T Regulatory Cells in Early Pregnancy in Mice

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ABSTRACT

To accommodate the semi-allogeneic fetus, a state of maternal immune tolerance to paternally-derived conceptus alloantigens is required. Tolerance is initially established when the same antigens are contacted following seminal fluid exposure, and increases in tolerogenic CD4+Foxp3+ T regulatory (Treg) cells are elicited. Clinical studies demonstrate the importance of seminal fluid contact in human pregnancy, where pathologies of pregnancy including preeclampsia are more likely with a short period of sexual cohabitation. Despite the pivotal role of Treg cells in pregnancy, the factors which regulate their response are yet to be fully understood. In this thesis, we describe experiments using mouse models that investigate regulators of the Treg cell pool and their impact on pregnancy success.

Initially, we examined the contribution of number of seminal fluid exposures to Treg cell generation. Our data demonstrate that repeated exposure to the same male alloantigens strengthens the Treg cell pool, and increases its stability. These changes were not observed after repeated mating to syngeneic males or following switching from syngeneic to allogeneic partners. Changes to the Treg cell population was also linked with greater resistance to inflammatory challenge in mid-gestation. These findings may provide a mechanistic explanation for clinical observations linking long-term seminal fluid exposure in women with improved outcomes of pregnancy.

We then assessed the contribution of a number of key immune regulatory factors on the female Treg cell response in early pregnancy. Prominent among the tolerance-inducing cytokines is IL10, which protects against fetal loss and alters key immune cells in early gestation including Treg cells. In this study we demonstrate that maternal as opposed to fetal IL10 deficiency causes increases susceptibility to fetal loss following inflammatory challenge in mid-gestation. The transcriptome of Treg cells is altered in IL10 deficiency with increased *Ctse* (cathepsin E), *Il1r1*, *Il12rb2* and *Ifng.* These findings highlight the pivotal contribution of maternal IL10 in facilitating robust Treg cell generation and immune protection of the fetus from inflammatory challenge.

Recent studies demonstrate that microRNAs (miRNA) also play a role in Treg cell generation. miR-155 and miR-223 are both regulated by seminal fluid following coitus and are postulated to play important roles in the peri-conception immune environment. Using *mir-155* null mutant mice, we demonstrate that miR-155 deficiency substantially alters the local Treg cell and antigen presenting cell populations after mating. Using *mir-223* null mice, we demonstrate that miR-223 also contributes to the peri-conception immune environment with alterations to the Treg cell and antigen presenting cell populations after mating. Furthermore, deficiency in either miR-155 or miR-223 increases susceptibility to fetal loss following pro-inflammatory challenge in mid-gestation. These findings indicate that both miR-155 and

miR-223 have pivotal roles in establishing the appropriate maternal immune environment during the peri-conception period, and in activating sufficient immune tolerance to protect against inflammatory challenge in later gestation.

Collectively, these data build understanding of key factors contributing to Treg cell generation and function in the peri-conception environment. The findings may be beneficial in informing new approaches to diagnosis and treatment of human gestational disorders associated with immune dysregulation.

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.

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Interleukin 10 deficiency alters the transcriptome of T regulatory cells in pregnant mice. Oral presentation in 08/2014. The Annual Scientific Meeting of the Endocrine Society of Australia and the Society for Reproductive Biology 2014

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miR223 Reduces the Percentage of Regulatory T Cells and Impairs the Pregnancy Outcomes in Mice. Oral presentation in 04/2014. The ASMR SA Annual Meeting

2013

Bihong Zhang, Hanan Wahid, Lachlan Moldenhauer, Sarah Robertson.

Repeated exposure to male seminal fluid expands uterine antigen presenting cell and regulatory T cell populations in mice. Oral presentation in 08/2013. The Annual Scientific Meeting of the Endocrine Society of Australia and the Society for Reproductive Biology 2013

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Effect of seminal fluid on miR-155, miR-146a and miR-23b expression in the uterus and uterine-draining lymph nodes. Oral presentation in 08/2013. The Adelaide Immunology Retreat-9

ABBREVