. (µg/mL)	Study used in
CD4	APC-Cy7	GK 1.5	BD	0.2	2.22	Ch 3, 4
	BUV496	GK 1.5	BD	0.2	2.22	Ch 6
	V450	RM4-5	BD	0.2	0.83	Ch 5
CD45	UV395	30-F11	BD	0.2	0.5	Ch 6
CD45.1	PECy7	A20	eBio	0.2	1	Ch 4
CD45.2	FITC	104	eBio	0.5	2.08	Ch 4
CD25	PE-Cy7	PC61	BD	0.2	2	Ch 6
	PE	7D4	BD	0.2	2.22	Ch 4,5
	FITC	7D4	BD	0.2	2.22	Ch 3, 5
	BB515	PC61	BD	0.2	2.22	Ch 3`
CD304 (Nrp1)	V421	3E12	Biolegend	0.025	0.33	Ch 3-6
CD44	BB515	IM7	BD	0.2	1.67	Ch 4
CD8	PE-Cy7	53-6.7	BD	0.2	2.22	Ch 3, 5
	APC-H7	53-6.7	BD	0.2	2.22	Ch 6
CD103	V711	M290	BD	0.2	2	Ch 6
CD196 (CCR6)	V650	140706	BD (optibuild)	0.2	0.8	Ch 6

BD = BD Biosciences, eBio = eBiosciences, Ch= Chapter

Table 2.3 Monoclonal intracellular antibodies used in flow cytometric analyses

anti-	Conjugate	Clone	Source	Stock conc. (mg/ml)	Used conc. (µg/mL)	Study used in
Foxp3	APC	FJK-16s	eBio	0.2	2.22	Ch 3-6
	PE	R16-715	BD	0.2	3.33	Ch 3
IFNγ	BV510	XMG1.2	Biolegend	0.1	2	Ch 3, 6
	PE-Cy7	XMG1.2	eBio	0.2	1.11	Ch 5
IL17	PE	eBio17B7	eBio	0.2	1.33	Ch 5
	AF647	TC11-18H10	BD	0.2	1	Ch 3
	PerCPCy5.5	TC11-18H10	BD	0.2	1.67	Ch 6
Tbet	AF647	4B10	BD	50 tests	0.5 (µL/well)	Ch 6
RORγt	V786	Q31-378	BD	0.2	2.22	Ch 6

BD = BD Biosciences, eBio = eBiosciences, Ch= Chapter

Table 2.4 Cytokines, neutralizing antibodies and hormones used in T cell polarization experiments

Polarizing condition	Reagent type	Reagent (cytokine, antibody, hormone)	Source	Final Concentration	Final dilution
Th0	Neutralizing antibody	IFNγ (XMG1.2)	BD	20 ng/mL	1/357
		IL-4 (11B11)	BD	20 µg/mL	1/411
	Hormone	Progesterone (+/-)	Sigma	0.5 µg/ml	1/1000
Th1	Neutralizing antibody	IL-4 (11B11)	BD	20 µg/mL	1/411
	Cytokine	1L-12	R&D systems	20 ng/mL	1/500
	Hormone	Progesterone (+/-)	Sigma	0.5 µg/ml	1/1000
Th17	Neutralizing antibody	IFNγ (XMG1.2)	BD	20 ng/mL	1/357
		IL4 (11B11)	BD	20 µg/mL	1/411
	Cytokine	rh TGFb	R&D systems	40 ng/mL	1/500
		IL-6	R&D systems	40 ng/mL	1/250
		rm IL-1β	R&D systems	20 ng/mL	1/500
		rm IL-23	R&D systems	20 ng/mL	1/500
	Hormone	Progesterone (+/-)	Sigma	0.5 µg/ml	1/1000

rh = recombinant human; rm = recombinant mouse

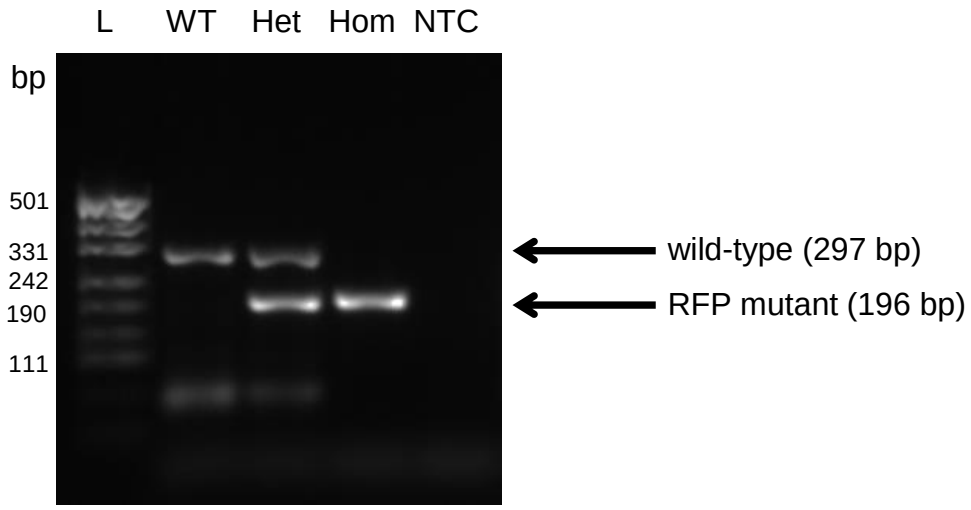

Figure 2.1 Rosa26RFP genotype determination by PCR and gel electrophoresis.

PCR products were run on a 2% agarose gel containing 1 x Gel Red™, alongside a DNA Ladder (L; pUC19) and no-template control (NTC). Genotypes of colony mice were determined by running PCR products against known control products of each genotype; Wild-type (WT; +/+), Heterozygous (Het; RFP/+) and Homozygous (Hom; RFP/RFP). Band sizes in base pairs (bp) of WT and RFP mutant are indicated with arrows.

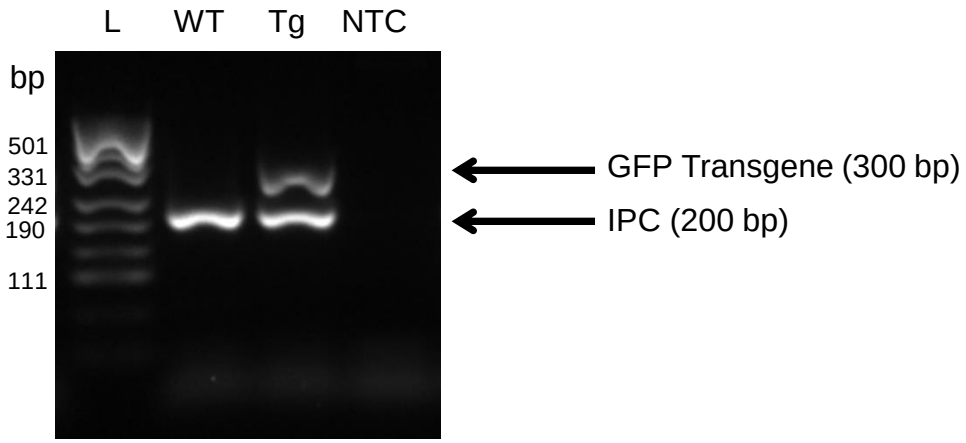

Figure 2.2 Foxp3GFPCre genotype determination by PCR and gel electrophoresis.

PCR products were run on a 2% agarose gel containing 1 x Gel Red™, alongside a DNA Ladder (L; pUC19) and no-template control (NTC). Genotypes of colony mice were determined by running PCR products against known control products of each genotype; Wild-type (WT), and GFP Transgene+ (Tg; GFP+) Band sizes in base pairs (bp) of internal positive control (IPC; present in all samples) and GFP Tg are indicated with arrows.

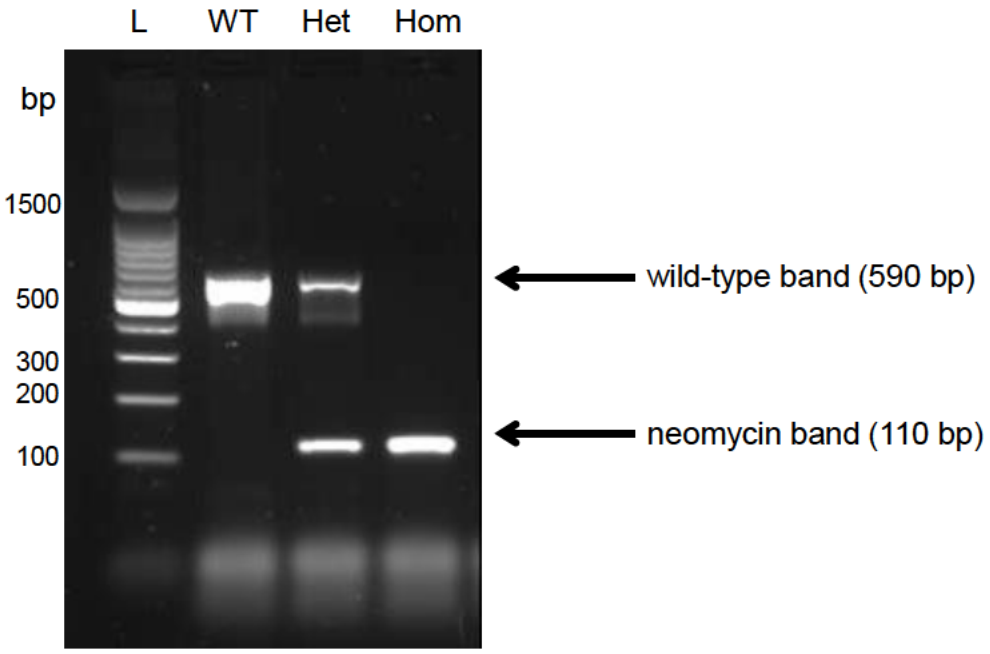

Figure 2.3 PRLacZ genotype determination by PCR and gel electrophoresis.

PCR products were run on a 2% agarose gel containing 1 x Gel Red™, alongside a DNA Ladder (L; Benchtop 100 bp DNA ladder). Genotypes of colony mice were determined by running PCR products against known control products of each genotype; Wild-type (WT; $PR^{+/+}$), Heterozygous (Het; $PR^{+/-}$) and Homozygous (Hom; $PR^{-/-}$). Band sizes in base pairs (bp) of WT and neomycin resistance cassette (present in PRlacZ mutants) are indicated with arrows.

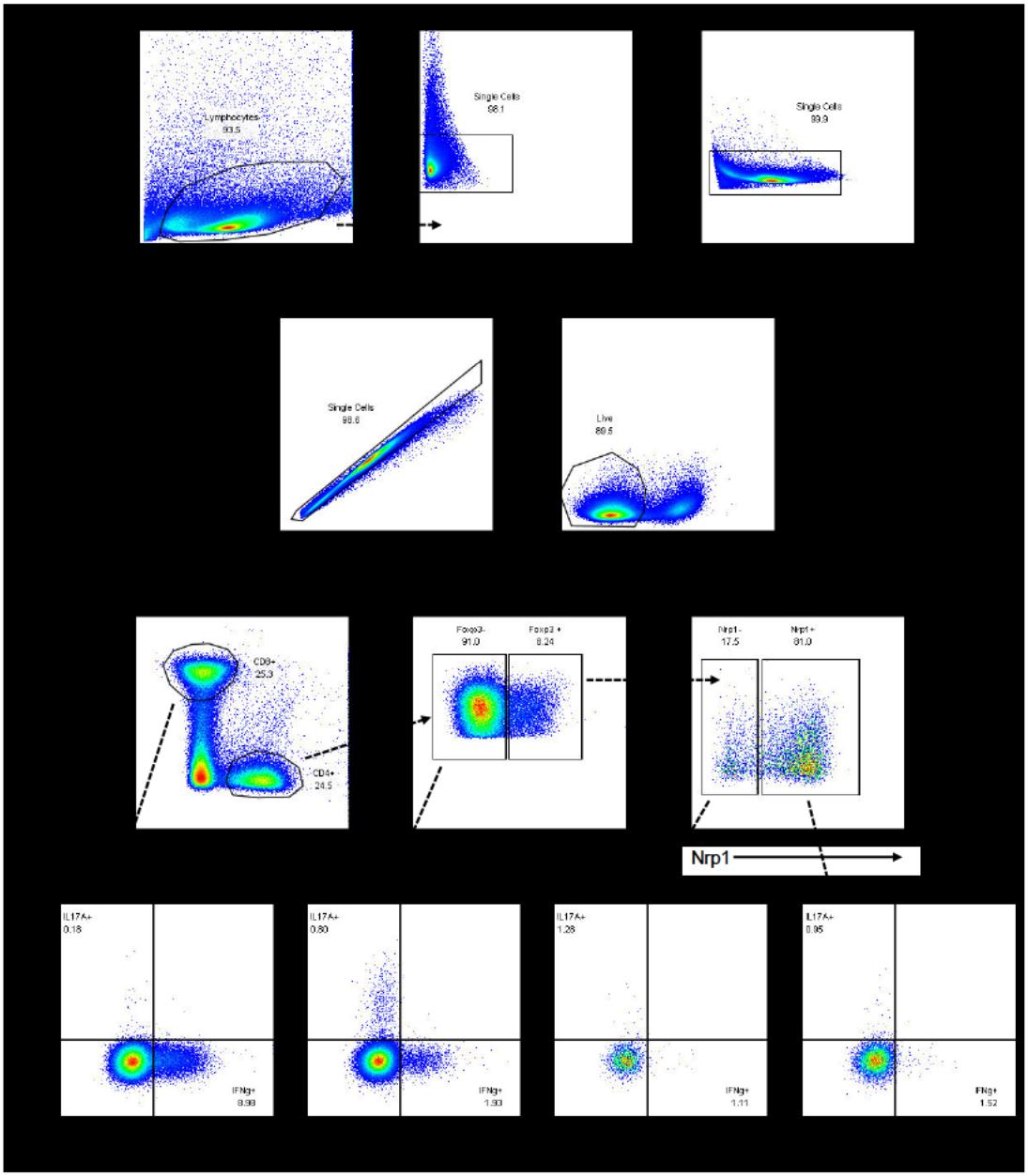

Figure 2.4 Representative flow cytometry gating strategy used to characterize T cell populations and measure inflammatory cytokine production in lymph nodes and spleen.

As described in 2.3.1, 1 x 10^6 spleen or LN cells were stained with surface and intracellular antibodies to enable classification of different T cell subsets. In experiments assessing cytokine production in T cells, ex vivo stimulation with PMA, ionomycin and GolgiStop for 4 hrs at 37°C was performed. Cell were then stained with a live/dead discrimination dye, washed, and stained with surface antibodies for CD8, CD4, CD25 and neuropilin-1 (Nrp1). Samples were fixed and permeabilsed overnight, then stained with intracellular antibodies for Foxp3, IL17A and IFNγ before FACS analysis. (A) Lymphocytes were gated based on their forward scatter (FSC-A) and side scatter (SSC-A) characteristics. Doublets were eliminated by SSC and FSC width (-W) vs height (-H) gating followed by FSC-H vs area (-A) proportional gating. Single cells that stained positive for fixable viability dye (Live/Dead) were excluded from analysis. (B) T cell subsets were identified based on expression of CD4 and CD8 amongst live cells. Treg cells were identified by gating on the $Foxp3^+$ proportion of $CD4^+$ cells, and nonTregs were classified as $CD4^+Foxp3^-$. Treg cell subsets were further defined based on expression of Nrp1. Thymic Treg cells (tTregs) were gated on as $Nrp1^+$ and peripheral Treg cells (pTregs) were gated on as $Nrp1^{-/lo}$. (C) Within CD8, nonTreg, pTreg and tTreg populations, intracellular inflammatory cytokine expression (IFNγ and IL17A) was assessed using quadrant gates.

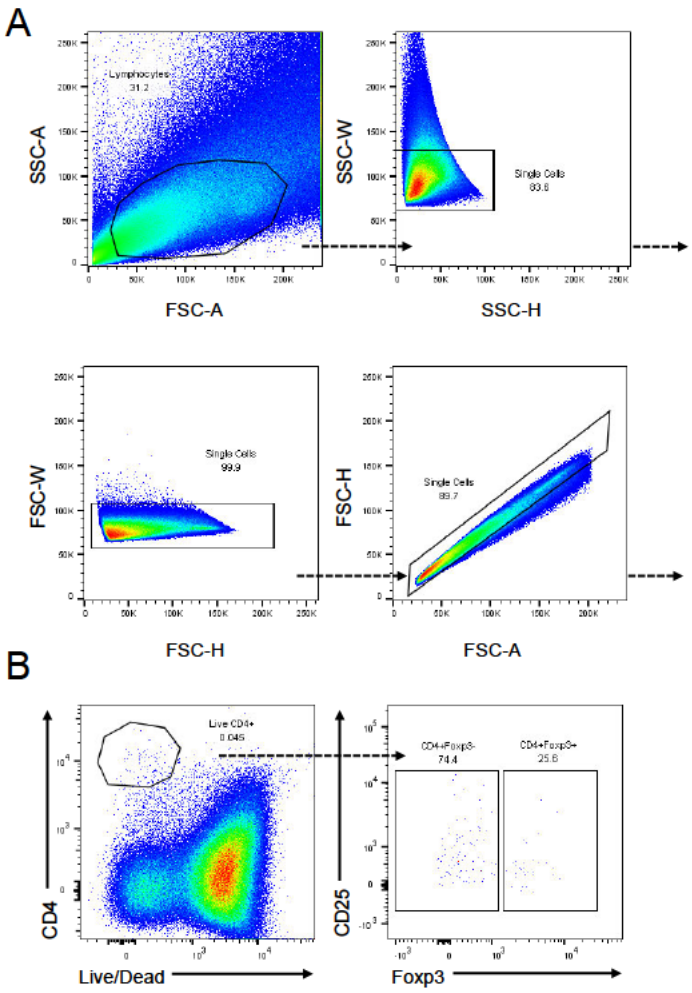

Figure 2.5 Representative flow cytometry gating strategy used to identify CD4+ T cells and Foxp3+ Treg cells in the uterus and decidua

As described in 2.3.1, 1 x 10^6 cells isolated from uterus (early or late gestation) or decidua (late gestation) were stained with surface and intracellular antibodies to enable classification of T cell subsets. Cells were first stained with a live/dead discrimination dye, washed, and stained with surface antibodies for CD4 and CD25. Samples were fixed and permeabilsed overnight, then stained with intracellular antibodies for Foxp3 before FACS analysis. (A) Lymphocytes were gated on based on their forward scatter (FSC-A) and side scatter (SSC-A) characteristics. Doublets were eliminated by SSC and FSC width (-W) vs height (-H) gating followed by FSC-H vs area (-A) proportional gating. (B) Cells that stained positive for fixable viability dye (Live/Dead) were excluded from CD4 gating. Live cells that expressed CD4, were assessed for Foxp3 expression. Treg cells were identified as $CD4^+Foxp3^+$ and nonTregs were identified as $CD4^+Foxp3^-$. Fluorescence minus one (FMO) were used in every experiment to set gates. Where possible, CD45 was also stained for and used to eliminate auto-fluorescent false positives from the analysis.

Chapter 3

Progesterone regulation of the Treg cell response in early pregnancy

3.1. INTRODUCTION

A number of studies have investigated the effect of progesterone (P4) on T cell and Treg cell parameters in mice (Lee *et al.* 2012, Hughes *et al.* 2013, Thangamani *et al.* 2015). While many of these studies were limited to in vitro work or applied to models of disease such as autoimmunity, they provide key evidence for P4 modulating $CD4^+$ T cell phenotype towards a tolerogenic, Treg or Th2-like response, and promoting $Foxp3^+$ Treg cell number, phenotype and function.

Preliminary experiments conducted in our laboratory (Green *et al*., Honours thesis) demonstrated an effect of P4 in regulating Treg cells in vivo using a hormone replacement model to mimic the hormone environment characteristic of early pregnancy. Female mice were ovariectomised and treated with a combination of E2 and P4 before T cells were measured by flow cytometry. This experiment showed that pregnancy hormones increase T cell and Treg cell numbers locally, in the uterus-draining para-aortic lymph nodes (udLN) and systemically, in the spleen. When the model was applied to *Pgr* null mutant ($PR^{-/-}$) female mice, reduced Treg cells were found in the udLN compared to heterozygous $PR^{+/-}$ females, suggesting PR is required to mediate these effects. This work demonstrates P4 can regulate Treg cell generation, trafficking and/or expansion in vivo and, along with the wider literature, highlights a potential role for P4 as a regulator of Treg cell during pregnancy.

To date there are a limited number of studies, utilising various mouse models of P4 administration or depletion, which examine the effect of P4 on Treg cells specifically in pregnancy (Mao *et al.* 2010, Furcron *et al.* 2015, Schumacher *et al.* 2017). These studies report findings implying that P4 boosts Treg cell proportions in vivo during pregnancy in a systemic manner, as well as in the uterus and decidual tissues.

Previous studies have mainly focused on the Treg cell response in mid-late pregnancy in the context of an extreme P4 perturbation known to cause abortion (Mao *et al.* 2010), or employed the abortion-prone CBA/J x DBA/2 mating model, which is known to produce poorly functioning Treg cells from the outset of pregnancy due to MHC antigen mis-match (Schumacher *et al.* 2017). The study by Mao et al. proposes a link between P4, Treg cells and pregnancy success. Administering a high, abortive dose (1.5 mg/mL) of the P4 antagonist RU486 in mid-gestation, caused decreases in uterine $CD4^+CD25^+$ T cell proportions and Foxp3 expression, as measured by western blot, prior to abortion occurring (Mao *et al.* 2010).

While important studies, these studies lack a focus on the events occurring in early pregnancy to shape the Treg cell response, including the priming of the T cell response in the local udLN in the preimplantation phase. Work from our laboratory has demonstrated the importance of priming $Foxp3^+$ Treg cells in the

udLN draining the female reproductive tract following the post-coital inflammatory response to seminal fluid, which importantly contains paternal antigen, cytokines and signalling molecules essential for activating maternal immune recognition of paternal and fetal antigens. Following activation and expansion, Treg cells are recruited into the uterus or retained in the udLN to mediate tolerance mechanisms necessary for pregnancy success (Aluvihare *et al.* 2004, Moldenhauer *et al.* 2009, Robertson *et al.* 2018).

P4-driven action on the uterus is essential for the processes of embryo implantation and decidualisation, which results in trophoblast invasion of the maternal uterine tissue (Wetendorf and DeMayo 2012). The priming of the maternal immune response prior to embryo implantation occurs in conjunction with this surge in P4 signalling. Immune cells, and particularly Foxp3^{+} Treg cells, are strongly implicated in the process of implantation. Adequate maternal tolerance mediated by Treg cells must be established for implantation and successful pregnancy to ensue (Guerin *et al.* 2011, Chen *et al.* 2013).

The aforementioned studies on P4 and Treg cells clearly indicate that Treg cells are sensitive to the effects of P4 and show a role of P4 in regulating Treg cells in pregnancy. However, the details of P4 regulation of Treg cells from the outset of pregnancy, including the timing of implant, mechanism of regulation, and significance for the success of pregnancy, are still largely not understood. We therefore hypothesised that peri-implantation P4 exposure is a determinant of maternal immune tolerance and the Treg cell response required to establish pregnancy.

To explore this hypothesis, we utilised an approach to investigate the effect of a perturbation in progesterone signalling during early pregnancy on the Treg cell response. Conventionally, genetic approaches have been used to alter P4 expression and signalling in vivo, such as reducing P4 bioavailability with the genetic P4 receptor (PR) deficient model. However, this approach is not useful for application in the context of pregnancy, since PR$^{-/-}$ females are infertile, display abnormal estrous cycles, and fail to exhibit normal sexual behaviour (Lydon *et al.* 1995). Hormone receptor manipulation has also been employed to alter hormone signalling, such as the use of RU486 (mifepristone). RU486 is a well-characterised classical PR antagonist that inhibits implantation at high doses and thus has been used extensively in mice and women. RU486 functions by binding to PRs as a competitive inhibitor. This in turn inhibits P4 signalling and the biological effects of P4 (Baulieu 1991). In the current study, we developed a model of reduced P4 bioavailability in the peri-implantation period using RU486, which we employed to investigate the role of early pregnancy P4 in regulating maternal tolerance and the Treg cell response.

3.2. EFFECT OF PERI-IMPLANTATION DISRUPTION OF PROGESTERONE SIGNALLING ON PREGNANCY SUCCESS

A model of reduced P4 signalling in early pregnancy, by administering RU486 to female mice following mating, was developed. Embryo implantation occurs on day 4 post-coitus (pc) in the mouse, therefore to cover the entire peri-implantation phase two doses of RU486 were administered, on day 1.5 and 3.5 pc. Initial experiments were performed using a range of doses, with the aim of finding an appropriate dose whereby pregnancy remains viable, but to achieve reduced P4 bioavailability and allow effects on the Treg cell response to be studied. C57Bl/6 (B6) female mice were mated to allogenic BALB/c stud males, and on day 1.5 and 3.5 pc, RU486 (0.5 - 8mg/kg) or vehicle control (0 mg/kg; sesame oil) was administered. Mice were euthanised on day 9.5 pc for assessment of pregnancy parameters at mid-gestation. Figure 3.1A shows a schematic of the experimental design. A dose-dependent decrease in the proportion of mated females that were pregnant (defined as the presence of at least one viable implantation) on day 9.5 pc (pregnancy rate) was observed with increasing doses of RU486 (Figure 3.1B, 3.1C). As expected, complete implantation failure was observed at the highest dose of RU486 (8 mg/kg) with no females in this treatment group carrying a viable pregnancy at mid-gestation compared to control-treated mice ($P<0.05$). A reduction in pregnancy rate compared to controls was also observed for females treated with moderate doses of 2 mg/kg or 4 mg/kg RU486, displaying 35% and 55% reduction in pregnancy rate, respectively ($P<0.05$). Notably, no difference in pregnancy rate was observed when comparing low doses of RU486 (0.5-1 mg/kg) to control treatment.

A similar trend in the data was observed when total implantations per mated female (Figure 3.1D) was assessed, whereby females treated with 2-8 mg/kg RU486 showed significant decreases in number of total implantations ($P<0.01$-0.0001) compared to control. This data highlights the early losses of implanted embryos experienced by females treated with moderate to high doses of RU486 and suggests that implantation failure likely occurred in these mice. Total implantations per pregnant female (with at least one viable implantation) on day 9.5 pc was quantified (Figure 3.1E). Females treated with 2 mg/kg RU486 showed a reduction in number of implantations ($P<0.01$) compared to controls. There appeared to be no reduction in the number of implantations for the 4 mg/kg group, however only 2/10 females were observed to be pregnant on day 9.5 pc in this group, with the majority of females experiencing early pregnancy losses as shown in Figure 3.1B and 3.1C. No differences in total implantations per mated or pregnant female (Figure 3.1D, 3.1E) was observed when comparing low doses of RU486 (0.5 or 1 mg/kg) to control treatment.

Number of viable (Figure 3.1F), non-viable (Figure 3.1G) and small (< 2mm) viable (Figure 3.1H) implantations per pregnancy was also measured following RU486 treatment in the peri-implantation period. Again, the moderate 2 mg/kg dose of RU486 caused a decrease in viable implantations/pregnancy (Figure 3.1F; $P<0.0001$). Mice treated with 1 mg/kg RU486 also exhibited an increase in non-viable implantations/pregnancy (Figure 3.1G; $P<0.05$). Interestingly, mice treated with moderate doses of RU486 exhibited a higher number of small viable implantations per pregnancy (Figure 3.1H; $P<0.05$ 2 mg/kg; $P<0.0001$ 4 mg/kg). There was also a trend towards an increase in small viable implantations in the 1 mg/kg dose ($P<0.1$). While they appeared viable on day 9.5 pc, these implantations were distinctively smaller in size and often paler in appearance to control implantations sites (Figure 3.1B, 3.1H), and so likely to be lost if pregnancy was allowed to progress.

Overall, this data demonstrates that peri-implantation disruption of P4 signalling with RU486 decreases pregnancy success in a dose-dependent manner.

3.3. EFFECT OF PERI-IMPLANTATION DISRUPTION OF PROGESTERONE SIGNALLING ON T CELL DYNAMICS AT MID-GESTATION (DAY 9.5 PC)

We next sought to examine immune parameters in mice treated with RU486 in the peri-implantation period by flow cytometry. Uterus-draining para-aortic lymph nodes (udLNs) and spleens from female mice mated and administered 0.5-8 mg/kg RU486, or control (Ch 3.1) were harvested on day 9.5 pc and processed for flow cytometric analysis of various $CD4^+$ T cell, $CD4^+Foxp3^+$ Treg cell, and $CD8^+$ T cell subsets. Cells were stimulated ex vivo for 4h with phorbol 12-myristate 13-acetate (PMA) and ionomycin to allow cytokine production in T cells to be measured, before being stained with antibodies against surface and intracellular antigens for flow cytometry.

3.3.1. $CD4^+$ T cells and Treg cells in the udLN

We began by looking at global changes occurring in $CD4^+$ T cell and Treg cell populations locally in the udLN following peri-implantation disruption of P4 signalling with RU486 (Figure 3.2). A decrease in total cell count was observed in females treated with 1 mg/kg RU486 compared to control (Figure 3.2A, $P<0.01$), and a trend towards a decrease was observed in females treated with the highest 8 mg/kg dose ($P<0.1$). In the $CD4^+$ T cell compartment, a 30-40% reduction in cell number was observed in females treated with low doses ($P<0.01$ 0.5 mg/kg, $P<0.05$ 1mg/kg) and moderate doses ($P<0.05$ 2 mg/kg, $P<0.05$

4 mg/kg) of RU486 compared to control mice (Figure 3.2B). No significant changes were observed in the proportion of $CD4^+$ T cell measured as a percentage of live cells (Figure 3.2C), indicating the relative size of the $CD4^+$ T cell compartment in regard to other live cells did not change significantly from that of control mice, and that decreases in $CD4^+$ cell numbers may be mirrored by decreases in other lymph node cell populations.

Treg cells were identified on the basis on Foxp3 expression, the Treg-specific transcription factor. Figure 3.2D shows representative FACS plots of Foxp3 staining in $CD4^+$ T cells in mice treated with RU486. Strikingly, a 55-65% decrease in the total number of $CD4^+Foxp3^+$ Treg cells was observed in mice treated with low doses of RU486, 0.5 mg/kg ($P<0.01$) and 1 mg/kg ($P<0.001$), and a 37-43% decrease was observed in mice treated with moderate doses of RU486, 2 mg/kg ($P<0.05$) and 4 mg/kg ($P<0.05$). A decrease in the proportion of $Foxp3^+$ Treg cells (as a % of the $CD4^+$ T cell population) was also observed for all low and moderate doses of RU486 (0.5-4 mg/kg) compared to control (Figure 3.2F, all $P<0.05$). Mice treated with 8 mg/kg RU486 showed no changes in the number and proportion of Treg cells, likely a reflection of the fact that unlike at other doses, these mice all experienced early implantation failure. Geometric mean fluorescence intensity (MFI) of Foxp3, an indicator of Foxp3 expression levels per cell, was measured in $CD4^+Foxp3^+$ cells and expressed as fold change MFI compared to control mice (Figure 3.2G). Mice treated with 1 mg/kg exhibited a small but significant decrease in Foxp3 MFI fold change ($P<0.01$). Likewise for 2 mg/kg ($P<0.05$), 4 mg/kg ($P<0.1$) and 8 mg/kg ($P<0.05$) RU486, a decrease in Foxp3 MFI fold change was observed.

Nrp1, a cell surface molecule regulated by TGFβ, has been identified as a reliable marker to distinguish between Treg cells of peripheral (pTreg) or thymic (tTreg) origin. tTreg cells, typically upregulate the expression of Nrp1 and therefore are $Nrp1^+$, whereas pTreg cells do not and are $Nrp1^{-/lo}$ (referred to herein as $Nrp1^-$) (Weiss *et al.* 2012). Figure 3.2D shows representative FACS plots of Nrp1 staining in $Foxp3^+$ Treg cells.

The proportion of pTreg and tTreg of the total Treg cell population was assessed, with tTreg cells making up 75%, and pTreg cells making up 25%, of the Treg cell pool in control mice at mid-gestation (Figure 3.2H). RU486 treated groups were largely consistent with control mice, with the exception of the 0.5 mg/kg group, which exhibited a 10% decrease in tTreg cell proportion and respective increase in pTreg cell proportion ($P<0.05$)). Interestingly, when the proportion of each Treg cell subset as a percentage of the total $CD4^+$ T cell pool were assessed, a decrease was observed specifically in tTreg cells and not pTreg cells in 0.5, 1 and 2 mg/kg groups (Figure 3.2I, 3.2J; $P<0.05$). This is consistent with the observed decline in tTreg cells as a % of total Treg cells following RU486 (Figure 3.2H). Thus, the observed decrease in

overall Treg cell proportions in mice treated with 0.5-2 mg/kg RU486 (Figure 3.2F) can be specifically attributed to a preferential loss of the tTreg subset in the udLN.

These data demonstrate that in the udLN, a decrease in number and relative proportion of Treg cells (specifically tTreg cells), accompanied by decreased Foxp3 expression in Treg cells, is observed in females receiving low to moderate doses of RU486 in the peri-implantation period.

To account for the fact that some mice analysed had likely lost their pregnancy early, around the time of implantation, the contribution of pregnant versus non-pregnant mice to the data and specifically to the observed changes in T cell and Treg cell parameters in the udLN was assessed. Although only a preliminary analysis could be conducted due to a low sample size, largely we observed the same trends in the data when we analysed all mice (Figure 3.2) or just pregnant mice (Figure 3.3). Figure 3.2A depicts pregnant and non-pregnant mice when Treg cell proportions are assessed, and shows similar patterns in the data when all mice or just pregnant mice are compared to controls. Figure 3.3B also shows Treg cell proportions but just among pregnant mice. Reduced Treg cell proportions were still observed for mice treated with RU486 compared with control mice. Similarly, statistically significance declines were observed for most RU486 treated groups when assessing Treg cell number (Figure 3.3C), Foxp3 MFI fold change (Figure 3.3D) and tTreg cell proportion (% of $CD4^+$). As some groups (2 and 4 mg/kg particularly) contained low numbers following removal of non-pregnant mice, statistical significance was not obtained, thus a combined analysis was also conducted whereby collectively the RU486 treated mice were compared to control by unpaired T-test. Combined analysis revealed a significant difference in the Treg parameters measured for pregnant mice on day 9.5 pc following RU486 treatment (Figure 3.3B - 3.3E), again consistent with results from individual groups of the full data set.

As same trends overall were observed when only pregnant mice were analysed, we were confident the combined analysis was relevant and generally reflective of pregnant mice particularly for the lower RU486 doses. Therefore, all mice were included for further analysis of T cell subsets.

3.3.2. $CD4^+$ T cells and Treg cells in the spleen

Next, we examined global changes in $CD4^+$ T cell and Treg cell populations systemically in the spleen on day 9.5pc following peri-implantation RU486 treatment (Figure 3.4). A significant decrease in total cell count was observed in females treated with 1, 2 and 8 mg/kg RU486 compared to control (Figure 3.4A, $P<0.01$ - 0.05), and a similar trend was observed in females treated with 4 mg/kg RU486 ($P<0.1$).

In the $CD4^+$ T cell compartment of the spleen, RU486 treatment caused changes contrary to that seen in the udLN. While splenic $CD4^+$ T cell numbers were not significantly reduced following RU486 treatment

(Figure 3.2B), the proportion of $CD4^+$ T cells amongst total viable cells was significantly increased in females treated with moderate (2 mg/kg $P<0.05$, 4 mg/kg $P<0.001$) and high (8 mg/kg $P<0.001$) doses of RU486 (Figure 3.4C). As the increase in the % of $CD4^+$ T cells could not be explained by changes in the numbers of $CD4^+$ T cells, it can be inferred that there is a decrease in other cell types, as highlighted by the decrease in total cell counts at these doses (Figure 3.4A). Thus, in the spleen, the $CD4^+$ T cell compartment is maintained 6 days following moderate-high RU486 treatment in the peri-implantation period.

Overall, the changes to Treg cell populations we observed in the udLN were mirrored in the spleen but to a lesser extent and not in all groups. A 40% decrease in the number of $CD4^+Foxp3^+$ Treg cells was observed in mice treated with 1 and 2 mg/kg RU486 only (Figure 3.4D, 3.4E, $P<0.05$). A decrease in the proportion of $Foxp3^+$ Treg cells (as a % of the $CD4^+$ T cell population) was also observed for mice treated with 2 and 4 mg/kg RU486 only (Figure 3.4F, $P<0.05$). Mice treated with 4 and 8 mg/kg exhibited a significant decrease in Foxp3 MFI fold change compared to control (Figure 3.4G, $P<0.05$-0.001). The proportion of pTreg and tTreg within the total Treg cell population was assessed, tTreg cells made up 82%, and pTreg cells made up 18% of the Treg cell pool in control mice at mid-gestation (Figure 3.2H). RU486 treated groups were largely consistent with control mice, with the exception of the 2mg/kg group, which exhibited an approximate 10% decrease in tTreg cell proportion and respective increase in pTreg cell proportion ($P<0.05$). When we assessed the proportion of each Treg cell subset amongst the total $CD4^+$ T cell pool, a decrease was observed in both tTreg cells and pTreg cells for the 2 mg/kg group (Figure 3.4I, $P<0.05$; Figure 3.4J, $P<0.01$). There was also a trend towards a decrease in the proportion of tTreg cells amongst $CD4^+$ T cells for the 1mg/kg and 4 mg/kg doses (Figure 3.4J, $P<0.1$). This indicates the observed decreases in splenic Treg cell number and proportion are explained by a reduction in tTreg cells.

Overall these data demonstrate that in the spleen, a decrease in Treg cell number, relative proportions and Foxp3 MFI was evident in RU486-treated mice, with the 2 mg/kg group mostly affected. Largely, groups treated with low-dose RU486 did not show significant changes to Treg cells in the spleen, aside from a decrease in Treg cell number and trend toward a decrease in tTreg cell (% of $CD4^+$ T cells) for the 1 mg/kg dose.

3.3.3. $CD4^+$ T effector cells in udLN and spleen

The inflammatory cytokines IFNγ and IL17 are hallmark effector cytokines produced by Th1 and Th17 cells respectively, and are commonly used as surrogate measures of the abundance of these T effector

(Teff) cell subsets. IFNγ and IL17 production among nonTreg ($CD4^+Foxp3^-$) cells was measured in the udLN and spleen following RU486-treatment in the peri-implantation period.

In control-treated females a small proportion of Teff cells were observed at mid-gestation in the udLN (Figure 3.5A). Treatment with moderate-high doses of RU486 (4 and 8 mg/kg) caused a significant increase in the number and proportion of IFNγ-producing Th1 cells in the udLN (Figure 3.5A, 3.5B). Approximately 3% of $CD4^+$ T cells produced IL17 in the udLN of control mice, and this level was not changed following RU486 treatment (Figure 3.5C, 3.5D). Interestingly, the number of Th17 cells was significantly decreased in the udLN following treatment with 1 mg/kg RU486 (Figure 3.5C, $P<0.01$), and a similar trend to this was observed for the 2 and 8 mg/kg groups ($P<0.1$).

Similar to the udLN, Teff cells in the spleen were observed in normal pregnancy at mid-gestation (Figure 3.5D). Approximately 10% of $CD4^+$ T cells in the spleen were capable of producing IFNγ following stimulation. Treatment with moderate-high doses of RU486 (4 and 8 mg/kg) caused a significant increase in the number and proportion of IFNγ-producing Th1 cells in the spleen (Figure 3,5E, 3.5F). The number of IFNγ-producing cells was approximately double that of control mice, and the % of IFNγ-producing T cells was raised to 15% - 20% of splenic CD4+ T cells (Figure 3.5E). As almost all of these mice weren't pregnant at this time point, this suggests that effects of higher doses of RU486 on implantation failure in the uterus are associated with a systemic inflammatory profile in T cells 6 days following treatment. Approximately 3% of $CD4^+$ T cells produced IL17 in the spleen of control mice, and this level remained unchanged following RU486 treatment (Figure 3.5D, 3.5F). A trend toward reduced Th17 cell numbers was observed for mice treated with 1 or 2 mg/kg RU486 (Figure 3.5F, $P<0.1$).

These data show that Teff cells are present at mid-gestation in the udLN and spleen. Overall the spleen contained a higher number and proportion of $CD4^+$ cells capable of producing IFNγ and IL17 than in the udLN, which contained relatively low numbers and proportions of Teff cells. RU486 treatment caused an increase in inflammatory Th1 Teff cells in both the udLN and spleen at high doses of RU486 (4 and 8 mg/kg). As most of these mice were non-pregnant on day 9.5pc likely due to implantation failure, the increase in inflammatory cytokine production in these T cells is associated with pregnancy loss 6 days earlier. RU486-treated mice that maintained a pregnancy on day 9.5 pc largely had Th1 levels comparable to controls.

3.3.4. Inflammatory cytokine production in Treg cell subsets

Inflammatory cytokine production has recently been reported in Treg cells and is as measure of effector, phenotypically plastic or unstable Treg cells under different conditions (Hori 2014). As P4 is hypothesised

to affect Treg cell phenotype, IFNγ and IL17 production was measured in pTreg and tTreg cells from udLN and spleen on day 9.5pc following RU486 treatment (Figure 3.6).

At mid-gestation, a small percentage of pTreg and tTreg cells from control mice produced measurable levels of IFNγ and IL17 in the udLN (Figure 3.6A-3.6C). Typically, pTreg cells produced more IFNγ and IL17 than tTreg cells. Overall there weren't substantial changes in inflammatory cytokine production following RU486 treatment with the exception of an increase in % of both pTreg and tTreg cells producing IFNγ at high 4 and 8 mg/kg doses of RU486 specifically in the udLN (Figure 3.6A). This is consistent with the findings of increased IFNγ production we observed in $CD4^+Foxp3^-$ Teff cells of mice treated with higher doses of RU486 (Figure 3.5). In addition, tTreg cells from mice treated with 4 mg/kg RU486 displayed an increased percentage of IL17 production in the udLN (Figure 3.6A, 3.6C). Similar to the udLN, in the spleen, a small percentage of pTreg and tTreg cells from control mice produced measurable amounts of IFNγ and IL17 (Figure 3.6D, 3.5E). Again, there wasn't substantial change in inflammatory cytokine production following RU486 treatment, other than in tTreg cells from mice treated with 4 mg/kg RU486 where an increased percentage of IL17 production was seen in the spleen (Figure 3.6A, 3.6E).

These data show that inflammatory cytokine production is present in a small proportion of pTreg and tTreg cells at mid-gestation, with the majority of these cytokines produced by pTreg cells. This is consistent with Treg cell plasticity and instability specifically being exhibited by pTreg cell subsets (Hori 2014). In mice treated with high doses of RU486 at peri-implantation, an inflammatory profile of increased IFNγ production and in some cases increased IL17 production, was evident in Treg cell subsets particularly in the udLN.

3.3.5. $CD8^+$ T cells

Finally, $CD8^+$ T cell numbers and proportions were measured in udLN and spleen following peri-implantation disruption of P4 with RU486. Notably, CD8 T cell numbers were decreased in the udLN (Figure 3.7A) with 1 mg/kg RU486 ($P<0.05$). In both LN and spleen, CD8 T cells as a % of live cells increased significantly with moderate-high doses of RU486 (Figure 3.7A, 3.7C). As this increase in % was not reflected by increased numbers, this indicates that loss of another cell subset was likely causing the changes in proportions observed here. This is consistent with the observed increase in proportion, not number of $CD4^+$ T cells in the spleen (Figure 3.4). We also measured the proportion of $IFN\gamma^+CD8$ T cells, and geometric MFI of IFNγ in $IFN\gamma^+CD8$ T cells, known as T cytotoxic (Tc) $CD8^+$ T cells. At mid-gestation in control mice 21% udLN and 34% splenic CD8 T cells were IFNγ-producing (Figure 3.7B, 3.7D). This level was similar across groups with the exception of 0.5 and 1 mg/kg RU486 groups in the spleen which showed an 5% increase in IFNγ positivity (39%) amongst CD8 T cells (Figure 3.7B, 3.7D). The MFI of

IFNγ in IFNγ$^+$CD8 T cells remained comparable across all treatment groups in LN and spleen (Figure 3.7B, 3.7D).

Overall, the FACS data on day 9.5 pc from mice treated with RU486 at peri-implantation demonstrates an immune imbalance following treatment with RU486. A distinction can be made between mice treated with low-doses of RU486 which were pregnant on day 9.5, and mice treated with higher doses of RU486 that experienced implantation failure and were not pregnant. Indeed, the data indicates differences in immune parameters in these two cases, as low-dose mice (0.5-2 mg/kg) tended to have an overall loss in cell numbers, with Treg cell number and proportions severely reduced, and high-dose mice (4-8 mg/kg) demonstrated an inflammatory profile in T cells overall, and a shift in the balance of immune cells towards increased CD4 and CD8 T cell proportions. In addition, the Treg cell compartment was also impaired with higher RU486 doses leading to lower Treg cell proportions and Foxp3 expression.

3.3.6. Net changes to immune parameters in mice treated with low-dose RU486 in peri-implantation

As we were interested in the net changes occurring in the immune system and specifically within the CD4$^+$ T cell compartment following RU486 treatment, we assembled pie charts of the flow cytometry data reflecting the relative proportions of cell subsets in the udLN of control and 1 mg/kg RU486 groups (Figure 3.8). We focused on this low dose of RU486 treatment as most of this group of mice were pregnant on day 9.5. Thus the 1mg/kg dose gives an indication of the immune changes during mid-gestation, following peri-implantation RU486 administration, while sustaining a pregnancy.

Pie charts were sized relative to the number of total cells (Figure 3.8A) and CD4$^+$ T cells (Figure 3.8B) in control and RU486 mice. For each chart, the relative proportions in each cell subset is indicated with percentages. Figure 3.8A depicts the whole immune cell compartment in the udLN on day 9.5 pc. A clear reduction in the size of the immune compartment is evident following RU486 treatment. The reduction affects all cell types, including larger subsets such as CD8$^+$ T cells and other cells, which represent live CD4$^-$CD8$^-$ cells that were not specifically identified in the study. This includes cells such as NK cells (and other innate lymphoid cells), B cells, dendritic cells (DCs), granulocytes, macrophages, and other T cell subsets. As CD4$^+$ T cells comprise only a small proportion of the total cell population, we also analysed the changes occurring specifically within the CD4$^+$ T cell compartment in Figure 3.8B. Reduction in size overall of the CD4$^+$ T cell compartment is again highlighted, with the reduction in size and proportion of the tTreg cell subset particularly evident (Figure 3.8B).

These data highlight the profound changes to the immune and specifically the $CD4^+$ T cell compartment by mid-gestation following peri-implantation administration of RU486.

3.4. EFFECT OF PERI-IMPLANTATION DISRUPTION OF PROGESTERONE SIGNALLING ON T CELL DYNAMICS AT IMPLANTATION

To investigate the timing of the induction in T cell changes observed in RU486-treated mice on day 9.5 pc, we measured key $CD4^+$ T cell and Treg cell parameters in mice treated with moderate doses of RU486 (2 mg/kg and 4mg/kg) or control, on day 4.5 pc, approximately 24 hours following the second dose of RU486 (Figure 3.9). In the udLN, we observed a trend towards a reduction in total cell numbers following treatment with 2 or 4 mg/kg RU486 (Figure 3.9A, $P<0.1$). When $CD4^+$ T cells were assessed (Figure 3.9B), a reduction in number was evident in mice treated with 2mg/kg RU486 ($P<0.05$) or 4 mg/kg RU486 ($P<0.1$). Similarly, a trend towards a reduction in Treg cell number was evident in the 4 mg/kg RU486 group. No significant differences or clear trends were found in the spleen between control and RU486-treated groups.

In the udLN, the patterns in the data on day 4.5 pc were largely consistent with the data from day 9.5 pc, demonstrating that less $CD4^+$ T cells and Treg cells are already evident at the time of implantation and therefore precede the changes at mid-gestation. Importantly, this suggests that the local T cell defect at mid-gestation in mice treated with low-dose RU486 is indicative of failed expansion and/or altered trafficking of T cells in the udLN in early pregnancy, rather than expansion followed by a subsequent decline.

3.5. EFFECT OF PERI-IMPLANTATION DISRUPTION OF PROGESTERONE SIGNALLING ON PROGESTERONE LEVELS AT IMPLANTATION AND MID-GESTATION

Next, to investigate the effect of RU486 on circulating P4 levels in early and mid-gestation, we measured P4 in the serum of mated females administered with low to moderate doses of RU486 on day 4.5 pc and day 9.5 pc by ELISA (Figure 3.10). P4 serum levels are known to rise by mid-gestation in normal pregnancy, due to increased P4 synthesis in the ovary (Murr *et al.* 1974). On day 4.5, no obvious differences in P4 serum levels were evident, albeit the low sample size of some groups in the analysis made it difficult to draw firm conclusions (Figure 3.10A). On day 9.5 pc, there was a significant decrease

in P4 serum levels in mice treated with 1 or 2 mg/kg RU486 (both $P<0.05$) compared to control. While somewhat unexpected due to the low doses of RU486 used and the early timing of the intervention, this data indicates that as well as eliciting antagonism of PR signalling, RU486 treatment had an effect on P4 synthesis resulting in lower levels of circulating P4 at mid-gestation.

3.6. DISCUSSION

The experiments in this study demonstrate that after a peri-implantation administration of RU486 to elicit disruption of P4 bioavailability, the immune compartment is significantly impaired at mid-gestation, even with low doses of RU486. A particular reduction in the $CD4^+$ cell and $Foxp3^+$ Treg cell compartment was evident in the local udLNs. The effects of RU486 on $CD4^+$ T cells and Treg cells was evident at both low and high doses, regardless of whether pregnancy progressed following treatment. The immune perturbation was already evident in the udLN specifically around the time of implantation, indicating a failure of normal expansion of $CD4^+$ T cells and Treg cells. Thus, we infer that adequate P4 bioavailability from the outset of pregnancy is required for robust Treg cell induction.

3.6.1. On the effect of reduced P4-signalling on pregnancy success at mid-gestation

In these studies RU486 was used in order to reduce P4 bioavailability in the peri-implantation period. RU486 is a competitive inhibitor and inhibits PR-dependent pathways by binding to the ligand binding-domain of PR to preventing DNA transcription from occurring (Baulieu 1991). RU486 has been used extensively in mouse models of pregnancy to elucidate the function of P4 (Baulieu 1991, Cheon *et al.* 2002). Notably, however, RU486 also binds to the glucocorticoid receptor (GR), and can act as an antagonist of glucocorticoid-dependent pathways (Baulieu 1991, Raaijmakers *et al.* 2009). Although selective progesterone receptor modulators (SPRMs) with increased specificity for PR exist, as these have mixed agonist/antagonist properties we decided to use RU486 for this study (Bouchard *et al.* 2011). GR-dependant mechanisms of action therefore cannot be ruled out in this study, however some effects of P4 are in fact mediated by GR signalling. P4 has been shown to promiscuously bind to the GR in immune cells, and GR, but not PR was found to mediate a direct effect of P4 on T cells in vitro and in pregnancy (Engler *et al.* 2017, Hierweger *et al.* 2019). P4 bioavailability is dependent on the concentration of P4 and the expression and availability of P4 receptors. As RU486 primarily antagonises PR (Baulieu 1997), we concluded that reduced P4 bioavailability was a key impact of the RU486 model used.

To our knowledge, this is the first study to use RU486 administered at low doses in mice, allowing a pregnancy to still progress despite the suppression of P4 bioavailability. P4 action in the uterus is dynamic throughout gestation, and particularly important at the start of gestation, prior to embryo implantation, for the processes of uterine receptivity and decidualisation (Franco *et al.* 2008). In women, P4 produced in the luteal phase prior to conception is essential to orchestrate endometrial transformation for decidualisation to occur (Evans *et al.* 2016). The priming of the maternal immune and Treg cell response also occurs in conjunction with these early events, largely in response to ovarian hormones and seminal fluid signals (Robertson *et al.* 2009). In the current study, RU486 was administered twice in the peri-implantation period to limit P4 bioavailability throughout the time of Treg cell priming by seminal fluid before implantation. By mid-gestation, pregnancy rate was decreased dose dependently in RU486-treated females, with the highest dose of RU486 used (8 mg/kg) known to cause complete implantation failure (Cheon *et al.* 2002). Importantly, at low doses, RU486 had no effect on pregnancy rate or number of implantations. Interestingly however, the mice treated with low doses of RU486 showed an increased number of noticeably smaller implantations by mid-gestation. While these implantations appeared viable at this time, their viability in late-gestation was not determined. In Chapter 4, the effect of low-dose RU486 in early pregnancy on fetal viability in late gestation was explored further.

3.6.2. On the effect of reduced P4-signalling on Treg cells and the maternal immune response

P4 is known to be a potent immunomodulatory agent capable of exerting effects on the immune environment throughout gestation. Analysis of mRNA gene expression in the uterus following administration of 8 mg/kg RU486 on day 3 pc showed inhibition of P4 signalling altered the expression of many genes encoding transcription factors, cell adhesion molecules, signal transduction molecules and molecules relating to angiogenesis (Cheon *et al.* 2002). In addition, several genes associated with regulatory pathways specific to immune function were also altered. Notably, the *H2-Ab1* gene, which encodes MHCII, was downregulated 2-fold in the uterus (Cheon *et al.* 2002). MHCII expression by antigen presenting cells (APCs) is necessary for activation of Treg cells in the context of tolerogenic signals (Maldonado and von Andrian 2010). APCs such as dendritic cells (DCs) are crucial for Treg cell induction in the local udLN in the peri-implantation period (Robertson *et al.* 2009). In addition to regulating antigen presentation, P4 regulates immune cell trafficking prior to pregnancy. In women, fluctuations in P4 and Estrogen (E2) over the course of the menstrual cycle causes Treg accumulation to the uterus (Kallikourdis and Betz 2007). RU486 was found to abrogate the down-regulation of CCL7 in a mouse model of delayed implantation whereby mice were ovariectomised on day 3.5 pc and administered combinations of P4, Estrogen (E2) and RU486 (Nautiyal *et al.* 2004). CCL7 is a chemoattractant for monocytes, neutrophils,

NK cells and DCs. Recruitment of these immune subsets occurs in the post-coital inflammatory response prior to implantation, and is then resolved once an anti-inflammatory immune profile is initiated (Guerin *et al.* 2011). These data suggest that RU486 may inhibit P4 regulation of immune cells in the peri-implantation period. P4 was also shown to affect Treg cells in mid- and late-gestation in mice. P4 administered vaginally to mice on days 13-17 pc increased Treg cell proportions in the decidua in late gestation (Furcron *et al.* 2015). Similarly, when RU486 was administered later in gestation as an abortive agent, clear effects on the maternal immune response were observed (Mao *et al.* 2010, Lajko *et al.* 2018). These effects were associated with a loss of P4 signalling in Treg cells (Mao *et al.* 2010).

In the current study, we focused on the effects of reduced P4 signalling during the peri-implantation period on the maternal T cell response in the udLN and spleen, to give an indication of P4 effects on T cells locally, and systemically. Many studies including those mentioned above, report key roles for Treg cells in the mouse uterus and decidua through flow cytometry analysis (Zenclussen *et al.* 2005, Samstein *et al.* 2012, Shima *et al.* 2015). Due to the uterus being a dynamic tissue and Treg cells being relative rare amongst all other cells types, we had technical difficulties identifying sufficient numbers of true Foxp3-expressing populations for analysis. Particularly, our analysis was complicated by background staining and auto-fluorescent cells, which became evident when CD45 (expressed by all hematopoietic cells) was added to the FACS panel. Upon analysis, the vast majority of cells expressing $CD4^+$ were in fact negative for CD45 expression. As other $CD45^+$ cells were evident, this likely indicates non-specific CD4 staining in some cells. Further investigation is therefore required to confidently identify lymphocyte subsets in the uterus. Despite this, the udLN is known to be a reliable measure of Treg cells mediating maternal tolerance, as it is the site of priming and source of Treg cells for active recruitment into the fetal maternal interface (Robertson *et al.* 2009). Therefore we used the data in the udLN to inform on potential uterine Treg cells dynamics.

In this study, using RU486 to reduce P4 bioavailability at peri-implantation caused profound changes in the T cell compartment by mid-gestation, notably reducing total cell numbers, $CD4^+$ T cells and $Foxp3^+$ Treg cells, particularly in the udLN. Overall Treg cell numbers in the udLN were dependent on the doses of RU486 administered, with low 0.5-1 mg/kg doses having the largest effect on Treg cell numbers, regardless of whether non-pregnant mice were eliminated from the analysis. Similarly, only low-moderate doses of RU486 (1 and 2 mg/kg) decreased cell numbers in the spleen. These results suggest that Treg cells during pregnancy are particularly sensitive to a low-level disruption in P4 signalling. The 0.5 and 1 mg/kg doses provide a good example of reduced P4 signalling on Treg cells in a pregnancy where viability is not overtly comprised at mid-gestation. The mechanism of P4 action in regulating Treg cells remains to be determined, but is investigated further in the following chapters in this Thesis.

In mice treated with high doses of RU486 resulting in early pregnancy failure, an increase in inflammatory T cell cytokine profile in $CD4^+$ and Treg cells, as well as an increase in $CD8^+$ T cell proportion was observed. These results could be explained by aberrant persistence of the inflammatory profile characteristic of early pregnancy, perhaps due to inefficient Treg cells to suppress and resolve this inflammatory response, or by excessive inflammation generated from the implantation failure itself (Dekel *et al.* 2010).

Overall, in mice treated with higher doses of RU486, no change in Treg cell numbers or proportions was evident. This may reflect the fact that the potential pregnancy ended ~6 days earlier, and the excessive inflammation required suppression by the local Treg cell response. Given these findings and the importance of Treg cells for implantation success, we postulate the immune perturbations following peri-implantation disruption in P4 signalling contribute to the pregnancy failure observed in early gestation following high-dose RU486 treatment.

An overall reduction in the size of the total immune cell compartment was evident by mid-gestation and could not be accounted for solely by the T cell subsets measured, indicating other cell subsets were affected following RU486 administration. These cells are likely to be macrophages, neutrophils, DCs and NK cells, as like T cells these subsets are responsive to P4 (Arruvito *et al.* 2008, Butts *et al.* 2008, Lu *et al.* 2015, Thiele *et al.* 2019). Future experiments are necessary to identify these cell types as well as to quantify changes to total $CD45^+$ and $CD3^+$ leukocyte populations following RU486 treatment.

Our study brings into question whether the effects of P4 on T cells are mediated through direct, T cell intrinsic pathways such as in the above study (Hierweger *et al.* 2019), or though indirect pathways via other immune cells known to be P4-responsive, such as DCs (Thiele *et al.* 2019). The role of P4 in mediating direct effects in T cells is investigated and discussed further in Chapter 5.

3.6.3. On the effect of reduced P4-signalling on P4 concentration

Interestingly, in the current study, P4 serum concentrations decreased in RU486-treated mice by mid-gestation. While confirming the model specifically affects P4 signalling, this suggests firstly that decreasing P4 signalling even with a low-dose of RU486 results in decreased P4 synthesis, and secondly that the effects of reducing P4 bioavailability appear to be prolonged. On the latter finding, RU486 is known to have a long metabolic half-life and thus may have a sustained effect in the model used (Baulieu 1991). RU486 is known to block the actions of P4 in the ovary, impairing normal ovarian function (Lydon *et al.* 1995). Despite the reduction in P4 levels following low-dose RU486 treatment in peri-implantation period, the serum P4 levels of the RU486 treated group were not substantially lower than the lowest levels

of P4 in WT females (95% CI=5.959 to 42.20). Indeed, some pregnancies were still viable with these lower concentrations of P4.

3.6.4. On the relevance of tTreg and pTreg cell subsets maternal immune tolerance

Nrp1 was used to distinguish between Treg cells of thymic (tTreg) and peripheral (pTreg) origin in this study. While there is a degree of overlap of expression of Nrp1 and the more traditionally used tTreg marker, Helios, Nrp1 is identified as a more accurate marker of tTreg cells in mice (Weiss *et al.* 2012). Despite key papers in the field of reproductive immunology showing the importance of paternal and fetal antigens in priming Treg cells during pregnancy (Zhao *et al.* 2007, Robertson *et al.* 2009, Kahn and Baltimore 2010, Rowe *et al.* 2012), distinct roles for tTreg versus pTreg cells in pregnancy are not well understood. Work from the Rudensky laboratory has suggested that pTreg cells are the essential Treg cell subset for pregnancy (Samstein *et al.* 2012). By using CNS1-deficient mice which lack pTreg cells specifically, they demonstrated increased resorption rate and impaired uterine spiral artery remodelling compared to CNS1-replete controls. The authors concluded that pTreg cells specifically are indispensable for optimal pregnancy success. However, the level of fetal loss seen was relatively minor (10%), with pregnancy progressing in the majority of implantations. Another study showed the memory Treg cells that proliferate following embryo implantation are in part specific to self-antigens and were thus tTreg cells (Chen *et al.* 2013). In support of tTreg cell involvement in pregnancy success, recent work from our laboratory has demonstrated that seminal fluid causes expansion predominantly in the tTreg cell population. tTreg cells from mated females on day 3.5 pc showed increased suppressive marker expression and progressive demethylation of the Treg specific demethylated region (TSDR) of the Foxp3 locus (Moldenhauer *et al.* 2019). tTreg cells make up the largest population of Treg cells in the non-pregnant state, and we observed that this is sustained throughout early-mid gestation. Interestingly, a recent human study revealed a distinct population of tTreg cells in first trimester decidual tissue on the basis of HELIOS expression (Salvany-Celades *et al.* 2019). However, as HELIOS is not a reliable marker to distinguish between tTregs and pTregs in humans, and is rather marker of Treg cell stability (Elkord 2016), the true origin of these cells is in question. Collectively, these studies indicate that both pTreg and tTreg subsets are important for overall pregnancy success, and hint toward differential functions of each subset in mediating pregnancy success. Importantly, the results presented herein demonstrate that tTreg cells, but not pTreg cells, are specifically affected by loss of P4 signalling.

Collectively these studies bring into question the antigen specificity of the tTreg cells in pregnancy. tTreg cells are known to respond primarily to self-antigen, but alloantigen specific Treg cells are largely responsive in pregnancy. Despite the commonly accepted pathway of tTreg development of priming by

thymic-resident APCs with tissue restricted self-Ags, strong evidence has emerged that they can also be activated against non-self, extra-thymically acquired antigens (Song *et al.* 2006, Hadeiba *et al.* 2012, Lee and Lee 2018). For example, the thymus was shown to play a role in the generation of Treg cells capable of inducing oral tolerance in experimental autoimmune encephalomyelitis (EAE) in an antigen specific manner (Song *et al.* 2006). It is well accepted that up to 10% of the T cell repertoire is alloantigen responsive and this population is thought to mediate organ rejection responses (Suchin *et al.* 2001). Despite their importance in mediating tolerance to allografts, the alloantigen reactivity in the Foxp3$^+$ Treg cell subset is not well understood. A recent study quantified the level of alloreactivity in T conv cells compared to Treg cells and found that Treg cells transferred to allogenic recipients can respond and expand in a TCR specific manner, in equivalent proportions to alloantigen responsive T conv cells (Lalfer *et al.* 2019). These findings, combined with their dominant presence in pregnancy, and evidence of local expansion in the udLN, suggests that tTreg cells implicated in maternal immune tolerance may be largely responding to alloantigen. This is consistent with the findings in the current study, in which a model of allogeneic mating is used, whereby a response to male alloantigens is generated. Here, we show the dominant tTreg cells subset is most affected by loss of P4 bioavailability in the peri-implantation period. In line with this, the proliferation and trafficking of presumably tTreg cells to reproductive tissues in response to hormones throughout the menstrual cycle in women, or the estrous cycle in mice, demonstrate that this Treg population is highly sensitive to hormonal regulation (Kallikourdis *et al.* 2007).

Overall, the results presented here and the wider literature suggest there are implications of P4 regulation on Treg cells for pregnancy success. Work from our laboratory and others have shown that the Treg cells induced in the peri-conception period are essential for mediating maternal immune tolerance necessary for later successful pregnancy outcomes (Lin *et al.* 2014, Prins *et al.* 2015). Recent evidence suggests that the mechanism by which this occurs may involve Treg cells setting a trajectory of events leading to appropriate decidual vessel remodelling, placental development and subsequent fetal growth (Croy *et al.* 2011, Cornelius *et al.* 2015, Care *et al.* 2018). Given that peri-implantation P4 regulation of Treg cells was found to be a key determinant of Treg cell number and proportions during mid-pregnancy in our model, we would expect this to have ramifications for later pregnancy success. Experiments to investigate the effect of reducing P4 signalling in the peri-implantation period on outcomes in later gestation, and the contribution of Treg cells to mediating the effects of P4 in pregnancy, are investigated in the following chapter (Chapter 4).

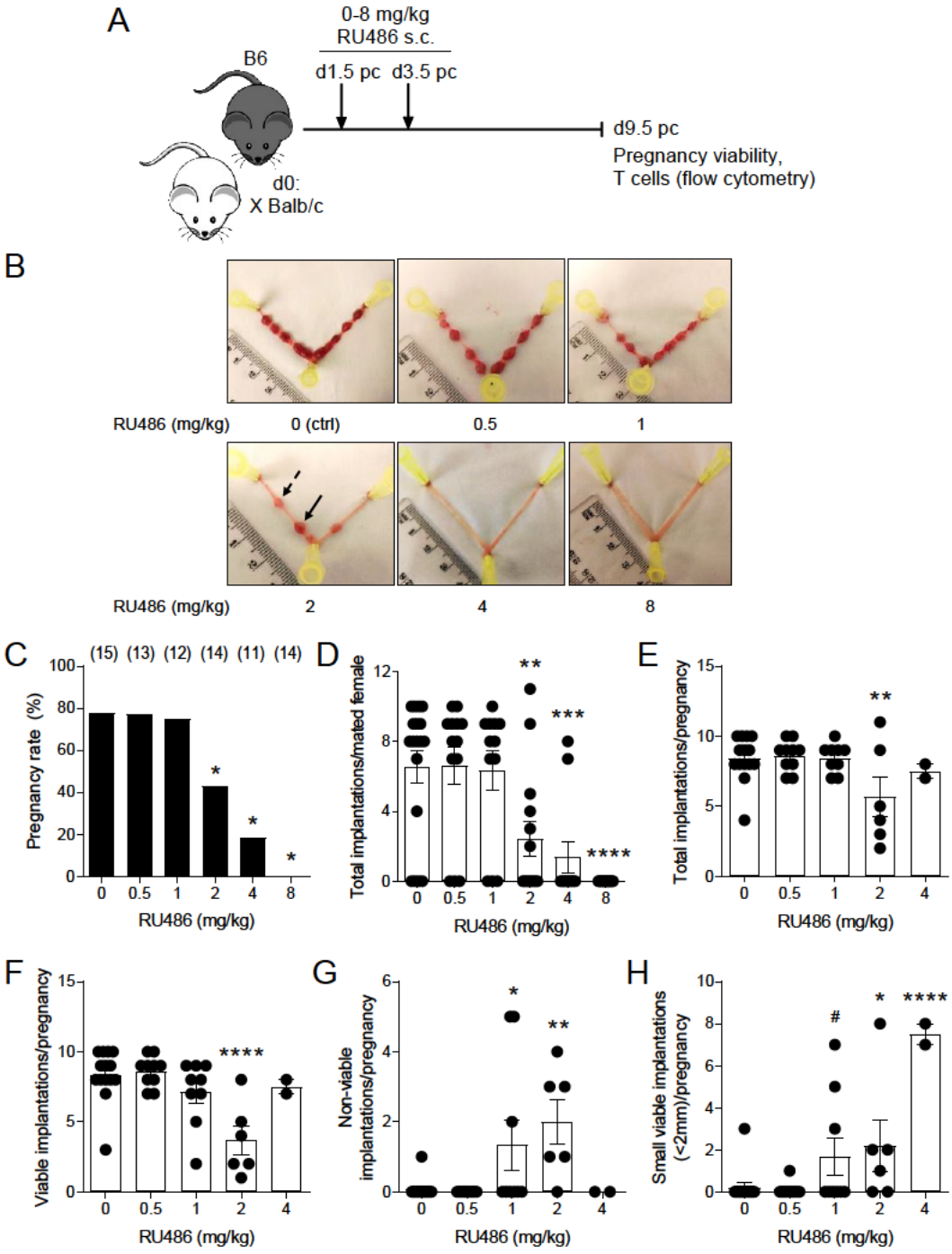

Figure 3.1 Disrupting progesterone signalling with RU486 in the peri-implantation phase elicits a dose-dependent decrease in pregnancy rate and number of implantations sites.

Female B6 mice were allogenically mated to BALB/c males and on day 1.5 and 3.5 post coitus (pc), RU486 (0.5 - 8 mg/kg) or vehicle control was administered. On day 9.5 pc, pregnancy progression was assessed. (A) Schematic of experimental design. (B) Representative images of uteri from RU486 treated mice. Filled arrow indicates a viable implantation and dotted arrow indicates a non-viable implantation. (C) Percentage of mated females that were pregnant with at least one implantation site on day 9.5 pc (pregnancy rate). (D) Total implantations per mated female, and (E) pregnant female. (F) viable implantations, (G) non-viable implantations, and (H) small viable implantations (>2mm in size) per pregnant female. n=11-14 mated females and n=2-14 pregnant females. Data are presented as mean ± SEM with individual females indicated by symbols. (B) Analysed by Chi-squared test. (D-G) analysed by one-way ANOVA compared to control; * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$, # $p<0.1$.

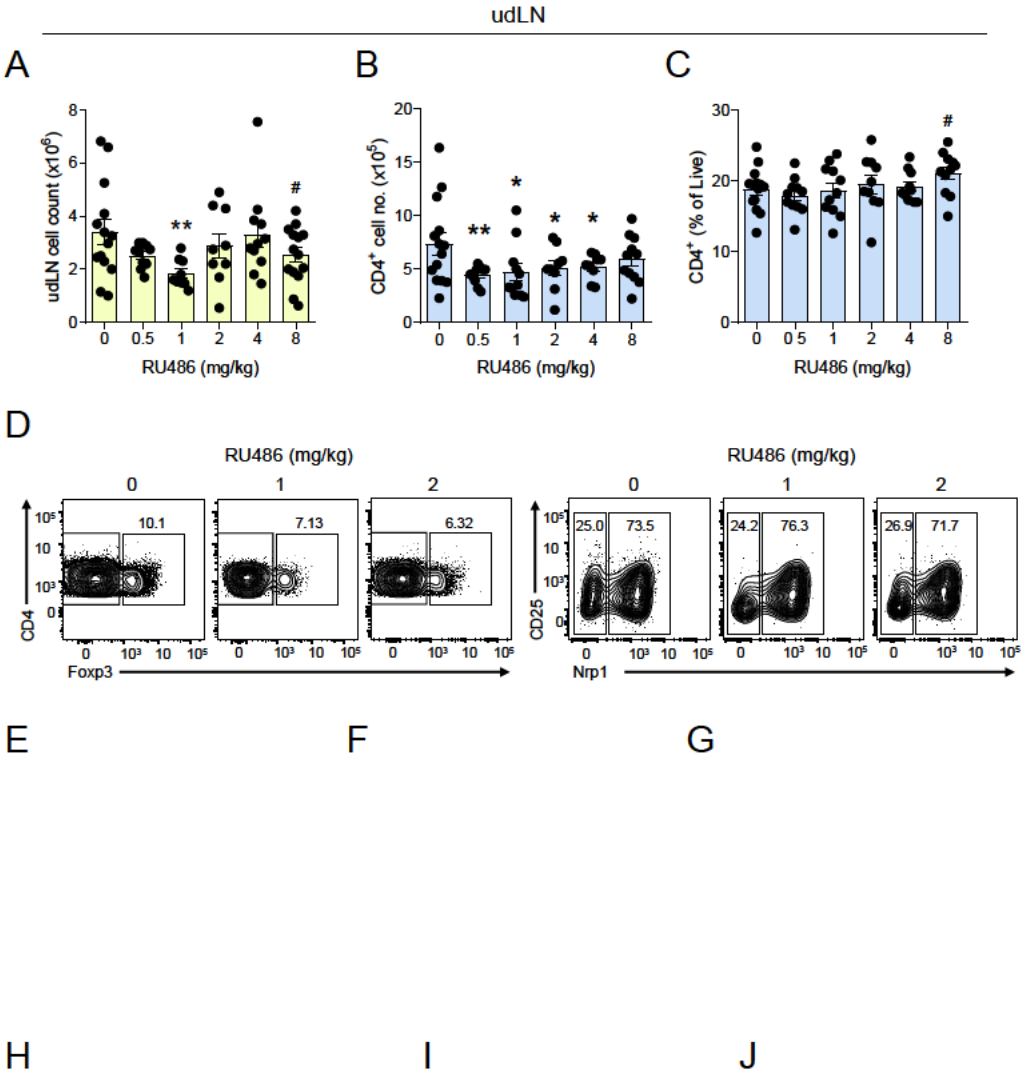

Figure 3.2 CD4+ T cell and Treg cell parameters in the uterus-draining LNs on day 9.5 pc following peri-implantation administration of RU486

Female B6 mice were allogenically mated to BALB/c males and on day 1.5 and 3.5 post coitus (pc), RU486 (0.5 - 8 mg/kg) or vehicle control was administered. On day 9.5 pc, T cells were measured by flow cytometry. (A) total cell count in the udLN, and (B) $CD4^+$ T cell number and (C) % of live cells was quantified. (D) Treg cells were identified as shown by representative FACS plots of Foxp3 staining in $CD4^+$ T cells following treatment with RU486. (E) Treg cell number, (F) % of $CD4^+$ T cells, and (G) Foxp3 Geo. MFI (as a fold change compared to control) was quantified. (D) Nrp1 expression in Treg cells was measured to quantify pTreg ($Nrp1^{-/lo}$) and tTreg ($Nrp1^+$) subsets. Relative proportions of $Nrp1^+$ and $Nrp1^-$ Treg cells of (H) total Treg cells, and of (I,J) $CD4^+$ T cells was assessed. n=8-14. Data are presented as mean ± SEM with individual mice indicated by symbols; one-way ANOVA compared to control;* $p<0.05$, ** $p<0.01$, *** $p<0.001$, $^{\#}$ $p<0.1$.

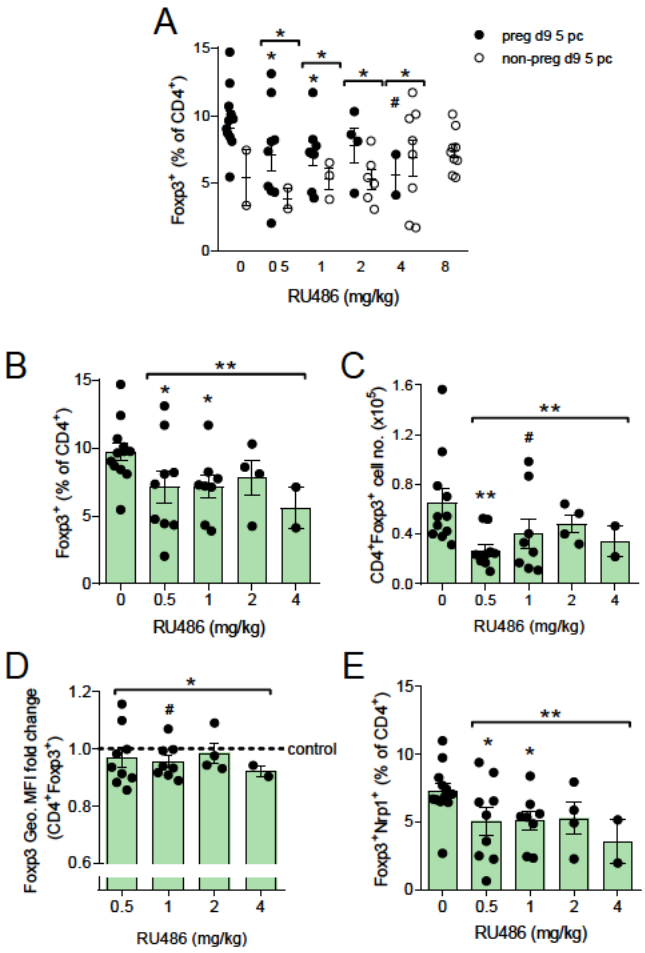

Figure 3.3 Uterus-draining LN Treg cell parameters based on pregnant or non-pregnant status on d9.5pc following peri-implantation administration of RU486.

Female B6 mice were allogenically mated to BALB/c males and on day 1.5 and 3.5 post coitus (pc), RU486 (0.5 - 8 mg/kg) or vehicle control was administered. On day 9.5 pc, T cells in the uterus-draining LNs (udLN) were measured by flow cytometry. (A) Treg cell % of $CD4^+$ cells in control and RU486-treated mice, with pregnant (at least one viable implantation) and non-pregnant mice on day 9.5 displayed for each group. (B-E) Selected Treg cell parameters for mice that were pregnant on day 9.5 pc (non-pregnant mice removed), showing (B) Treg cell % of $CD4^+$ T cells, (C) Treg cell number, (D) Foxp3 geometric MFI (as a fold change compared to control) and (E) % of $Nrp1^+$ Treg cells of total $CD4^+$ T cells. n=8-14 mice total, n=2-12 mice pregnant. Data are presented as mean ± SEM with individual mice indicated by dots. (A) One-way ANOVA compared to control; comparisons of total mice indicated by symbols with lines, and comparisons of pregnant groups alone indicated by symbols. (B-E) One-way ANOVA compared to control indicated by symbols; Unpaired T-test of control compared to RU486-treated indicated by symbols and lines. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, $^{\#}$ $p<0.1$

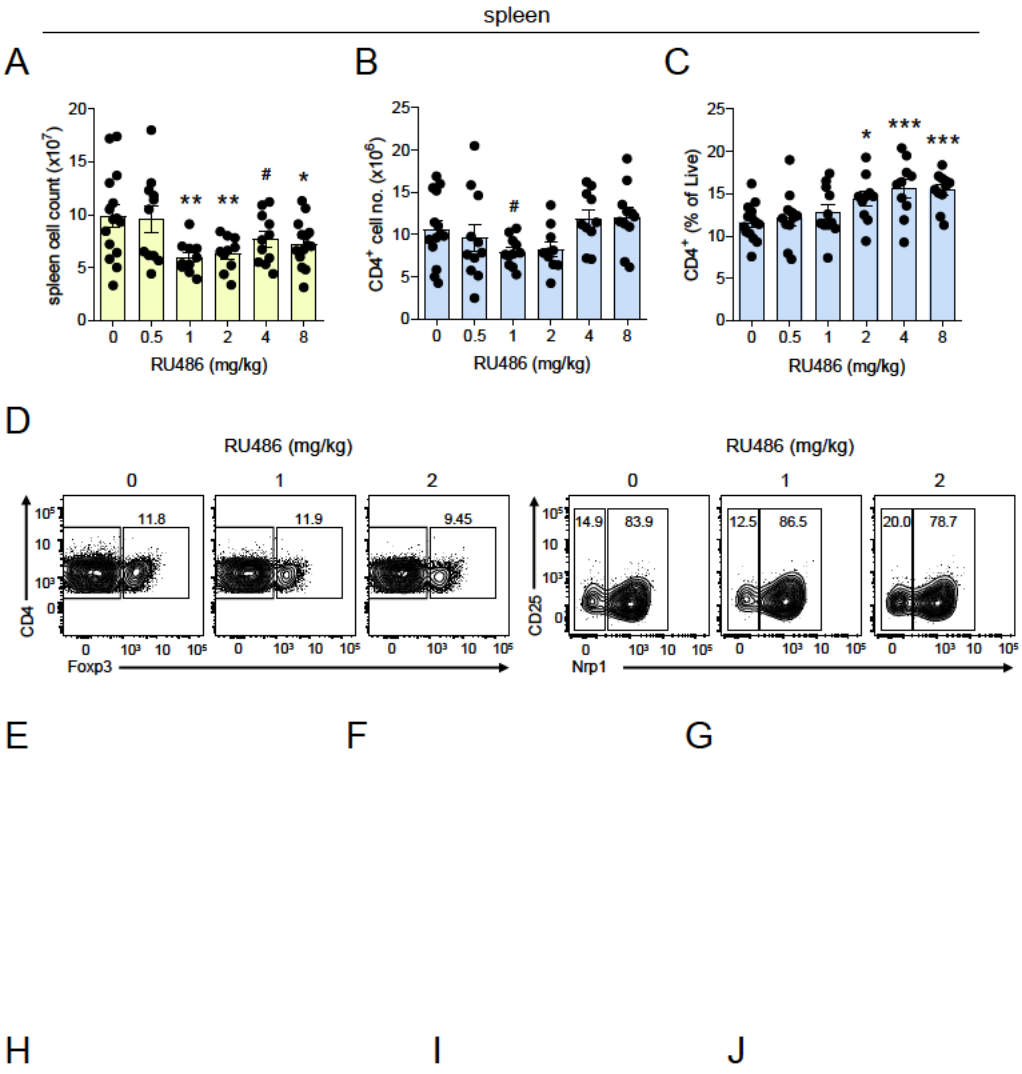

Figure 3.4. CD4$^+$ T cell and Treg cell parameters in the spleen on day 9.5 pc following peri-implantation administration of RU486

Female B6 mice were allogenically mated to BALB/c males and on day 1.5 and 3.5 post coitus (pc), RU486 (0.5 - 8 mg/kg) or vehicle control was administered. On day 9.5 pc, T cells were measured by flow cytometry. (A) total cell count in the spleen, and (B) CD4$^+$ T cell number and (C) % of live cells was quantified. (D) Treg cells were identified as shown by representative FACS plots of Foxp3 staining in CD4$^+$ T cells following treatment with RU486. (E) Treg cell number, (F) % of CD4$^+$ T cells, and (G) Foxp3 geometric MFI (as a fold change compared to control) was quantified. (D) Nrp1 expression in Treg cells was measured to quantify pTreg (Nrp1$^{-/lo}$) and tTreg (Nrp1$^+$) subsets. Relative proportions of Nrp1$^+$ and Nrp1$^-$ Treg cells of (H) total Treg cells, and of (I,J) CD4$^+$ T cells was assessed. n=8-14. Data are presented as mean ± SEM with individual mice indicated by symbols; one-way ANOVA compared to control;* p<0.05, ** p<0.01, *** p<0.001, $^{\#}$ p<0.1.

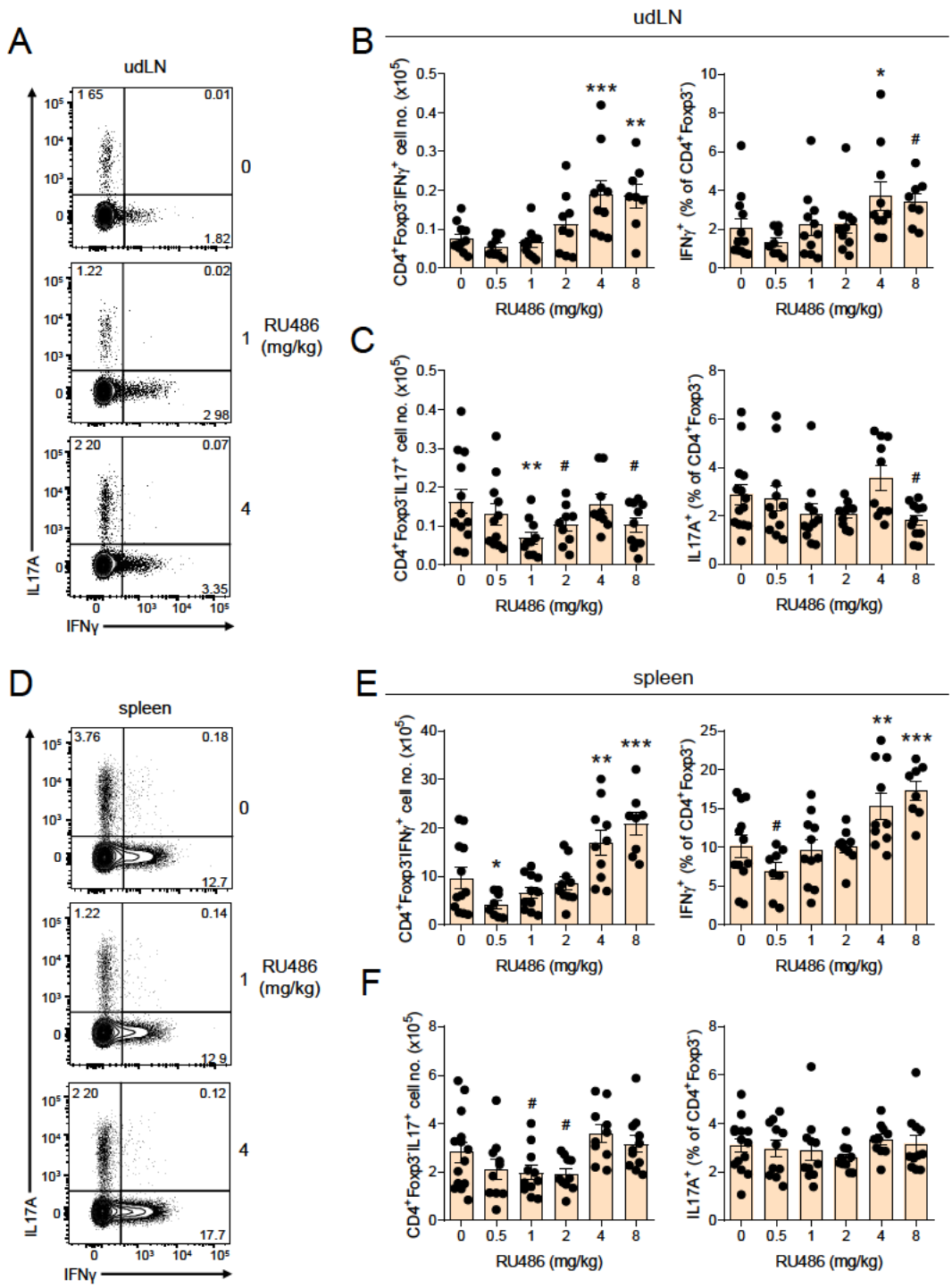

Figure 3.5 Inflammatory cytokine production in CD4+ T cells from uterus-draining LNs and spleen on day 9.5 pc following peri-implantation administration of RU486.

Female B6 mice were allogenically mated to BALB/c males and on day 1.5 and 3.5 post coitus (pc), RU486 (0.5 - 8 mg/kg) or vehicle control was administered. On day 9.5 pc, 1 x 10^6 cells were stimulated with PMA and Ionomycin for 4 h before quantification of T cell cytokine production by flow cytometry. (A,D) Representative FACS plots showing IFNγ and IL17A staining in (A) udLN and (D) spleen CD4+Foxp3- T cells of control or RU486-treated females. (B,C) Quantification of (A) showing number and proportion of CD4+ (B) IFNγ and (C) IL17+ T effector cells in the udLN. (E,F) Quantification of (D) showing number and proportion of CD4+ (E) IFNγ and (F) IL17+ T effector cells in the spleen. n=8-14. Data are presented as mean ± SEM with individual mice indicated by symbols; one-way ANOVA compared to control; * $p<0.05$, ** $p<0.01$, *** $p<0.001$, # $p<0.1$.

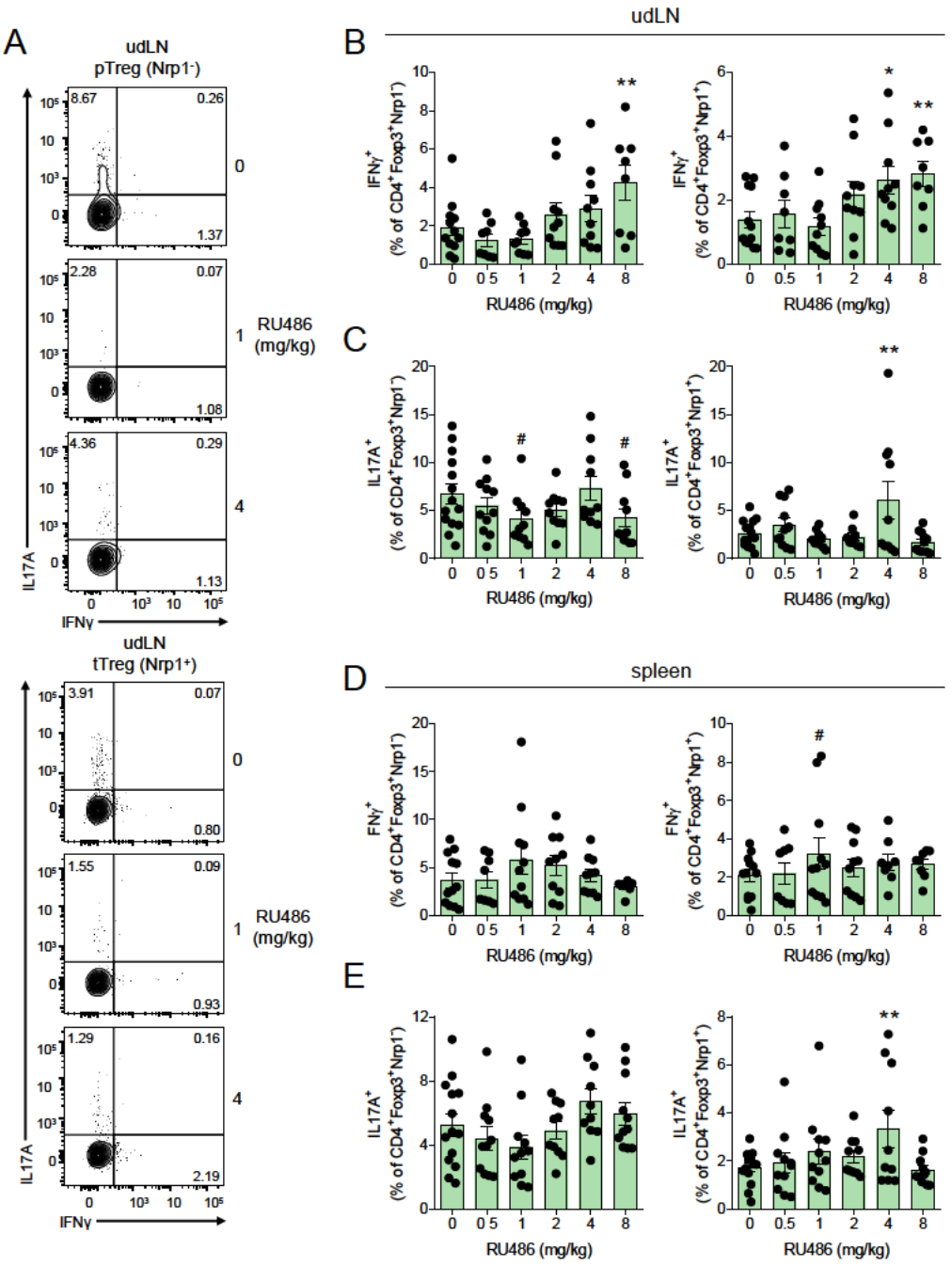

Figure 3.6 In flammatory cytokine production in Treg cells from uterus-draining LNs and spleen on day 9.5 pc following peri-implantation administration of RU486.

Female B6 mice were allogenically mated to BALB/c males and on day 1.5 and 3.5 post coitus (pc), RU486 (0.5 - 8 mg/kg) or vehicle control was administered. On day 9.5 pc, 1 x 10^6 cells were stimulated with PMA and Ionomycin for 4 h before quantification of T cell cytokine production by flow cytometry. (A) Representative FACS plots showing IFNγ and IL17A staining in udLN $CD4^+Foxp3^+Nrp1^-$ (pTreg) or $CD4^+Foxp3^+Nrp1^+$ (tTreg) cells of control or RU486-treated females. (B-C) Quantification of (A) showing number and proportion of $CD4^+$ (B) IFNγ and (C) $IL17^+$ T effector cells in the udLN. (D-E) Quantification of (A) showing number and proportion of $CD4^+$ (D) IFNγ and (E) $IL17^+$ T effector cells in the spleen. n=8-14. Data are presented as mean ± SEM with individual mice indicated by symbols. Data are presented as mean ± SEM; One-way ANOVA compared to control; * $p<0.05$, ** $p<0.01$, *** $p<0.001$, # $p<0.1$.

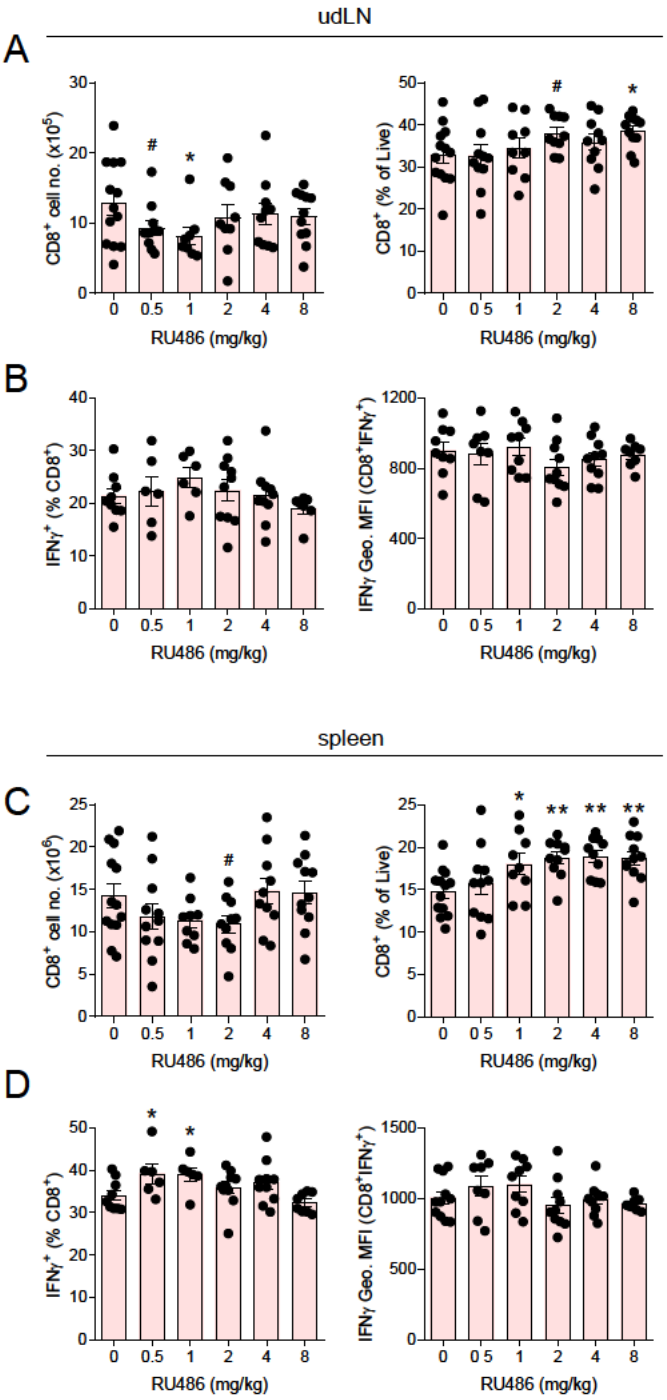

Figure 3.7 CD8$^+$ T cell parameters in the uterus-draining LNs and spleen on day 9.5 pc following peri-implantation administration of RU486.

Female B6 mice were allogenically mated to BALB/c males and on day 1.5 and 3.5 post coitus (pc), RU486 (0.5 - 8 mg/kg) or vehicle control was administered. On day 9.5 pc, 1 x 10^6 cells were stimulated with PMA and Ionomycin for 4 h before quantification of T cells and cytokine production by flow cytometry. CD8+ T cell parameters for the (A-B) udLN and (C-D) spleen were assessed. (A, C) Number and proportion (% of live cells) of CD8$^+$ T cells. (B,D) Proportion of IFNγ-expressing CD8$^+$ T cells (% of total CD8$^+$ population) and IFNγ geometric MFI in CD8$^+$IFNγ$^+$ T cells. n=8-14. Data are presented as mean ± SEM with individual mice indicated by symbols. Data are presented as mean ± SEM; One-way ANOVA compared to control; * p<0.05, ** p<0.01, *** p<0.001, $^{\#}$ p<0.1.

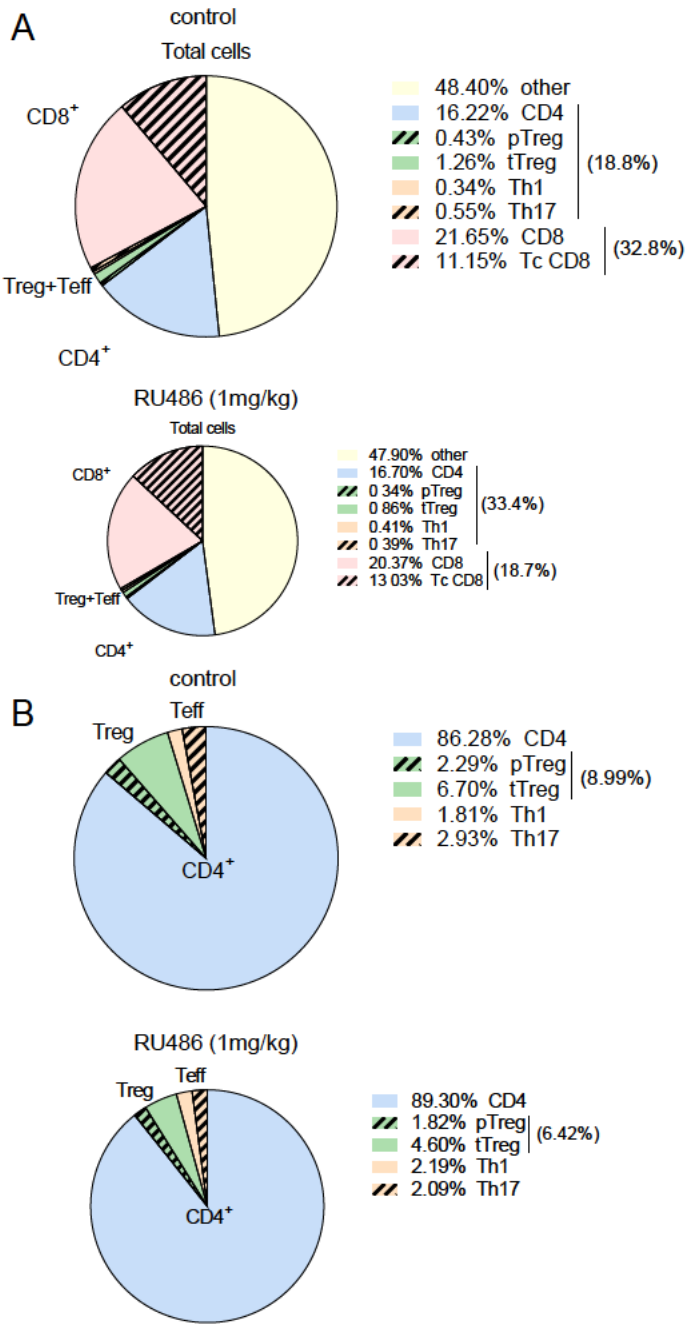

Figure 3.8 Net changes relative to control in total cell and CD4$^+$ T cell compartments in uterus-draining LNs on day 9.5 pc following administration of low-dose RU486 in peri-implantation.

Female B6 mice were allogenically mated to BALB/c males and on day 1.5 and 3.5 post coitus (pc), RU486 (1 mg/kg) or vehicle control was administered. On day 9.5 pc, total cells, CD4$^+$ and CD8$^+$ T cells and cell subsets were quantified. (A, B) Charts depicting net changes in % and relative size of total cell populations of CD4 (total), CD4 Teff (Th1, Th17), CD4 Treg (pTreg, tTreg), CD8 (total), CD8 T cytotoxic (Tc) cells, and other (Live CD4$^-$CD8$^-$) cells in (A) control and (B) RU486-treated mice. (C, D) Charts depicting net changes in % and relative size of total populations specifically in the CD4 T cell compartment, showing CD4 (total), Teff (Th1, Th17), and Treg (pTreg, tTreg) cells in (C) control and (D) RU486-treated mice. Data are presented as mean values of T cell proportions (A,B) as a % of live and (C,D) as a % of CD4$^+$ cells and pie charts are sized relative to (A,B) total cell counts for and (C,D) CD4$^+$ T cell counts. Data are representative of total populations of cells or each T cell subset for treatment vs control mouse groups (n=9-13 mice/group) as quantified in Figures 3.2, 3.5 and 3.7.

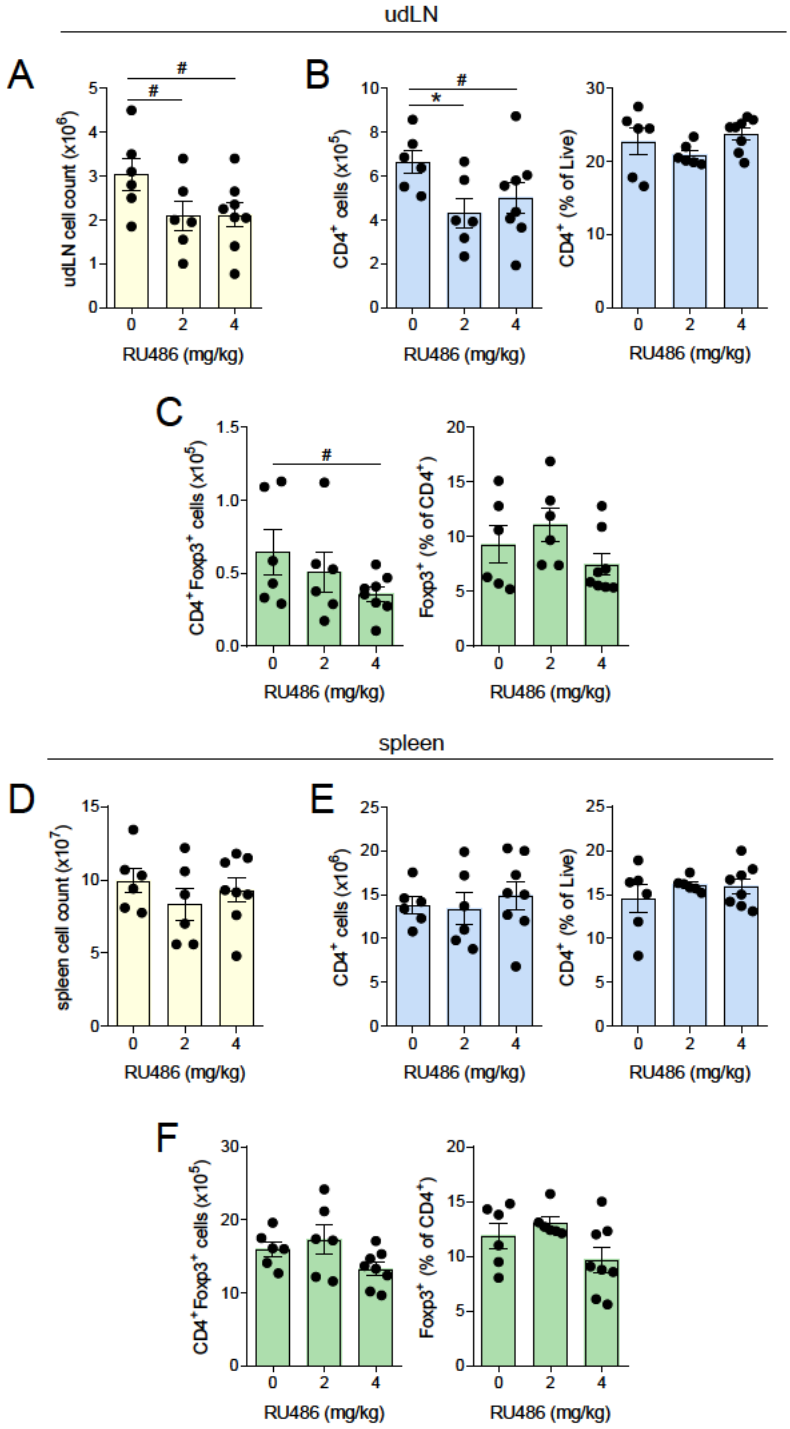

Figure 3.9 CD4$^+$ T cell and Treg cell parameters in the uterus-draining LNs and spleen on day 4.5 pc following peri-implantation administration of RU486.

Female B6 mice were allogenically mated to BALB/c males and on day 1.5 and 3.5 post coitus (pc), RU486 (2 or 4 mg/kg) or vehicle control was administered. On day 4.5 pc, T cells were measured by flow cytometry. (A) Total cell count and (B) CD4$^+$ T cell number and % of live cells in the udLN. (C) CD4$^+$Foxp3$^+$ Treg cell number and % of CD4$^+$ T cells in the udLN. (D) Total cell count and (E) CD4$^+$ T cell number and % of live cells in the spleen. (F) CD4$^+$Foxp3$^+$ Treg cell number and % of CD4$^+$ T cells in the spleen. n=6-8. Data are presented as mean ± SEM with individual mice indicated by symbols; one-way ANOVA compared to control;* $p<0.05$, $^{\#}$ $p<0.1$.

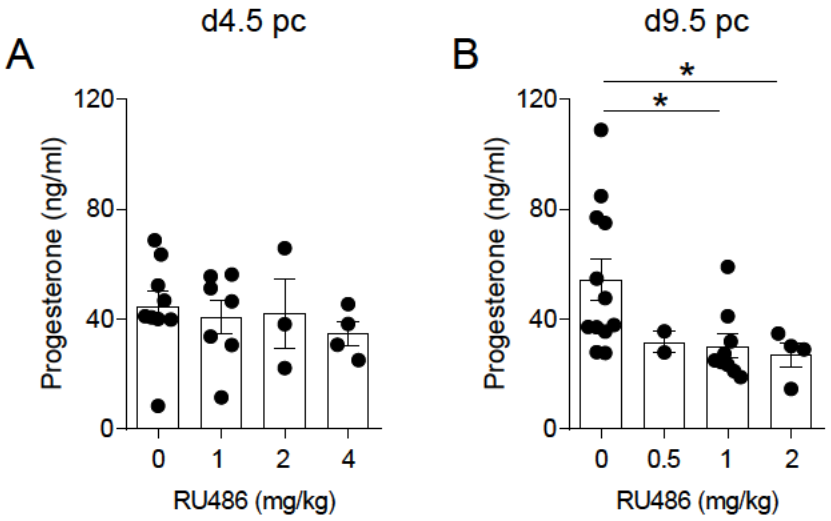

Figure 3.10 Progesterone serum concentrations on day 4.5 and 9.5 pc following peri-implantation administration of RU486.

Female B6 mice were allogenically mated to BALB/c males and on day 1.5 and 3.5 post coitus (pc), RU486 (0.5 - 8 mg/kg) or vehicle control was administered. On day 4.5 or 9.5 pc, mice were euthanised and blood was harvested. Serum was isolated from blood for P4 analysis by ELISA. (A) Serum P4 levels on day 4.5 pc in mated mice treated with low to moderate doses of RU486, or control. (B) Serum P4 levels on day 9.5 pc in mice confirmed pregnant following RU486 treatment, or control. (A) n=3-9. (B) n=2-12. Data are presented as mean ± SEM with individual mice indicated by symbols. Data are presented as mean ± SEM; One-way ANOVA compared to control; * $p<0.05$.

Chapter 4

The role of peri-implantation progesterone exposure and contribution of Treg cells to pregnancy outcome in late-gestation

4.1. INTRODUCTION

Successful pregnancy is dependent on several environmental and physiological factors, including endocrine and immune factors, which influence outcomes. P4 is known to coordinate complex events in the "window of receptivity" at the start of pregnancy including the important processes of embryo implantation and decidualisation (Franco *et al.* 2008, Wetendorf and DeMayo 2012). Following decidualisation, fetal trophoblast invasion of the maternal decidua occurs and leads to spiral artery remodelling and placental development. These P4-regulated events are complex and involve numerous regulatory pathways influencing angiogenesis, cell proliferation, and immune regulation (Whitley and Cartwright 2010). The placenta is a dynamic organ, responsible for providing nutrient and oxygen supply to the fetus to facilitate fetal growth and mediate maternal-fetal crosstalk. Placental development must be established correctly for efficient placental function, because if this process or those preceding it are dysregulated, adverse consequences arise for the mother and fetus. Obstetric disorders such as intrauterine growth restriction (IUGR), preeclampsia, spontaneous abortion and preterm birth (PTB) are characterised by defective placentation (Brosens *et al.* 2011). Furthermore, these pathologies are reported to have origins pre-pregnancy and at conception, and growing evidence suggest that these early events go on to shape the overall course of the pregnancy and fetal outcomes (Brosens *et al.* 2011, Roberts and Redman 2017). Thus, to properly understand pregnancy complications and develop new rational interventions to target these pathologies, it is essential to understand the initiating events at the time of conception.

Adequate P4 is needed from the outset of pregnancy, and particularly in the luteal phase prior to conception, for the early processes preceding placental development to occur. (Evans *et al.* 2016). In women, luteal phase deficiency (LPD) is defined as insufficient P4 to maintain a secretory endometrium and to adequately support embryo implantation and development (Mesen and Young 2015). The details and mechanisms of P4 action in early pregnancy for overall pregnancy success are not well understood.

The maternal immune response is also implicated in the pregnancy complications mentioned above, since all these pathologies are associated with inflammation or a breakdown of fetal-maternal tolerance (Romero *et al.* 2014, Roberts and Redman 2017). For instance, the failure of maternal immune tolerance and decidualisation are thought to contribute to intrauterine growth restriction in human pregnancy (Dunk *et al.* 2019). Treg cells are known to control excessive inflammation, and their importance in mediating tolerance to paternal alloantigens necessary for embryo implantation has been proven in mice (Aluvihare

et al. 2004, Robertson *et al.* 2018). Recent evidence has emerged for a role of Treg cell tolerance induced at the start of pregnancy, in setting the course of reproductive events in later gestation, including fetal growth, onset of labour and timing of birth (Steinborn *et al.* 2012, Care *et al.* 2018, Dunk *et al.* 2019). The role of Treg cells in mediating late pregnancy success has been studied in mice in the context of a second-hit inflammatory challenge such as models of inflammation-induced fetal loss (Lin *et al.* 2014, Prins *et al.* 2015). These studies demonstrate the importance of having adequate Treg cell expansion and activation to establish appropriate maternal tolerance to resist susceptibility and resolve inflammation induced by later gestation inflammatory insults.

Importantly, the preparation of the Treg cell response for pregnancy occurs prior to conception in response to both hormone fluctuations and seminal fluid factors (Kallikourdis and Betz 2007, Robertson *et al.* 2018). In the previous chapter we found that peri-implantation P4 exposure is a determinant of Treg cell expansion and the maternal immune response. Our work and the wider literature suggest that P4 shapes the number and phenotypes of immune cells in favour of a strong Treg cell response. However, the significance of this regulation for the overall success of the pregnancy is not well-understood.

The current study aimed to investigate whether P4 regulation of Treg cells at the outset of pregnancy was important for the overall success of pregnancy. We hypothesised that early pregnancy P4 regulation of Treg cells is a key determinant for overall pregnancy success. In this study, a low-dose of RU486 (1 mg/kg) was administered to mice, utilising the model developed in the previous chapter. Mated females administered this dose of RU486 were comparable to control females in terms of pregnancy rate and number of implantations at mid-gestation, however a significant perturbation to the maternal immune system was evident, including a decline in the Treg cell population. Thus, RU486 was again administered at 1 mg/kg, to investigate the implications of reduced P4 signalling on Treg cells and later pregnancy success. The following experiments use the RU486 model of reduced P4 signalling, developed in Chapter 3, to study the importance of reduced P4 signalling for pregnancy outcomes, and the significance of Treg cells in mediating the effects of P4 in establishing healthy implantation and pregnancy progression.

4.2. EFFECT OF PERI-IMPLANTATION DISRUPTION OF PROGESTERONE SIGNALLING ON PREGNANCY OUTCOMES IN LATE GESTATION

In the previous chapter, low doses of RU486 were sufficient to perturb the immune response and cause a significant reduction in Treg cells at mid-gestation. Given these findings, and that an adequate Treg cell

response is important for shaping later pregnancy outcomes (Robertson *et al.* 2018, Dunk *et al.* 2019), we hypothesised that mice treated with low-dose RU486 would progress to have poor maternal and fetal outcomes in late gestation. Thus late-gestational outcomes in mice administered the low-dose 1 mg/kg RU486 were investigated here (Figure 4.1).

4.2.1. Net changes to immune parameters in mice treated with low-dose RU486 in peri-implantation

Mated female mice were administered RU486 or control and on day 16.5, pregnancy rate and gestational weight gain was assessed. Strikingly, a 34% decrease in pregnancy rate was observed following low-dose RU486 treatment in the peri-implantation period (Figure 4.1A). Additionally, RU486 females gained significantly less weight over the course of the gestational period, indicative of fewer pregnant females overall and less fetuses per pregnant female (Figure 4.1B). These data suggest that pregnancy loss occurs from mid-gestation, since mice administered 1 mg/kg RU486 had normal pregnancy rate when measured on day 9.5pc (Figure 3.1).

4.2.2. Maternal outcomes in RU486 treated mice in the presence or absence of a late-gestational inflammatory challenge

In mice that were pregnant on day 16.5 pc, late-gestational maternal and fetal outcomes were subsequently measured, including in the presence or absence of an inflammatory agent, lipopolysaccharide (LPS). LPS is a cell wall component of gram negative bacteria which induces systemic and local inflammatory reactions through the TLR4 pathway (Park and Lee 2013). It has been extensively used in our laboratory and others as a inflammatory insult to study pregnancy outcomes in mouse models studying the immune response to pregnancy (Robertson *et al.* 2006, Nadeau-Vallee *et al.* 2017). Dependent on the time of administration, LPS leads to preterm birth and fetal resorption and death. The 2 μg dose of LPS used in this study was chosen based on previous studies in our laboratory and was optimised to cause preterm birth in approximately 50% of B6 mice administered LPS in late-gestation.

Pregnant mice were treated with RU486 or vehicle control and then injected with 2 μg LPS (or control; PBS) i.p. on day 16.5 pc. Mice were video monitored to determine the timing of birth, with mice that had not delivered by day 18.5 pc euthanized for late gestational outcomes to be measured. Figure 4.1C shows a schematic of the experimental design. Compared to PBS-treated controls which did not undergo preterm birth, 50% of dams injected with LPS gave birth prematurely (Figure 4.1D). Unexpectedly, RU486 treated females administered with LPS did not deliver preterm, suggesting that disruption of P4 signalling in early gestation abrogates susceptibility to LPS induced preterm birth (Figure 4.1D). Females from all groups

had at least one viable fetus per litter, with the exception of one pregnant female treated with RU486 and LPS observed to have complete late fetal resorptions (Figure 4.1D).

4.2.3. Fetal outcomes in RU486 treated mice in the presence or absence of a late-gestational inflammatory challenge

Next fetal outcomes in mice treated with RU486 (or control), and LPS (or control) were assessed (Figure 4.2). Figure 4.2A shows fetal outcome expressed as a percentage for each group. On day 18.5 pc, uteri were examined for the presence of viable fetuses, non-viable fetuses, early (small) resorptions and late (large) resorptions. In the LPS-treated females that delivered preterm, fetal viability was also assessed in Figure 4.2A. While control (-RU486-LPS) fetuses had approximately 95% viability in utero on day 18.5 pc, fetal viability was decreased in RU486 treated (+RU486) dams, showing approximately 25% early resorptions. Most noticeably, the LPS-treated (+LPS) group contained mixed outcomes with a large proportions of pups delivered preterm (of which most were viable), a proportion of non-viable fetuses in utero on day 18.5 pc, and a small proportion of fetuses resorbed late (between day 16.5 and 18.5 pc). The final group treated with RU486 and LPS (+RU486+LPS) showed a combination of effects from both the RU486 and LPS with mainly early resorptions and non-viable fetuses observed. Surprisingly there were generally higher proportions of small resorptions present in the +RU486+LPS group compared with the +RU486 group, however as these resorptions would have occurred prior to LPS treatment, they represent losses that would have occurred at the same time as the +RU486 group, and thus the difference between these groups may represent a statistical anomaly (Figure 4.2A). If +RU486 and +RU486+LPS groups are combined, approximately 50% of total fetal outcomes are early resorptions (data not shown).

Figure 4.2B shows representative images of uteri from control and RU486 treated mice, highlighting the presence of early fetal resorptions following RU486 treatment in the peri-implantation period. Due to the small size of these resorptions and lack of developed placenta, they are likely implantation sites that failed to develop from mid-gestation. This data is consistent with the increased number of ‘small’ implantation sites present on day 9.5 pc following RU486 treatment presented in the previous chapter (Figure 3.1).

The results in Figure 4.2A and 4.2B were quantified specifically for viable pups (Figure 4.2C) and resorptions + non-viable pups (Figure 4.2D) per litter. A significant decrease in viable pups per litter (Figure 4.2C) was observed in +LPS dams compared to control dams ($P<0.05$), which was further decreased in the +RU486+LPS group compared to control ($P<0.0001$), +LPS ($P<0.05$) and +RU486 ($P<0.01$) groups. Resorptions and/or non-viable pups per litter were measured (Figure 4.2D) with an increase observed in RU486 treated mice compared to controls ($P<0.01$) as shown in Figure 4.2B. Combining early and late resorptions, and non-viable pups per litter, +RU486+LPS dams showed a

significant increase in resorptions and/or non-viable pups per litter compared to controls ($P<0.0001$) and +RU486 dams ($P<0.05$).

Fetal and placental weight were measured for each group and compared by linear mixed model ANOVA, using viable litter size (normalized to 6 fetuses per litter) as a covariate. This analysis allows for fetal weight to be collectively normalised to viable litter size and thus provides a method to confidently assess the effect of treatment on fetal and placental parameters (Chin *et al.* 2016, Nadeau-Vallee *et al.* 2017). Notably, in RU486 treated mice, a significant 12% reduction in fetal weight was observed compared to control mice (1344 ± 15 vs 1189 ± 16). In +LPS and +RU486+LPS mice, fetal weight was decreased compared with control mice, and comparable to +RU486 mice. There was also a decrease in placental weight in LPS-treated mice (Figure 4.2F, $P<0.001$ compared to control), which along with the observed decrease in fetal weight (Figure 4.2E) is consistent with previous results from our laboratory using LPS in late-gestation. No change in placental weight was observed for RU486 treated mice, regardless of LPS treatment (Figure 4.2F). The observed reduction in fetal weight in RU486 treated dams led to an overall decrease in the fetal:placental weight ratio in RU486 treated mice compared to controls, initiating decreased placental efficiency in these pregnancies (Figure 4.2G, $P<0.001$). The placentae in this experiment were collected and formalin-fixed for sectioning and future analysis of placental structure by Masson's trichrome staining.

Overall, the results from these experiments indicate that a perturbation in P4 signalling at the peri-implantation stage of pregnancy with a low dose of RU486 has a profound impact on outcomes in later gestation, most notably manifesting in a reduction in overall pregnancy rate, and a 12% reduction in fetal weight on day 18.5 pc, indicative of intra-uterine growth restriction (IUGR). Interestingly, a disruption in P4 signalling in early pregnancy appear to induced resistance to LPS-induced preterm birth. RU486 treated mice however were still susceptible to the effects of LPS in causing late fetal death and resorption.

4.3. EFFECT OF PERI-IMPLANTATION DISRUPTION OF PROGESTERONE SIGNALLING ON GESTATION LENGTH

Given that RU486 treated mice were resistant to LPS-induced PTB, we next investigated whether they delivered on-time, by allowing treated mice to progress to birth. Pregnant mice were treated with RU486 or vehicle control on day 1.5 and 3.5 pc and then injected with 2 μg LPS (or control; PBS) i.p. on day 16.5 pc. From day 16.5 pc, mice were video monitored to determine the timing of birth (Figure 4.3). Strikingly,

RU486 treated mice had a significant increase in gestation length by an average of 1 day (Figure 4.3 A, B), regardless of LPS treatment (P<0.01-0.05 vs control). Moreover, a large subset of RU486 mice (-/+ LPS) did not deliver until day 21 or 22 pc (Figure 4.3 A, B). This is visually demonstrated in Figure 4.3A, which shows the percentage dams pregnant following LPS treatment. By day 19 pc 50% of control or LPS-treated mice had delivered, whereas 50% of RU486 treated mice (-/+ LPS) had delivered by approximately day 20. By day 20.5 pc, almost all of the remaining 50% of control or LPS-treated mice had delivered, whereas approximately 40% of RU486 treated mice had not. The remaining 40% of RU486 treated mice went on to deliver on days 21-22.5 pc. Surprisingly, LPS in this experiment did not cause a significant decrease in gestation length. However, this was due to the fact that while 50% of these mice gave birth prior to term (day 18.5 pc), they gave birth between day 18-18.5 and thus were considered to be “late-preterm” (Figure 4.3 A, B).

Viable pups per dam at birth were also assessed (Figure 4.3C) demonstrating that upon birth, less viable pups were present following RU486 treatment in peri-implantation, again regardless of LPS treatment (P<0.01 vs control). This is best explained by the increase in small resorptions following RU486 treatment presented in Figure 4.2, but also can be explained by the high rate of fetal death observed in RU486 treated mice that delivered on day 21 or 22 pc (data not shown).

Overall these data demonstrate that peri-implantation disruption of P4 signalling with low-dose RU486 caused pregnant dams to deliver approximately 1 day later than control mice. Furthermore, the increase in gestation length following RU486 treatment was associated with less viable pups at birth.

4.4. EFFECT OF PERI-IMPLANTATION DISRUPTION OF PROGESTERONE SIGNALLING PROGESTERONE LEVELS IN LATE-GESTATION

P4 signalling is a known regulator of the timing of birth, and in mice P4 levels must decline to initiate the events that lead to labour, such as cervical ripening and uterine contractions (Shynlova and Lye 2014). Given that RU486 treated mice had an extended gestation length, delivering on average 1 day later than controls, we hypothesised their delayed labour could be correlated to P4 levels in late gestation. Thus P4 serum levels were measured in a subset of RU486 treated mice on days 16.5 and d18.5 pc by ELISA. In control mice a decrease in P4 levels was observed from day 16.5 to day 18.5 pc (Figure 4.4A, 4.4B; P<0.05). This data is consistent with published studies demonstrating a P4 decline proceeding birth (Barkley *et al.* 1979). Conversely, most RU486 treated mice did not exhibit a decline in P4 levels between

day 16.5-18.5 pc (Figure 4.4A,B) and P4 in the majority of RU486 treated mice was higher than those in control mice on day 18.5 pc (Figure 4.4A, $P<0.05$). This data was combined with the data on P4 concentrations in early and mid-gestation presented in Chapter 3 (Figure 3.10) to enable visualisation of P4 levels at multiple time points across gestation (Figure 4.4B). Whereas control mice showed a rise in P4 by mid-gestation, which declined in late gestation, RU486 treated mice showed a defect in P4 at mid-gestation ($P<0.05$ compared to control). Interestingly, the P4 serum concentrations at mid-gestation in RU486 treated mice were maintained throughout late gestation, and P4 failed to decline by day 18.5 ($P<0.05$ compared to control).

This data indicates that early pregnancy treatment with RU486 results in an abnormal P4 profile that persists over the course of gestation, leading to perturbation of the characteristic P4 decline in late-gestation. As the decline in P4 is necessary for parturition and the onset of labour, this finding may contribute to the mechanisms by which RU486 treated mice exhibited an extended gestation length.

4.5. EFFECT OF TREG TRANSFER ON PREGNANCY OUTCOMES IN MICE WITH PERI-IMPLANTATION DISRUPTION OF PROGESTERONE SIGNALLING

Given these results, we hypothesised that the loss of Treg cells, initiated during the peri-implantation period in response to disrupted P4 signalling, and evident by mid-gestation, were implicated in the cause of the poor maternal outcomes observed in late-gestation. Therefore we investigated the extent to which Treg cells can account for the effects of P4, using a Treg cell transfer approach, with the expectation of improving pregnancy success in P4 disrupted pregnancies.

CD4 and CD25 are commonly used to identify Treg cells based on surface markers alone, as Treg cells cannot be isolated for downstream application using intracellular markers such as Foxp3. The majority of $CD4^+CD25^+$ cells are known to represent true $Foxp3^+$ Treg cells. Isolated $CD4^+CD25^+$ Treg cells have been used in numerous studies to investigate the ability of Treg cells to rescue pregnancy complications (Zenclussen *et al.* 2005, Zenclussen *et al.* 2006). For example, the Zenclussen laboratory used 2×10^5 isolated $CD4^+CD25^+$ cells to rescue the phenotype of abortion-prone mating between CBA/2 and DBA/J mice (Zenclussen *et al.* 2006).

A similar approach was taken in the current study, whereby $CD4^+CD25^+$ 'Treg' cells were isolated from donor B6.SJL-Ptprca Pepcb/BoyJ (Ly5.1) females in mid-pregnancy for transfer into P4-disrupted pregnant females. By using Ly5.1 females as donors, the congenic marker Ly5 was employed to enable

the tracking of the donor Treg cells in vivo once transferred into the standard B6 mouse, which expresses the Ly5.2 isoform. Mid-pregnant females were used as a source of systemically primed and 'P4-experienced' T cells. $CD4^+CD25^+$ Treg cells from spleen and various LNs were isolated using StemCell™ Treg isolation kit as described in Chapter 2.3.3. This kit also allows for $CD4^+CD25^-$ cells to be isolated during the process, which were used as 'nonTreg' controls in these experiments.

4.5.1. Treg and nonTreg cell isolation from pregnant donors

Initially, the efficiency of donor T cell isolation was optimised and measured by flow cytometry. Figure 4.5 shows representative FACS plots of T cell subsets prior to and following isolation of Treg and nonTreg cells from a pregnant Ly5.1 donor female on day 14.5 pc. Prior to isolation, spleen and LN cells contained $CD4^+$, $CD8^+$ and $CD4^-CD8^-$ cell subsets (Figure 4.5A). Following isolation of $CD4^+CD25^+$ or $CD4^+CD25^-$ cells, $CD4^+$ T cells represented over 90% of cells, with a minor population (<5%) of $CD4^-CD8^-$ cells remaining (Figure 4.5A). Following $CD4^+CD25^+$ isolation, consistently 75-85% of the total cells isolated were double positive for $CD4^+CD25^+$, as assessed by flow cytometry (data not shown). Figure 4.5A shows the $CD4^+$ population was largely (80.4%) $CD25^+$ and $Foxp3^+$, demonstrating a true enrichment of the Foxp3 positive Treg population using the $CD4^+CD25^+$ isolation method. Following isolation of $CD4^+CD25^-$ cells, amongst $CD4^+$ T cells, over 95% were $Foxp3^-CD25^-$. Figure 4.5B shows CD44 and Nrp1 expression in $Foxp3^+CD25^+$ Treg and $Foxp3^-CD25^-$ nonTreg cells following isolation of either $CD4^+CD25^+$ or $CD4^+CD25^-$ cells. The vast majority of all T cells were CD44 positive following cell isolation from mid-gestation pregnant female mice, indicating their activated status.

In the $CD4^+CD25^+$ cell isolation, where the majority of cells were $Foxp3^+CD25^+$ (Figure 4.5A), both $Nrp1^+$ tTreg and $Nrp1^-$ pTreg subsets were identifiable, in the expected proportions (Figure 4.5B). Amongst the <1% of $Foxp3^+CD25^+$ cells contaminating transferred $CD4^+CD25^-$ cells, most were $Nrp1^+$ and $CD44^+$ and represented bone fide, activated Treg cells. The 10% of cells in the $CD4^+CD25^+$ cell isolation that were $Foxp3^-CD25^-$ (Figure 4.5A), were largely $CD44^+$ and $Nrp1^-$ and thus likely represented activated T cells (Figure 4.5B). This was also the case in the 95% of nonTreg cells yielded from the $CD4^+CD25^-$ cell isolation. In Treg and nonTreg isolations, a small contaminating population of CD8-expressing cells were present (Figure 4.5A). These $CD4^+CD8^{lo}$ double positive cells are a known minor population found in spleen and lymph nodes (Overgaard *et al.* 2015).

This data shows that true Treg and nonTreg cells can be isolated using a using the $CD4^+CD25^+$ isolation kit. However, as there were small populations of other cells affecting the purity of the isolation in each case, the recovered populations should be considered as enriched, rather than pure populations. To overcome the issue of purity in the cell preparations, future experiments could involve further purification

by FACS sorting of desired cell subsets. For the purposes of the current experiments however, it was judged sufficient to use the enriched Treg and nonTreg populations. In each experiment, following Treg and nonTreg isolation, a sample of cells were stained and analysed by flow cytometry to measure purity. Total cell numbers transferred into recipient mice were adjusted for purity based on surface staining with CD4 and CD25 antibodies. Typically, 80% and 95% purity was obtained for $CD4^+CD25^+$ and $CD4^+CD25^-$ populations, respectively.

4.5.2. Identification of transferred donor Treg cells in congenic recipient mice on day 6.5 pc

We aimed to transfer the Treg cells back to P4-disrupted females just prior to the time of implantation, when the P4-disruption begins to affect the endogenous T cell population (as shown in Chapter 3). Initially, we wanted to measure the success of the transfer by identifying the introduced cells in vivo. On day 3.5 pc, females were administered with 2 x 10^5 $CD4^+CD25^+$ Treg cells or $CD4^+CD25^-$ nonTreg cells from Ly5.1 mid-pregnant mice, or vehicle control (PBS), by i.v. tail vein injection. On day 6.5 pc, approximately 72 hrs following adoptive cell transfer, mice were killed, pregnancy status was assessed, and donor cells were measured in the spleen, udLN, inguinal LN and blood using the marker Ly5 (CD45) to distinguish between donor (CD45.1) and recipient (CD45.2) T cells. Figure 4.6 shows the gating strategy used to identify donor Treg and nonTreg cells. By 72 hrs, donor CD45.1 Treg or nonTreg cells were undetectable in the blood (data not shown). Donor CD45.1 Treg and nonTreg cells were detectable in the spleen and LNs, including in the udLN, consisting of a small but trackable population of cells amongst endogenous recipient cells (Figure 4.6). Consistent with the cell isolations (4.5.1), the $CD4^+CD25^+$ cells introduced were largely $Foxp3^+$. A minor population of cells in $CD4^+CD25^+$ transferred mice were $Foxp3^-$ and represented nonTreg cells.

This data demonstrates that Treg and nonTreg cells are successfully introduced into pregnant donor females following i.v. transfer, and migrate from the blood to secondary lymphoid organs within 72 hrs. Given this, we presumed the introduced T cells had the potential to be recruited to the uterus under physiological conditions.

4.5.3. Late-gestational maternal outcomes following Treg and nonTreg transfer to P4-disrupted females

Allogenically mated B6 females were administered with 1 mg/kg RU486 (or control) on day 1.5 and 3.5 pc as used previously. Then, on day 3.5 pc, approximately 8 hours following the final RU486 treatment, females were administered i.v. with 2 x 10^5 $CD4^+CD25^+$ Treg cells, $CD4^+CD25^-$ nonTreg cells from mid-gestation Ly5.1 donors, or vehicle control. On day 18.5 pc, late-gestation maternal and fetal outcomes

were measured. Figure 4.7A shows a schematic of the experimental design. Pregnancy rate (Figure 4.7B) and maternal outcome expressed as a percentage of total dams (Figure 4.7C), was measured in RU486 (+RU486) or control (-RU486) treated females administered Treg cells (+Treg), nonTreg cells (+nonTreg) or vehicle control (-Treg).

Consistent with our previous data, a significant reduction in pregnancy rate was observed in P4-disrupted females (+RU486-Treg) compared with control females (-RU486-Treg) ($P<0.01$). In this experiment, pregnancy rate was reduced by 58% (Figure 4.7B). Strikingly P4-disrupted mice administered Treg cells (+RU486+Treg) at implantation showed a restoration in pregnancy rate, comparable to controls. Unexpectedly, in P4-disrupted mice administered nonTreg cells (+RU486+nonTreg) we also observed a restoration in pregnancy rate. However, when assessing maternal outcomes in +RU486+nonTreg cell mice, substantial fetal resorption per dam was present, with over 60% of dams experiencing a severe fetal resorption rate of >75% per dam (Figure 4.7C). While both RU486 control mice receiving vehicle (-RU486-Treg) and Treg cells (-RU486+Treg) experienced minor or no (>25%) fetal resorption overall per dam, P4-disrupted (+RU486-Treg) mice experienced moderate fetal resorption (>25 and >75%) in approximately 80% of dams. In comparison, the RU486 treated group administered Treg cells saw an improvement in maternal outcome whereby approximately 50% of outcomes were normal with none or a minor rate of fetal resorption (<25%) per dam. The other 50% of outcomes consisted of mainly moderate (>25 and <75%) resorptions, with a small proportion of dams experiencing >75% fetal resorption (Figure 4.7C).

4.5.4. Late-gestational fetal outcomes following Treg and nonTreg transfer to P4-disrupted females

Next, fetal outcomes were measured in P4-disrupted females transferred Treg or nonTreg cells (Figure 4.8). Fetal outcome expressed as a percentage of total fetuses (Figure 4.8A) revealed the percentage of fetal resorption following RU486 and/or T cell treatment. Based on their size and appearance in the uterus, approximately 40% of fetuses from RU486 treated mice (+RU486) were resorbed in early or mid-gestation (Figure 4.8A and 4.8B). A 10% improvement in overall fetal viability was evident in Treg treated mice, particularly due to a reduction in fetsues resorbed in mid-gestation. Conversely, nonTreg cell treated mice experienced severe fetal resorption with approximately 60% of fetuses resorbed early (Figure 4.8A).

Figure 4.8B depicts representative uteri showing implantations and resorptions from control (-RU486-Treg) and RU486 treated (-RU486-Treg) dams. The early fetal resorptions characteristic of RU486 treatment at peri-implantation were visible in late-gestation (Figure 4.2B and 4.8B), appearing as small red marks in the uterus. In some mice treated with RU486 and nonTreg cells (+RU486+nonTreg), larger

fetal resorptions were present (Figure 4.8B). In each case, these resorptions had a developed placenta therefore pregnancy failed after placental development, likely around day 12-14.5 pc.

Figure 4.8C-4.8E shows the number of viable pups and resorptions per litter. A decrease in viable pups per litter compared to controls (Figure 4.8C) was again observed following RU486 treatment (+RU468-Treg; $P<0.05$ compared to -RU486-Treg). A respective increase in the % fetal resorption per litter (Figure 4.8E) was also observed between these two groups $P<0.05$). An improvement in both the number of viable pups per litter and the % fetal resorption per litter was observed following Treg transfer into RU486 treated females (+RU486+Treg) (Figure 4.8C and 4.8D). Most notably, mice treated with both RU486 and nonTreg cells (+RU486+nonTreg) experienced a significant reduction in viable pups per litter compared to control (-RU486-Treg, $P<0.001$) and +RU486+Treg mice ($P<0.1$). Respectively, an increase in both number of resorptions (Figure 4.8D) and % fetal resorption per litter (Figure 4.8E) in +RU486+nonTreg mice was evident compared to -RU486-Treg mice ($P<0.001$, Figure 4.8E) and +RU486+Treg mice ($P<0.05$, Figure 4.8E).

Fetal and placental weight were measured for each group and again compared by linear mixed model ANOVA, using viable litter size as a covariate (normalized to 7.2). Fetal and placental parameters measured were consistent between both -Treg and +Treg control groups. As observed previously (4.2.3), a significant reduction in fetal weight of 24% in this experiment (1348 ± 20 vs 1028 ± 33), was observed in fetuses from RU486 treated females (Figure 4.9A). This reduction in fetal weight in dams with RU486 treatment in early pregnancy again led to a trend towards reduced fetal:placental weight ratio (Figure 4.9C, $P<0.1$ compared with -RU486-Treg or -RU486+Treg groups).

Strikingly, Treg cell transfer to RU486 treated mice partially restored the reduction in fetal weight by 16%, with the +RU486+Treg group showing a significant increase in fetal weight compared to +RU486-Treg cell treated mice ($P<0.001$; 1223 ± 20 vs 1028 ± 33). Despite this improvement, fetal weight was still slightly decreased compared to -RU486-Treg mice ($P<0.001$) and -RU486+Treg mice ($P<0.01$). However, the fetal:placental weight ratio was restored in +RU486+Treg mice (Figure 4.9C, P=NS compared to -RU486-Treg and +RU486+Treg groups).

Similar to the +RU486-Treg mice, a reduction in fetal weight was again evident in nonTreg cell-treated mice (+RU486+nonTreg) compared with both control groups (Figure 4.9A; $P<0.01$). Fetal weight in nonTreg cell-treated mice was also reduced compared with RU486 treated mice administered Treg cells (+RU486+Treg; $P<0.05$), but was comparable to the +RU486-Treg group (P=NS). Interestingly, mice treated with both RU486 and nonTreg cells exhibited an increase placental weight (Figure 4.9B), significant when compared with either +RU486-Treg ($P<0.001$) or +RU486+Treg ($P<0.001$) groups. The

perturbed fetal and placental weights in nonTreg cell treated mice caused a reduction in fetal:placental weight ratio (Figure 4.9C), compared to control groups ($P<0.001$) and also the +RU486+Treg group ($P<0.001$).

Overall, the results from these experiments demonstrate that Treg cell transfer prior to implantation can rescue pregnancy rate and improve late-gestational maternal and fetal outcomes in mice after perturbed P4 signalling during the peri-implantation period. Importantly, Treg cell transfer was sufficient to improve the fetal weight reduction observed following P4 signalling perturbation, enabling the fetal:placental weight ratio to be restored in these mice. Conversely, nonTreg cell transfer to RU486 treated mice caused increased adverse maternal and fetal outcomes, with significant incidence of fetal resorption. Transfer of nonTreg cells to RU486 treated females also caused a reduction in fetal weight and increase in placental weight, skewing the fetal:placental weight ratio and thus indicating efficiency of the placenta was compromised. However, these experiments are limited by having no nonTreg cell control group (-RU486+nonTreg), and therefore conclusions on the effects of nonTreg cells on pregnancy outcomes cannot be determined from the current data.

4.6. EFFECT OF TREG TRANSFER ON T CELL SUBSETS IN LATE GESTATION IN MICE WITH PERI-IMPLANTATION DISRUPTION OF PROGESTERONE SIGNALLING

Next, flow cytometry was performed on a subset of mice that underwent pregnancy outcome analysis on day 18.5 pc to assess the effect of Treg and nonTreg transfer on endogenous T cell parameters in late-gestation (Figure 4.10), as well as to identify the presence of the donor cells in late-gestation (Figure 4.11). Due to the low number of animals in these experiments the following results are considered preliminary data. $CD4^+$ T cell and Treg cell parameters were measured on day 18.5 pc in RU486 treated mice following transfer of Treg or nonTreg cells (Figure 4.10). There were no differences between control mice treated with Treg cells (-RU486+Treg) or vehicle (-RU486-Treg) for any of the $CD4^+$ T cell and Treg cell parameters measured in the LN and spleen in Figure 4.10 (data not shown). Therefore, these groups were combined for the analysis to strengthen the statistical comparisons between data.

In the udLN, we observed a trend towards a decrease in total cell numbers (Figure 4.10A) in RU486 treated mice ($P<0.1$ compared to control group) and a significant decrease in +RU486+nonTreg treated mice ($P<0.05$). Interestingly, Treg cell transfer to RU486 treated females led to an overall restoration of udLN cell number compared with control (P=NS), and an increase in cell number compared to the +RU486-Treg group ($P<0.05$). No changes in number of $CD4^+$ T cells in the udLN was observed (Figure

4.10B) with the exception of the +RU486+nonTreg group, which showed a reduction in $CD4^+$ T cell number compared to control and +RU486+Treg cell mice. Interestingly, despite no change to $CD4^+$ T cell number, the proportion of $CD4^+$ T cells out of total cells was increased in +RU486-Treg mice ($P<0.05$ compared to control).

For the +RU486+nonTreg treated mice, nonTreg cell transfer appeared to cause a reduction in total $CD4^+$ T cell and Treg cell numbers (Figure 4.10C). No changes to the number of Treg cells was observed for any of the groups, and no changes in the proportion of Treg cells was observed between any groups in the udLN (Figure 4.10C). In the spleen, there were no significant changes observed for any of the T cell or Treg cells parameters measured (Figure 4.10D-4.10E).

Overall, this data suggests that RU486 treated mice may have a deficit in leukocyte numbers specifically in the udLN, and that Treg cell transfer at peri-implantation restores endogenous cell populations to control levels. The deficit in cell number did not correlate to the T cell populations measured, indicating P4 signalling disruption affects other cell types. This observation could be one explanation for the mechanisms of Treg transfer restoring pregnancy outcomes in P4-disrupted mice. However, due to a low sample size, further experiments are required to draw robust conclusions regarding these data.

Lastly, the presence of donor Treg and nonTreg cells on day 18.5 pc was assessed by flow cytometry, using the gating strategy described in Figure 4.6 to identify donor CD45.1 cells in recipient udLN and spleens (Figure 4.11). Overall, the donor Treg cells typically made up less than 2% of the total endogenous cell population. As expected, no CD45.1 cells were detected in mice injected with PBS vehicle (-Treg).

In mice transferred with $CD4^+CD25^+$ Treg cells (either +RU486 or -RU486 mice) a detectable population of donor $Foxp3^+$ cells was evident in the udLN and spleen on day 18.5 pc (Figure 4.11A and 4.11C). Additionally, a similar number of donor $CD4^+Foxp3^-$ cells were detected (Figure 4.11B and 4.11D). Whether these represent the fraction of $Foxp3^-$ cells amongst $CD4^+CD25^+$ isolated cells, or the fraction of $CD25^-$ isolated cells in the Treg cell transfer preparation is unknown. In mice transferred with $CD4^+CD25^-$ nonTreg cells, no $Foxp3^+$ cells were detected in the udLN or spleen (Figure 4.11A and 4.11C). As expected, $Foxp3^-$ donor cells were detected in udLN and spleen of mice transferred with $CD4^+CD25^-$ nonTreg cells (Figure 4.11B and 4.11D).

This data shows that donor T cell subsets isolated from pregnant mice and transferred into recipient mice at peri-implantation are detectable 15 days later at the end of gestation, in the draining LNs and spleen, thus confirming the efficiency of the transfer and the persistence of the transferred cells throughout gestation.

4.7. EFFECT OF TREG TRANSFER ON SERUM PROGESTERONE LEVELS IN LATE GESTATION IN MICE WITH PERI-IMPLANTATION DISRUPTION OF PROGESTERONE SIGNALLING

Finally, serum P4 concentrations were measured by ELISA in a subset of mice on day 18.5 pc following Treg or nonTreg cell transfer (Figure 4.12). Control mice (-RU486-Treg) showed approximately 5 ng/mL P4 on day 18.5 pc, which is consistent with the previous data on P4 levels measured (Figure 4.4). For the other control group administered Treg cells (-RU486+Treg), while only two mice were analysed, P4 levels were around 15 ng/mL (Figure 4.12), which was also within the range of P4 concentrations previously observed (Figure 4.4). Again, RU486 treatment in peri-implantation (+RU486-Treg) caused significantly higher P4 levels in late gestation (Figure 4.12; $P<0.01$ compared to control), consistent with the data in Figure 4.4. Similarly, nonTreg cell treatment to RU486 treated mice (+RU486+nonTreg) also resulted in increased P4 levels compared to controls (Figure 4.12; $P<0.01$). Interestingly, upon Treg cell transfer to RU486 treated mice (+RU486+Treg), a trend towards significantly higher P4 serum levels was observed compared to control mice (Figure 4.12; $P<0.1$). However, this was not at the same level of significance as the other RU486 treated groups that failed to show a P4 decline, and 4 out of the 6 mice showed comparable P4 levels to the -RU486+Treg control group. Therefore, Treg cell transfer may restore the P4 decline in a subset of mice.

As Treg cell transfer restored fetal viability in some mice (Figure 4.8), we went on to examine correlations between P4 serum concentrations on day 18.5 pc and fetal parameters following Treg cell transfer (Figure 4.12B). A strong negative correlation was observed in the data overall when P4 levels in dams were compared to percentage fetal viability in respective litters ($r=-0.8077$, $P<0.0001$). Interestingly, the mice that exhibited P4 levels within the normal range (<15 ng/mL) on day 18.5 pc, clustered together with high fetal viability (Figure 4.12B). This included the 4 +RU486+Treg mice that showed improved P4 levels on day 18.5 (Figure 4.12A) which clustered alongside control mice. Mice that exhibited low fetal viability and high P4 levels included most of the RU486 treated mice and most of the RU486 and nonTreg cell treated mice. No significant correlation was observed when the number of implantations per litter was compared with P4 concentration, however significant correlations were made when number of viable pups per litter ($r=0.7186$, $P=0.0002$) and number of resorptions per littler ($r=0.7332$, $P<0.0001$) were compared with P4 concentration on day 18.5 pc

Overall these data suggest that Treg cell transfer may partially restore the late gestation P4 decline in some mice. However, increasing the number of mice in this analysis will be necessary to confirm this. Overall, low P4 serum levels correlated with high fetal viability, whereas failure of P4 to decline was

associated with low fetal viability. This suggests a common underlying causal pathway may regulate both luteal P4 synthesis and fetal viability in pregnancy, and our findings suggest that Treg cells may be implicated in this pathway.

4.8. DISCUSSION

The experiments in this study demonstrate that upon peri-implantation disruption of P4 bioavailability with low-dose RU486, late-gestational outcomes were negatively affected. While pregnancies following RU486 treatment were viable at day 9.5 pc, they went on to fail from mid-gestation, with an overall 34-58% reduction in pregnancy rate observed in late gestation. In females that retained their pregnancies, fetal growth restriction was evident, with a 12-24% reduction in fetal weight following RU486 treatment. Additionally, RU486 treated females failed to exhibit a normal P4 decline in late gestation, and proceeded to have an extended gestation length of 24 hrs on average compared to controls. Low-dose RU486 was found to perturb the maternal immune response, leading to a reduction in Treg cell number and proportion, as described in the previous chapter. Here, when Treg cells were transferred to RU486-treated females, pregnancy rate was restored, and fetal viability and fetal weight were significantly improved. Collectively from these results, we conclude that Treg cells can account for the effects of peri-implantation P4 disruption on pregnancy success at late-gestation, and that P4-driven Treg cell action in early gestation is necessary for optimal fetal growth and viability throughout gestation.

4.8.1. On The effect of reduced P4-signalling on pregnancy loss, and the maternal T cell response

In these studies, RU486 was used to explore the effect of a peri-implantation reduction in P4 bioavailability on late gestational outcomes. To our knowledge, this is the first study to limit P4 signalling in the peri-implantation period, while still retaining a pregnancy, to study the role of P4 signalling throughout gestation. Notably, the RU486 model used in this study caused fetal loss from mid-gestation (>day 10 pc). The mechanisms by which this occurs remains to be elucidated, but it is clear that Treg cells are implicated. As described in Chapter 3, low-dose RU486 administration caused profound changes in the T cell compartment by mid-gestation, notably reducing total cell numbers, $CD4^+$ T cell numbers and $Foxp3^+$ Treg cell numbers and proportions of the $CD4^+$ T cells. In mice treated with high doses of RU486 resulting in early pregnancy failure, an increase in inflammatory T cell cytokine production in $CD4^+$ T cells and Treg cells, as well as an increase in $CD8^+$ T cell proportions was evident.

Despite the decline in P4 concentration that occurred on day 9.5 pc following RU486 treatment (Chapter 3), and given that the overall adaptation of the $CD4^+$ T cell compartment and the involvement of activated Treg cells are imperative for pregnancy success (Chapter 3), we hypothesised that the immune perturbations following RU486 treatment were a contributing factor for pregnancy failure in early and mid-gestation and that P4 acts via Treg cells to mediate pregnancy success. Importantly, transferring a modest number of pregnancy and P4-experienced Treg cells back to RU486 treated females significantly improved maternal and fetal outcomes, and appeared to restore P4 levels in a proportion of females. Thus, we concluded that P4s effects on Treg cells were at least partially responsible for the pregnancy loss initially, and that some of P4s effects on early pregnancy success are thus mediated by Treg cells.

Interestingly, in the model used, RU486 treated mice did not have increased susceptibility to the effects of LPS. On day 18.5 pc RU486 treated mice treated with LPS did not exhibit an increase in LPS-mediated fetal death. An additive effect was observed however whereby these mice had a combination of both early and late resorptions, resulting in overall poorer fetal outcomes. Unexpectedly, RU486 treated dams were also protected from LPS-induced preterm birth, which is discussed in detail below (4.6.3)

4.8.2. On the role of Treg cells in mediating pregnancy success

As transfer for $CD4^+CD25^+$ Treg cells on day 3.5 pc could significantly improve both maternal and fetal outcomes following RU486 treatment at peri-implantation, this raises the pressing question of how Treg cells act to promote pregnancy success. It is important to note that not all of the cells transferred in this model were CD25 and Foxp3-expressing. However, as nonTreg cell transfer to RU486 treated females led to significantly adverse fetal outcomes compared to Treg cell transfer, this suggests that the predominant population of $CD25^+Foxp3^+$ cells were mediating the improved outcomes observed. Notably though, these experiments were limited by the absence of a nonTreg cell control group (-RU486+nonTreg), and thus the effects of nonTreg cell transfer alone are hard to discern.

Following Treg cell transfer, donor Treg cells were found to have migrated to the udLN and peripheral sites such as spleen and inguinal LNs. The small number donor Treg cells we identified wouldn't have accounted for all transferred cells, we expect they were systemically introduced and also presumed that they were able to home to the uterus given the appropriate signals. Overall, Treg cells have an essential role in mediating fetal-maternal tolerance systemically and at the fetal-maternal interface, as evidence by numerous studies (Aluvihare *et al.* 2004, Moldenhauer *et al.* 2009, Rowe *et al.* 2012, Chen *et al.* 2013). The expansion of Treg cells is most important in the peri-implantation period, as evidenced by Treg depletion studies using transgenic mouse models or administering αCD25 antibody to females prior to mating (Aluvihare *et al.* 2004, Darasse-Jèze *et al.* 2006). Treg cells are known to elicit their tolerogenic

effects in diverse ways in vivo, and one or multiple of the following explanations could explain the effects of Treg cells in our model.

Firstly, through general immune suppression, Treg cells could be modulating the systemic or local immune environment to cause a shift in the balance of immune cells towards tolerance and away from inflammation. Cell contact-independent mechanisms of Treg cell action are common, including the secretion of immunomodulatory cytokines TGFβ and IL10 to promote tolerogenic phenotypes in target cells (Shevach 2009).

Secondly, through correcting the failed T cell priming at the start of pregnancy, Treg cell transfer could be acting to restore the natural expansion of T cells and therefore improve pregnancy outcomes. Our results demonstrated a failure of $CD4^+$ T cell expansion by day 4.5 pc. The thymus is known to be P4 sensitive, with PRs expressed by medullary thymic epithelial cells (TECs) (Tibbetts *et al.* 1999). TECs mediate the self-antigen repertoire that is presented to T cells, resulting in central tolerance (Cheng and Anderson 2018). This may have implications for the generation of new T cells and also Treg cells involved in pregnancy tolerance in our model where the actions of PR were blocked with RU486. In support of this, our data showed an overall loss of $CD4^+$ T cells and $Foxp3^+$ Treg cells systemically and in the udLNs following peri-implantation administration of RU486 (Chapter 3). Treg cell transfer may be replacing the Treg cells lost in this process, restoring maternal immune tolerance. Additionally, Treg cells interact with tolerogenic DCs synergistically to promote maternal tolerance pathways in vivo. Following Ag exposure in the uterus, DCs present paternal Ag to activate Treg cells in the udLN. In turn, Treg cells interact with DCs via contact-dependent mechanisms to ensure their tolerogenic phenotype (Shevach 2009). Following a high dose of RU486 treatment, Cheon *et al*., observed decreased expression of mRNA encoding MHCII, which may indicate an impairment of uterine DC ability to present Ag to activate $CD4^+$ T cells (Cheon *et al.* 2002). A role for tolerogenic DCs, regulated by Galectin-1, an immunoregulatory glycan-binding protein, in inducing expansion of IL10-secreting Treg cells in pregnancy was reported by Blois *et al.,* (Blois *et al.* 2007). This study revealed involvement of P4 in the Gal-1 pathway, ultimately highlighting the interplay between DCs, Treg cells and P4 for pregnancy success. Human decidual APCs were shown to interact with decidual NK cells to promote Treg cell induction and immunosuppression in vitro (Vacca *et al.* 2010). In our study, the initial impairment of $CD4^+$ T cell expansion in the udLN following RU486-treatment may relate to the decrease in total udLN cell numbers in late gestation in these mice. Given that late gestation cell numbers could be restored following Treg cell transfer, but not nonTreg cell transfer, supplementing with Treg cells on day 3.5 pc may sustain total cell number and allow for expansion over the course of gestation. This may indicate a potential for failure of DC-Treg interactions to cause poor priming and subsequently pregnancy failure, an impairment that can be overcome following

introduction of pregnancy primed Treg cells. In the current study, an overall loss of cells in the udLN was observed following low-dose RU486 treatment, so it is possible that DCs were too affected. In support of this, a recent study demonstrated that upon deletion of PR in DCs, Treg cell generation during pregnancy was impaired, resulting in a IUGR phenotype (Thiele *et al.* 2019).

Thirdly, in the RU486 model used, we saw a failure of pregnancy from mid-gestation (>day 10 pc) and a significant reduction in fetal weight. The timing of the pregnancy failure suggests that the process leading to placental development is impaired following RU486 treatment. Implantation and trophoblast invasion set the course for spiral artery remodelling and placental development to occur. The effects of both RU486 and Treg cell treatments coincide with the timing of these early events. The fact that Treg cells can improve both resorption rate and fetal weight hints to potential involvement of Treg cells in placental development. The role of Treg cells in regulating the vascular adaptations to pregnancy that underlie placental and fetal development is an area of keen interest in the field of reproductive immunology. Reduced Treg cell numbers or impaired function is strongly associated with preeclampsia in women (Sasaki *et al.* 2007, Steinborn *et al.* 2008, Prins *et al.* 2009, Hsu *et al.* 2012). Recently, a study from our laboratory showed that a reduction in Treg cells in early mouse pregnancy is causal to uterine artery dysfunction (Care *et al.* 2018). Rat models of preeclampsia demonstrate that Treg cells suppression of inflammation reduces blood pressure and pathophysiology associated with preeclampsia, whilst Th17 cells causes preeclampsia-like symptoms (Cornelius *et al.* 2015, Cornelius *et al.* 2016). In women, a recent study linked defective P4-mediated decidualisation and failed maternal immune tolerance with suboptimal uterovascular remodelling in pregnancies complicated by IUGR (Dunk *et al.* 2019). While evidence suggests Treg cells are involved in vascular remodelling and placental development the mechanisms remain undefined. Treg effects may be mediated through interactions with other immune cells such as NK cells and macrophages, which are known to be key modulators of vascular remodelling. Uterine NK (uNK) cells regulate decidual blood vessel remodelling for appropriate trophoblast invasion and placental development to occur (Ashkar *et al.* 2000) and T cells are thought to interact with uNK cells to influence this process, as mice deficient in both T cell and NK cells do not undergo normal spiral artery remodelling (Croy *et al.* 2011). Here, we show that Treg cell transfer to P4-disrupted mice improved fetal weight whereas the transfer of nonTreg cell did not improve outcomes. In fact, nonTreg cells had a deleterious effect, causing an increase in placental weight, and skewing the fetal:placental weight ratio, indicating impairment of placental efficiency. Thus, our current findings support the literature that Treg cells specifically influence the vascular remodelling process and interact with decidua immune cells and suggest that this occurs P4-mediated pathways. However, it is important to note that our experiments were limited by the absence of a -RU486+nonTreg cell control, and as such, the effects of nonTreg cells

alone could not be determined. Further experiments are therefore required to understand the effect of nonTreg cells in normal and P4-disrupted pregnancies.

Finally, since P4 synthesis was impaired but Treg cell transfer was able to restore pregnancy success, Treg cells effects in the ovary must be considered. In support of this, preliminary data showed Treg transfer restored P4 levels in the majority of mice in late gestation. Interestingly, the correlation between P4 concentration and fetal viability hints to a potential common underlying causal pathway regulating these parameters. As Treg cell transfer not only restored pregnancy viability and the number of viable pups, but may restore the P4 decline in late pregnancy, this suggests that Treg cells are implicated in the common pathway. Studies have shown Treg cells are present in the ovarian stroma and regulate tolerance to reproductive tissue-restricted self-antigens, controlling the local immune environment (Warren *et al.* 2014, Tung *et al.* 2017). Additionally, other immune cells particularly macrophages, are abundant in the ovary and are known to regulate corpus luteum development at implantation (Care *et al.* 2013). As substantially higher concentrations of P4 are found in ovarian lymph compared to ovarian blood (Staples *et al.* 1982), this suggests that an important component ovarian P4 regulation is through immune mediated mechanisms. Aged B6 mice exhibit a prolonged gestation length associated with elevated plasma P4 levels. Interestingly, P4 levels were inversely correlated with fetal viability in this study (Holinka *et al.* 1978). This, combined with the results in the present study, suggests Treg cells may be implicated in reproductive ageing processes in the ovary that are important in the timing of birth. It is unknown what role Treg cells play in the ovary during pregnancy, but our findings highlight their potential importance.

4.8.3. On progesterone and the timing of birth

P4 is implicated in the timing of birth, as it maintains myometrial quiescence until the end of pregnancy, when, in mice, luteal regression occurs causing P4 levels to decline and the events leading to labour to progress (Shynlova and Lye 2014). In women, where P4 is primarily placenta-derived, a “functional” P4 withdrawal occurs leading to the events proceeding labour (Nadeem *et al.* 2016, Keelan 2018). A decline in P4 concentrations leads to labour whereas P4 administration blocks labour, even in mouse models of LPS-induced PTB (Furcron *et al.* 2015). Conversely, administration of high doses of RU486 later in gestation causes PTB (Dudley *et al.* 1996). In our model, the low-dose RU486 administered at the start of pregnancy caused mice to deliver on average 1 day later than control mice, regardless of LPS treatment. Given gestation length in the inbred B6 mouse strain is a relatively short period of 19 days, the 5% increase observed in overall gestation is significant. To our knowledge, this is the first study to show a peri-implantation disruption in P4 signalling has implications for timing of birth. Interestingly, these mice show abnormally high P4 levels in late-gestation. A significant decline in P4 serum concentration is seen

2 days before parturition in normal mouse pregnancy (Murr *et al.* 1974, Barkley *et al.* 1979). This decline is consistent with the luteal regression that controls P4 withdrawal and subsequent events including uterine contractility (Romero *et al.* 2014). In RU486 treated mice, the persistent P4 levels on day 18.5 suggest a failure to undergo luteolysis. The prolonged gestation length exhibited in aged 12 month old B6 mice was associated with elevated plasma P4 levels (Holinka *et al.* 1978). This can be attributed to failed luteolysis, since the corpus luteum exhibits functional decline during pregnancy from 10-11 months of age in mice (Harman and Talbert 1970).

The abnormal timing of birth following peri-implantation disruption of P4 signalling may be explained by the role of P4 in establishing the program of decidual quiescence at the start of pregnancy. Appropriate decidual quiescence, or the "decidual clock" is thought to control the timing of birth and is regulated by molecular, cellular and immunological pathways (Norwitz *et al.* 2015). The immunological pathways at play ensure that the maternal immune system does not reject the fetus despite close contact with extravillous trophoblast cells which are of fetal origin. Treg cells are implicated in this process since they elicit tolerance to fetal antigens to avoid maternal immune rejection. P4 may function to regulate a program of immunological tolerance that must be maintained until parturition. Timing of delivery is linked to the balance between P4 and inflammation in late gestation (Keelan 2018). Labour is characterised by a myometrial switch from a quiescent state to a contractile state, along with the initiation of a pro-inflammatory signalling cascade mediated by the inflammatory cytokines IL1, IL6 and IL8 (Romero *et al.* 2014). P4 represses expression of these inflammatory pathways to maintain uterine quiescence (Tan *et al.* 2012). These inflammatory cytokines trigger expression of key genes involved in cervical ripening and uterine contractions including oxytocin receptor, connexion 43 and prostaglandin receptors and synthases (Romero *et al.* 2014). A switch from tolerance to immune activation and inflammation is now appreciated as a common mechanism to labour, occurring in both term and pre-term pregnancies. An increasing number of studies have reported that labour is associated with decreases in the number and/or change in phenotype of the Treg cells, along with increases in T effector cell proportions in mice (Koucky *et al.* 2014, Furcron *et al.* 2015) and women (Kisielewicz *et al.* 2010, Schober *et al.* 2012). Additionally, changes to innate immune populations such as macrophages and neutrophils appear to be important in regulating the timing of birth (Gomez-Lopez *et al.* 2014).

In the current study, given the immune dysfunction that persisted throughout gestation in RU486 treated mice, including in the Treg cell compartment, it is plausible that immune factors influenced their timing of birth. Notably, RU486 treated mice had a reduction in total udLN cells, including Treg cells, at mid-gestation (Chapter 3). As the immune system is thought to prime the "decidual clock" (Norwitz *et al.* 2015), the immune dysfunction observed here may act to disrupt it. A possible mechanism by which this occurs

may be through Treg effects in the ovary as discussed above (4.6.2), because Treg transfer did restore the P4 decline in some dams. As the dams that exhibited normal P4 decline also showed high fetal viability, similar to controls, we would have expected these mice to have a normal gestation length. Indeed, fetal viability was associated with timing of birth, with control dams with litters of high viability delivering on-time, and RU486 treated dams with litters of lower viability delivering later.

Additionally, in the current study, on day 18.5 pc total udLN cell number was reduced in RU486 treated dams compared to controls, but was restored following Treg cell transfer. Whether Treg cell transfer can rescue the timing of birth in the RU486 model remains to be determined but is an area of investigation within our laboratory.

Given the protective effect of Treg cells on pregnancy success in our study and others, whether Treg cells can be utilised to restore the timing of birth in the model of LPS-induced PTB is another outstanding question. Interestingly, Rag1-/- (T and B cell-deficient) mice showed increased susceptibility to LPS-induced PTB, which was rescued upon mid-gestation transfer of $CD4^+$ T cells, which migrate to udLNs and differentiate into $Foxp3^+$ Treg cells (Bizargity *et al.* 2009). Additionally, in a recent study, prior transfer of wildtype Treg cells to miR155-/- mice rescued their susceptibility to inflammation-induced fetal loss and fetal growth restriction (Schjenken *et al.* 2020). Therefore, boosting anti-inflammatory Treg cells in early pregnancy may provide protection against subsequent inflammatory challenges such as inflammation-induced PTB, and further experiments are required to test this hypothesis.

The overall role of Treg cells in late gestation is investigated further in Chapter 6, whereby a Treg fate-mapping model is employed to track the transcriptional fate of Treg cells throughout gestation.

4.8.4. Clinical relevance

This study is clinically relevant to miscarriage, recurrent pregnancy loss and unexplained infertility in women, which are known to be associated with poor maternal immune adaptation and in particular a loss of Treg cells (Yang *et al.* 2008, Lee *et al.* 2011). Miscarriage is also associated in some women with luteal phase deficiency (LPD) whereby a short or dysregulated luteal phase causes insufficient P4 in early pregnancy resulting in impaired implantation (Mesen and Young 2015). Adequate luteal phase P4 is key for P4-mediated uterine functions in early pregnancy, and may be key for establishing Treg cell populations to ensure correct immune environment is present allowing placental development to occur (Evans *et al.* 2016). Thus, our findings could be one mechanism by which luteal phase insufficiency causes later miscarriage. Additionally, our findings may help to explain why luteal phase support (via P4

supplementation) given to women undergoing IVF cycles assists pregnancy success (van der Linden *et al.* 2011).

The RU486 model used in this study additionally caused a fetal growth restriction phenotype evident in late gestation, which is clinically relevant to IUGR. IUGR complicates up to 10% of pregnancies in Western countries and is implicated with lifelong health consequences for children (Longo *et al.* 2013). Our findings are also relevant to preeclampsia which is often associated clinically with IUGR (American College of Obstetricians and Gynecologists 2013). Inadequate placental development and/or function is a leading cause of IUGR and PE. Failure of decidualisation and maternal immune tolerance are also implicated in the cause of IUGR (Dunk *et al.* 2019). Immune maladaptation and inflammation appear to be central feature of preeclampsia, and are hypothesised to be causal to the disease (Redman and Sargent 2010). Given that Treg cell transfer can improve pregnancy loss and fetal growth restriction in this study, this highlights the potential for future novel therapies involving Treg cells to treat pregnancy complications (Robertson *et al.* 2019).

In conclusion, these set of experiments demonstrate that Treg cell regulation by P4 in the peri-implantation period is essential for subsequent pregnancy success in late gestation. This study adds to growing evidence revealing the importance of early events in shaping the course of pregnancy and later maternal and fetal outcomes. The studies presented in the chapters to follow investigate the molecular mechanisms of P4 regulation of Treg cells and Treg cell stability during pregnancy.

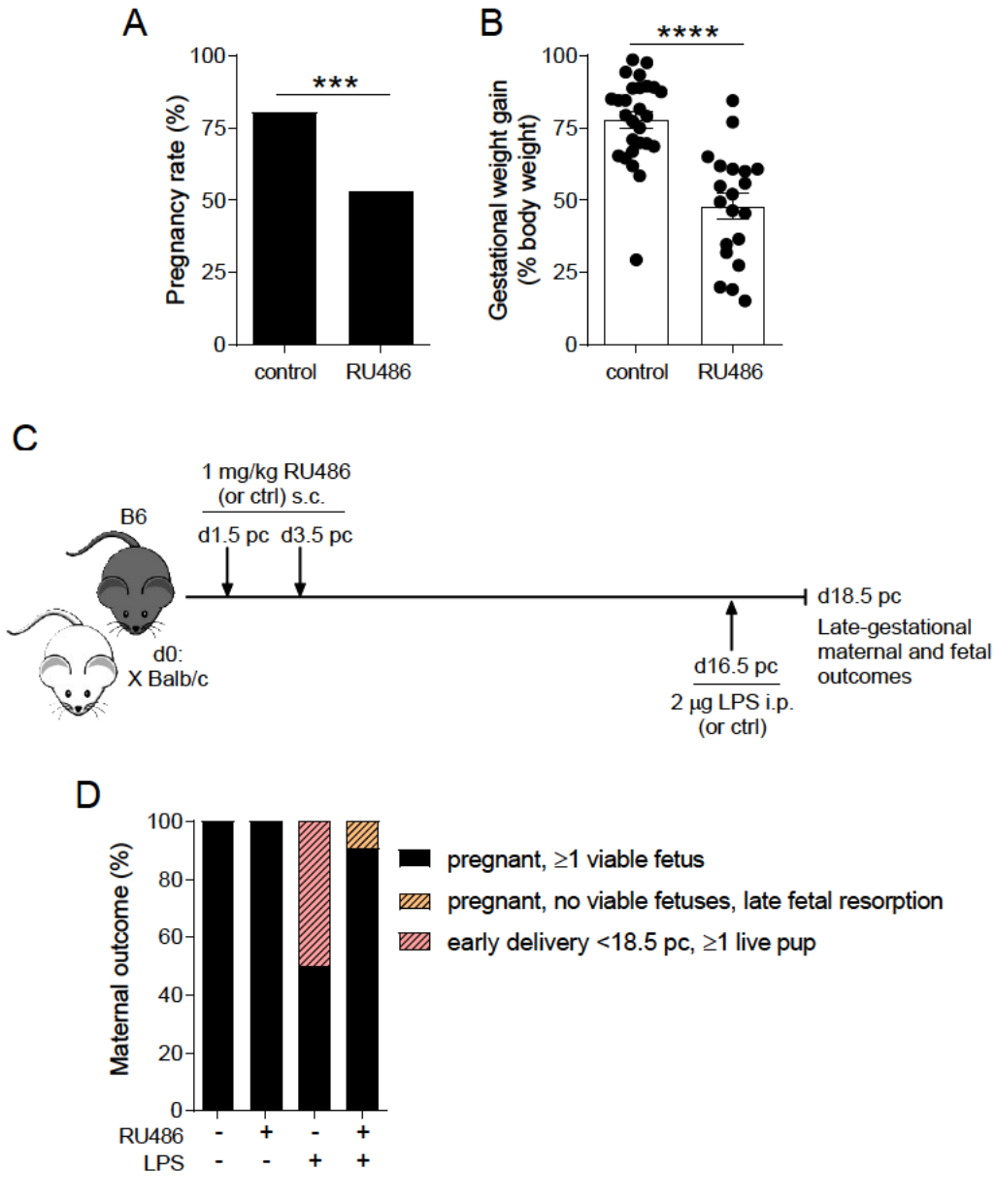

Figure 4.1 Reduced P4 signalling in early pregnancy with low-dose RU486 decreases pregnancy rate in late gestation and alters maternal outcomes following a late-gestational inflammatory challenge

Female B6 mice were allogenically mated to BALB/c males and on day 1.5 and 3.5 post coitus (pc), RU486 (1 mg/kg) or vehicle control was administered. On day 16.5 pc, (A) pregnancy rate rate (≥ viable fetus) and (B) gestational weight gain was measured in control and RU486-treated mice. (C) Schematic of experimental design. On day 16.5 pc, pregnant females were injected i.p. with 2 µg lipopolysaccharide (LPS) or control (PBS) and rate of preterm birth (delivery prior to day 18.5 pc) following LPS injection was measured. On day 18.5 pc, mice that had not delivered were considered 'term' and were euthanized for late gestation maternal and fetal outcome assessment. (D) Maternal outcome (expressed as a % of total pregnancies) on day 18.5 pc for females treated with RU486 (or control) and LPS (or control), as indicated by -/+ symbols. (A) n= 36 control and 49 RU486-treated mated mice. (D) n=10-14 pregnant mice. Mice classified as pregnant on day 16.5 were confirmed pregnant at autopsy. (A) Data analysed by Chi-squared test; (B) Data are presented as mean ± SEM with individual mice indicated by symbols, Unpaired T-test; * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

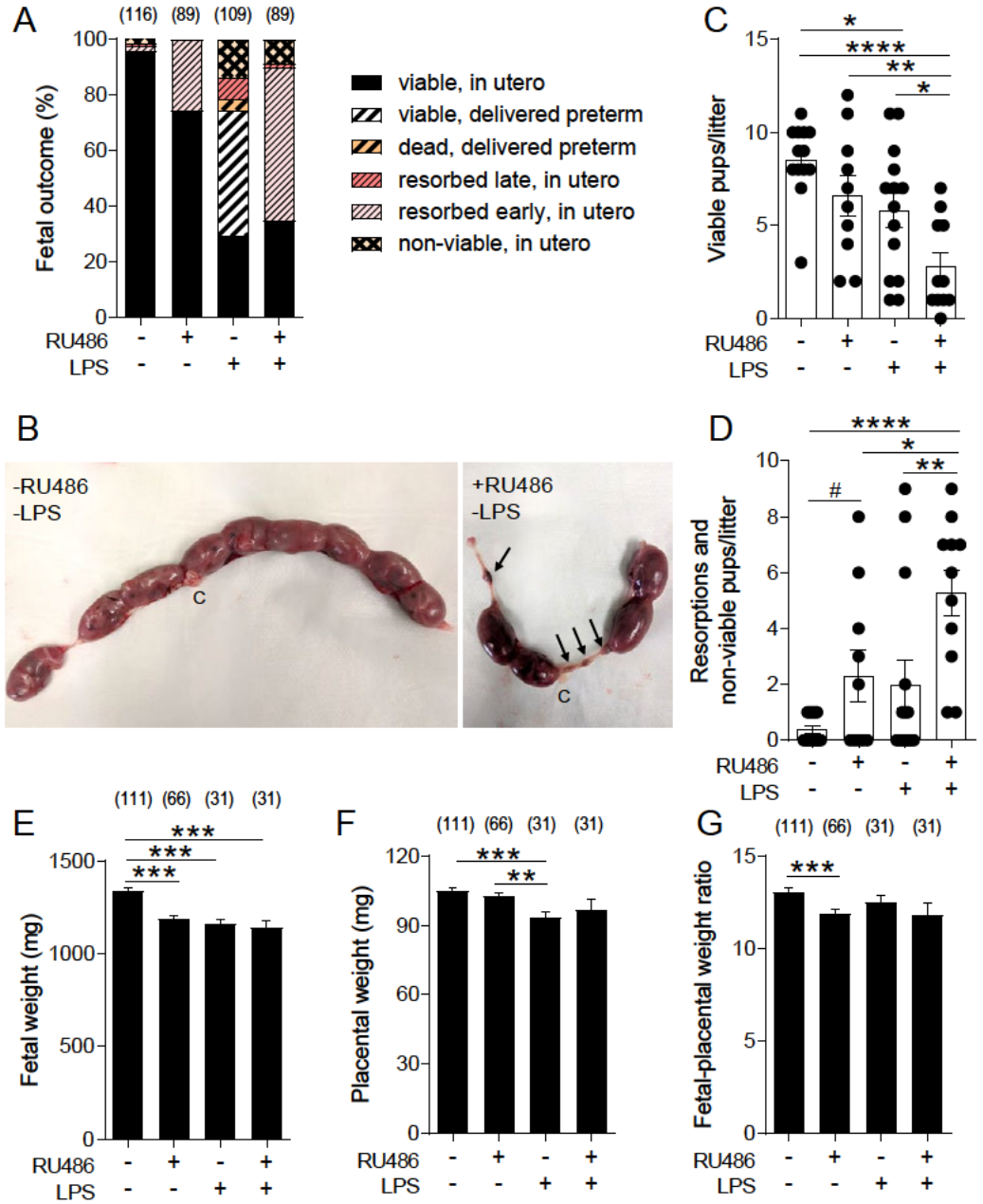

Figure 4.2 Reduced P4 signalling in early pregnancy with low-dose RU486 increases fetal resorption and decreases viable fetal weight in late-gestation

Female B6 mice were allogenically mated to BALB/c males and on day 1.5 and 3.5 post coitus (pc), RU486 (1 mg/kg) or vehicle control was administered. On day 16.5 pc, pregnant females were injected i.p. with 2 µg lipopolysaccharide (LPS) or control (PBS) and rate of preterm birth (delivery prior to day 18.5 pc) following LPS injection was measured. On day 18.5 pc, fetal outcomes were measured in both dams that delivered prior to day 18.5 pc, and dams that had not delivered by day 18.5 pc (A) Fetal outcome (expressed as a %) on day 18.5 pc for fetuses/pups from dams treated with RU486 (or control) and LPS (or control). (B) Representative images of implantations in uteri from control and RU486-treated mice on day 18.5 pc. (C,D) Quantification of (A) for (C) viable pups per litter, and (D) resorptions and/or non-viable pups per litter. (E) fetal weight, (F) placental weight and (G) fetal:placental weight ratio was measured in dams treated with RU486 (or control) and LPS (or control). Treatment is indicated by -/+ symbols. (A, E-G) n=31-111 viable fetuses. (C,D) n=10-14 litters. (C-D) Data analysed by one-way ANOVA and presented as mean ± SEM with individual mice indicated by symbols. (E-G) Data analysed by linear mixed model ANOVA with viable litter size as covariate and presented as estimated marginal mean ± SEM.

* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$, # $p<0.1$.

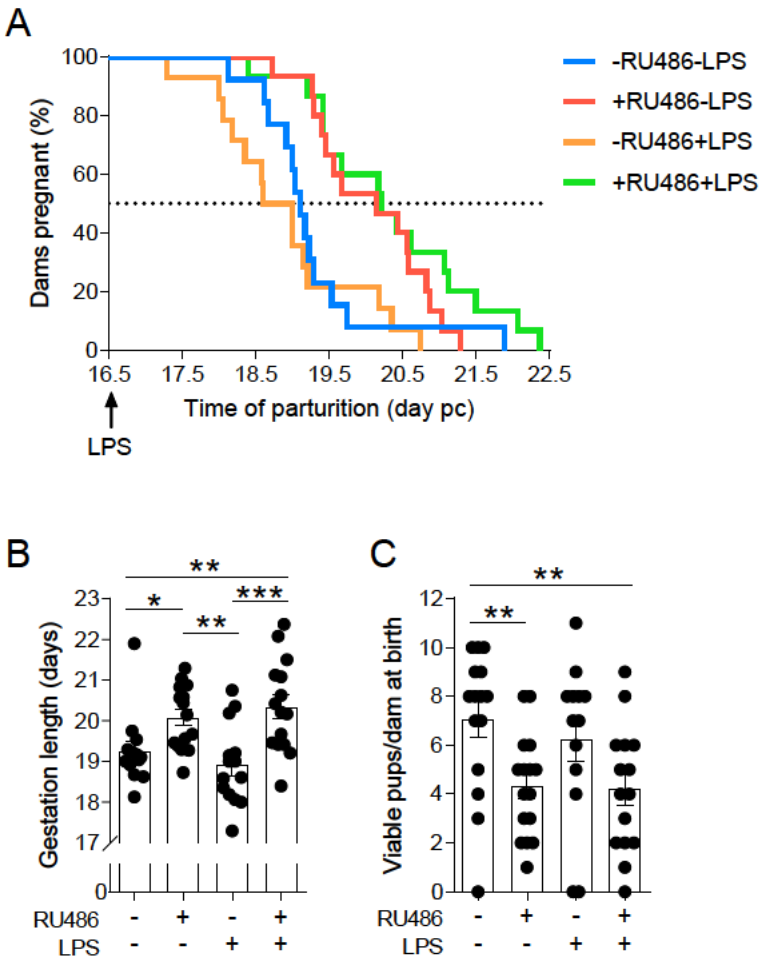

Figure 4.3 Reduced P4 signalling in early pregnancy with low-dose RU486 increases gestation length and decreases viable pups at birth

Female B6 mice were allogenically mated to BALB/c males and on day 1.5 and 3.5 post coitus (pc), RU486 (1 mg/kg) or vehicle control was administered. On day 16.5 pc, pregnant females were injected i.p. with 2 µg lipopolysaccharide (LPS) or control (PBS) and were allowed to progress to birth. Parturition time and fetal viability at birth were subsequently measured. (A) Survival curves of pregnant dams (%) treated with RU486 (or control) and LPS (or control), following LPS treatment on day 16.5, showing time of parturition (day pc) in individual mice. (B) Gestation length (days) and (C) viable pups (at birth) per dam, for dams treated with RU486 (or control) and LPS (or control). Treatment is indicated by -/+ symbols. n= 13-15 dams. (B,C) Data analysed by one-way ANOVA and presented as mean ± SEM with individual mice indicated by symbols. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

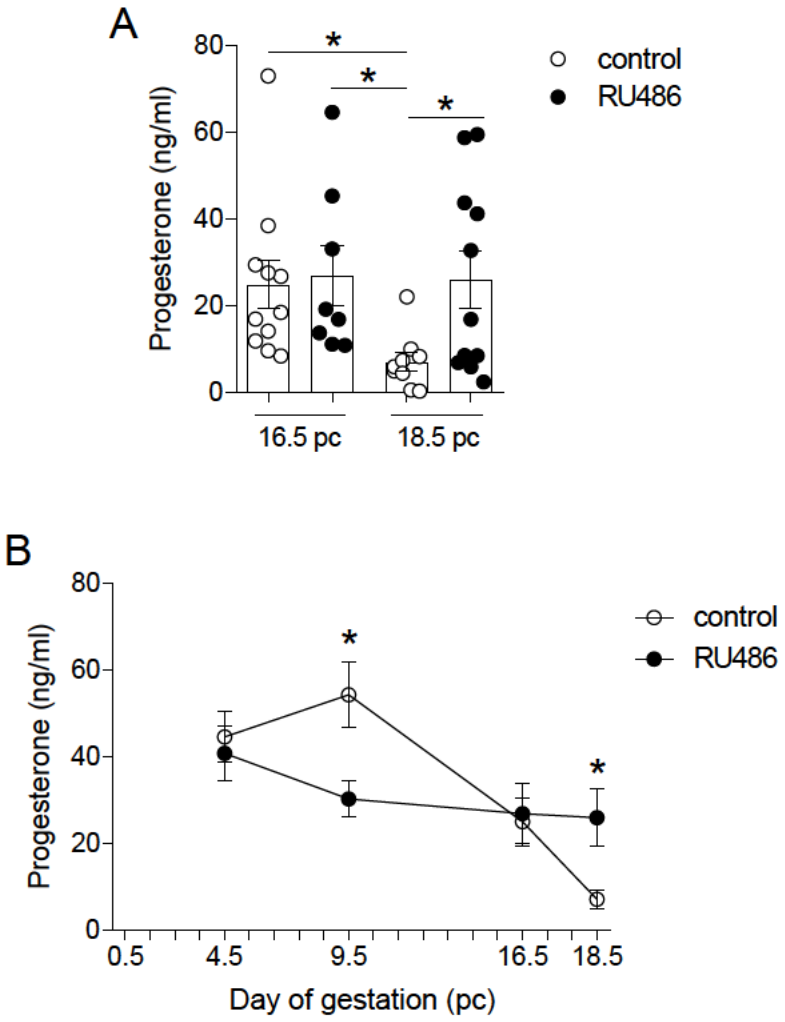

Figure 4.4 Progesterone serum concentrations in late-gestation following peri-implantation administration of RU486.

Female B6 mice were allogenically mated to BALB/c males and on day 1.5 and 3.5 post coitus (pc), RU486 (1 mg/kg) or vehicle control was administered. On day 16.5 and 18.5 pc dams were euthanised and blood was harvested. Serum was isolated from blood for P4 analysis by ELISA. (A) Serum P4 levels on day 16.5 pc and day 18.5 pc in mated mice treated with 1 mg/kg RU486. (B) Serum P4 concentration on various gestational days throughout pregnancy following treatment with 1 mg/kg RU486 or control. Late-gestational data is presented in combination with data from early- (day 4.5 pc) and mid- (day 9.5 pc) gestation pregnant mice from Figure 3.10. (A) n=8-11 mice. Data analyzed by one-way ANOVA and presented as mean ± SEM with individual mice indicated by symbols. (B) n=3-9 mice on day 4.5pc, n=9-12 on day 9.5 pc and n=8-11 mice on days 16.5-18.5 pc. Data presented as mean ± SEM. Comparisons were made between control and RU486-treated mice at each gestational time point. Unpaired T-test. * $p<0.05$.

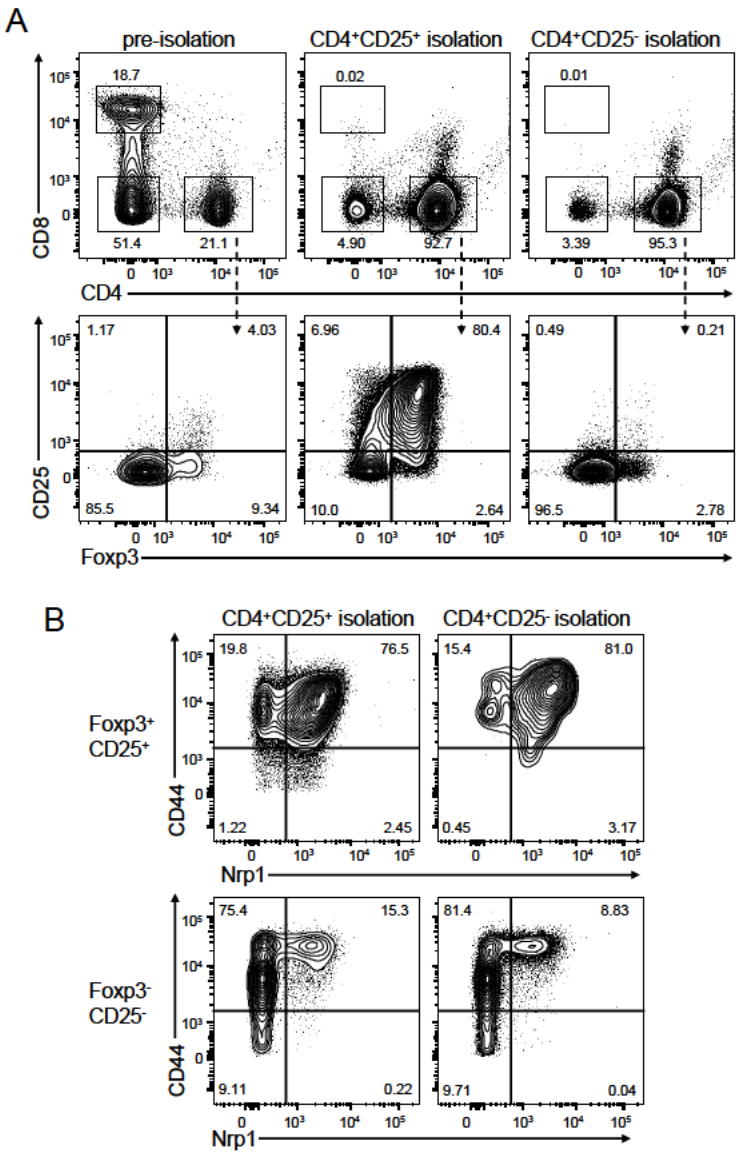

Figure 4.5 T cell profile before and after isolation of $CD4^+CD25^+$ Treg and $CD4^+CD25^-$ nonTreg cells from spleen and LNs of Ly5.1 females in mid-gestation.

Female Ly5.1 mice were mated to BALB/c males and on day 11.5-14.5 pc, pregnant mice were euthanised and spleen and LNs (uterus-draining, inguinal, brachial, axillary and mesenteric) were harvested for $CD4^+CD25^+$ Treg and $CD4^+CD25^-$ nonTreg isolation. (A) Representative FACS plots of T cell subsets before (pre-isolation) and after isolation of $CD4^+CD25^+$ and $CD4^+CD25^-$ cells, showing CD4 and CD8 expression amongst live cells, and CD25 and Foxp3 expression amongst $CD4^+$ cells. (B) Representative FACS plots of T cell subsets following $CD4^+CD25^+$ and $CD4^+CD25^-$ isolation, showing CD44 and Nrp1 expression amongst $Foxp3^+CD25^+$ Treg cells and $Foxp3^-CD25^-$ nonTreg cells.

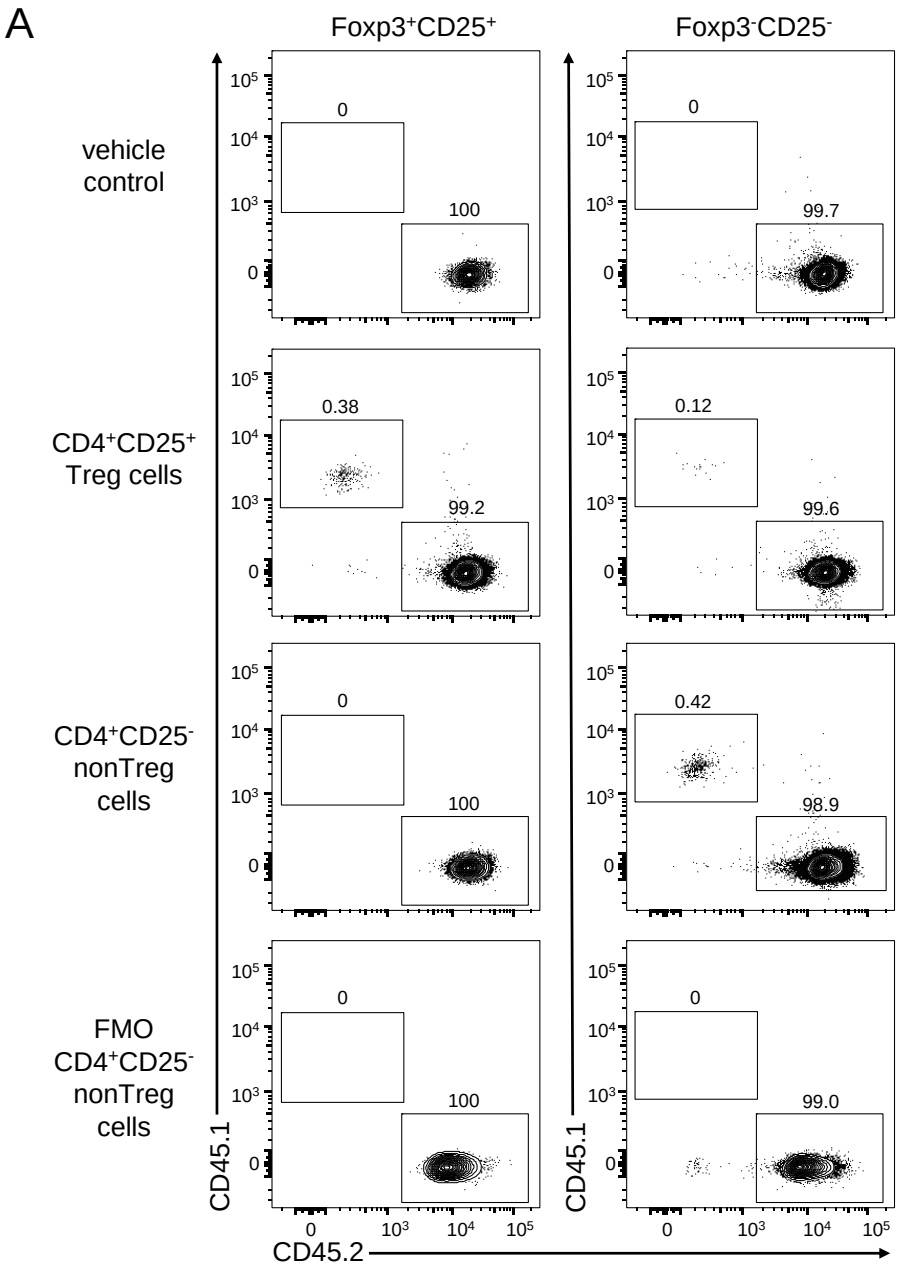

Figure 4.6 Identification of donor Treg and nonTreg cells in pregnant congenic recipient mice on day 6.5 pc following adoptive transfer.

Donor Ly5.1 female mice were mated to BALB/c males and on day 11.5-14.5 pc, pregnant mice were euthanised, spleen and LNs (uterus-draining, inguinal, brachial, axillary and mesenteric) were harvested and $CD4^+CD25^+$ Treg and $CD4^+CD25^-$ nonTreg cells were isolated. Recipient female B6 (Ly5.2-expressing) mice were mated to BALB/c males, and on day 3.5 pc, females were injected i.v. with 2 x 10^5 donor Ly5.1 Treg cells ($CD4^+CD25^+$), nonTreg cells ($CD4^+CD25^-$) or vehicle control (PBS). On day 6.5 pc, recipient mice were euthanised and donor cells were measured by flow cytometry. (A) Representative FACS plots showing udLN $Foxp3^+$ Treg and $Foxp3^-$ nonTreg cell populations, in mice transferred with vehicle control (PBS), $CD4^+CD25^+$ Treg cells and $CD4^+CD25^-$ nonTreg cells. Donor ($CD45.1^+$) and recipient ($CD45.2^+$) among $Foxp3^+$ Treg and $Foxp3^-$ nonTreg cell populations were identified using antibodies against CD45.1 and CD45.2. A fluorescence minus one (FMO) control for a $CD4^+CD25^-$ nonTreg cell transferred mouse is shown, demonstrating positive staining of the donor population when the CD45.1 antibody is present.

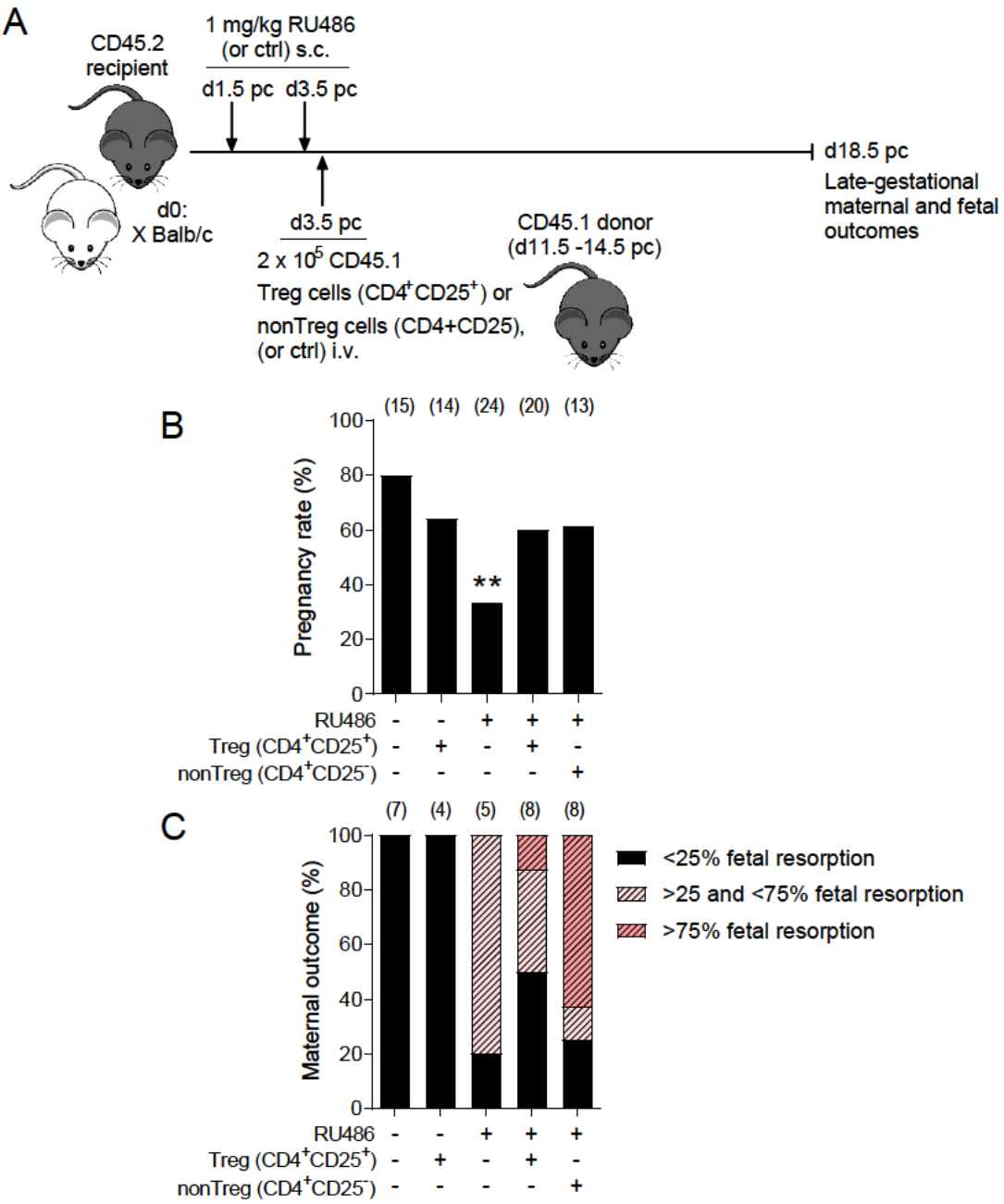

Figure 4.7 Treg cell transfer restores both pregnancy rate and improves late-gestational maternal outcomes in mice with peri-implantation disruption in P4 signalling.

Female B6 mice were mated to BALB/c males and on day 1.5 and 3.5 post coitus (pc), RU486 (1 mg/kg) or vehicle control was administered. On day 3.5 pc, approximately 8 hours following the final RU486 dose, females were injected i.v. with 2 x 10^5 Treg cells ($CD4^+CD25^+$), nonTreg cells ($CD4^+CD25^-$) or vehicle control (PBS). On day 18.5 pc, dams were euthanised and pregnancy rate (≥ viable fetus) and maternal outcomes were measured. (A) Schematic of experimental design. (B) Pregnancy rate and (C) maternal outcome (expressed as a %) on day 18.5 pc for females treated with RU486 (or control) and Treg cells, or nonTreg cells, or control. Treatment is indicated by -/+ symbols. (B) n=13-24 mated females. (B,C) n=4-8 pregnant females. (B) Data analysed by Chi-squared test; * $p<0.05$, ** $p<0.01$.

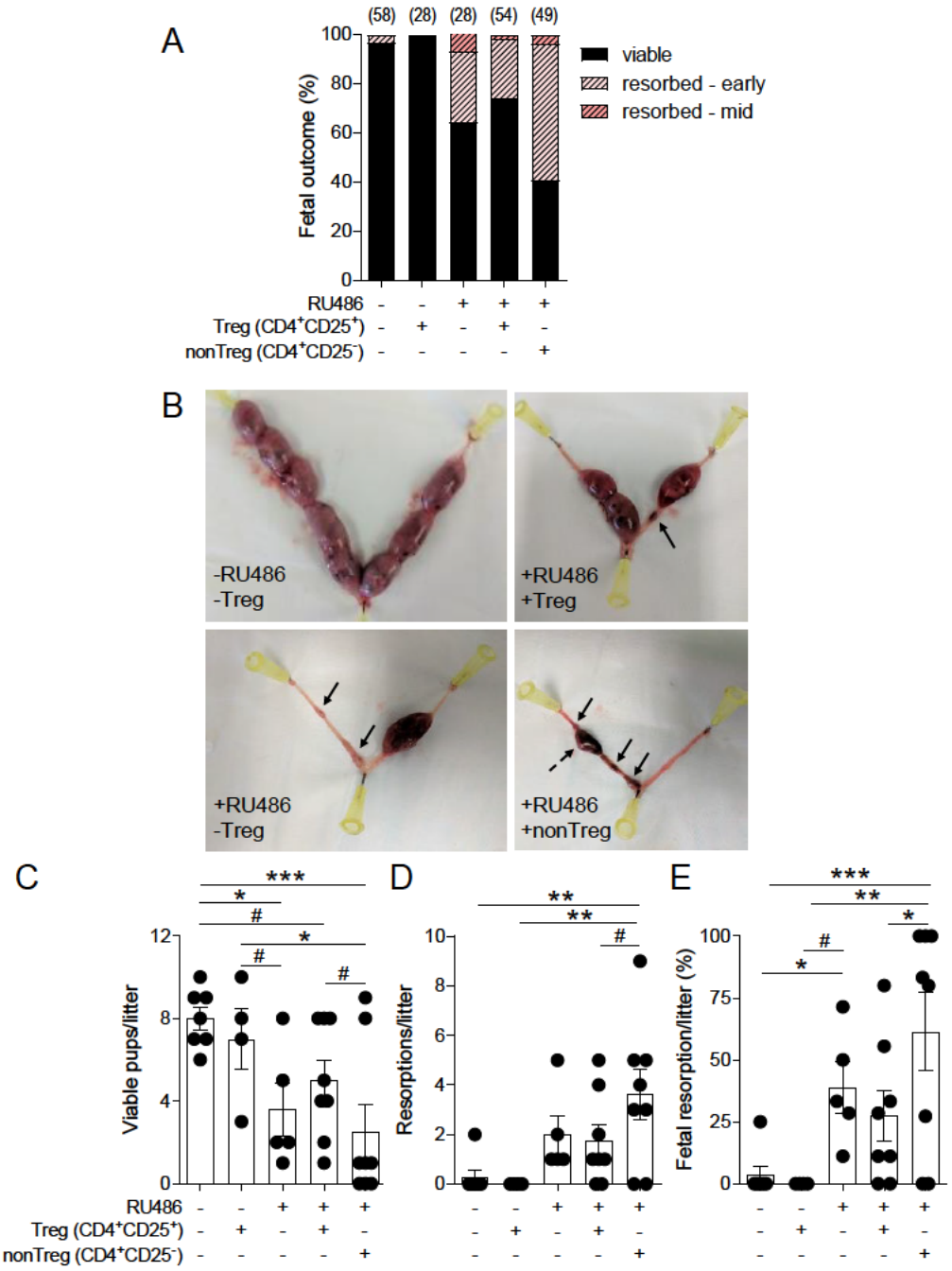

Figure 4.8 Treg cell transfer, but not nonTreg cell transfer, improves late-gestational fetal outcomes in mice with peri-implantation disruption in P4 signalling.

Female B6 mice were mated to BALB/c males and on day 1.5 and 3.5 post coitus (pc), RU486 (1 mg/kg) or vehicle control was administered. On day 3.5 pc, females were injected i.v. with 2 x 10^5 Treg cells ($CD4^+CD25^+$), nonTreg cells ($CD4^+CD25^-$) or vehicle control (PBS). On day 18.5 pc, dams were euthanised and fetal outcomes were measured. (A) % fetal outcome on day 18.5 pc for fetuses from dams treated with RU486 (or control) and Treg cells, nonTreg cells or control. (B) Representative images of uteri from control (-RU486-Treg), RU486-treated (+RU486-Treg), RU486- and Treg-treated (+RU486+Treg) and RU486- and nonTreg-treated (+RU486+nonTreg) mice. Filled arrows indicate small resorptions and dotted arrows indicate large resorptions. (C-E) Quantification of (A,B) for (C) viable pups, (D) resorptions and (E) % fetal resorption per litter. Treatment is indicated by -/+ symbols. n=28-58 implantations. n=4-8 litters. (C-E) One-way ANOVA; mean ± SEM with individual mice indicated by symbols. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, # $p<0.1$.

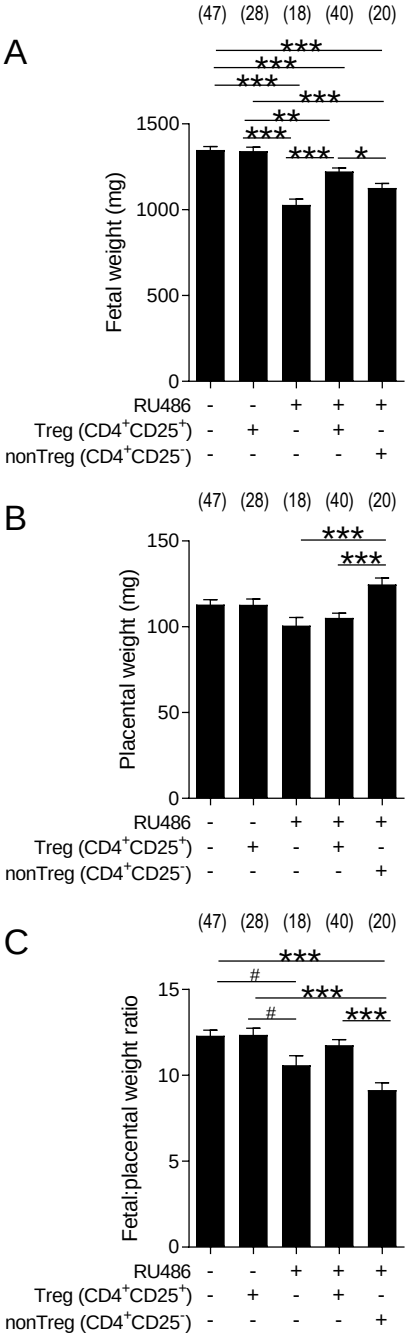

Figure 4.9 Treg cell transfer improves fetal weight in late-gestation in mice with peri-implantation disruption in P4 signalling.

Female B6 mice were mated to BALB/c males and on day 1.5 and 3.5 post coitus (pc), RU486 (1 mg/kg) or vehicle control was administered. On day 3.5 pc, females were injected i.v. with 2 x 10^5 Treg cells ($CD4^+CD25^+$), nonTreg cells ($CD4^+CD25^-$) or vehicle control (PBS). On day 18.5 pc, dams were euthanised and fetal outcomes were measured. (C) Fetal weight, (B) placental weight and (C) fetal:placental weight ratio was measured in dams treated with RU486 (or control) and Treg cells, nonTreg cells or control. Treatment is indicated by -/+ symbols. n=18-47 fetuses. n=4-8 litters. Data analysed by linear mixed model ANOVA with viable litter size as covariate and presented as estimated marginal mean ± SEM. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001, $^{\#}$ p<0.1.

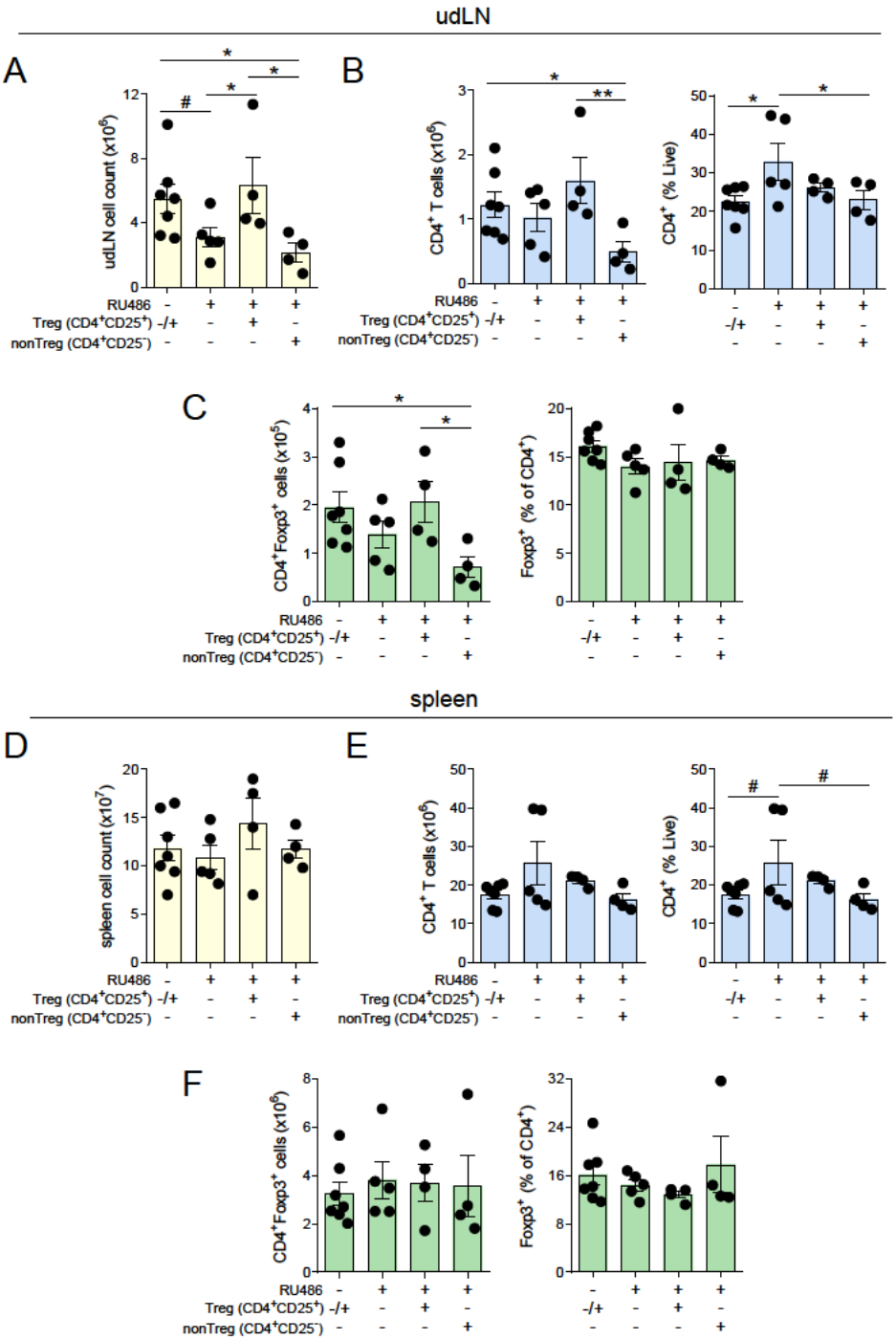

Figure 4.10 CD4$^+$ T cell and Treg cell parameters in uterus-draining LNs and spleen on day 18.5 pc following transfer of Treg and nonTreg cells to RU486-treated mice in peri-implantation.

Female B6 mice were mated to BALB/c males and on day 1.5 and 3.5 post coitus (pc), RU486 (1 mg/kg) or vehicle control was administered. On day 3.5 pc, females were injected i.v. with 2 x 10^5 Treg cells (CD4$^+$CD25$^+$), nonTreg cells (CD4$^+$CD25$^-$) or vehicle control (PBS). On day 18.5 pc, T cells were measured by flow cytometry. (A) Total cell count and (B) CD4$^+$ T cell number and % of live cells in the udLN. (C) CD4$^+$Foxp3$^+$ Treg cell number and % of CD4$^+$ T cells in the udLN. (D) Total cell count and (E) CD4$^+$ T cell number and % of live cells in the spleen. (F) CD4$^+$Foxp3$^+$ Treg cell number and % of CD4$^+$ T cells in the spleen. Treatment is indicated by -/+ symbols. n=4-7. Data are presented as mean ± SEM with individual mice indicated by symbols; one-way ANOVA; * p<0.05, $^{\#}$ p<0.1.

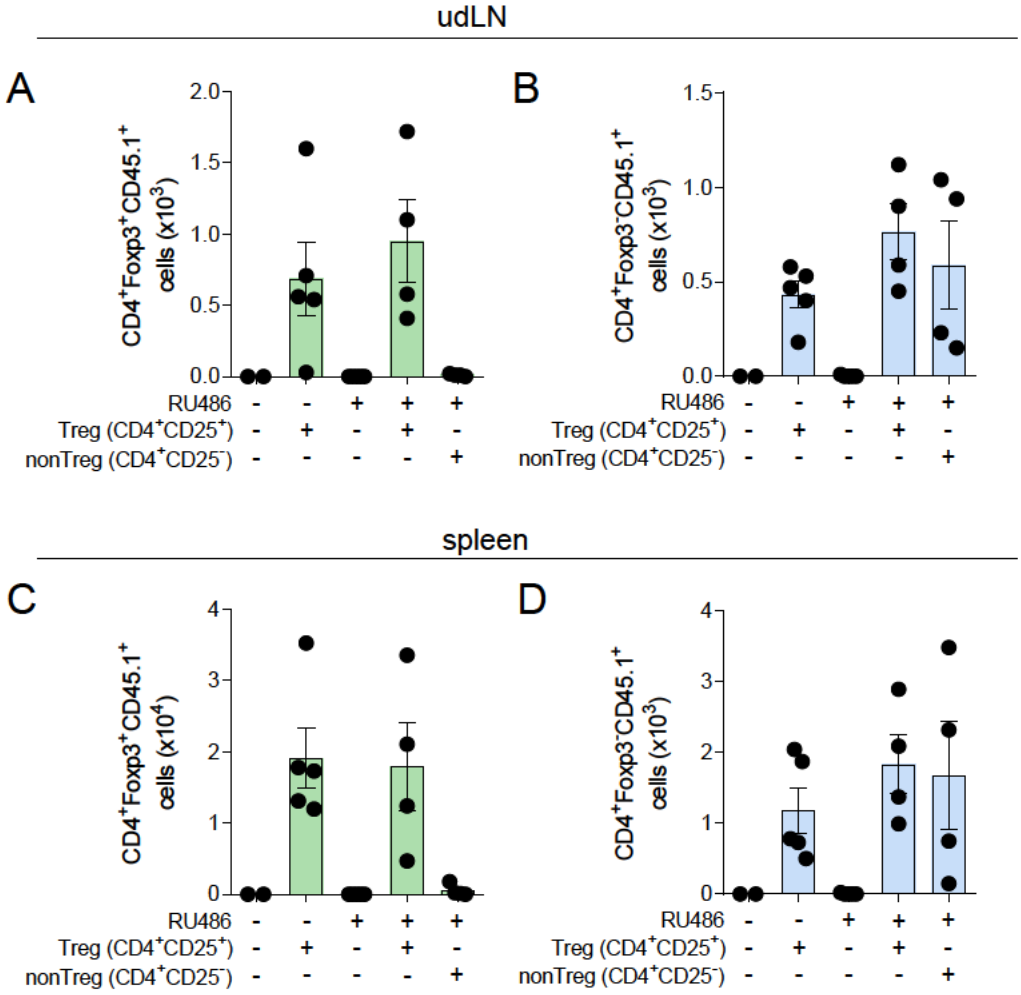

Figure 4.11 Identification of donor Foxp3⁺ Treg and Foxp3⁻ nonTreg cells in recipient mice on day 18.5 pc following adoptive transfer to RU486-treated mice in peri-implantation.

Female B6 mice were mated to BALB/c males and on day 1.5 and 3.5 post coitus (pc), RU486 (1 mg/kg) or vehicle control was administered. On day 3.5 pc, females were injected i.v. with 2 x 10^5 Ly5.1 Treg cells ($CD4^+CD25^+$), nonTreg cells ($CD4^+CD25^-$) or vehicle control (PBS). On day 18.5 pc, CD45.1 donor T cells were measured by flow cytometry. (A, C) Total numbers of $CD4^+Foxp3^+CD45.1^+$ donor Treg cells in the (A) udLN and (B) spleen. (B, D) Total numbers of $CD4^+Foxp3^-CD45.1^+$ donor nonTreg Treg cells in the (B) udLN and (D) spleen. Treatment is indicated by -/+ symbols. n=2-5. Data are presented as mean ± SEM with individual mice indicated by symbols.

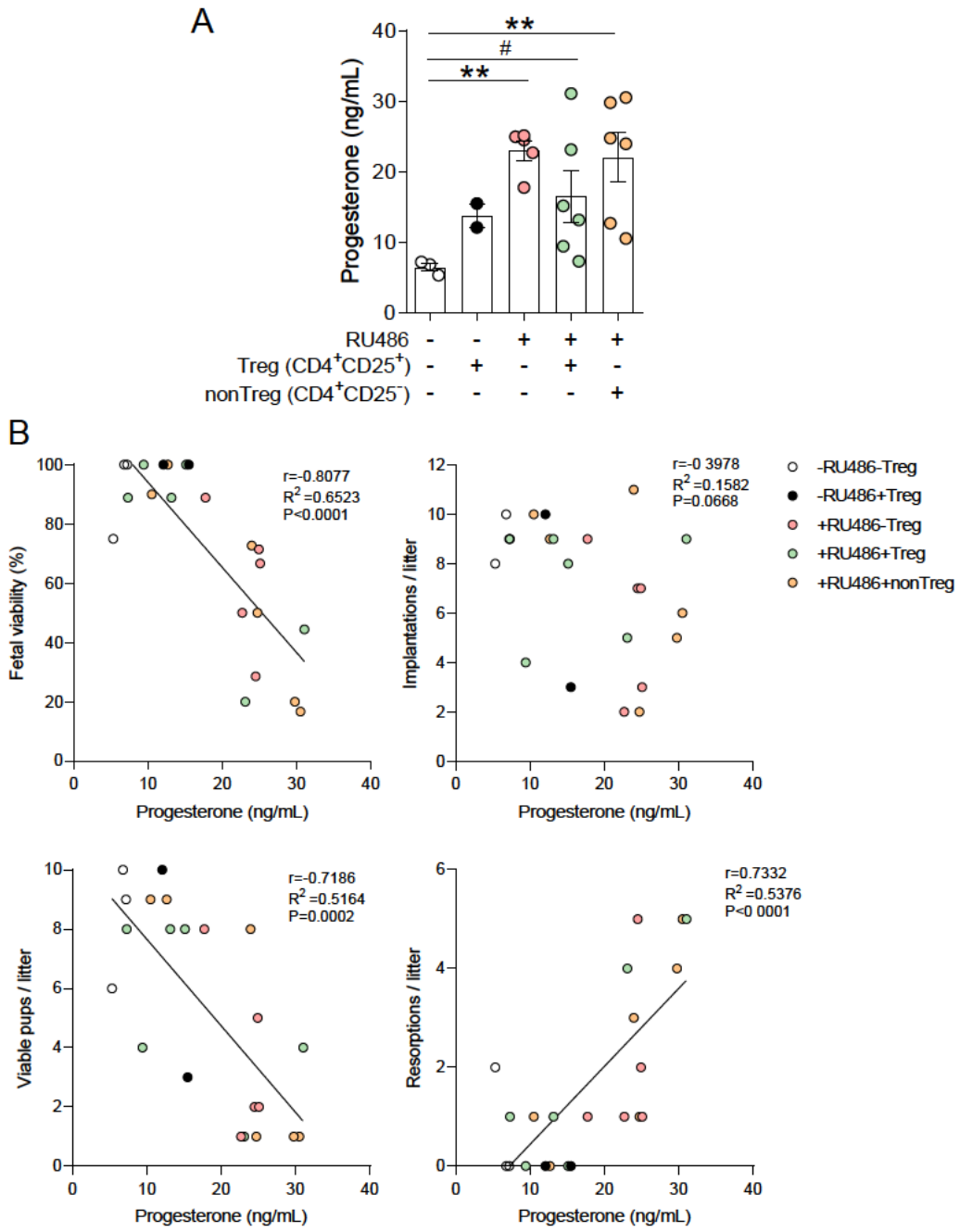

Figure 4.12 Progesterone serum concentrations and correlations with fetal viability on day 18.5 pc following Treg and nonTreg cell transfer to RU486-treated females at peri-implantation.

Female B6 mice were mated to BALB/c males and on day 1.5 and 3.5 post coitus (pc), RU486 (1 mg/kg) or vehicle control was administered. On day 3.5 pc, females were injected i.v. with 2 x 10^5 Treg cells ($CD4^+CD25^+$), nonTreg cells ($CD4^+CD25^-$) or vehicle control (PBS). On day 18.5 pc, dams were euthanised and blood was harvested. Serum was isolated from blood for P4 analysis by ELISA. (A) Serum P4 concentration in mice treated with RU486 or control, and Treg cells, nonTreg cells or control. (B) Correlations between P4 concentration in dams and fetal parameters in respective litters on day 18.5 pc. Percentage fetal viability, and number of implantations, viable pups, and resorptions per litter are each compared to P4 concentration. (A) n=2-6 mice. Data analysed by one-way ANOVA and presented as mean ± SEM with individual mice indicated by symbols. (B) n=22 data points. Data analysed by Pearson's correlation. r, R^2 and exact P values are reported per comparison. If correlations were statistically significant (P<0.05), a line of best fit was applied to the data. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001, $^{\#}$ p<0.1.

Chapter 5

Exploring mechanisms of progesterone action in $CD4^+$ T cells and Treg cells

5.1. INTRODUCTION

The mechanisms of P4 action in many target tissues and cells in the non-pregnant and pregnant state remain to be defined. This is due to the complex nature of P4 signalling, which is highly regulated, concentration dependant, and mediated by multiple receptors and regulatory pathways. Furthermore, P4 action is sex-dependent, and in women it differs between pregnant and non-pregnant states (De Leon-Nava *et al.* 2009). While classical genomic P4 action is reported to be largely mediated by nuclear P4 receptors (PRs) (Lydon *et al.* 1995, Mulac-Jericevic and Conneely 2004), other non-classical mechanisms of P4 action in target cells have been found (Dosiou *et al.* 2007, Thomas *et al.* 2007). Many of these are effects are mediated through cytoplasmic pathways that are attributed to the actions of membrane PRs (mPRs) and pathways activated by progesterone receptor membrane components (PGRMCs). Additionally, some of the actions of P4 appear to be mediated via the glucocorticoid receptor (GR), for which it has moderate binding affinity (Grosser *et al.* 1971, Kontula *et al.* 1983).

T cells have been demonstrated to be directly regulated by P4 in mice and humans through in vitro studies, and this regulation is thought to be important for pregnancy success (Miyaura and Iwata 2002, Mao *et al.* 2010, Schumacher *et al.* 2017). In 1983, synthetic progestins were first found to cause immunosuppressive effects in human PBMCs, which the authors attributed to a "suppressor" population of T cells (Kontula *et al.* 1983). Progesterone was shown to favour the development of Th2 cells and promote IL4 production in Th1 cell clones (Piccinni *et al.* 1995). Lymphocytes from pregnant women were also found to secrete an immunomodulatory protein known as progesterone-inducible blocking factor (PIBF), which binds to a novel IL4 receptor, altering the Th1/Th2 balance (Szekeres-Bartho and Wegmann 1996, Kozma *et al.* 2006).

The question of which receptors mediate P4 effects in T cells is a controversial issue. Human T cells from maternal PMBC cultures with P4 showed decreased production of inflammatory cytokines IFNγ, TNFα, and increased expression of IL4 (Lissauer *et al.* 2015). This effect was thought to be mediated by mPRs that were found to be expressed by CD4 and CD8 T cells by qPCR. Conversely, PR expression was not detectable in human T cells (Lissauer *et al.* 2015). mPRs have been found to mediate the non-genomic rapid effects of P4 in T cells, such as increasing intracellular calcium levels and acidification (Ehring *et al.* 1998, Ndiaye *et al.* 2012). Through mPR binding, P4 can modulate cytokine expression through modulation of calcium and potassium channel signalling (Ehring *et al.* 1998, Dosiou *et al.* 2007). Similar responses to P4 are reported in mouse T cells, with inhibition of Th1 development observed (Miyaura and

Iwata 2002, Hughes *et al.* 2013). Hughes found that P4 could repress IFNγ expression in $CD4^+$ T cells leading to reduced T effector (Teff) activity, and suppress T cell-dependent antibody responses, and in this case the effect was mediated by nuclear PR in $CD4^+$ T cells (Hughes *et al.* 2013).

Despite these studies on PBMCs, pregnancy lymphocytes and $CD4^+$ T cells, few studies have examined the action of P4 specifically in $Foxp3^+$ Treg cells. P4 was found to expand $Foxp3^+$ Treg cells from human cord blood-derived naïve precursors in (Lee *et al.* 2011). Lee *et al.,* also demonstrated that P4 promoted differentiation of murine $Foxp3^+$ Treg cells following culture of naïve $CD4^+$ T cells under Treg cell polarising conditions (IL2 and TGFβ). Inducible Tregs (iTregs) generated in culture with P4 were found to have increased suppressive activity and enhanced capacity to control the mouse autoimmune disease experimental autoimmune encephalomyelitis (EAE) (Lee *et al.* 2012). The effect of P4 was thought to be mediated by PR, as RU486 was able to block the effect of P4 on human cord blood Treg cell expansion (Lee *et al.* 2011). Engler *et al.,* showed again that P4 expands mouse Treg cells in vitro, but in contrast to the previous study, the authors showed that the effect was mediated by GR and not PR (Engler *et al.* 2017). The authors went on to investigate a role for GR-mediated P4 action on T cells in pregnancy, and found that GR in T cells was needed to confer pregnancy protection against autoimmunity, but not for successful pregnancy outcome.

In the studies presented in this Thesis, we found that a disruption of P4 signalling with the P4 antagonist, RU486, caused a reduction in $CD4^+$ T cell and Treg cell populations (Chapter 3) and subsequently caused late gestation pregnancy loss and complications. The Treg cell reduction was implicated in pregnancy loss, since transferring Treg cells back to RU486-treated mice improved pregnancy viability and fetal parameters (Chapter 4). These studies raise the question of how P4 affects Treg cells in our model, and how P4 may drive pregnancy success though its actions on T cells.

As moderate to high doses of RU486 were able to increase inflammatory cytokine expression in nonTreg ($CD4^+Foxp3^-$) and Treg ($CD4^+Foxp3^+$) cells in vivo (Chapter 3), we hypothesised that a mechanism of P4 action on T cells during pregnancy is to constrain inflammatory cytokine production. It is well appreciated that the Th1/Th2 cytokine balance is controlled during pregnancy to favour a 'Th2/Treg' profile, and P4 is has been shown to mediate this effect in the studies cited above, promoting Th2-type cytokines and Treg cell differentiation over Th17/Th1 subsets (Lee *et al.* 2011, Lissauer *et al.* 2015). Furthermore, Treg cell stability is tightly controlled by environmental signals, with inflammatory stimuli shown to drive Treg cell transdifferentiation (Zhou *et al.* 2009). As tolerance induction via Treg cells is essential for pregnancy, P4 may be a driver of Treg cell stability during pregnancy through cytokine modulation. In this study, we

investigated the regulation of Treg and Teff cell phenotype by P4 through in vitro polarisation assays, and explored which receptors may be mediating the effects of P4 in T cells.

5.2. EFFECT OF P4 ON TREG AND T EFFECTOR PHENOTYPE IN VITRO UNDER TH1- AND TH17-POLARISING CONDITONS

P4 favours Th2/Treg differentiation and is thought to influence Treg cell stability (Lee *et al.* 2012), but the mechanism by which this occurs is not defined. In this study, the effect of P4 on Treg cell phenotype was investigated in vitro, under conditions that favour the differentiation of inflammatory T effector (Teff) subsets; Th1 and Th17 cells, which are characterised by the production of effector cytokines IFNγ and IL17, respectively. Splenocytes were harvested from B6 female mice for cell culture. As determined by daily vaginal smearing, females were in the estrus stage of their estrous cycle at the time of the experiment, to control for endogenous hormonal effects on T cells. Splenocytes were activated using plate-bound αCD3 and soluble αCD28 and cultured under non-polarising (Th0) conditions or under Th1- or Th17-polarising conditions, in the presence of 0.5 μg/mL P4 (+P4) or EtOH control (-P4). The 0.5 μg/mL dose of P4 was used as it is physiologically relevant to pregnancy and in the range of what previous studies have used to examine the in vitro effect of P4 on mouse T cells (Lee *et al.* 2012, Engler *et al.* 2017). After 48 hours, cells were removed from culture and restimulated with PMA/ionomycin for 4 hours. Cells were subsequently stained with antibodies for flow cytometry analysis of nonTreg ($CD4^+Foxp3^-$) and Treg ($CD4^+Foxp3^+$) cell production (MFI and percentages) of inflammatory cytokines IFNγ and IL17A as a measure of Th1 and Th17 Teff cell phenotypes, respectively (McGeachy *et al.* 2007, Cimmino *et al.* 2008, Hirota *et al.* 2011).

Figure 5.1A shows a schematic of the experimental design. In nonTreg cells, proportions of $IFN\gamma^+$ Th1 and IL17A Th17 cells were detected following culture under Th0, Th1 and Th17 conditions (Figure 5.1B). Significant IFNγ expression (Th1 cells) was detected in all cultures, and particularly in Teffs from Th1-polarisation cultures (Figure 5.1B). For the quantification of each parameter in the presence of P4, data was expressed as fold change +P4, compared to control (-P4) cultures (Figure 5.1C). Notably, percentages of $IFN\gamma^+$ Teff cells were significantly decreased when P4 was added to Th0 and Th17 cultures, as evidenced by an average 0.25-fold decrease in $IFN\gamma^+$ Teffs +P4 (Figure 5.1B, 5.1C; $P<0.001$). Similarly, a decrease was observed after P4 addition in fold change of MFI of IFNγ in nonTreg cells from Th0, Th1 and Th17 cultures (Figure 5.1C; $P<0.01$-0.001). This demonstrates that P4 suppresses IFNγ

expression in Teff cells cultured under inflammatory polarising conditions. Interestingly, the effect of P4 was the least pronounced in Th1 cultures.

Only minor Th17 differentiation was observed following culture under Th17-polarising conditions (Figure 5.1B). This is likely due to the short culture time used in this experiment, as Th17 cells differentiate optimally after a period in longer culture (Stritesky *et al.* 2008). Despite this, we did assess the percentage of IL17A$^+$ Teff cells and the MFI of IL17A in nonTreg cells in conditions favouring Th0, Th1 and Th17 differentiation. No difference was observed in proportion of IL17A$^+$ cells amongst nonTreg cells cultured under Th17 conditions in the presence of P4. A small number of Th17 cells were identified following Th0 and Th1 cultures. The addition of P4 caused a slight decrease in the fold change MFI of IL17A in Th0 cultures and a slight increase in the fold change MFI of IL17A in Th1 cultures (Figure 5.1C). Notably, these changes only occurred in a very small number of cells that produced IL17A following Th0 and Th1 differentiation. Due to the poor Th17 differentiation overall, conclusions cannot be made regarding the role of P4 in Th17 differentiation in this experiment.

Next, IFNγ and IL17A expression amongst CD4$^+$Foxp3$^+$ Treg cell populations in Th0, Th1 and Th17 cultures in the presence or absence of P4 was measured (Figure 5,1D, 5.1E). Treg cell inflammatory cytokine production following polarisation towards Th1 and Th17 cells serves as a measure of in vitro stability of Treg cells. Some Treg cells capable of producing inflammatory cytokines under certain conditions can undergo transdifferentiation into Teff subsets (Zhou *et al.* 2009, Komatsu *et al.* 2014). Overall, not many Treg cell were present following culture, which wasn't unexpected as the conditions used favoured differentiation towards other T cell subsets. These Foxp3$^+$ cells likely represented Treg cells present in the spleen prior to cell culture. In Foxp3$^+$ Treg cell populations, proportions of IFNγ$^+$ "Th1-like" and IL17A$^+$ "Th17-like" cells were detected following culture under Th0, Th1 and Th17 conditions (Figure 5.1D). Notably, percentages of IFNγ$^+$ Th1-like Treg cells were significantly decreased when P4 was added to Th0, Th1 or Th17 cultures, as evidenced by significant fold change decreases in IFNγ$^+$ Treg cells on average +P4 (Figure 5.1D, 5.1E; $P<0.001$). The greatest effect was observed in the Th0 cultures, whereby on average a 0.5-fold decrease in Treg cells expressing IFNγ was found. Similar to the findings in nonTreg populations, a decrease was also observed in fold change of MFI of IFNγ in Foxp3$^+$ Treg cells cultured under all conditions in the presence of P4 (Figure 5.1E; $P<0.05$-0.001). This demonstrates that P4 suppresses IFNγ expression in Treg cells under non-polarising and inflammatory polarising conditions. Again, the effect of P4 was the least pronounced in Th1-polarised Treg cells.

Overall, these results show that P4 can modulate cytokine production in both $CD4^+$ nonTreg and Treg cells by suppressing IFNγ expression under normal conditions (Th0) and conditions favouring the differentiation of Th1 and Th17 cells. $Foxp3^+$ Treg cells were found to express inflammatory cytokines under these polarising conditions, showing characteristics of instability in vitro. As P4 was able to suppress IFNγ expression in Treg cells cultured under non-polarising and inflammatory conditions, P4 may act to promote Treg cell stability in vivo during pregnancy. Due to the minor Th17 differentiation observed, further experiments may need to be performed to determine whether P4 affects IL17A expression following culture under Th17 conditions.

Our findings presented in the previous chapters demonstrate an effect of the PR antagonist, RU486, in regulating T cells subsets in vivo. Additionally, there is some evidence of P4 modulating T cell cytokine production through the PR (Hughes *et al.* 2013, Thangamani *et al.* 2015). Therefore, we hypothesised that PR was mediating the direct effect of P4 in suppressing IFNγ expression in T cells.

To address this, splenoctyes from PR-replete ($PR^{+/-}$) and deficient ($PR^{-/-}$) mice were cultured in Th0-, Th1- and Th17-polarising conditions as above, in the presence or absence of P4. After 48 hours, cells were removed from culture and flow cytometry analysis of nonTreg and Treg cell was performed to assess inflammatory cytokine production. As observed with B6 splenocytes, P4 suppressed IFNγ production in $PR^{+/-}$ nonTreg and Treg cells under non-polarising, Th1 and Th17 conditions (Figure 5.2A, 5.2B). Strikingly, the same effect of IFNγ suppression was observed in Teff and Treg cells when splenocytes from $PR^{-/-}$ mice were cultured with P4 (Figure 5.2A, 5.2B). This result demonstrates that P4 suppression of IFNγ production in Teff and Treg cells is not mediated by nuclear PR. Thus, other mechanisms of P4 action on T cells to modulate cytokine expression must be considered.

These findings argue against the question of whether T cells express classical PRs, which is a matter of debate in the literature. To address this, mouse spleen, LN and uterus cells from PR-replete mice were stained with a commercially available monoclonal antibody targeting both isoforms of the PR (PR-FITC; Abcore) to assess PR expression in various immune subsets. $PR^{-/-}$ cells and FITC fluorescence minus one (FMO) stains were used as controls. No positive staining was observed in $CD45^+CD3^+$ T cells (data not shown). This data can be interpreted as evidence that mouse T cells do not express detectable PR, as discussed below. However, as we were unable to find a positive control for this antibody in our experiments thus far, it needs to be tested against other cell types known to be positive for PR such as uterine epithelial and stromal cells (Franco *et al.* 2008, Simon *et al.* 2009).

To further explored what receptors could be mediating the effects of P4 on T cells, we FACS-sorted naïve and activated $CD4^+$ T cells, and Treg cell subsets from non-pregnant and pregnant mice to investigate

the expression of P4 receptor genes by qPCR. We aimed to measure the expression of genes encoding PR (*Nr3c3*), glucocorticoid receptor (GR; *Nr3c1*), membrane progesterone receptors (mPRs; *Paqr7, Paqr8*), and progesterone receptor membrane component 1 (PGRMC1; *Pgrmc1*). Unfortunately, due to the low amount of RNA obtained from the sorted T cells, and the relatively low abundance of hormone receptor expression in T cells (Heng *et al.* 2008), we were unable to isolate high enough quality RNA to confidently detect expression of any of these receptors (data not shown).

5.3. INVESTIGATION OF THE CAPACITY OF T CELLS TO BIND A MEMBRANE-IMPERMEABLE FORM OF P4

Following the previous experiments, we continued to explore potential molecular mechanisms by which P4 could exert effects on T cells. Recent evidence has uncovered a potential role for mPRs in modulating responses in T cells, with mPR expression identified in human $CD4^+$ and $CD8^+$ T cells (Lissauer *et al.* 2015). Furthermore, RU486 has been identified as an antagonist of mPR actions in T cells (Chien *et al.* 2009). Here, we investigated whether mouse T cells have the capacity to respond to P4 at the membrane, by flow cytometry using a membrane-impermeable form of P4, P4-BSA-FITC (P4-FITC) (Gaetjens and Pertschuk 1980). P4-FITC has been used to show P4 membrane binding capacity of other cell lineages, that respond to P4 via mPRs expressed on the cell surface (Karteris *et al.* 2006, Ndiaye *et al.* 2012, Nader *et al.* 2018). To control for non-specific staining effects caused by BSA conjugation, we used a BSA-FITC conjugate, at equimolar concentration. Spleen and LNs were isolated from B6 females and rested for 3 hours at 37ºC before FACS staining. Cells were surface stained, then incubated with 10 µg/mL P4-FITC or BSA-FITC for 1 hour, before intracellular staining and flow cytometry. Initially, we measured FITC fluorescence in various T cell and Treg cell populations stained with P4-FITC and BSA-FITC samples (Figure 5.3A). FITC staining is shown in $CD8^+$ T cells, $CD4^+Foxp3^-$ nonTreg cells, $CD4^+Foxp3^+$ Treg cells, and Treg cell subsets based on Nrp1 expression (tTreg and pTreg) (Figure 5.3A). While some background staining was evident in cells incubated with BSA-FITC, a clear positive shift was observed in samples incubated with P4-FITC, in all T cell subsets measured (Figure 5.3A, 5.3B), indicating these cells are capable of binding P4 at the plasma membrane. Representative histograms show the shift in FITC fluorescence in nonTreg and Treg cells from spleen and LN incubated with P4-FITC and BSA-FITC, demonstrating capacity to bind P4 at the cell membrane (Figure 5.3B).

As shown by the previous studies presented in this thesis, P4 action in early pregnancy is important for Treg cell regulation. Thus, we examined the capacity of Treg cells, and other $CD8^+$ and $CD4^+$ T cells, to bind P4 at the membrane in early pregnancy and in virgin mice. We hypothesised that T cells in early pregnancy would have increased capacity to bind P4 at the membrane due to an increase in the expression of membrane receptors capable of binding P4.

Spleen, udLN and peripheral LNs (inguinal, brachial, axillary, and mesenteric) were harvested from non-pregnant virgin B6 mice in estrus and from mice on day 3.5 pc following allogeneic mating to BALB/c males. Cells were stained and incubated with P4-FITC and BSA-FITC as above, before flow cytometry analysis. Figure 5.4A shows FITC fluorescence in Treg cells from mice in estrus and on day 3.5 pc following incubation with P4-FITC and BSA-FITC. Again a positive shift in fluorescence can be demonstrated following P4-FITC incubation (Figure 5.4A). To quantify the staining in Figure 5.4A, P4 binding capacity (% $P4\text{-}FITC^+$) of cell populations (Figure 5.4B) and P4-FITC MFI (Figure 5.4C) were determined by subtracting FITC fluorescence of BSA-FITC was from P4-FITC fluorescence.

In the spleen and other LNs, proportions of $P4\text{-}FITC^+$ cells were identified in all cell subsets tested and these proportions did not change between estrus and day 3.5 pc (Figure 5.4B). For example, in the spleen an average of 10% of $CD8^+$ T cells were $P4\text{-}FITC^+$, and levels were comparable between non-pregnant and pregnant mice. This was also observed when MFI of P4-FITC was assessed, with no change in MFI between cells from estrus and day 3.5 pc mice (Figure 5.4C).

In the udLN, proportions of $P4\text{-}FITC^+$ cells were identified amongst all cell subsets tested (Figure 5.4B). Interestingly, the proportion of $P4\text{-}FITC^+$ cells increased in early pregnancy specifically in udLN $CD4^+$ nonTreg, total Treg cells and in tTreg and pTreg cells (Figure 5.4B; $P<0.1\text{-}0.05$). Strikingly, Treg cell populations from udLN of early pregnant mice had the highest capacity to bind P4 at the membrane, with a 2-3 fold increase in the proportion of $P4\text{-}FITC^+$ Treg cells, representing 10-20% of total Treg cells in udLNs (Figure 5.4B). Similarly to these proportions, the MFI of P4-FITC was also increased specifically in total Treg cells, tTreg and pTreg cells from udLN of early pregnant mice (Figure 5.4C; $P<0.1\text{-}0.05$). Interestingly, the strongest effect of P4-FITC binding was observed in tTreg cells in the udLN (Figure 5.4B, C).

Overall, these data show that $CD8^+$ and $CD4^+$ T cells, and $CD4^+Foxp3^+$ Treg cells have the capacity to bind a membrane impermeable form of P4 and thus must express receptors capable of binding P4 at the plasma membrane. Furthermore, these data demonstrate that in early pregnancy, $CD4^+$ nonTreg and Treg cell from udLNs show increased capacity to bind P4 at the plasma membrane, and furthermore udLN Treg cells contain the highest proportion of cells positive for membrane-bound P4. This suggests that

mouse CD4+ T cells and Foxp3+ Treg cells in the local draining LNs may upregulate receptors to mediate the effects of P4 at the membrane in early pregnancy. The most likely candidate receptors to mediate the effects of P4 at the membrane are mPRs, which have been shown to bind P4-FITC at the plasma membrane in various cells (Ndiaye *et al.* 2012, Nader *et al.* 2018) (Figure 5.5).

5.4. DISCUSSION

The experiments in this study investigated the role of P4 in regulating Treg cell phenotype in vitro and explored mechanisms of direct P4 action in T cells by investigating the capacity of T cells and Treg cells to bind to P4 at the cell surface. We demonstrate that P4 suppresses IFNγ expression in Treg and Teff cells cultured under inflammatory-polarising and non-polarising conditions. Therefore, P4 may act to promote Treg cell stability and constrain Teff differentiation in vivo during pregnancy. Interestingly, the effect of IFNγ suppression by P4 was not mediated by nuclear PR signalling, since $PR^{-/-}$ Teff and Treg cells responded the same way to P4 as PR-replete mice. Given these findings, other mechanisms of P4 action in T cells were explored. We found that T cells were able to respond to P4 at the plasma membrane, binding membrane-impermeable P4-FITC. Further, the capacity of CD4+ T cells and Treg cells to bind P4 at the membrane was increased in early pregnancy. Thus mouse Treg cells likely express receptors capable of mediating the effects of P4 at the membrane, and these receptors may be upregulated in early pregnancy. Here, we discuss the significance of these findings, and possible mechanisms of both direct and indirect action of P4 on T cells and Treg cells, for pregnancy success. The various P4 receptors and mechanisms of action of P4 in T cells are summarised in Figure 5.5.

5.4.1. Direct effects of P4 on the T cell response

The findings presented in this study add to many studies showing a direct role of P4 in altering T cell phenotype. P4 has been found to modulate T cell cytokine production in vitro in mice and humans, favouring Th2 cytokines and suppressing inflammatory Teff cytokines (Lee *et al.* 2011, Hughes *et al.* 2013, Hierweger *et al.* 2019). This study shows that in addition to CD4+ T cells, cytokine production specifically in Foxp3+ Treg cells is modulated by P4, particularly suppression of IFNγ production.

The culture system used in this study is a well-characterised method of in vitro polyclonal T cell activation. Although these were splenocyte cultures and not cultures of purified T cells, the activation process of using plate bound αCD3 and soluble αCD28 antibodies essentially circumvents antigen-specific activation by APCs in the culture microenvironment. αCD3 activates T cell receptor (TCR) complexes in the absence of antigenic peptide from APCs, and αCD28 stimulates T cells, bypassing CD80/CD86 costimulatory

signals from APCs. Although APCs in the culture (macrophages, DCs B cells) could theoretically interact with the T cells, the T cells are activated and differentiated in the absence of APC signals and thus we would expect them to behave the same way in the absence of APCs. It is expected therefore, that along with their capacity to differentiate indirectly in response to cytokines, T cells have the capacity to respond directly to P4 in this system. Others routinely use splenocyte cultures for T cell polarisation studies (Kara *et al.* 2013, Hierweger *et al.* 2019). Furthermore, others report the same effect of P4 on T cells in splenocyte cultures and in purified T cell cultures (Engler *et al.* 2017). Thus we can be confident that P4 directly interacts with T cells in our culture experiments. Indeed, we went on to find that P4 interacts directly with T cells by binding receptors on the plasma membrane.

The question of what receptor pathways mediate the direct effects of P4 on T cells was explored in this study and is summarised in Figure 5.5. Firstly, we found that nuclear PR did not mediate the direct effects of P4 on Treg and Teff cell cytokine production, since the absence of PR in T cells ($PR^{-/-}$ splenocytes) had no effect on the ability of P4 to suppress IFNy production in T cells, as was observed in PR-replete mice. Whether Treg cells actually express classical PRs is a controversial issue (Ndiaye *et al.* 2012, Hierweger *et al.* 2019).

PR was found to be expressed in T cells from mice and women (Szekeres-Bartho *et al.* 1990, Mao *et al.* 2010, Hughes *et al.* 2013). Mao et al., showed PR to be expressed in $CD4^+$ $CD25^+$ and $CD4^+CD25^-$ T cells by western blot. They also claim to show PR expression in Treg cells by flow cytometry, but concerningly the authors failed to show their antibody staining or any controls used. Other studies have shown a specific role for PR in T cell regulation. Hughes et al. showed in mice that PR suppresses mouse $CD4^+$ Teff activity likely though suppression of the *Ifng* gene. Contrary to our results, this study showed that *Ifng* mRNA induction was impaired in WT cells but not in $PR^{-/-}$ cells (Hughes *et al.* 2013). These findings cannot be readily reconciled with the findings from the current study. However, there were differences in the culture systems used, with the Hughes study using a 5 day culture with αCD3 and αCD28 and no cytokine polarisation. Thangamani et al. showed PR binding elements (PREs) in the human *VDR* gene (encoding the Vitamin D receptor). P4 directly upregulated *VDR* expression in human and mouse $CD4^+$ T cells for regulation by Vitamin D (Thangamani *et al.* 2015). Interestingly, Vitamin D is known to regulate IFNγ production in T cells, and VDR was found to directly bind to the *Ifng* promoter to suppress Th1 cell generation (Cippitelli and Santoni 1998, Thangamani *et al.* 2015). These findings are consistent with our results of IFNγ suppression in T cells by P4, but again contradict our observation that the effect was not mediated by nuclear PR signalling. In the Thangamani study, a combination of Vitamin D and P4 caused the greatest reduction of $IFN\gamma^+CD4^+$ human cord blood T cells. But importantly, when VDR was knocked down with siRNAs, a reduction in $IFN\gamma^+$ cells was still observed (Thangamani *et al.*

2015). This implies that the P4-VDR pathway is one of multiple mechanisms of P4 action that can suppress IFNγ in T cells, and that there are other PR-independent mechanisms at play. Furthermore, PR-independent mechanisms of P4 action on *VDR* expression may also exist.

Overall, our findings are in agreement with many others, who find no detectable expression of nuclear PR in T cells or show no evidence of PR action in T cells using specific receptor antagonists (Kontula *et al.* 1983, Mansour *et al.* 1994, Lissauer *et al.* 2015, Engler *et al.* 2017). We used a FITC-conjugated monoclonal PR antibody for flow cytometry and found no expression of PR in T cells (data not shown). The disagreement in the literature could be due to the fact that the majority of studies showing PR expression in human T cells were in pregnant women or in cord blood (Szekeres-Bartho *et al.* 1990, Thangamani *et al.* 2015). This might be due to temporal upregulation of PR in response to hormones, since *Pgr* expression is known to be regulated by both estradiol and progesterone (Quadros and Wagner 2008, Diep *et al.* 2016). Indeed the Immunological Genome Project found PR to be expressed at very low levels in T cells (Heng *et al.* 2008). More investigation is needed to determine the biological relevance of PR expression in T cells for immunology and reproduction. Importantly, when using antibodies for PR (or any hormone receptor), it is imperative that the correct controls are used to control for background and non-specific staining, to provide confidence in published data regarding PR expression in immune cells.

Not all effects of P4 can be explained by classical nuclear PR signalling. For example, P4 is known to produce rapid non-genomic effects in target cells. These effects of P4 are evident in PR-deficient cells and the PR knockout ($PR^{-/-}$) mouse model (Karteris *et al.* 2006, Thomas *et al.* 2007, Ndiaye *et al.* 2012, Lu *et al.* 2015). The rapid effects of P4 are known to be mediated by mPRs or membrane associated proteins such as PGRMCs. mPRs are a novel family of G-protein coupled receptors (GPCRs) known as the progestin and adipoQ receptor (PAQR) family (Figure 5.5).

mPRs are expressed in many tissues including the ovaries, myometrium and brain (Karteris *et al.* 2006, Charles *et al.* 2010, Ndiaye *et al.* 2012). Furthemore, mPRs (mPRα and mPRβ) are expressed by human $CD4^+$ and $CD8^+$ T cells and P4 binding to mPRs in T cells activates G proteins (Dosiou *et al.* 2007, Thomas *et al.* 2007, Ndiaye *et al.* 2012). mPRα may also be expressed by human peripheral blood $Foxp3^+$ Treg cells during pregnancy (Areia *et al.* 2015). Although they are known to cause rapid effects including acidification and calcium flux, these cellular modifications can lead to genomic effects. G protein activation following mPR binding is one way in which P4 may mediate its actions in T cells. G proteins regulate mitogen-activated protein kinase (MAPK) function, which can go on to affect cellular transcription, inhibiting Th1 development (Miyaura and Iwata 2002). mPR signalling has been proposed as an immunosuppressive pathway that leads to increased calcium signalling and direct blocking of K^+ channels,

which are linked with TCR signalling (Ehring *et al.* 1998). P4 inhibited Ca^{2+} signalling in human T cells following TCR engagement, by specifically blocking K^+ channels. This leads to a reduction in nuclear factor of activated T cell (NF-AT)-driven gene expression (Ehring *et al.* 1998). As NF-AT is regulated by MAPK and is an intermediate of TCR action leading to IL2 production, inhibition of this pathway would inhibit production of T cell cytokines. Importantly, NF-AT regulates IFNγ gene expression in T cells (Kiani *et al.* 2001). This fits with our findings of P4 suppression of IFNγ production following TCR engagement by αCD3, and suggests that mPR action in T cells could modulate direct effects of P4 on T cells in pregnancy.

Other than mPRs, a second family of non-classical progesterone receptors exists; the b5-heme/steroid protein family. The best-characterised proteins from this family are PGRMC1 and PGRMC2, which are cytoplasmic proteins shown to anchor to the plasma membrane (Thomas *et al.* 2014). T lymphocytes express PGRMC1 and this receptor has been proposed to mediate some of the actions of P4 in T cells (Ndiaye *et al.* 2012). PGRMC1 was shown to transport mPRα to the cell surface in human $PR^{-/-}$ breast cancer cell lines. Thus PGRMCs may work in concert with mPRs to mediate P4 effects in target cells (Thomas *et al.* 2014).

The field of membrane PR research is relatively new and more work is needed to understand the regulatory pathways and cellular effects of mPRs and it's associated proteins, elicited upon P4 binding. Our study demonstrates that $CD4^+$ and $CD8^+$ T cells can bind P4 at the membrane and that $CD4^+$ and $Foxp3^+$ Treg cells are sensitive to regulation by P4 in early pregnancy as they have an increased capacity to bind membrane P4. This suggests that mouse T cells express mPRs, and that their expression is upregulated in pregnancy, specifically in $CD4^+$ and Treg cells. Early pregnancy may prime Treg cells to respond to membrane-bound P4. Therefore mPR regulation of $CD4^+$ T cells and Treg cells could be one mechanism of P4 regulation of the immune system in early pregnancy, contributing to the induction of fetal-maternal tolerance. P4-mPR action in T cells during pregnancy may help to explain the studies presented in this thesis, demonstrating that the P4 antagonist RU486 decreases Treg cell numbers and consequently impacts upon pregnancy success. Indeed, RU486 antagonises mPR mediated effects on T cells (Chien *et al.* 2009).

The question of whether P4 influences Treg cell subsets differently is relevant for understanding P4-Treg dynamics during pregnancy. Given most Treg cells in early pregnancy are tTreg cells (Moldenhauer *et al.* 2019) and the tTreg cell subset showed the strongest binding to P4 at the membrane (Figure 5.4), this Treg subset may be particularly responsive to the effects of P4. This is in line with studies in women showing pre-pregnancy effects of P4 on Treg cells, which are presumably of thymic origin (Kallikourdis

and Betz 2007). However, P4 also generates suppressive human and murine iTreg cells in vitro (Lee *et al.* 2011, Lee *et al.* 2012). Whether P4 differentially affects Treg cells with distinct antigen specificities (e.g. self, allo, fetal) in mouse and human pregnancy remains to be determined.

Another candidate mediator of P4 action in T cells is the steroid hormone receptor GR. GR is widely expressed by immune cells, including T cells. P4 binds to GR with one third of the affinity that corticosterone binds to GR (Grosser *et al.* 1971, Kontula *et al.* 1983). Also similar to P4, glucocorticoids inhibit Th1 cell differentiation (Miyaura and Iwata 2002).

P4 promotes Treg cells in vitro and this effect could be blocked by RU486. However, the effect was found to be mediated by P4 binding the GR in T cells, and not through PR (Engler *et al.* 2017). The authors go on to show that although GR expression on T cells is necessary for the protective effects of pregnancy on autoimmunity, it is not required for the maintenance of pregnancy (Engler *et al.* 2017). Recently, the same group demonstrated that P4 selectively regulates the T cell response in vitro by promoting T cell death, preferentially in T conventional cells (Hierweger *et al.* 2019). This caused P4 to selectively expand Treg cells, which were refractory towards P4-induced T cell death. Interestingly this effect was found to be mediated by P4 binding to GR (Hierweger *et al.* 2019). Although not measured directly in this study, in our in vitro experiments we observed overall high levels of cell death following the 48 hour cell culture as shown by standard live/dead staining in flow cytometry. The cell death was observed independently of P4 however, as both +P4 and -P4 cultures showed cell death (data not shown). Some cell death is normal from T cell activation with αCD3 in vitro, and notably, our culture conditions differed significantly from the above study.

It is feasible that the IFNγ suppression observed in the current study could be mediated through GR, since GR has previously been shown to repress *Ifng* transcription. Deletion of GR specifically in T cells causes increased IFNγ expression (Brewer *et al.* 2003). These findings are also relevant to our model of RU486 administration in pregnancy, presented in Chapters 3 and 4 of this Thesis. As RU486 is also known to be a GR antagonist, reasonably it could be blocking GR in our model. However, as RU486 caused a decrease in pregnancy success through Treg-cell mediated mechanisms, it seems unlikely that the effect is mediated through GR, as GR in T cells has been found to be dispensable for pregnancy success (Engler *et al.* 2017). Indeed, the importance of IFNγ suppression in T cells for overall pregnancy success and the Treg cell response needs to be determined and is discussed below and further in Chapter 7.

5.4.2. P4 as a regulator of Treg cell phenotypic plasticity and stability

Our study adds to a large body of literature showing that P4 can modulate cytokine expression in T cells. Here we demonstrate that P4 modulates cytokine expression in $CD4^+$ T cells and specifically in $Foxp3^+$ Treg cells by suppressing IFNγ production, under non-polarising, Th1-polarising or Th17-polarising conditions. P4 is known to inhibit Th1 differentiation by suppressing *Ifng* expression in mouse and human $CD4^+$ T cells in vitro (Miyaura and Iwata 2002, Lissauer *et al.* 2015, Thangamani *et al.* 2015). In our experiments the effect of P4 suppression of IFNγ, was subtle overall, particularly in Teff cells, however P4 causes dose dependent effects in T cells, and as we used a relatively low, physiologically relevant dose (0.5 μg/mL) (Lee *et al.* 2011, Lee *et al.* 2012), we would expect the IFNγ suppression to increase with higher concentrations of P4. Indeed some of P4 effects on T cells including Foxp3 induction were more evident using higher physiologically relevant doses of P4 in culture (Lee *et al.* 2012). However, the dose used here is well within the range of what is known to produce effects in T cells (Engler *et al.* 2017).

Although the biological significance of P4 suppression of IFNγ in T cells and Treg cells is not fully defined, evidence points to a key role in maternal tolerance of the fetus during pregnancy. P4 may function to modulate T cell cytokine expression during pregnancy to limit Teff differentiation and ensure Treg cell tolerance is maintained. The importance of Treg cells for pregnancy success is well appreciated. Inhibition of Th1 differentiation is necessary for fetal maternal tolerance, as committed Th1 differentiation blocks $Foxp3^+$ expression in pregnancy and leads to antigen-specific fetal loss (Xin *et al.* 2014). In women, the Treg/Teff balance must be maintained for pregnancy success, with reduced Treg cell activity and increase Teff activity reported in many pregnancy pathologies (Ng *et al.* 2002, Steinborn *et al.* 2008, Prins *et al.* 2009, Lee *et al.* 2011, Wu *et al.* 2014, Care *et al.* 2018). Peripheral blood lymphocytes exhibit downregulation of IFN-related genes in the luteal phase compared with the follicular phase of the menstrual cycle, when P4 levels are dominant. This may ensure that pregnancy tolerance can be established though correct T cell phenotypes (Dosiou *et al.* 2004). Additionally in pregnant women, P4 causes hypermethylation of *IFNG* in $CD8^+$ memory T cells (Yao *et al.* 2017).

IFNγ expression in $Foxp3^+$ Treg cells is relevant for Treg cell stability, as Treg cells known to exhibit instability were shown to express Teff cytokines, convert to Teff cells, and cause pathology in various inflammatory diseases (Zhou *et al.* 2009). P4 may act as a molecular switch to control Treg and Teff phenotypes for pregnancy tolerance through controlling Treg cell stability. Treg cell stability in pregnancy is explored further in Chapter 6, using a model to measure the fate of Treg cells during pregnancy. This is relevant for Treg cell functional suppressive capacity, as the stability of Treg cells dictates their ability to exhibit a suppressive effector phenotype, with stable Treg cells exhibiting a 'Treg cell gene signature'

required for suppressive function (Zheng et al. 2010, Ohkura et al. 2012). As Treg cell phenotypic plasticity is associated with a loss of suppressive capacity (Jung et al. 2017), P4 is expected to promote Treg cell suppressive capacity. However, distinct populations of phenotypically plastic Treg cells exhibiting high functional suppressive capacity exist (Pandiyan and Zhu 2015, Levine et al. 2017), requiring this hypothesis to be specifically tested. Nonetheless, P4 may promote Treg cell suppressive capacity through other mechanisms, such as through promoting Foxp3 expression (Lee et al. 2012). Our findings in Chapter 3 and 4 show early pregnancy P4 is important for Treg cell action on pregnancy success, and a recent study in our laboratory shows early mouse pregnancy is characterised by the expansion of tTreg cells expressing CTLA4, a marker of Treg suppressive competence (Moldenhauer et al. 2019). Therefore early pregnancy P4 likely promotes stable, suppressive Treg cells for pregnancy success.

Unfortunately, in this study we couldn't confidently assess effect of P4 on modulating Th17 cytokine production due to the poor polarisation towards Th17 with the culture conditions used. If repeated, to measure Th17 polarisation, a culture time longer than 48 hours should be used (Stritesky *et al.* 2008). Other studies have shown that P4 suppresses IL17A production in Th17 cells (Xu *et al.* 2013), with one study showing P4 decreases expression of Th17-associated genes (*IL17F, IL21, RORC*) in human cord blood $CD4^+$ T cells cultured under Th17-polarising conditions (Lee 2011). Thus, P4 may suppress both Th1 and Th17 cells in vivo during pregnancy to ensure Treg cell tolerance in maintained.

Overall this study reports that P4 directly regulates T cell cytokine production, repressing inflammatory IFNγ expression in both Treg and Teff cells. Furthermore, our findings add to the current literature demonstrating a role for P4 in skewing T cell cytokine expression towards a Th2/Treg profile and away from a Th1/Th17 profile. This study also shows that Treg cells increase their capacity to bind P4 at the plasma membrane in early pregnancy, highlighting a potential role for mPRs in mediating the biological effects of P4 in T cells. The overall relevance of these findings for pregnancy need to be investigated, but they may help to explain the interplay between P4 and Treg cells during pregnancy and the impact on pregnancy success, as investigated in the previous chapter in this Thesis.

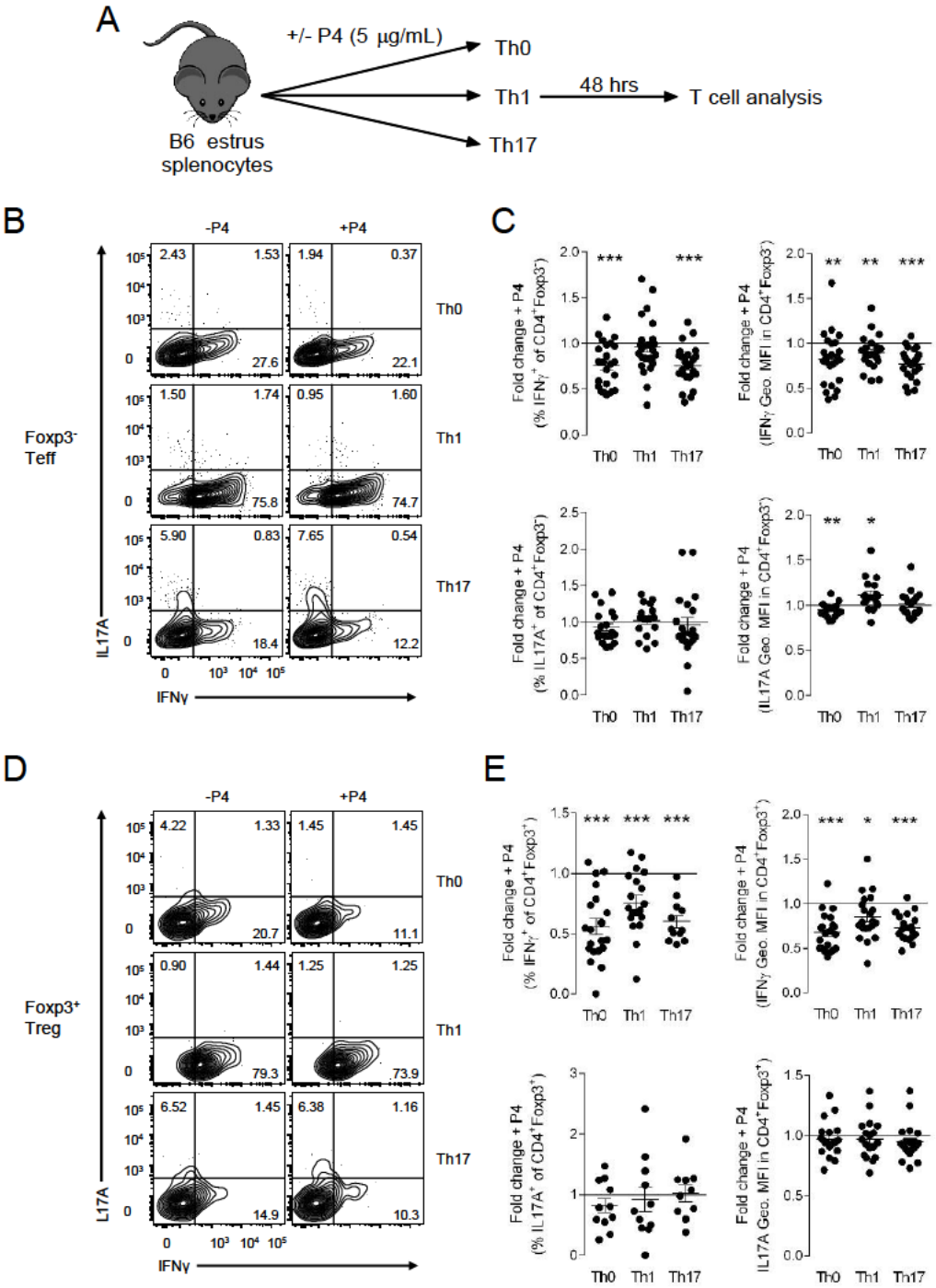

Figure 5.1 P4 suppresses IFNγ production in Teff and Treg cells in vitro.

Splenocytes from B6 female mice in estrus were cultured under Th1-polarising (αIL4, IL2), Th17-polarising (αIFNγ, αIL4, IL6, TGFβ, IL1β, IL23) or non-polarising (Th0; αIFNγ, αIL4) conditions in the presence (+P4) or absence (-P4) of P4 (0.5 µg/mL) for 48 hours followed by stimulation with PMA and ionomycin for 4 hours and subsequent quantification of T effector (Teff) and Treg cell cytokine production by flow cytometry. (A) Schematic of experimental design. (B,D) Representative flow cytometric analysis of IFNγ and IL17A expression in (C) Teff ($CD4^+Foxp3^-$) and (E) Treg ($CD4^+Foxp3^+CD25^+$) cells cultured under Th0-,Th1- or Th17-polarising conditions in the presence or absence of P4. (C,E) Quantification of (B,D) showing proportion and geometric MFI of IFNγ and IL17A in (C) Teff cells and (E) Treg cells, expressed as fold change +P4 compared to respective -P4 culture. (C,E) n=15-21. Each symbol represents an individual mouse/condition. Data are pooled from 5 individual experiments and are presented as mean fold change ± SEM. One samples T test where -P4 culture=1; * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

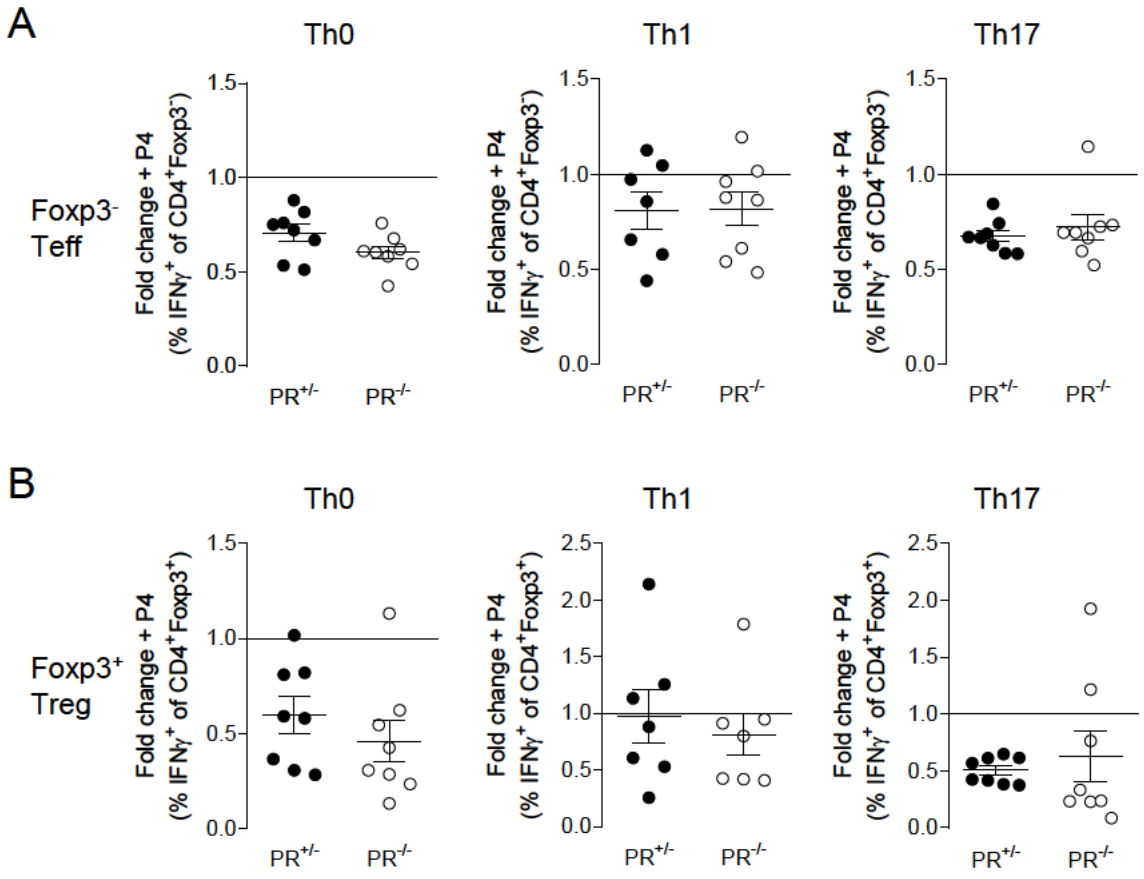

Figure 5.2 P4 suppresses IFNγ production in Teff and Treg cells from $PR^{-/-}$ mice

Splenocytes from PR replete ($PR^{+/-}$) and PR deficient ($PR^{-/-}$) female mice in estrus were cultured under Th1-polarising (αIL4, IL2), Th17-polarising (αIFNγ, αIL4, IL6, TGFβ, IL1β, IL23) or non-polarising (Th0; αIFNγ, αIL4) conditions in the presence (+P4) or absence (-P4) of P4 (0.5 µg/mL) for 48 hours followed by stimulation with PMA and ionomycin for 4 hours and subsequent quantification of T effector (Teff) and Treg cell cytokine production by flow cytometry. (A) Proportion of $IFN\gamma^+$ Teff ($CD4^+Foxp3^-$) and (B) Treg ($CD4^+Foxp3^+CD25^+$) cells from $PR^{+/-}$ and $PR^{-/-}$ mice cultured under Th0-,Th1- or Th17-polarising conditions in the presence or absence of P4. Data expressed as fold change +P4 compared to respective -P4 culture. n=7-8. Each symbol represents an individual mouse/condition. Data are pooled from 2 individual experiments and are presented as mean fold change ± SEM. One samples T test where -P4 culture=1; * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

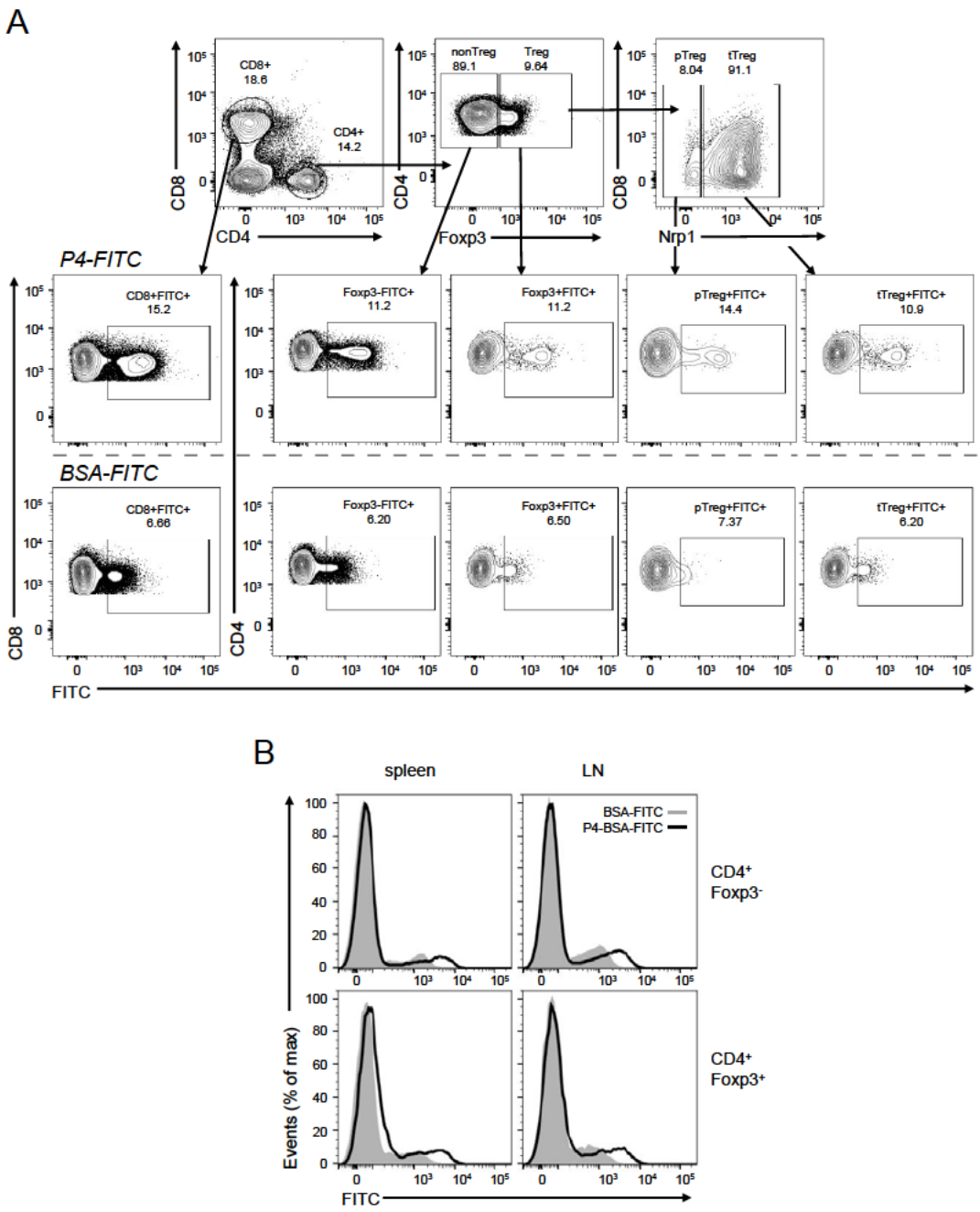

Figure 5.3 CD4$^+$ and CD8$^+$ T cells and Foxp3$^+$ Treg cells have the capacity to bind to a membrane-impermeable form of P4.

Spleen and LN cell suspensions from virgin estrus females were rested at 1 x 10^7 cells/mL in cRPMI media for 3 hours at 37˚C. Then, 1 x 10^6 cells were stained for surface antibodies and incubated with a membrane-impermeable form of progesterone, P4-BSA-FITC (P4-FITC; 10 µg/mL) or BSA-FITC to control for background fluorescence. Cells were subsequently stained with intracellular antibodies and characterised by flow cytometry. (A) Flow cytometry gating strategy to assess P4-FITC fluorescence (membrane P4 binding capacity) in T cell populations in spleen and LNs. FITC fluorescence was examined in CD4$^+$, CD8$^+$, total Treg, pTreg and tTreg cell subsets stained with P4-FITC or BSA-FITC. Background BSA-FITC fluorescence was subtracted from P4-FITC fluorescence to determine true P4 staining at the membrane (P4-FITC$^+$ cells). Fluorescence minus one (FMO) controls were used to set the gate for FITC$^+$ cells. (B) Representative histograms of FITC fluorescence in CD4$^+$ nonTreg (Foxp3$^-$) and Treg (Foxp3$^+$) cells from spleen and LN of non-pregnant mice stained with P4-FITC and BSA-FITC.

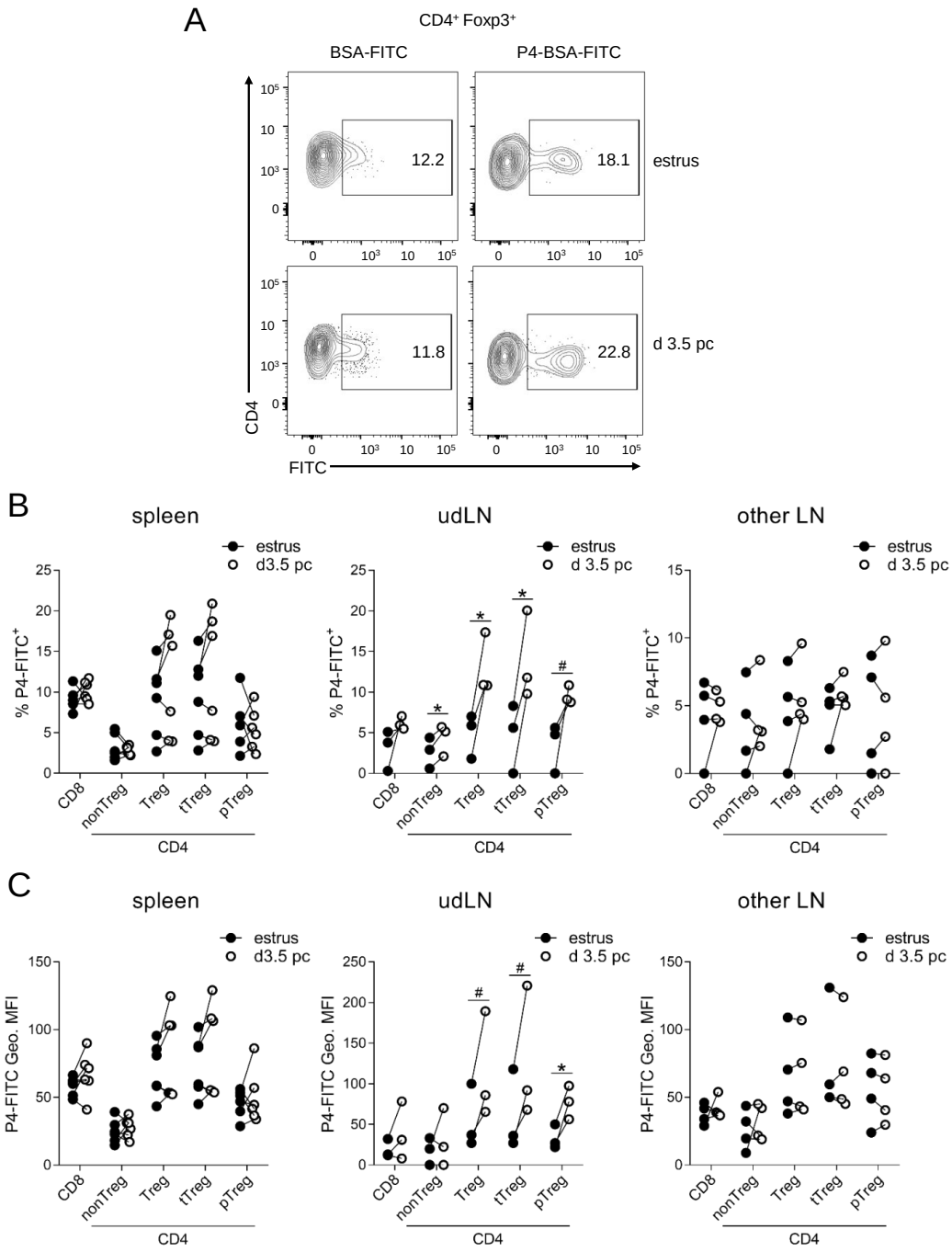

Figure 5.4 CD4$^+$ T cells and Treg cells from udLN of mice in early pregnancy have an increased capacity to bind to P4 at the plasma membrane.

Spleen and LN cell suspensions from virgin estrus females and females on day 3.5 pc (blastocysts confirmed present) were rested at 1 x 10^7 cells/mL in cRPMI media for 3 hours at 37˚C. Then, 1 x 10^6 cells were stained for surface antibodies and incubated with P4-FITC (10 µg/mL) or BSA-FITC before intracellular staining and quantification by flow cytometry. (A) Representative analysis of P4-FITC and BSA-FITC staining in udLN Treg cells of estrus and day 3.5 pc mice. (B, C) Quantification of (A) for spleen, udLN and other LNs, expressed as (B) P4 binding capacity (% P4-FITC positivity) and (C) P4-FITC geometric mean fluorescence intensity (Geo. MFI), as measured by subtracting BSA-FITC fluorescence from P4-FITC fluorescence. (B, C) Data are from 3 independent experiments (n=3-6) and are paired per experiment. Each symbol represents an individual mouse for spleen samples. LNs were pooled from 2-4 mice per experiment. Paired T test; * p<0.05, $^{\#}$ p<0.1.

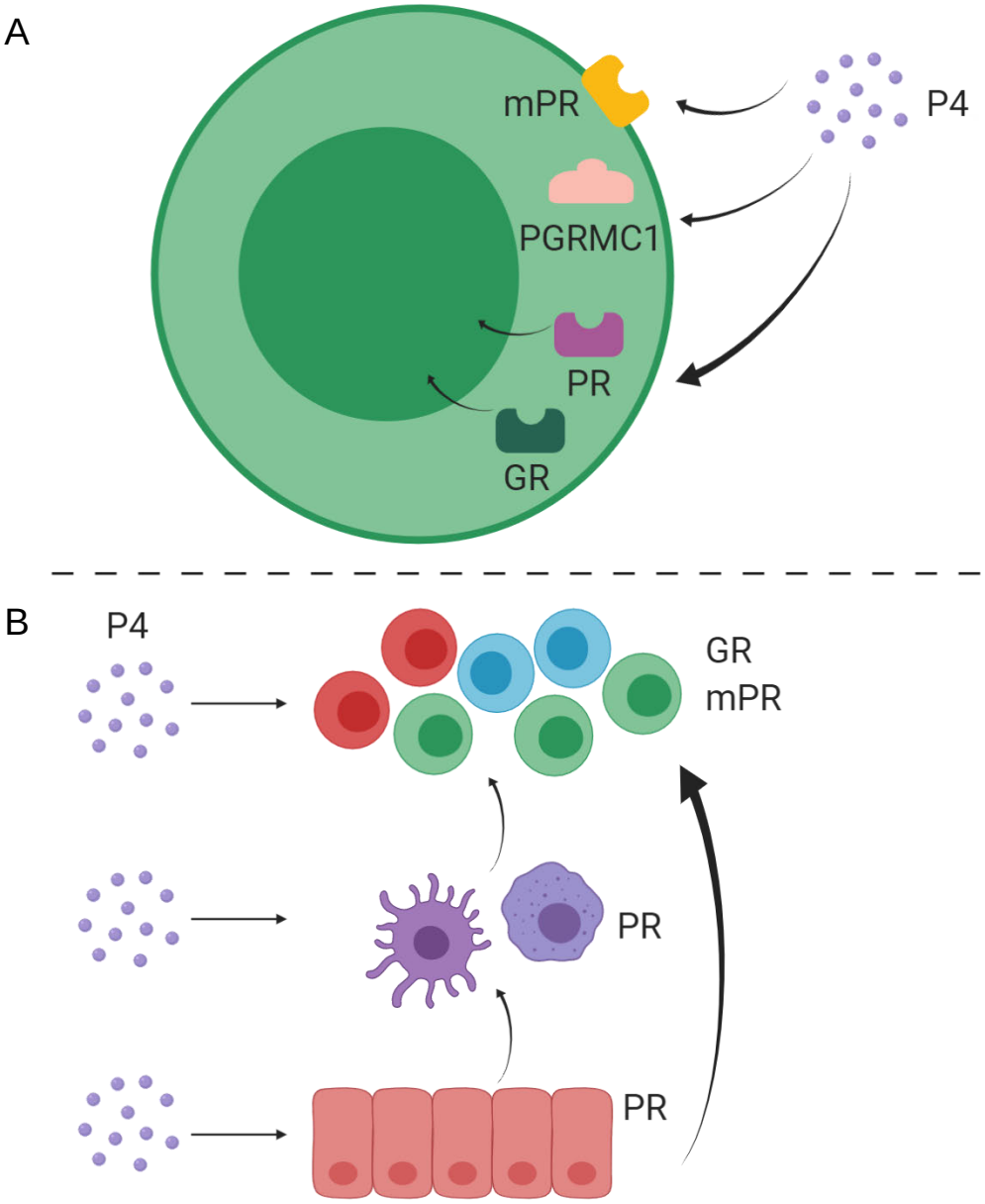

Figure 5.5 Progesterone receptors and mechanisms of action in T cells.

(A) Direct progesterone (P4) action on target cells is mediated by numerous surface and intracellular receptors. Genomic effects of P4 are mediated by the classical steroid hormone receptors Progesterone Receptor (PR) and Glucocorticoid Receptor (GR), which translocate to the nucleus following ligand binding to regulate target gene expression. Other effects of P4 are mediated membrane P4 receptors (mPRs) such as mPRα and mPRβ which are G protein coupled receptors that mediate non-genomic, rapid effects such as calcium flux and acidification. mPRs may work in concert with cytoplasmic, membrane associated proteins such as Progesterone Receptor Membrane Component 1 (PGRMC1) which also mediate rapid P4 effects. (B) Progesterone influences $CD4^+$ T cells through both direct and indirect mechanisms. Our experiments (and others) support that direct mechanisms of P4 as in (A) are likely mediated via GR and mPR in $CD4^+$ T cells and Treg cells. Indirect mechanisms include P4 action on other target cells which then interact with T cells. These include immune cells such as DCs and macrophages, and epithelial and stromal cells, all of which respond to P4 via PR action. Image created with Biorender.com.

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Chapter 6

Investigating Treg cell stability and fate in pregnancy

CHAPTER 6

6.1. INTRODUCTION

Treg cells are primarily identified by their robust expression of the Treg-cell lineage defining transcription factor, Foxp3 (Fontenot *et al.* 2003). Foxp3 is a multi-functional transcription factor that establishes commitment to the Treg cell fate through repression of effector pathways and induction of the Treg cell transcriptional program (Gavin *et al.* 2007). In addition to Foxp3 expression, Treg cell lineage commitment also requires a Treg-specific pattern of CpG hypomethylation which is induced following T cell receptor (TCR) stimulation (Ohkura *et al.* 2012). For example, the highly conserved Treg-specific demethylated region (TSDR) of the *Foxp3* promoter, while hypermethylated in Foxp3$^-$ CD4$^+$ cells, is hypomethylated in Treg cells (Zheng *et al.* 2010). These epigenetic changes lead to activation of the Treg cell gene signature required for Treg suppressive function, such as expression of *Ctla4, Il2ra, Tnfrsf18* and *Ikzf2* which encode CTLA4, CD25, GITR and Eos, respectively (Zheng *et al.* 2010, Ohkura *et al.* 2012).

It is well known that CD4$^+$ T helper (Th) cells, including Foxp3$^+$ Treg cells can possess a high degree of phenotypic plasticity (the ability to express transcription factors and/or cytokines typically associated with other cell lineages) in certain environmental conditions, leading to functional heterogeneity (Voo *et al.* 2009, Levine *et al.* 2017). For example, some Treg cells express Tbet, the Th1 signature transcription factor, and Tbet$^+$Foxp3$^+$ Treg cells can possess immunosuppressive effector function (Levine *et al.* 2017). Additionally, phenotypically plastic Treg cells may contribute to disease pathogenesis in inflammatory diseases (Jung *et al.* 2017). Despite exhibiting phenotypic plasticity, Treg cells were historically viewed as a stable lineage. Foxp3$^+$ Treg cells were found to be stable in homeostatic and inflammatory conditions (Rubtsov *et al.* 2010), however recent evidence demonstrates that some Treg cells display lineage instability; the loss of identity and conversion to other cell fates (Zhou *et al.* 2009, Miyao *et al.* 2012, Hori 2014, Komatsu *et al.* 2014). For example, Treg fate mapping experiments using transgenic mouse models revealed Foxp3$^+$ Treg cells can undergo transdifferentation and convert to Th1 (exTreg) cells in type 1 diabetes (Zhou *et al.* 2009). exTreg cells may derive from Treg cells that lose Foxp3 expression (Zhou *et al.* 2009), and from conventional T cells that transiently express Foxp3 without fully committing to the Treg lineage (Miyao *et al.* 2012). Strikingly, exTreg cells exhibit pathogenicity and are shown to drive disease progression in a range of models of inflammatory or lymphopenic environments including type 1 diabetes, inflammatory arthritis and inflammation of the lung and liver (Zhou *et al.* 2009, Miyao *et al.* 2012, Hori 2014, Komatsu *et al.* 2014). Foxp3$^+$ Treg cells were reported to have low CD25 expression and produced inflammatory T effector (Teff) cytokines prior to loss of Foxp3 expression and conversion to exTreg cells (Komatsu *et al.* 2014).

Foxp3^{+} Treg cells are essential for mediating fetal maternal tolerance at implantation in allogeneic pregnancy, as evidenced by pregnancy loss after Treg depletion in a range of mouse models (Aluvihare *et al.* 2004, Shima *et al.* 2010, Teles *et al.* 2013). Induction of Treg cells through *Foxp3* expression, and maintenance of their phenotype and suppressive function, are important determinants of pregnancy outcome (Rowe *et al.* 2012, Xin *et al.* 2014, Prins *et al.* 2015). It is clear an appropriate Treg:Teffector (Teff) balance is essential for pregnancy success from mouse studies of Teff-mediated pregnancy loss (Rowe *et al.* 2012, Xin *et al.* 2014), and human studies that show the Treg/Teff balance is skewed in favour of inflammatory Teff cells in pathologies of pregnancy including preeclampsia, preterm birth and unexplained infertility (Kwak-Kim *et al.* 2003, Prins *et al.* 2009, Rowe *et al.* 2011, Schober *et al.* 2012, Wu *et al.* 2014). This suggests that maintenance of the Treg cell lineage is essential to mediate fetal maternal tolerance and limit Teff responses. Analysis of Treg cells during normal and perturbed mouse pregnancy suggests that robust Foxp3 expression is important for pregnancy success (Xin *et al.* 2014, Prins *et al.* 2015), implying that Treg cell lineage stability is likely to be critical. For example, a recent study from our laboratory showed tTreg cells proliferate in early pregnancy and express high CTLA4, a Treg cell marker of suppressive competence, enabling Treg cell downregulation of APC costimulatory molecules to constrain anti-fetal T cell responses. Treg cell stability in pregnancy has not been definitively investigated. We hypothesise that Treg stability must be induced and maintained for robust fetal maternal tolerance, and that disrupted tolerance might result from instability in Treg cells.

There are two key time points in normal gestation where Treg cells would likely be exposed to environmental signals known to cause Treg cell transdifferentation – implantation and parturition. These specific events are associated with inflammation and proliferation of paternal/fetal antigen-responsive T cells and are heavily influenced by the maternal hormonal and cytokine environment (Johansson *et al.* 2004, Rowe *et al.* 2012, Romero *et al.* 2014). Importantly, the switch towards T cell activation is thought to contribute to the inflammatory process driving both term and preterm labour. T cells are hypothesised to contribute to parturition and labour (Koucky *et al.* 2014, Romero *et al.* 2014), but whether Treg stability is associated with susceptibility to preterm labour, is unknown.

The studies in this thesis show the potential for P4 to be a key regulator of establishing T cell plasticity in vivo during early pregnancy. In Chapter 3, we showed T cells exhibited phenotypic plasticity through expression of inflammatory IFNγ and IL17A during normal pregnancy. IFNγ expression in T cells and Treg cells increased upon early pregnancy P4 signalling disruption with high doses of RU486. Furthermore, in Chapter 5, P4 was found to repress IFNγ expression in Teff and Treg cells in vitro. The significance of these findings for overall T cell lineage stability during pregnancy is currently unknown. We thus predict

that P4 is a key regulator of Treg cell stability, and that reduced luteal phase P4 will increase susceptibility to Treg cell instability and phenotype switching in inflammatory gestational environments.

In this chapter, we aimed to evaluate Treg cell stability in pregnancy. We hypothesised that in healthy pregnancy Treg cells would be stable throughout gestation until late pregnancy, when some Treg cells may show instability in line with the T cell activation that occurs at parturition. We also hypothesise that environmental factors controlling the local and systemic immune response in pregnancy drive Treg cell stability in healthy pregnancy but that this process is disrupted in pregnancy pathologies, leading to Treg cell plasticity and instability.

The best way to definitively measure Treg cell stability is to use transgenic models allowing the fate of Treg cells to be tracked in vivo. Various Treg fate-mapper models have been used to study Treg cell stability in other contexts such as autoimmunity and inflammation (Zhou *et al.* 2009, Miyao *et al.* 2012). A similar model was used in this study. In this Chapter, the Treg cell fate-mapper model is characterised and used for preliminary experiments to investigate Treg cell stability in early and late gestation. In addition, a 16-colour flow cytometry panel was developed to enable the phenotypic characterisation of current and exTreg cells in pregnancy.

6.2. FOXP3GFPCRE TREG CELL REPORTER MICE

We used a well characterised Treg cell reporter mouse strain, Foxp3GFPCre, which reports endogenous *Foxp3* expression and also allows conditional modifications in the Treg cell lineage when crossed to the appropriate strain (Zhou *et al.* 2008). Before crossing the strain to Rosa26RFP mice, we initially confirmed that GFP and Foxp3 were co-expressed in the Foxp3GFPCre strain as expected, using flow cytometry (Figure 6.1). In this strain, mice have a bacterial artificial chromosome (BAC) transgene encoding GFP and humanised Cre recombinase (Cre) under the control of the Foxp3 promoter. Cre expression is driven by the *Foxp3* promoter, thus enabling GFP to report $Foxp3^+$ Treg cells. Flow cytometry staining of $CD4^+$ T cells in Foxp3GFPCre LNs demonstrates positive GFP staining in lymph nodes in GFP^+ mice but not in GFP^- mice (Figure 6.1A). Notably, the same percentages of Treg cells were obtained when analysing GFP expression or Foxp3 expression with a commercial antibody (Figure 6.1A). Additionally, GFP expression was closely aligned with Foxp3 expression, and associated with expression of key Treg cell markers CD25 and Nrp1, as expected (Figure 6.2B). These results are consistent with outcomes of the published study from the laboratory that produced these mice (Zhou *et al.* 2008).

6.3. TREG CELL FATE-MAPPER MICE TO TRACK TREG STABILITY IN VIVO

We next crossed Foxp3GFPCre mice with Rosa26RFP mice to create Foxp3GFPCre.R26RFP Treg fate-mapper mice. Figure 6.2A outlines the transgenic modifications of the two strains and the resulting modifications upon crossing them. Rosa26RFP mice possess a loxP-flanked STOP cassette downstream of the constitutive promoter Rosa26, which is expressed in all cells. As Foxp3GFPCre mice express GFP which is fused to a Cre recombinase, the Cre will be actively transcribed only in Foxp3$^+$ (GFP$^+$) cells. Upon crossing these two strains, Cre will cause the recombination of the 2 loxP sites surrounding the STOP codon which precedes RFP transcription. This recombination results in the excision of the STOP codon and recombination of the ends of the loxP sites, allowing RFP to be expressed. As Rosa26 is constitutively expressed, RFP expression is turned on permanently in Cre-expressing cells. Thus, GFP identifies cells currently expressing Foxp3$^+$, while RFP identifies cells that either currently or previously expressed Foxp3. Therefore, current Treg cells are identified as GFP$^+$RFP$^+$ and exTreg cells that have become unstable and lost Foxp3 expression are identified as GFP$^-$RFP$^+$ (Figure 6.2A).

We first characterised the Foxp3GFPCre.R26RFP mouse model at “steady-state”. LNs and spleen were harvested from non-pregnant 8-14-week-old mice and analysed by flow cytometry (Figure 6.2B). Upon gating on CD4$^+$ T cells, GFP and RFP expression was examined to detect Treg and exTreg cells (Figure 6.2B). We observed RFP expression to be induced in GFP$^+$ mice in the spleen and LNs. No RFP expression was detected in GFP$^-$ mice, confirming that RFP expression was limited to recombined cells in which Cre has been expressed (Figure 6.2B). As expected, the majority of RFP expression was found in GFP$^+$ Treg cells, marking current Treg cells as GFP$^+$RFP$^+$. Interestingly, a small percentage (~5%) of CD4$^+$ T cells in spleen and udLN were RFP$^+$GFP$^-$ indicating they had previously expressed Foxp3, but had since lost Foxp3 expression and were thus considered exTreg cells (GFP$^-$RFP$^+$) (Figure 6.2B). These GFP$^-$RFP$^+$ exTreg cells were observed in similar proportions to previous reports (Zhou *et al.* 2009, Miyao *et al.* 2012). They presumably represent a population of exTregs that have converted from unstable Treg cells under normal conditions, or alternatively, a population of naïve CD4$^+$ T cells that previously expressed Foxp3 at a low level, without having fully committed to the Treg cell transcriptional program of differentiation (Miyao *et al.* 2012). Similar results regarding GFP and RFP expression were obtained in peripheral LNs (data not shown).

6.4. EVALUATION OF TREG CELL STABILITY IN PREGNANCY

Once the Treg fate-mapper model was validated to detect Treg cell instability by measuring Treg and exTreg cells, we applied the model to evaluate Treg cell stability in pregnancy, initially in normal uncomplicated pregnancy. The following experiments use the Treg cell fate-mapper model to evaluate Treg cell stability in early and late gestation allogeneic pregnancy. Due to breeding difficulties with the colony (outlined in 2.1.2) and time constraints, we were unable to obtain sufficient experimental mice to conduct a robust analysis. The following experiments typically feature 4-5 mice per group and therefore the analysis is considered a preliminary proof-of-concept. Further experiments with additional mice will be necessary to allow definitive conclusions to be drawn.

6.4.1. Pregnancy outcomes in Foxp3GFPCre.R26RFP mice

Due to breeding problems in the Foxp3GFPCre.R26RFP colony as outlined in Chapter 2 (2.1.2), we first aimed to characterise pregnancy outcomes in experimental Treg fate-mapper mice to verify they were suitable for use in our pregnancy experiments. Foxp3GFPCre.R26RFP mice were allogenically mated to BALB/c males and pregnancy rate and outcomes were measured in late-gestation (day 16.5 and 18.5 pc). Figure 6.3A shows the number of mated mice that were pregnant or non-pregnant on day 16.5-18.5. This data correlated to a 71.4% plug to pregnancy rate, which is considered normal and consistent with the results presented in this thesis for pregnancy rate in standard B6 mice (Figure 6.2A). Furthermore, Foxp3Cre.R26RFP mice exhibited a normal number of total, viable and resorbed implantations (Figure 6.2B), again consistent with other results presented in this thesis. Fetal and placental parameters were also assessed and compared between d16.5 and 18.5 pc for total fetuses. Significant fetal growth was observed between day 16.5 and 18.5 pc (Figure 6.2C), whereas placenta weight remained unchanged (Figure 6.2D). These observations are characteristic features of late gestation mouse pregnancy. As Foxp3CreGFP.R26RFP mice exhibited normal pregnancy rate and outcomes, they were considered an appropriate model to measure Treg cell stability during pregnancy.

6.4.2. Assessment of Treg cell stability in early and late normal pregnancy

We next evaluated Treg cell stability in normal pregnancy, at early and late time points using flow cytometry. A 14-colour flow cytometry panel was developed to assess Treg stability and measure markers of Treg, and Th1 and Th17 identity, and phenotype throughout pregnancy. As well as detection of standard T cell markers CD45, CD4 and CD8, and Treg lineage markers GFP and RFP, the panel enabled detection of inflammatory cytokines IFNγ and IL17A and lineage-defining transcription factors of Th1 and

Th17 cells, Tbet and Rorγt respectively. Further, the panel enabled characterisation of expression of the Treg signature markers CD25, Nrp1, CCR6 and CD103.

Foxp3GFPCre.R26RFP mice were allogenically mated to BALB/c males and were euthanised in early (day 4.5 pc) and late (day 16.5 and 18.5 pc) gestation for flow cytometric analysis of Treg cell stability through quantification of Treg and exTreg cells in immune compartments. A non-pregnant (NP) group was also included for comparison to the pregnant groups. Figure 6.4A shows a schematic of the experimental design. Following isolation of leukocytes, cells were stimulated ex vivo for 4 hours with phorbol 12-myristate 13-acetate (PMA) and ionomycin to allow cytokine production to be measured, before staining with antibodies against surface markers, and intracellular antigens for flow cytometry analysis. Figure 6.4B shows representative FACS plots from the udLN on the different gestational days to illustrate current and ex Treg cells. GFP and RFP expression was detected in the udLN (Figure 6.4B) and spleen (data not shown) at all timepoints, indicating Treg (GFP^+RFP^+) and exTreg cells (GFP^-RFP^+). We also assessed expression of GFP and RFP in the uterus but were unable to detect sufficient $CD45^+CD4^+GFP^+$ or RFP^+ cells to quantify for robust analysis (data not shown). Thus, results presented here are for the udLN and spleen.

Initially, immune parameters including $CD4^+$ T cells, Treg and exTreg cells were quantified for the udLN (Figure 6.5) and spleen (Figure 6.6). In the udLN, an increase in total cell count was observed in late gestation on day 16.5 pc and day 18.5 pc compared to NP controls (Figure 6.5A, $P<0.05$). In the $CD4^+$ T cell compartment, a similar trend was observed (Figure 6.5A), with an increase in $CD4^+$ T cells compared to NP on day 16.5 pc ($P<0.1$) and 18.5 pc ($P<0.05$). Furthermore, an increase in $CD4^+$ T cell number on day 18.5 compared to day 4.5 pc was observed ($P<0.05$). In the $CD8^+$ T cell compartment (Figure 6.5A), an increase in cell number within the $CD8^+$ T cell compartment was observed only on day 18.5 compared to NP ($P<0.05$) and day 4.5 pc ($P<0.01$).

Treg and exTreg cells were assessed on the basis of GFP and/or RFP expression. A 2-5 fold increase in GFP^+RFP^+ Treg cell number was observed on day 18.5 pc compared to NP, day 4.5 pc and day 16.5 pc controls (Figure 6.5B, $P<0.1$-0.01). Furthermore, a 10% increase in the Treg cell proportion of $CD4^+$ T cells was evident on day 18.5 pc, compared with NP, day 4.5 pc and day 16.5 pc (Figure 6.5B, $P<0.01$-0.001). Nrp1 expression was assessed on the Treg cell population to determine the proportion of Treg cells that were of thymic origin ($Nrp1^+$ tTreg cells) and peripheral origin ($Nrp1^-$ pTreg) (Weiss *et al.* 2012). pTreg cells consistently made up approximately 20-25% of the Treg cell pool at the time points measured in virgin mice and throughout gestation (Figure 6.5B). This indicates the increase in Treg cells on day 18.5 was likely represented by both pTreg and tTreg subsets.

Interestingly, the number and proportion of GFP$^-$RFP$^+$ exTreg cells also increased over the course of gestation, suggesting that to some extent, Treg cells exhibit instability and convert to exTreg during pregnancy (Figure 6.5C). Specifically, there was a trend towards an increased number of exTreg cells on day 16.5 pc (3-fold increase, P<0.1) and 18.5 pc (6-fold increase, P<0.01) compared with baseline NP controls. A significant increase in exTreg cell number was also observed on day 18.5 pc compared to day 4.5 pc (P<0.05). The proportion of exTreg cell amongst CD4$^+$ T cells was increased specifically on day 18.5 pc compared to NP, day 4.5 pc and day 16.5 pc (P<0.1-0.05). Finally, Nrp1 was measured to indicate the potential pTreg or tTreg origin of the exTreg cells (Figure 6.5C). Whereas the majority of Treg cells were represented by the Nrp1$^+$ tTreg subset, the majority (55-60%) of exTreg cells were Nrp1$^-$ over the course of gestation, indicating their pTreg cell origin. As some Nrp1$^+$ exTreg cells were observed, this may suggest that both pTreg as well as tTreg cells are susceptible to becoming exTreg cells in late gestation.

The results in the spleen mirror the udLN for most parameters, but changes in T cells over pregnancy are smaller in scale (Figure 6.6). Unlike the udLN, total cell number remained consistent in the spleen, with the exception of a trend towards an increase on day 4.5 compared to NP controls (Figure 6.6A, P<0.01). A trend towards an increase in CD4$^+$ T cells was observed on day 18.5 pc compared to other time points (Figure 6.6A, P<0.1), while no changes in the CD8$^+$ T cell compartment were observed in the spleen (Figure 6.6A).

Similar to in the udLN, in the spleen a 2.5-fold increase in GFP$^+$RFP$^+$ Treg cell number was observed on day 18.5 pc compared to NP, day 4.5 pc and day 16.5 pc controls (Figure 6.6B, P<0.05). The Treg cell proportion amongst CD4$^+$ T cells was increased 2-fold on day 18.5 pc compared to NP and day 4.5 pc time points (P<0.05). In the spleen, 25-30% of Treg cells were represented by pTreg cells at all time points measured (Figure 6.6B). When GFP$^-$RFP$^+$ exTreg cells in the spleen were assessed, a trend towards an increase in number on day 18.5 pc (Figure 6.6C, <0.1), and a 2-fold increase in proportion of CD4$^+$ T cells on day 18.5 pc (Figure 6.6C, <0.1-0.05) was observed. exTreg cells were represented by similar numbers of Nrp1$^-$ and Nrp1$^+$ cells (45-60% Nrp1$^-$ of total exTreg cells), suggesting that both pTreg and tTreg cells were susceptible to becoming exTreg cells in late gestation (Figure 6.6C).

Overall these results show that pregnancy induces generation of Foxp3$^-$ exTreg cells. exTreg cells were not detectable beyond baseline levels in early pregnancy, but were elevated in late gestation. An increase in both Foxp3$^+$ Treg cells and Foxp3$^-$ exTreg cells in late gestation was evident in the local uterine draining udLN and to a lesser extent, in the spleen. Interestingly, Treg and exTreg cells were often increased from day 16.5 to 18.5 pc. Additionally in late gestation in the udLN, overall T cell numbers were increased, and

this was mirrored by increases in all $CD4^+$ and $CD8^+$ subsets measured. These changes were significant compared to groups of NP mice, and day 4.5 pc pregnant mice, reflecting a specific expansion of T cell subsets from early gestation onwards. However, further experiments must be conducted to repeat the analysis and enable robust conclusions to be drawn. Additionally, future studies should build on these initial findings to assess Treg stability in various perturbations modelling complications of pregnancy.

6.4.3. Phenotyping exTreg and Treg cells throughout gestation

Next, we sought to characterise of phenotype of Treg, exTreg and nonTreg cells over the course of pregnancy. An in-depth quantitative analysis has not yet been conducted due to the preliminary nature of the data collected thus far, therefore the following is a qualitative description of the panel and markers measured. The flow cytometry panel was designed to measure Treg, exTreg and Teff cell phenotypic markers including Nrp1, CD25, CD103 and CCR6 (Figure 6.7A). In addition to CD25 and Nrp1 which have been used throughout the studies in this thesis, the chemokine receptor CCR6 (CD196), and the integrin CD103 were analysed. Treg and Th17 cells both express CCR6, and it is implicated in their transdifferentiation (Yamazaki *et al.* 2008, Mercer *et al.* 2014) and CD103 is upregulated on activated Treg cells retained at mucosal sites (Annacker *et al.* 2005, Suffia *et al.* 2005). CD103 has previously been shown to be expressed by udLN Treg cells in non-pregnant and pregnant (day 10.5 pc) mice (Chen *et al.* 2013) and may indicate Treg cells with uterus-homing capacity. Figure 6.7A shows example histograms depicting the expression of these markers in udLN Treg, exTreg and nonTreg cells in a pregnant dam on day 18.5 pc. Differences in the expression profiles of the T cell subsets is evident, for example, Treg cells are predominantly $CD25^+$ and nonTreg cells are $CD25^-$ whereas some exTreg cells expressed moderate levels of CD25 (Figure 6.7A).

The antibody panel also allows assessment of inflammatory cytokines and transcription factors associated with Th1 and Th17 cells (Figure 6.7B). udLN exTreg cells from the pregnant dam on day 18.5 pc subset produced inflammatory cytokines IFNγ and IL17, and expressed Rorγt and Tbet, indicating their potential effector function (Figure 6.7B). Interestingly, a proportion of Treg cells also expressed Tbet and Rorγt (Figure 6.7B).

In future, a full analysis comparing the Treg, exTreg and nonTreg subsets at each time point in pregnancy and formally quantifying the changes in each subset over the course of gestation will be conducted, and is expected to reveal novel phenotypic information pertaining to Treg cell stability and phenotypic plasticity in pregnancy. In particular, data on the quantity and phenotype of Treg and exTreg cells at the end of gestation may inform of their potential role in protecting from inflammatory challenge and influencing the timing of labour and birth.

6.5. DISCUSSION

In this study, a mouse model was developed to assess Treg cell stability in pregnancy through measuring current Treg cells and previous Treg cells (exTreg cells) that arise from Treg lineage instability and transdifferentiation. Further, a multi-colour flow cytometry panel was developed to quantify not only exTreg and Treg cells, but to assess their phenotype through measuring Treg and Teff cell markers, inflammatory cytokines and lineage-associated transcription factors. The experiments in this study, while preliminary, indicate that some Treg cells exhibit instability that increases in late gestation pregnancy, with highest number and proportion on day 18.5 pc. Interestingly, Treg cells also increased throughout gestation, peaking on day 18.5 pc. However, more data is needed to add confidence to these conclusions, and the details and significance of these findings must be addressed. Further analysis on phenotypes of exTreg and Tregs will allow for thorough characterisation of these subsets and determine whether their phenotypes change over the course of the pregnancy, and will aim to determine if Tregs and exTregs adopt an inflammatory Th1 or Th17 effector phenotype in late pregnancy. The significance of Treg cell instability in pregnancy in the context of the wider literature is discussed below.

6.5.1. On Treg instability and exTreg cells in pregnancy

Upon establishment and commitment to the Treg transcriptional programs, *bone fide* Treg cells have been shown to be a stable lineage (Rubtsov *et al.* 2010). However, Treg cells undeniably exhibit plasticity and instability in various in vitro and in vivo inflammatory or lymphopenic settings. Initially, the inflammatory cytokine IL6 was shown to act in conjunction with IL1 to downregulate Foxp3 expression, dependent on STAT3 signalling (Yang *et al.* 2008). Further studies using lineage tracing methods identified some Tregs that lost Foxp3 expression and subsequently gained effector function (Zhou *et al.* 2009, Miyao *et al.* 2012, Komatsu *et al.* 2014, Hua *et al.* 2018). These so-called exTreg cells were shown to drive disease pathology in mouse models of autoimmune diseases such as inflammatory arthritis (Komatsu *et al.* 2014). This implicated Treg cell instability during inflammation as a potential contributor to the cause of inflammatory pathologies. Whether Treg cells exhibit instability in normal pregnancy, and importantly in inflammatory pregnancy complications, such are preeclampsia and preterm birth, is currently unknown. Of relevance to pregnancy, Treg cell stability was recently evaluated in the context of allotolerance, using a model of corneal transplantation. Strikingly, Treg cell instability and conversion to exTreg cells was associated with loss of allotolerance (Hua *et al.* 2018).

Our study used a well-characterised Treg cell lineage-tracer mouse model to track the fate of Treg cells during pregnancy, combining a fate reporter with a Foxp3Cre reporter strain. The FoxpGFPCre strain used to make the fate model here was developed by the Bluestone laboratory (Zhou *et al.* 2008) and used to investigate Treg cell instability (Zhou *et al.* 2009). Similar to our findings, Zhou *et al*., found a small proportion of Treg cells lost Foxp3 expression in steady-state conditions in the thymus and peripheral tissues. These exTreg cells were found to have an activated-memory phenotype and produce inflammatory cytokines, and were over represented in inflamed tissues during autoimmune diseases (Zhou *et al.* 2009). Here, we found that exTreg cells increased in late pregnancy. Whether these exTreg cells derived from bone fide Treg cells or from recently activated $CD4^+$ T cells undergoing lineage decisions remains to be investigated. Notably, the late pregnancy immune environment is associated with inflammation and immune activation, with induction of the inflammatory mediators IL1, IL6 and IL8 (Romero *et al.* 2014). As these pro-inflammatory cytokines bring about conditions that favour T cell plasticity, this may explain the presence of exTreg cells at this time point in gestation. While Treg instability has been shown in both $Nrp1^-$ pTreg and $Nrp1^+$ tTreg cells (Zhou *et al.* 2009), the pTreg cell subset is thought to be more susceptible to losing Foxp3 expression as their peripheral induction leads to weaker lineage commitment with only partial demethylation of the Foxp3 locus, consequently leaving these pTreg cells sensitive to the environmental cytokine milieu (Ohkura *et al.* 2012, Sawant and Vignali 2014, Hua *et al.* 2018). Therefore, the pTreg cells subset may be more amendable to transcriptional changes and plasticity upon the correct signals. In line with this, in our study most (~60%) exTreg cells were pTreg-derived. However, a large proportion (~40%) were $Nrp1^+$ indicating their thymic origin. As thymic Treg cells represent usually a more committed, suppressive Treg cell population, this could indicate instability of previously fully-committed Treg cells during pregnancy.

Additionally, newly generated $Foxp3^+$ Treg cells which have not fully committed to the Treg cell program are more susceptible to switching if given the correct inflammatory stimuli, which we may also be observing in the current study. Miyao et al. (2012) found that some $CD4^+Foxp3^-$ cells transiently express Foxp3 upon TCR stimulation. This minor subset was not immunosuppressive and could subsequently lose Foxp3 expression. Thus, these exTreg cells were attributed it to a minor uncommitted population of $CD4^+$ T cells that briefly express Foxp3 without committing to the lineage (Miyao *et al.* 2012). In line with the Miyao study and the concept that true bone fide Treg cells are inherently stable (Rubtsov *et al.* 2010), our results may support the interpretation that during pregnancy, when a switch to inflammation occurs (such as in late gestation), newly fetal antigen-experienced T cells that would otherwise commit to the Treg cell lineage are driven down an alternate differentiation pathway. However, regardless of whether the observed exTregs resulted from committed $Foxp3^+$ Treg cells, or from newly activated $CD4^+$ T cells

that temporarily upregulated Foxp3 without fully committing to the Treg cell lineage, the fact they converted to Foxp3$^-$ cells reveals a loss of potential Treg cell commitment in these cells in late pregnancy. Even temporary upregulation of Foxp3 is driven by a stimulus, and over the course of pregnancy there is continual Treg cell differentiation in response to key Treg-inducing signals (Rowe *et al.* 2012, Robertson *et al.* 2018). In addition, the large degree of anergy amongst the general CD4$^+$ T cell pool in pregnancy promotes Treg cell generation and prevents Teff activation (Rowe *et al.* 2012, Kalekar *et al.* 2016). Despite this, our results suggest that by the end of gestation, a distinct Foxp3- population is present, highlighting the plasticity and sensitivity of this system and raising the question of the functional role of these cells. Furthermore, these exFoxp3-expressing cells are likely capable of producing inflammatory cytokines and adopting a Teff cell fate, particularly given the right stimuli and therefore the fate rather than the origin of these cells are of pressing interest as they may influence pregnancy outcomes. Expanding knowledge on the origin and fate of exFoxp3-expressing cells in pregnancy may change the traditional simplistic 'Treg vs Teff' view of pregnancy. It is likely that T cells implicated in pregnancy are poised to respond to changing environmental signals such as inflammatory stimuli, and this may be one mechanism of immune-mediated quality control of pregnancy (Robertson 2010).

Whether our findings show committed Treg cell transdifferentiation or instability of newly generated Treg precursors remains to be determined, but addressing instability only in committed Treg cells remains a challenge with available models. Rubtsov et al used a tamoxifen-inducible cre model to show stability in bone fide Treg cells in physiological conditions (Rubtsov *et al.* 2010). While interesting, this model poses some challenges for application in pregnancy studies. Firstly, tamoxifen is a selective estrogen receptor modulator and causes abortions when administered during pregnancy (Ved *et al.* 2019). Secondly, as cre recombinase is induced upon tamoxifen administration, the recombination is transient and thus the fate of Treg cells only at specific time points can be tracked. Finally, it has become clear that not only Foxp3 expression defines the Treg cell lineage, and establishment of a Treg-specific methylation program is equally important for Treg lineage commitment (Ohkura *et al.* 2012). Thus, our future studies addressing Treg cell stability using Foxp3 fate models will also investigate the methylation profile of exTreg, Treg and nonTreg cell, using cell sorting and methylation analysis.

ExTreg cells are phenotypically distinct from both Treg cells and T conventional cells and are capable of producing inflammatory effector cytokines such as IL17 (Komatsu *et al.* 2014). Continuing work in this study will enable determination of whether the exTregs identified in pregnancy align more closely to Treg or Teff phenotypes. Our preliminary data likely indicates that in general, subsets of exTreg cells in pregnancy produce inflammatory cytokines and express transcription factors associated with Th17 or Th1

cells. Following phenotypic characterisation of the exTreg cells, the next logical step would be to test their pro-inflammatory potential in vitro and in pregnancy.

6.5.2. On the physiological significance of Treg cell stability in pregnancy

Treg cells are essential for pregnancy tolerance through their anti-inflammatory actions, which suppress effector and inflammatory immune responses that are detrimental to fetal and placental development (Shima *et al.* 2010, Rowe *et al.* 2012, Xin *et al.* 2014). The balance between inflammatory and anti-inflammatory immune responses that are important for maintaining Treg cell phenotypic strength and stability appear to also be important for controlling pregnancy success. For instance, committed Th1 differentiation blocks Foxp3 induction from fetal antigen-specific maternal $CD4^+$ T cells and triggers antigen specific fetal loss (Xin *et al.* 2014). In women, increased Th1 cytokine responses are reported in recurrent pregnancy loss and implantation failure (Kwak-Kim *et al.* 2003). Treg cell stability is therefore likely essential for overall pregnancy success to provide protection from inflammatory challenge and limit inflammatory responses. If this is the case, then Treg cell instability may be associated with inflammatory maternal immune environments that lead to pregnancy complications such as placentation disorders (Redman and Sargent 2010, Brosens *et al.* 2011).

T cell stability is regulated by complex environmental stimuli that control cytokine networks. In the context of pregnancy, the main immune environmental controllers are maternal hormones, seminal fluid, and paternal and fetal antigens. Seminal fluid (SF) is particularly important in the inductive phase of the immune response to pregnancy, as the female reproductive tract is first exposed to seminal fluid at coitus. SF contains abundant cytokines such as TGFB, signalling molecules, and paternal alloantigen that go on to initiate the female immune response (Robertson *et al.* 2002). The process of leukocyte recruitment and activation that follows ultimately leads to the generation of Treg cells in the uterine tissues and LNs. The importance of SF for maternal Treg cell generation has been described (Guerin *et al.* 2011, Robertson *et al.* 2013). Acting in concert with SF are maternal hormones, notably P4. The previous chapters in this Thesis have outlined key roles for P4 in regulating T cell abundance and phenotype in pregnancy. We showed that P4 was able to decrease IFNγ expression in Treg and Teff cells, suggesting that P4 likely regulates $CD4^+$ T cell stability. Luteal phase P4 that determines uterine receptivity appears to also play a key role in determining the capacity for Treg cell generation in early pregnancy through creating an anti-inflammatory cytokine environment. Overall, the quality of the SF and P4 responses from the outset of pregnancy are crucial for establishing a regulatory program of Treg cell induction. Therefore if poor quality P4 or SF is present, this may result in a suboptimal environment for Treg cell generation and instead favour Treg plasticity and Teff cell generation. An example of this is environmental control over plasticity

between Treg and Th17 cells. Treg and Th17 cell differentiation programs exhibit molecular antagonism, allowing for plasticity of these two T cell subsets (Yang *et al.* 2008). Importantly, TGFβ is required for both Treg and Th17 differentiation, but Foxp3 inhibits Th17 generation through antagonising RORγt and ROR, whereas IL6 overrides the effect of Foxp3 and in conjunction with IL1, induces genetic reprogramming of Treg cells (Yang *et al.* 2008, Zhou *et al.* 2008). Whether Treg or Th17 cells are induced in early pregnancy may depend on the specific cytokine environment, which is influenced by a multitude of factors including seminal fluid and maternal hormones. In the current study, our preliminary results indicate that in healthy early pregnancy, Treg cells do not exhibit instability. This result is not unexpected, as in a recent study from our laboratory, we found that exposure to seminal fluid at mating caused epigenetic changes specifically to hypomethylate the CNS1 region of the foxp3 locus in tTreg cells to reinforce the Treg cell phenotype (Moldenhauer *et al.* 2019). As methylation and regulation of Foxp3 TSDRs is important for Treg cell functionality and maintenance of the Treg cell lineage (Ohkura *et al.* 2012, Yue *et al.* 2019), it would be expected that the events in early pregnancy drive the Treg cell lineage towards further commitment and thus greater stability. However, in the context of a perturbation in maternal tolerance in the period of embryo implantation, such as eliminating the seminal plasma component of seminal fluid (Guerin *et al.* 2011), Treg cells would not be induced to the same extent and thus may be susceptible to switching in the context of inflammatory signals. Further, the work from this thesis suggests that limiting P4 signalling prior to implantation also peturbs fetal maternal signalling and the Treg cell response. Thus, using an intervention of P4 depletion in early pregnancy may also destabilise the methylation profile of the Treg TSDR and lead to Treg cell instability. These specific questions are areas of future investigation using the current and related mouse models in this laboratory.

In addition to early pregnancy, Treg cell stability is also relevant in later gestation. T cells appear to be implicated in the timing of birth, where a switch to inflammation is evident as part of the normal events of parturition (Romero *et al.* 2014). As inflammation is now appreciated as a hallmark feature of many pregnancy complications including preeclampsia and preterm birth (Redman and Sargent 2010, Brosens *et al.* 2011), Treg cells may become unstable and susceptible to transdifferentiation into Teffs in pregnancy complications and at birth, in term and preterm pregnancies. Whether the inflammatory switch at the end of gestation is associated with Treg cell lineage instability has not directly been addressed. In humans and mice, the transcriptional regulator Helios is recognised as a marker of Treg cell stability (Kim *et al.* 2015), and Helios-deficient Treg cells develop an unstable phenotype associated with reduced Foxp3 expression, production of inflammatory cytokines and conversion to Teff phenotypes (Kim *et al.* 2015, Nakagawa *et al.* 2016). Interestingly, Helios expression in $CD4^{+}CD25^{hi}$ T cells was found to be reduced in term human pregnancy (Salvany-Celades *et al.* 2019) and well as in cases of miscarriage

(Inada *et al.* 2015). Helios downregulation may indicate less stable Treg cells amenable to exhibiting instability and plasticity. Therefore, these studies support the biological plausibility that Treg instability can occur at term pregnancy and in pregnancy pathologies. Given the potential for exTreg cells to drive disease pathogenesis, defining Treg cell stability at the end of gestation, and in pregnancy pathologies, will be important. Our preliminary results here indicate an increase in exTreg in late pregnancy. Interestingly, other T cell subsets were also increased in late pregnancy, indicating generalised proliferation. This included Treg cells, which were also at their highest numbers in late gestation, a finding reported previously for fetal antigen-specific Tregs (Rowe *et al.* 2012). Despite this, Treg cells appear unable to control the inflammatory load in late gestation, calling into question the mechanism of how the switch to the inflammatory cascade of parturition occurs, despite the expanded memory population of Treg cells in late gestation. The exTreg cells present may indicate systemic T cell proliferation and a failure to commit to the Treg program or alternatively a subpopulation of Treg cells converted to exTreg cells in late-gestation. Whether the exTreg cells contribute to the growing inflammatory load leading to labour through production of inflammatory mediators needs to be determined. Interestingly, preliminary data of both Treg and exTreg cells from a female on day 18.5 pc showed a considerable proportion to express inflammatory cytokines and transcription factors of Th17 and Th1 cells. Detailed phenotyping and the significance of the Treg and exTreg cell increases in late gestation will be determined through further analysis. Additionally, Treg cell fate in the context of preterm birth will be investigated with models of inflammation-induced preterm birth, such as the LPS model used in Chapter 4 of this Thesis.

In conclusion, the preliminary data from this study shows that Treg cells are stable in normal early pregnancy, but that in late pregnancy exTreg cells accumulate. However, repeating these experiments with an increased sample size are necessary to confirm these preliminary observations, and further analysis is required to gain more insight into kinetic changes across gestation and assess the migratory, effector and phenotypic capacity of exTreg cells at different stages of pregnancy.

The significance of exTreg cells in the timing of birth and success of the pregnancy remains a key unanswered question. The model developed in this study will enable future determination of whether P4 and SF composition are key regulators of Treg cell stability, and whether Treg cells exhibit instability in pregnancy pathologies such as inflammation-induced miscarriage and preterm birth. Ultimately, the significance of Treg cell instability and phenotypic plasticity for the outcome of normal and complicated pregnancy will be addressed in future work.

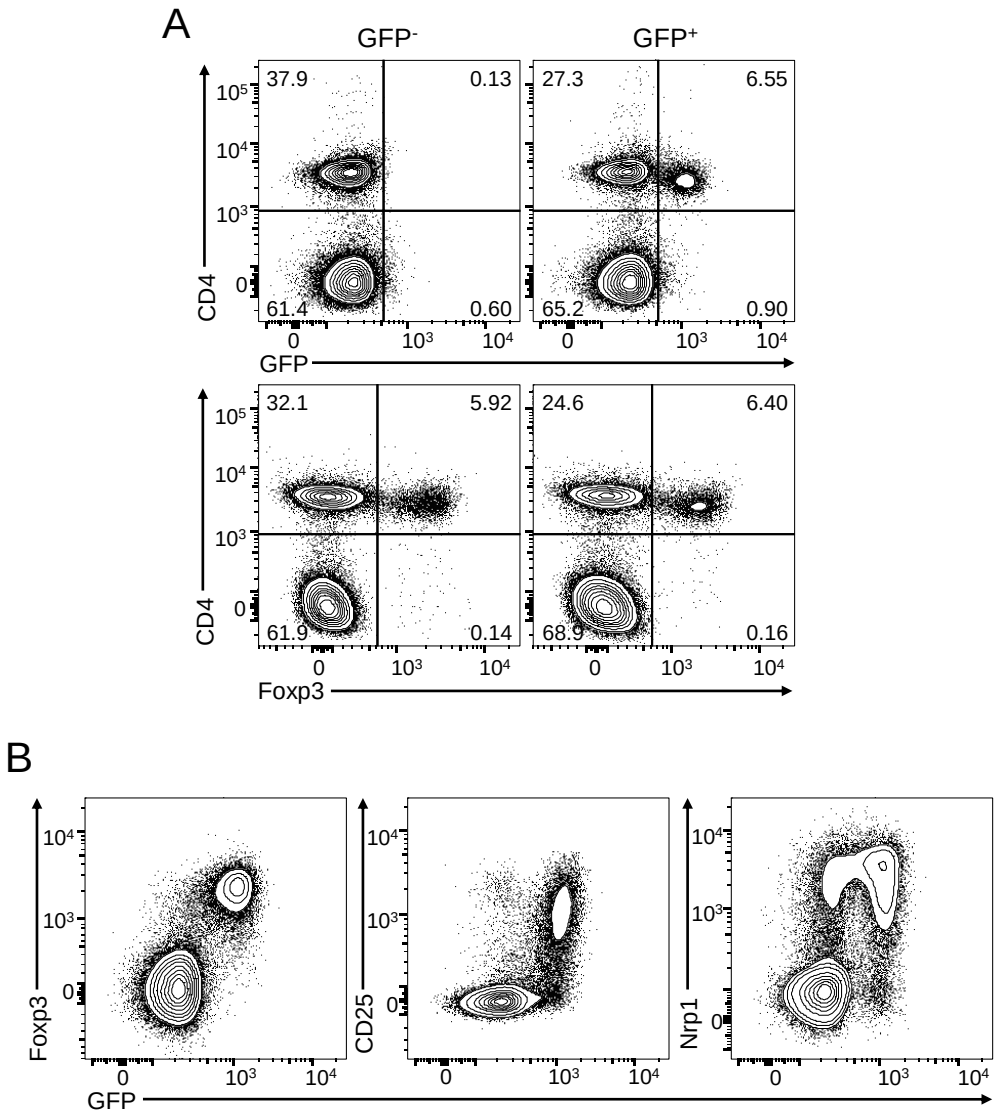

Figure 6.1 GFP identification of Foxp3$^+$ Treg cells in Foxp3GFPCre mice.

Foxp3GFPCre mice express green fluorescent protein (GFP) under the control of the *Foxp3* promoter allowing for the detection of Foxp3$^+$ Treg cells by GFP. (A) FACS plots showing GFP and Foxp3 expression amongst CD4$^+$ T cells in lymph nodes of GFP$^-$ and GFP$^+$ mice. (B) FACS plots of CD4$^+$ T cells in lymph nodes of GFP$^+$ mice showing GFP expression correlates with Foxp3 expression, and Treg cell markers CD25 and Nrp1.

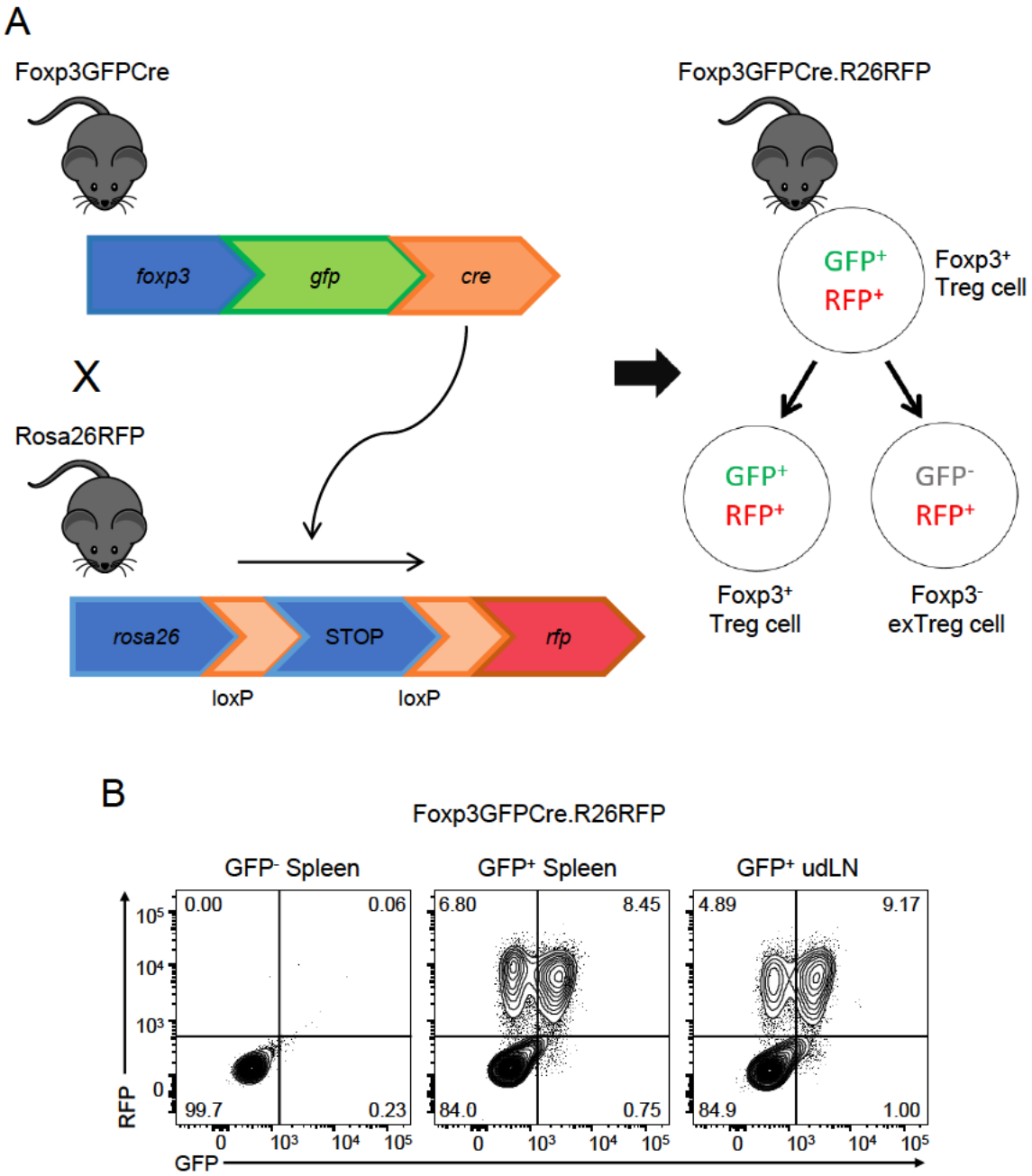

Figure 6.2 Treg cell fate-mapper model to evaluate Treg cell stability

Foxp3GFPCre mice were crossed to Rosa26RFP mice to create Foxp3GFPCre.R26RFP mice. (A) When these strains are crossed, Cre recombinase, driven by the *Foxp3* promoter, causes excision of the STOP codon in the Rosa26 locus, resulting in permanent RFP expression specifically in Foxp3-expressing cells. Current Treg cells are detected by GFP expression, which is fused to Cre and driven by the *Foxp3* promoter. Thus, this dual reporter strain can detect current Treg cells (GFP^+RFP^+) and exTreg cells (GFP^-RFP^+) that have divided and lost expression of Foxp3. Mice were bred for several generations to create Foxp3GFPCre.R26RFP mice that were homozygous for RFP. Experimental females were GFP^+ and. RFP/RFP. (B) Detection of exTreg cells in non-pregnant Foxp3GFPCre.R26RFP mice in udLN and spleen by flow cytometry. GFP^- mice were used to control for GFP and RFP transgene expression.

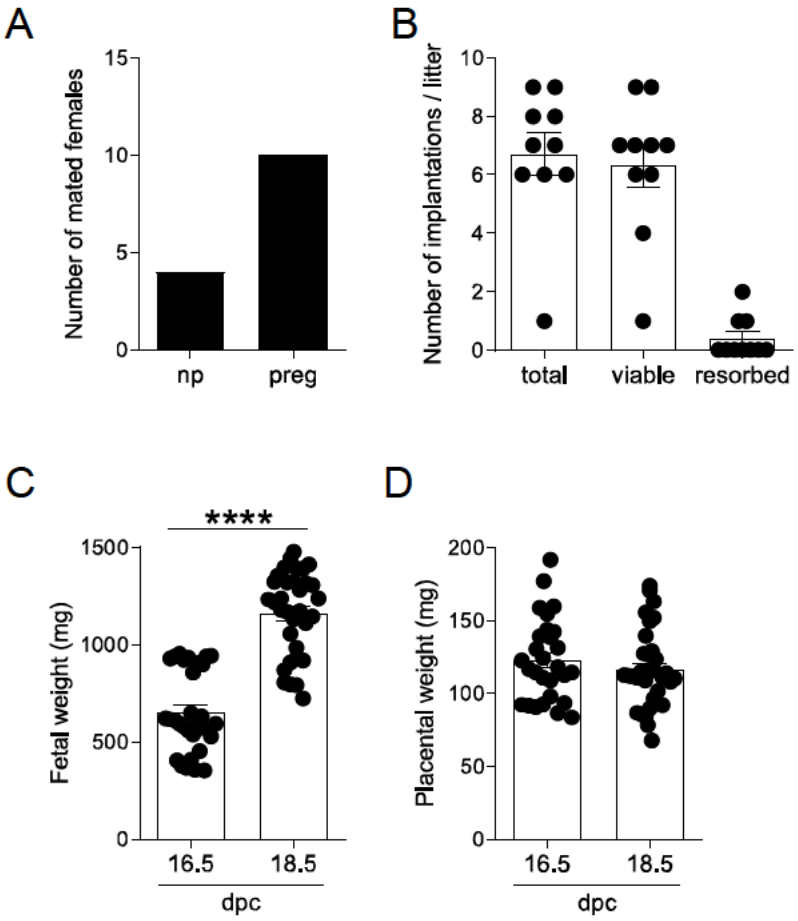

Figure 6.3 Assessment of pregnancy outcomes in Foxp3GFPCre.R26RFP mice

Female Foxp3GFPCre.R26RFP mice were allogenically mated to BALB/c males. On day 16.5 and 18.5 post-coitus (pc) mice were euthanised and pregnancy outcomes were measured. (A) pregnancy rate, expressed as number of mated females that were either pregnant (preg) or non-pregnant (np) on day 16.5-18.5 pc. (B) Number of implantations per litter, showing total implantations, viable implantations and resorptions on day 16.5-18.5pc. (C) Fetal and (D) placental weight for viable fetuses, comparing 16.5 to 18.5 days pc (dpc). (B) n= 10; (C, D) n=30-33. Data are presented as mean ± SEM with individual (B) mice, (C) fetuses and (D) placentas indicated by symbols; Unpaired T test; * $p<0.0001$.

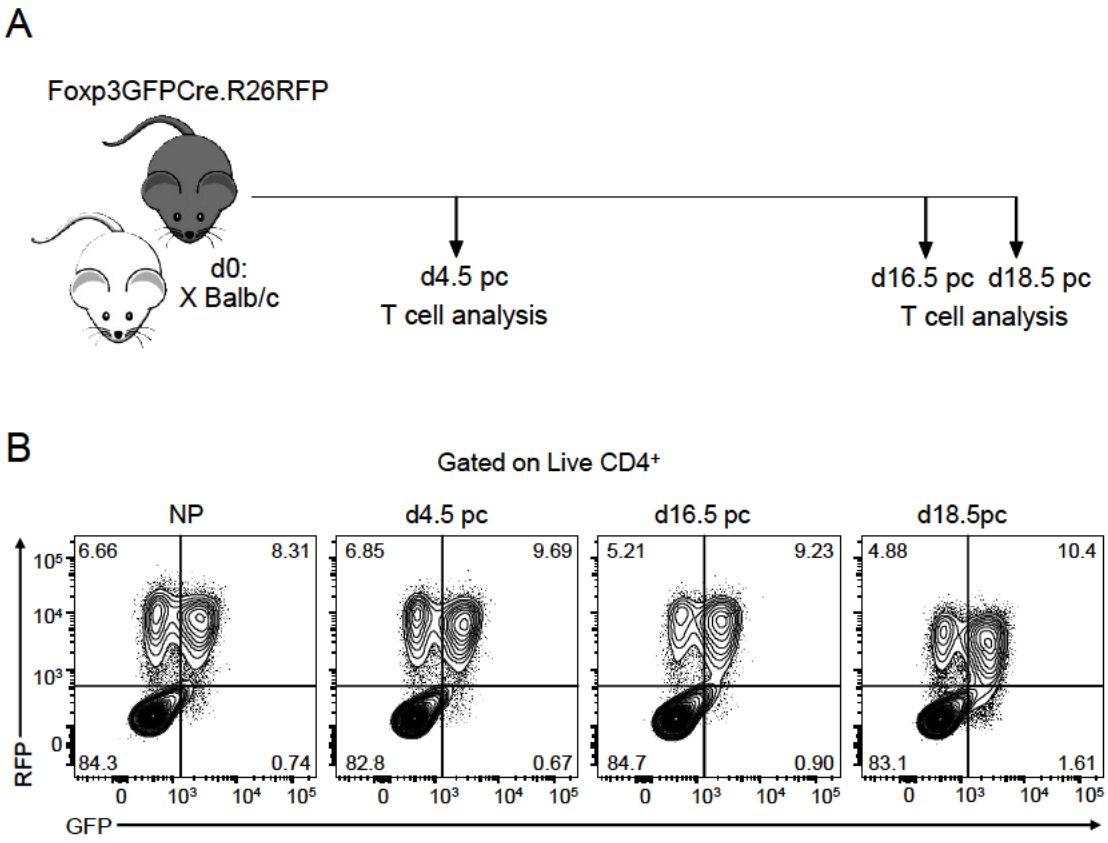

Figure 6.4 Assessment of Treg cell stability in early and late gestation in Foxp3GFPCre.R26RFP mice

Female Foxp3GFPCre.R26RFP mice were allogenically mated to BALB/c males. On day 4.5, 16.5 and 18.5 post-coitus (pc) mice were euthanised and flow cytometry was performed to assess Treg stability and quantify Tregs, exTregs and nonTregs in udLN and spleen. Non-pregnant (NP) mice were used as controls. (A) Schematic of experimental design. (B) Representative flow cytometry plots showing proportions of Treg cells (GFP^+RFP^+), exTreg cells (GFP^-RFP^+) and nonTreg cells (GFP^-RFP^-) amongst live $CD4^+$ T cells in the udLN across gestation and in non-pregnant Foxp3GFPCre.R26RFP mice.

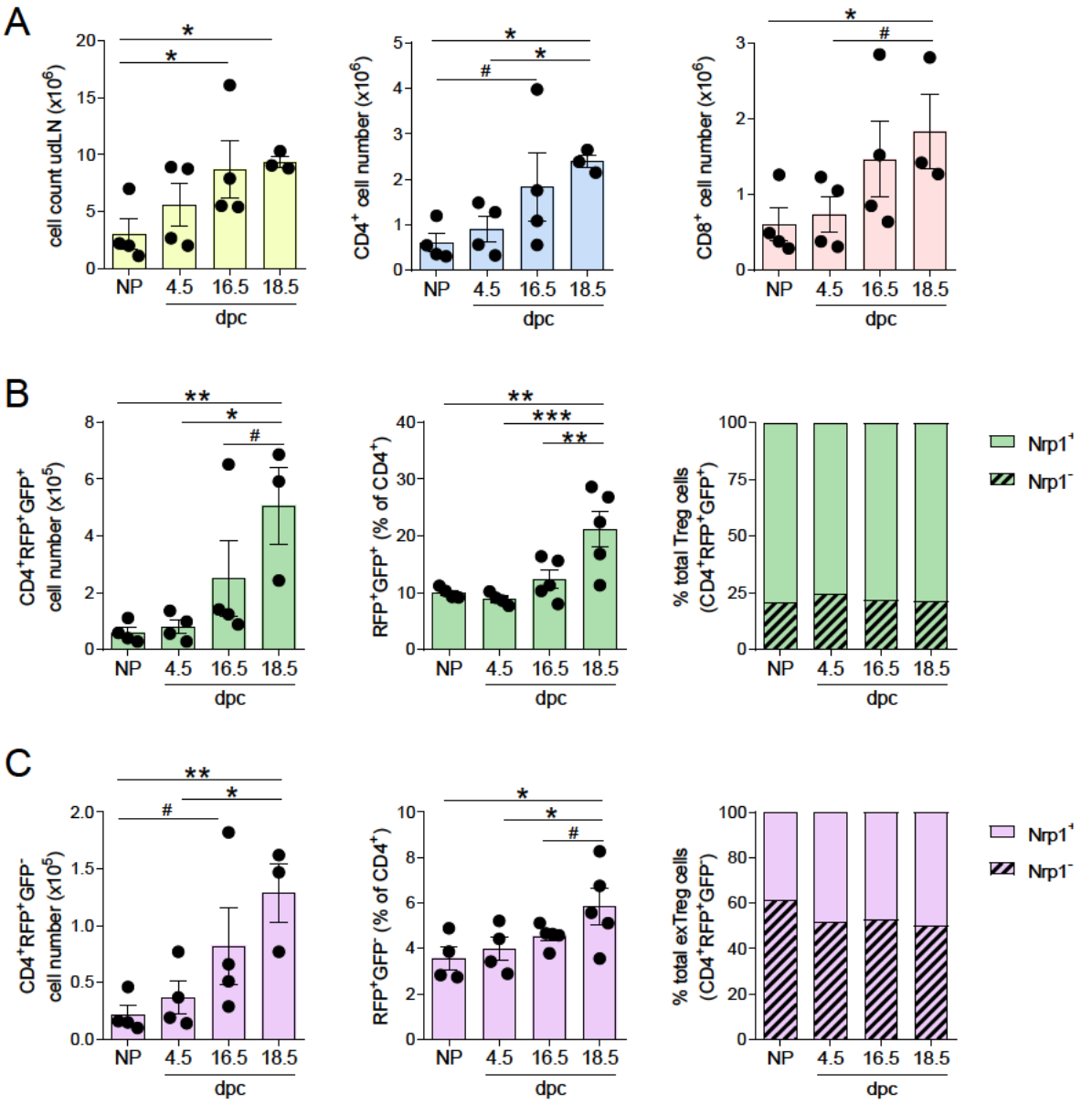

Figure 6.5 Treg and exTreg cell subsets increase in the uterus-draining lymph nodes (udLN) as pregnancy progresses in Foxp3GFPCre.R26RFP mice.

Female Foxp3GFPCre.R26RFP mice were allogenically mated to BALB/c males. On day 4.5, 16.5 and 18.5 post-coitus (pc) mice were euthanised and flow cytometry was performed to assess Treg stability and quantify Tregs, exTregs and nonTregs in the udLN. Non-pregnant (NP) mice were used as controls. (A) Number of total, $CD4^+$, and $CD8^+$ T cells in the udLN. (B, C) Quantification of Figure 6.4 for (B) Treg (GFP^+RFP^+), and (C) exTregs (GFP^-RFP^+). Proportions of $Nrp1^-$ and $Nrp1^+$ of total (B) Treg cells and (C) exTreg cells. n=3-5. Data are presented as mean ± SEM with individual mice indicated by symbols; one-way ANOVA; ** $p<0.01$, * $p<0.05$, # $p<0.1$.

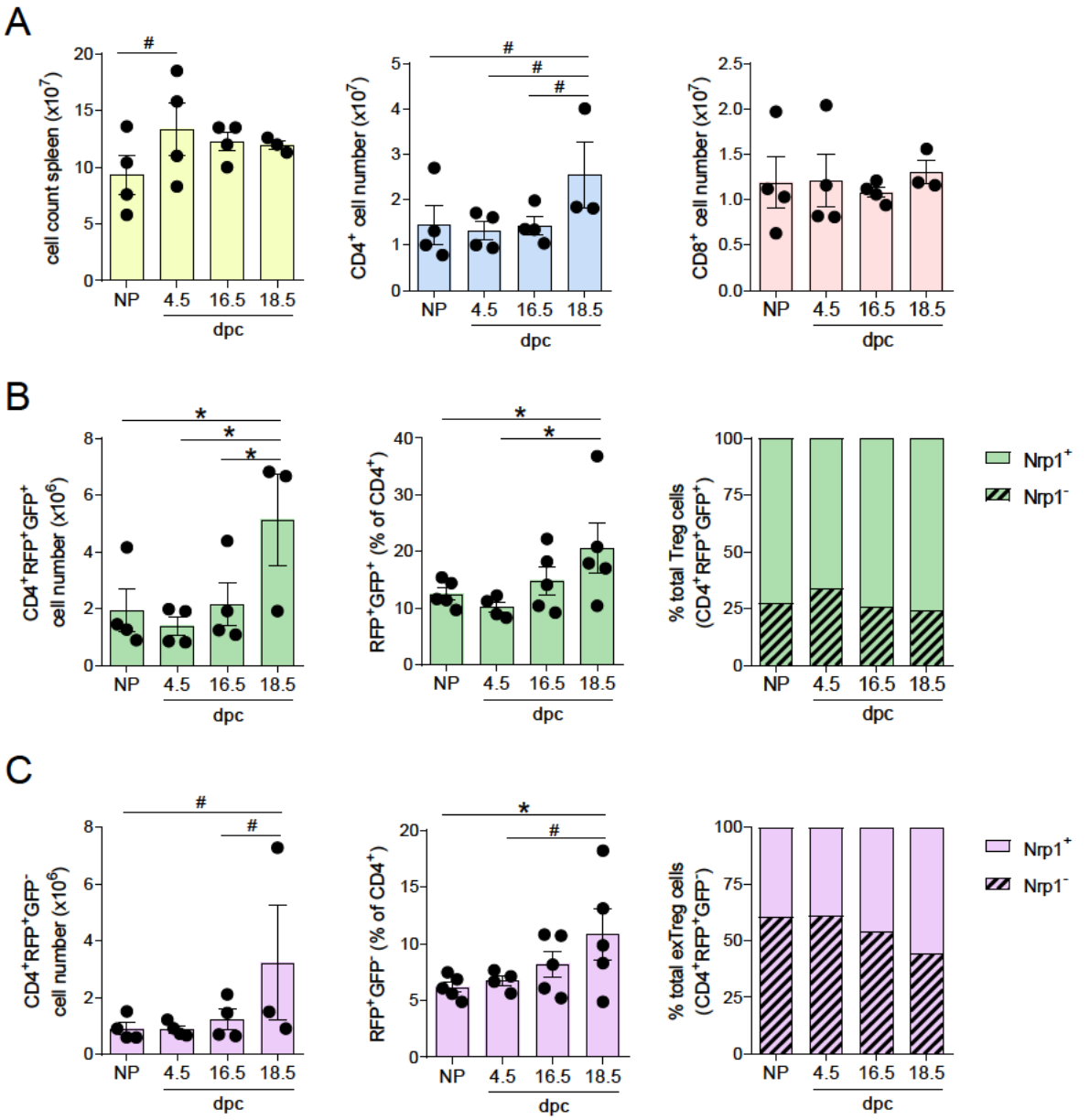

Figure 6.6 Treg and exTreg cell subsets increase in the spleen as pregnancy progresses in Foxp3GFPCre.R26RFP mice.

Female Foxp3GFPCre.R26RFP mice were allogenically mated to BALB/c males. On day 4.5, 16.5 and 18.5 post-coitus (pc) mice were euthanised and flow cytometry was performed to assess Treg stability and quantify Tregs, exTregs and nonTregs in the spleen. Non-pregnant (NP) mice were used as controls. (A) Number of total, $CD4^+$, and $CD8^+$ T cells in the spleen. (B, C) Quantification of Figure 6.4 for (B) Treg (GFP^+RFP^+), and (C) exTregs (GFP^-RFP^+). Proportions of $Nrp1^-$ and $Nrp1^+$ of total (B) Treg cells and (C) exTreg cells. n=3-5. Data are presented as mean ± SEM with individual mice indicated by symbols; one-way ANOVA; ** $p<0.01$, * $p<0.05$, $^{\#}$ $p<0.1$.

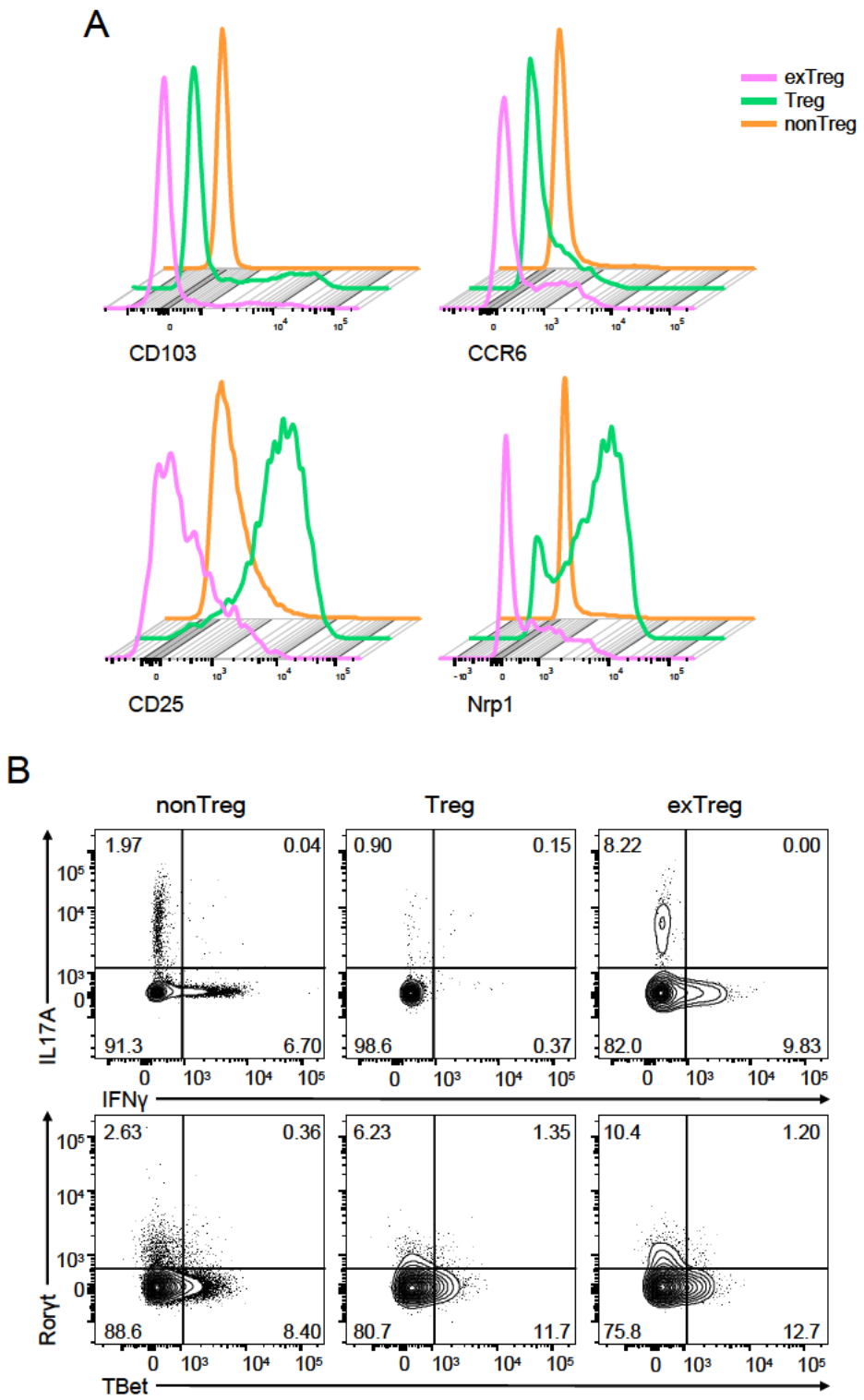

Figure 6.7 Phenotypic and effector characterisation of Treg, exTreg and nonTreg cells in pregnancy.

Female Foxp3GFPCre.R26RFP mice were allogenically mated to BALB/c males. On day 18.5 post coitus (pc) mice were euthanised and flow cytometry was performed to characterise Treg, nonTreg an exTreg phenotype in the udLN and spleen. In each T cell subset, expression of phenotypic markers, capacity to express inflammatory cytokines, and expression of T effector (Teff) lineage transcription factors was measured. (A) Representative overlaid FACS histograms showing expression of CD25, Nrp1, CD103 and CCR6 in Treg (green), exTreg (pink) and nonTreg (orange) cells in each subset. (B) Representative FACS plots showing staining of inflammatory cytokines IL17A and IFNγ, and Teff transcription factors Roryt and Tbet in each subset.

Chapter 7

General discussion and conclusions

7.1. INTRODUCTION

Treg cells are identified as essential mediators of fetal-maternal tolerance and pregnancy success, however the mechanisms through which Treg cells mediate their actions, and the factors that control the strength, quality and stability of the maternal Treg cell response to pregnancy are poorly defined. Progesterone (P4) is a candidate regulator of Treg cells, through largely unknown mechanisms. Furthermore, the relationship between P4 and Treg cells for pregnancy success is not well understood. The overall aims of this project were to; (1) investigate how early pregnancy progesterone exposure regulates Treg cells in vivo, and the significance of this for pregnancy success; (2) investigate mechanisms of progesterone action in $CD4^+$ T cells and Treg cells; and (3) investigate Treg cell stability during pregnancy.

Initial investigations found that reducing P4 bioavailability in the peri-implantation period caused immune perturbation in the udLN at the time of implantation, indicating a failure of normal $CD4^+$ T cell expansion pre-implantation. These immune perturbations persisted at least until mid-gestation, whereby reduced $CD4^+$ T cells and Treg cells and a change in their phenotype towards an inflammatory profile were evident. Assessment of gestational parameters revealed pregnancies initiated with reduced P4 bioavailability were suboptimal, with a reduction in ongoing pregnancy rate from mid-gestation, and amongst pregnant females in late-gestation, a reduced number of viable fetuses and lower fetal weight. In addition, pregnancies with reduced P4 bioavailability during the peri-implantation period failed to show the normal P4 decline in late gestation and had an extended gestation length compared to control females. Notably, transfer of Treg cells at the time of implantation significantly improved pregnancy rate, along with viable implantations and fetal weight in late gestation. Furthermore, Treg cell transfer may restore the timing of the expected late gestational P4 decline, comparable to that seen in control mice. Taken together, these data show that Treg cells partially account for the effects of P4 on pregnancy success. These findings suggest that adequate P4 bioavailability from the outset of pregnancy is a requirement for robust Treg cell induction, and reveal Treg cells as an effector mechanism for P4 action in early pregnancy. A limitation of this work is that Treg transfer experiments had a small sample size, and thus further repeat experiments will be required to confirm these findings.

Investigation of mechanisms of P4 action in $CD4^+$ T cells using an in vitro T cell polarisation assay demonstrated P4 as a regulator of Treg and T effector cell phenotype, though suppressing IFNγ expression in the $CD4^+$ subsets. This T cell intrinsic effect was found to be progesterone receptor-

independent, prompting further investigation of alternate pathways of P4 action in T cells. $CD4^+$ T cells were found to bind P4 at the membrane, and this binding capacity was increased in $Foxp3^+$ Treg cells in early pregnancy. From these findings we concluded that P4 has direct effects on T cell phenotypic stability, which are mediated in part through non-classical membrane signalling pathways that may play a role in Treg cell tolerance in early pregnancy. Taken together with the previous findings, we concluded that both direct and indirect effects (for example, through direct action on other immune cell subsets) of P4 on T cells are highly likely to occur in pregnancy, allowing P4 to orchestrate tolerogenic T cell responses for pregnancy success.

Finally, investigation of Treg cell stability in pregnancy was established with a mouse model enabling $Foxp3^+$ Treg cell fate to be measured in vivo. Preliminary findings show largely stable Treg cells in pregnancy, along with a population of Treg cells that express Foxp3 throughout pregnancy. This demonstrates the relevance of studying Treg cell stability in pregnancy and provides a basis for future studies to investigate the role of P4 in regulating Treg cell stability throughout gestation. However, these findings are considered preliminary due to small numbers of animals, and as such, further experiments are required to enable firm conclusions to be drawn from these experiments.

Overall, these studies identify P4 as a key environmental determinant of Treg cell abundance, phenotype and functional competence during pregnancy, and show that Treg cells are an effector mechanism of P4 action in early pregnancy enabling successful implantation and fetal development for overall gestational success. P4-Treg cell interactions are therefore highly like to be relevant in pregnancy complications such as preeclampsia, intrauterine growth restriction (IUGR) and preterm birth, in which immune and endocrine abnormalities are strongly associated.

7.2. P4-TREG CELL ACTIONS TO ORCHESTRATE PREGNANCY SUCCESS

These studies add to the work of others (Mao *et al.* 2010, Lee *et al.* 2011) in defining P4 as a determinant of Treg abundance and phenotype in pregnancy. Notably, Treg cells mediate some essential effects of P4 on pregnancy success, since transfer of Treg cells to females with reduced P4-signalling delivered a clear improvement in late gestational fetal viability and weight. This is the first study to demonstrate Treg cells are an effector mechanism of P4 action. Thus, appropriate P4 signalling at the outset of pregnancy is essential for Treg cell actions, and consequently pregnancy success. The mechanisms by which Treg cells mediate pregnancy success are still not clearly defined, but recent work defined a role for P4 in

promoting Treg cell number and *Foxp3* expression in naïve CD4$^+$ T cells in vitro (Lee *et al.* 2012, Hughes *et al.* 2013, Thiele *et al.* 2019). From these studies it is clear that P4-regulated Treg cells are essential for both adequate maternal immune tolerance, and normal fetal growth.

The process of implantation is instrumental to ensure the subsequent events of trophoblast invasion and placentation occur in an optimal manner to sustain pregnancy. Animal studies show that if this process is compromised or dysregulated, through poor maternal immune adaptation or endocrine disruption, then adverse outcomes for placentation and fetal development result (Norwitz 2006, Cornelius *et al.* 2016, Care *et al.* 2018). In women, compromised implantation manifests as pregnancy disorders such as preeclampsia, characterised by failed vascular adaptation to pregnancy (Brosens *et al.* 2011, Roberts and Redman 2017). Findings from this thesis build on previous understanding of early pregnancy immune adaptations as mediators of implantation success. In particular, Foxp3$^+$ Treg cells are increased in the udLN and uterus in response to seminal fluid exposure following coitus, in women and mice (Robertson *et al.* 2009, Guerin *et al.* 2011, Robertson *et al.* 2013). Trafficking and induction of Treg cells occurs in the presence of an appropriate cytokine and hormone environment, including abundant TGFβ contained in seminal fluid. Treg cells induced in early pregnancy are hypothesised to be important for constraining the post-coital inflammatory response, to ensure implantation success (Robertson *et al.* 2018). P4 is highly induced in the luteal phase of the menstrual cycle just after conception occurs, and has a known effect of constraining pro-inflammatory immune responses. As shown in these studies in mice, early pregnancy P4 regulation of Treg cells is important for implantation and overall pregnancy success. Given that P4 action during the time of embryo receptivity in the female coincides with local immune induction following mating and seminal fluid exposure, it is feasible that P4 action is necessary and complementary to the actions of seminal fluid, to drive the early events of pregnancy immune adaptation. These studies support a role for P4 in resolving the post-coital inflammatory response through the induction of Treg cells. In this thesis we demonstrated that the magnitude of the Treg response is determined by the quality of the P4 response. Thus peri-conceptional P4 is an essential determinant of implantation success through actions on Treg cells.

The mechanisms of Treg cell action for pregnancy success are largely undefined, but the studies in this thesis support several extant hypotheses for Treg action during pregnancy to ensure a tolerogenic immune environment and appropriate placental and fetal development. A local draining LN Treg cell response appears key for suppressing detrimental inflammatory anti-fetal immune responses (Darasse-Jèze *et al.* 2006, Rowe *et al.* 2011), and Treg cells interact with T cells, dendritic cells (Fallarino *et al.* 2003, Schumacher *et al.* 2012), uterine NK (uNK) cells (Ghiringhelli *et al.* 2005, Vento-Tormo *et al.* 2018) and macrophages (Salvany-Celades *et al.* 2019) to modulate the maternal immune environment in the

udLN and decidua. Treg cell-derived IL10 and TGFβ are powerful modulators of immune responses through constraining the phenotypes of these immune subsets (Shevach 2009). In the studies in this thesis, reduced P4 signalling led to a reduction in $CD4^+$ T cells and $Foxp3^+$ Treg cells at mid-gestation, originating from failed $CD4^+$ T cell expansion at implantation. In addition to T cell changes, total live cell numbers were reduced suggesting other subsets were also affected by the P4 signalling disruption. Transfer of Treg cells into mice with reduced P4 signalling notably restored both total cell counts and $CD4^+$ T cell numbers. It is therefore likely that P4-experienced Treg cells are required to regulate other immune subsets that contribute to pregnancy success.

Treg cells may be recruited from the uterus and draining LNs to the endometrium in early pregnancy to promote decidualisation and mediate events leading to placentation. Treg cells may facilitate the tissue remodelling that occurs during embryo implantation and trophoblast invasion, through interacting with remodelling macrophages (Vacca *et al.* 2010), uNK cells, DCs (Plaks *et al.* 2008) and trophoblast cells (Du *et al.* 2014). Recently, Treg cells were shown to be important in the vascular adaptations required to support pregnancy, for example rescuing hypertensive phenotype in preeclamptic rats (Cornelius *et al.* 2015, Care *et al.* 2018). The studies in this thesis demonstrate that P4-experienced Treg cells are needed at the time of implantation for normal placental, and fetal development to occur. Notably this is consistent with the wider literature demonstrating the requirement for robust Treg cell induction in early pregnancy for later pregnancy success (Aluvihare *et al.* 2004, Shima *et al.* 2010, Robertson *et al.* 2018), and contributes new information that adequate P4 signalling is a key driver of this Treg induction for optimal fetal development. In our studies, the effect of P4 disruption on uterine and decidual Treg cells could not be determined due to the relative paucity of T cells in the uterus, and technical limitations such as choice of enzymatic digestion techniques. Although the udLN are a good measure of the capacity for Treg cell recruitment to the fetal-maternal interface (Robertson *et al.* 2009, Chen *et al.* 2013, Engler *et al.* 2017), future studies must include phenotypic analysis of uterine, decidual and placental Treg cells to gain a full picture of Treg functionality in pregnancy.

The studies in this thesis are not only relevant to the role of Treg cells in implantation success and fetal development, but they also inform on the role of T cells in the timing of birth. $CD4^+$ T cells are known to increase over the course of gestation and may play a key role in the induction of labour. In humans and mice, labour is initiated by inflammatory signals known as pathogen-associated molecular patterns (PAMP)s and damage associated molecular patterns (DAMPs) arising from both the fetus and mother, which activate maternal toll-like receptors (TLRs) and drive an inflammatory signalling cascade at the fetal-maternal interface which ultimately culminates in cervical ripening and uterine contractions (Romero *et al.* 2014). This inflammatory pathway involves the recruitment and activation of key immune cells to the

fetal-maternal interface, including macrophages, monocytes, neutrophils and T cells. These cells produce signalling molecules such as NF-kB which drives expression of prostaglandins, oxytocin receptor and Cyclooxygenase 2, and pro-inflammatory cytokines such as IL1Bβ, IL6, IL8 and TNFα (Gomez-Lopez *et al.* 2014). This inflammatory cascade occurs in both normal and preterm labour, but importantly, preterm labour involves pathological activation of the normal physiological process, for example via bacteria-dervied DAMPs (Romero *et al.* 2014). Normal and preterm labour is characterised by increased inflammation and Teff cells at the fetal-maternal interface, and changes to Treg cell phenotype and function in mice (Gomez-Lopez *et al.* 2014, Koucky *et al.* 2014, Furcron *et al.* 2015) and women (Kisielewicz *et al.* 2010, Schober *et al.* 2012). Activated T cells and their effector cytokines are shown to cause preterm labour (Frascoli *et al.* 2018, Arenas-Hernandez *et al.* 2019). Sufficient Treg cells with suppressive function may be necessary at the end of gestation to constrain inflammation caused by Teffs and potentially other activated immune cells. Therefore, a decrease in Treg cells in late gestation may result in excessive inflammation and labour. In these studies, we found $CD4^+$ T cells and specifically Treg cells were increased at the end of normal gestation compared to in non-pregnant and early-pregnant mice. Interestingly, our results reveal that despite P4 levels declining in late gestation prior to parturition, Treg cell numbers and proportions in the udLN actually increase during this time. Therefore, while P4 is a key regulator of Treg cells in early and mid gestation, it is unlikely that P4 is a major driver of Treg cell generation prior to parturition, and other influencing factors must be at play. One explanation for the increase in Treg cells prior to parturition could be simply due to their migration from other sites in response to chemotactic signals.

In addition to Treg cells, small proportion of exFoxp3-expressing exTreg cells were present in late gestation in the udLN and spleen, suggesting some Treg instability occurs in normal pregnancy. Whether the Teffs implicated in the timing of birth convert from $Foxp3^+$ precursors, as well as whether Treg cell instability occurs in inflammation induced-preterm birth, are pressing questions requiring further investigation.

Assessment into the effect of P4 regulation of T cells and the timing of birth interestingly showed that reduced P4 signalling in the peri-implantation period caused prolonged gestation. In these studies we did not determine whether Treg cell transfer could correct gestational length and the timing of birth in mice with progesterone signalling disruption. However, several lines of evidence from our experiments suggest this is possible. Treg cell transfer restored fetal weight and viability, normal total and $CD4^+$ T cell counts in the udLN and spleen, and notably restored the normal late-gestation P4 decline in some females. P4 regulation of Treg cells in early pregnancy may be essential to orchestrate correct timing of birth, however

further experiments are needed to determine whether this is the case and through which mechanisms this occurs.

Finally, these findings may support the hypothesis that T cells act in the ovary to mediate their effects on fetal development and timing of birth, as Treg transfer restoring P4 levels in late gestation hint of a potential role in mediating luteal regression. Further experiments must be performed specifically to determine whether adoptive Treg cell transfer can restore the normal timing of gestation, and to investigate the role of T cells in the ovary and luteal regression in pregnancy.

In these studies, allogeneic pregnancy was used to the study the significance of P4 regulation of Treg cells in pregnancy. Given the absolute necessity of Treg cells in early allogeneic but not syngeneic mouse pregnancy (Aluvihare *et al.* 2004, Shima *et al.* 2010), it is likely that an essential function of Treg cells in the processes of implantation and placental development is primarily to prevent the anti-fetal immune responses that can occur in allogeneic pregnancy resulting from the mating of MHC-mismatched mice. At the fetal-maternal interface Treg cell derived signals likely mediate local immune phenotypes to ensure fetus-derived cells are tolerated. Although the antigen-specificity of Treg cells in pregnancy is not well understood, paternal-derived fetal antigen specific peripherally-induced pTreg cells are shown to be important for pregnancy success (Rowe *et al.* 2012, Samstein *et al.* 2012). However, thymic-derived Treg cells are also found to respond to alloantigens (Suchin *et al.* 2001, Lalfer *et al.* 2019) and in our laboratory we recently reported that tTreg cells are the primary Treg cell responders in early pregnancy and undergo commitment to the Treg cell phenotype through further *Foxp3* Treg-specific demethylated region (TSDR) methylation (Moldenhauer *et al.* 2019). In the studies in this thesis both pTreg and tTreg cells were affected by disruption in P4 signalling in early pregnancy, but tTreg cells, which make up ~70% of the Treg cells in udLN and spleen, were preferentially depleted in cell number and proportion of the $CD4^+$ T cell pool. Further work must identify the differential functions and requirements for pTreg and tTreg cells in pregnancy, to truly understand the complexities of maternal Treg tolerance.

When Treg cell stability was assessed in pregnancy, we found that Treg cell populations largely maintained stability in normal pregnancy, with the exception of a small, equal proportion of pTreg and tTreg cells which lost *Foxp3* expression and became exTreg cells by late gestation. This finding is not unsurprising, as the high P4 concentrations throughout most of gestation would be expected to promote Treg cell stability. For example, our in vitro findings identify P4 as a regulator of T cell phenotypic stability through suppression of inflammatory IFNγ expression in Teff and Treg cells. This adds to previous findings of P4 promotion of *Foxp3* expression in naïve $CD4^+$ T cells and prevention Teff generation in vitro (Lee *et al.* 2012, Engler *et al.* 2017), and findings of P4 promoting anti-inflammatory IL10 production in T cells

(Mao *et al.* 2010). Taken together, P4 is an environmental determinant of CD4$^+$ T cell phenotypic stability, influencing T cell towards tolerogenic fates. Given that inflammation promotes Treg cell instability (Zhou *et al.* 2009, Miyao *et al.* 2012, Hori 2014, Komatsu *et al.* 2014) this provides a strong basis for the hypothesis that Treg instability is exacerbated in inflammatory pregnancy complications such as preeclampsia and preterm birth, or in pregnancies with reduced progesterone signalling. Determining in vivo regulation of Treg cell stability by P4, and the fate and role of exTregs in normal and pathological pregnancies, requires further work using Treg cell fate mapping models. Furthermore, determining the overall significance of Treg cell instability in pregnancy complications will be an important component of future research.

7.3. MECHANISMS OF P4 REGULATION OF T CELLS DURING PREGNANCY

Work in this thesis demonstrates that P4 has a potent effect on promoting Treg cell abundance and phenotypic stability, but whether the effects of P4 on Treg cells, and on CD4$^+$ T cells in general, occur through direct cell-intrinsic mechanisms, indirect mechanisms though interaction with other cells, or both, are not well defined. Our investigations highlighted direct effects of P4 in regulating T cell phenotype in vitro through repression of IFNy expression, but showed they weren't mediated by classical signalling through the nuclear progesterone receptor (PR). This is consistent with the findings of others (Lee *et al.* 2011, Hughes *et al.* 2013, Hierweger *et al.* 2019), who show direct effects of P4 on T cells. While there remains some inconsistencies in the literature regarding T cell expression of classical PR, it is likely expressed at extremely low levels, perhaps in a hormone-dependent manner (Quadros and Wagner 2008, Diep *et al.* 2016).

Upon investigation of non-classical P4 signalling pathways in T cells we found some direct effects are likely mediated through membrane-dependant pathways such as novel membrane PRs, which are expressed by T cells and Treg cells (Dosiou *et al.* 2007, Areia *et al.* 2015). We found T cells in the udLN of mice in early pregnancy had an increased capacity to bind P4 at the cell surface membrane, suggesting this mechanism of P4 action may be important for the P4-Treg interactions required for pregnancy success. Notably, there is also strong evidence for GR to mediate the effects of P4 in T cells (Engler *et al.* 2017, Hierweger *et al.* 2019), and our studies cannot rule out RU486 is a GR-antagonist as well as a PR antagonist. Therefore, our model of reduced P4 signalling may be targeting the GR in T cells to downregulate P4 signalling within T cell subsets.

In addition to direct effects of P4 on T cells, it is expected that indirect effects on P4 on T cells are essential for pregnancy success, and this is also evident in our model using RU486. Notably, our studies showed that RU486 reduced other immune cells as well as T cells, as a reduction in live cells was evident, and could not be accounted for by the T cell changes alone. Further experiments will be needed to determine which immune subsets are most affected by RU486 treatment, but monocytes/granulocytes and NK cells are candidates. T cell non-specific effects of RU486 are indeed possible, as P4 signalling occurs directly in stromal and epithelial cells (Simon *et al.* 2009, Franco *et al.* 2012, Xu *et al.* 2013) and other immune subsets, including NK cells, macrophages and γδ T cells (Szekeres-Bartho 2009, Lu *et al.* 2015, Shah *et al.* 2019) and thus could be mediating the effect on T cells this indirect manner. One strong candidate of this regulation is DCs, which respond to P4 via nuclear PR (Butts *et al.* 2007). In a mouse model of conditional PR deletion in DCs, Theile *et al.* recently demonstrated P4 regulation of DCs via PR is essential for Treg cell induction in the uterus at mid-gestation (Thiele *et al.* 2019). A DC-T cell signalling impairment could explain the effect of reduced P4 signalling on fetal development in our in vivo model. Furthermore, lack of PR on DCs caused a IUGR phenotype (Thiele *et al.* 2019), which we also observed upon investigation of late fetal outcomes in late gestation.

Further work is needed to fully elucidate the mechanisms of P4 action in T cells, and how effects of P4 on other immune cells, namely DCs, have secondary effects for Treg cells and maternal tolerance. Understanding the mechanisms of P4 action on immune subsets will allow exploitation of ways to naturally boost Treg cell number and function.

7.4. CLINICAL PERSPECTIVE

Insights on the fundamental immunobiology of pregnancy derived from these mouse studies can be applied to human pregnancy, to gain a better understanding of the basic biology of normal and complicated pregnancies. Overall, the studies in this thesis support growing evidence that early pregnancy perturbations or interventions can have a profound impact on outcomes in later gestation, and add to the evidence that these effects can be mediated through suboptimal maternal immune adaptation to pregnancy. This has relevance to many obstetric complications, which are increasingly recognised both for their peri-conceptional origins and their strong association with maternal inflammatory responses (Brosens *et al.* 2011).

Our findings demonstrate a link between a lack of P4 bioavailability at conception and poor immune adaptation to pregnancy, and the potential for this to lead to later gestation pregnancy complications. Notably, this has direct relevance to pregnancies compromised by luteal phase deficiency (LPD), whereby a short or dysregulated luteal phase, leads to inadequate P4 concentrations/bioavailability and subsequent fertility and pregnancy complications such as IUGR. Here we demonstrated that reduced P4 signalling at implantation causes reduced Treg cells at mid-gestation, leading to IUGR in late gestation. The IUGR phenotype could notably be improved when a modest number of Treg cells were transferred into mice with peri-implantation P4 signalling disruption. This result shows an essential role of Treg cells in mediating appropriate fetal growth, dependent on peri-conceptional P4. These results raise important questions about whether luteal phase P4 in women is a rate-limiting determinant of Treg generation that subsequently impairs capacity to withstand an inflammatory challenge in later gestation.

The incidence of LPD is difficult to estimate due to unreliable and inconsistent diagnostic criteria, and difficult to treat due to an incomplete understanding of the pathophysiology of LPD (Mesen and Young 2015). Suspected LPD is treated by improving follicular development with ovulation induction agents and supplementing with P4. Luteal phase support is also provided to women using assisted reproductive technologies (ART), as these women experience an effective LPD (Mesen and Young 2015). In women receiving an embryo in a non-ovulatory cycle (e.g. donor embryo), an absent corpus luteum (CL) explains the need for luteal phase support. Women that undergo ovarian hyperstimulation prior to oocyte retrieval also experience a dysregulated luteal phase, for reasons not well understood (van der Linden *et al.* 2011). In ART women, the luteal phase is mimicked with P4 supplementation to facilitate a receptive endometrium to implantation. Extensive research on luteal support has been directed towards optimum supplementation of the luteal phase to maximise live births, however, achieving the correct timing and dosage of P4 in combination with other hormones such as estrogen is challenging (Versen-Höynck *et al.* 2019). P4 supplementation has been trialled in various routes of administration including, oral, vaginal and intramuscular. Vaginally administered P4 significantly increased implantation rates compared to orally administered P4, which has poor bioavailability (Friedler *et al.* 1999, Licciardi *et al.* 1999). However, synthetic progestins such as dydrogesterone has improved bioavailability and when administered orally, showed comparable pregnancy rate to vaginal progesterone (Chakravarty *et al.* 2005, Ganesh *et al.* 2011). Recently, higher early luteal P4 concentrations were reported to lead to increased live birth rates in IVF women supplemented with dydrogesterone (Netter *et al.* 2019). Notably, some studies report excessive P4 impairs embryo implantation and decidualisation in mice and causes reduced live birth rates in women who undergo IVF/ICSI (Liang *et al.* 2018, Thomsen *et al.* 2018). Therefore, optimal P4 levels

appropriate to the individual pregnancy appear essential for optimal pregnancy progression (Thomsen *et al.* 2018).

Our findings on the importance of P4 priming and education of Treg cells for pregnancy success may explain why women supplemented with P4 or synthetic progestins have increased implantation and live birth rates (Netter *et al.* 2019). Importantly, no studies have specifically investigated the link between Treg cells, LPD and IVF. Future studies focusing on understanding the link between luteal phase P4, early pregnancy immune adaptations and processes of implantation and placentation will likely be important to increase understanding of the pathophysiology of LPD and of immune involvement in establishing pregnancy.

Given the importance of P4 for adequate Treg cell number and phenotypic strength for pregnancy success, it is plausible that excessive inflammation or inadequate P4 could cause insufficient Treg cells to adequately overcome the post-coital inflammatory response. This has potential to lead to impaired decidualisation and embryo implantation, for instance shallow implantation and poor maternal vascular adaptation, that cause placental dysfunction and later manifest as obstetric disorders such as preterm birth or preeclampsia (Robertson *et al.* 2019). Reduced numbers of Treg cells and increased numbers of Th17 cells are implicated in the pathophysiology of preeclampsia, with Treg cells shown to be vital for vascular adaptations to pregnancy in mice and rats (Cornelius *et al.* 2015, Cornelius *et al.* 2016, Care *et al.* 2018). Whether Treg cells exert similar functions in vascular adaptations in women remains to be proven, but seems likely based on the animal data. Notably, IVF pregnancies without a CL are at a higher risk of developing preeclampsia and perturbed maternal circulation in early pregnancy (Versen-Höynck *et al.* 2019)

Our findings inform on the potential of Treg cells to influence the timing of birth. T cells are implicated in preterm birth, whereby a switch to effector cells is evident (Gomez-Lopez *et al.* 2014). Interestingly, our findings that P4-disrupted mice had prolonged gestation, delivering on average 24 hours later than control dams, may indicate the failed immune adaptation in early pregnancy caused the normal immune contribution to parturition to be disrupted. As Treg cell transfer appears to restore the P4 decline prior to parturition and restore normal immune cell numbers in the draining LNs, Treg cells may be implicated in influencing the timing of normal birth. However, further investigation is necessary to address this hypothesis.

Overall, this work adds to growing evidence that Treg cell tolerance established in early pregnancy is crucial for fetal development and outcomes in later gestation. Therefore, these studies provide a basis for research efforts to further investigate clinical associations between Treg cells in early pregnancy and late gestation outcomes in women, and add to the evidence base warranting development of rational inventions for pregnancy complications that focus on maternal immune tolerance. If Treg cell deficiencies can be diagnosed during early pregnancy in women, and then boosted when deficiency is evident, this may have promise for women experiencing repeated pregnancy loss or at risk of developing pregnancy complications. This strategy could lead to the basis of novel treatments for inflammatory pregnancy disorders that can be traced back to poor immune tolerance (Robertson *et al.* 2019). Treatments to boost Treg cell number, stability and functional competence, such as low dose IL2, CD28 superligand, P4 supplementation and cell-based approaches, used alone or in combination, warrant evaluation as potential therapeutic strategies in preeclampsia, for example (Robertson *et al.* 2019). In addition, chimeric antigen receptor (CAR) therapy to generate alloantigen specific T cells for treatment of autoimmune diseases (MacDonald *et al.* 2016), has real promise for treatment of reproductive disorders if the appropriate antigens for T cell activation are recognised. In the case of pregnancy, there is the added challenge of male partner-specific antigens, which call into question the feasibility of this approach. The many factors that contribute to immune tolerance in pregnancy such as genetics, diet, endocrine status and partner MHC compatibility, highlight the need for personalised medicine in the treatment of reproductive disorders. Future efforts must therefore account for these differences with the ultimate goal of tailoring immune or endocrine-based therapies to the specific needs of the individual.

7.5. CONCLUSION

In summary, the studies in this thesis demonstrate P4 regulation of Treg cells in early pregnancy is essential for the immune adaptations required to allow for pregnancy success. Furthermore, P4 is an endogenous determinant of Treg cell abundance and phenotype, and this regulation occurs through both direct and indirect mechanisms of action to impact pregnancy establishment and progression. Overall, P4 is a central modulator of the quality and strength of the immune adaptation to pregnancy through actions on $CD4^+$ T cells and Treg cells. We therefore conclude that luteal phase and early pregnancy P4 is an essential rate-limiting driver of a robust Treg cell anti-inflammatory immune response necessary for optimal reproductive success. This work adds to growing evidence that Treg cell tolerance established in

early pregnancy is crucial for fetal development and later gestational outcomes, including the timing of birth. These studies advance fundamental knowledge of the immunobiology of pregnancy, and provide insight that may be relevant to defining the origins of some clinical complications of fertility and pregnancy disorders that are related to inadequate maternal immune responses and/or poor P4 signalling. Ultimately, we hope these advances will aid future research efforts to investigate links between P4 and immune tolerance in women, and inform development of novel treatments to alleviate these pregnancy complications.

Chapter 8

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8.1. REFERENCES

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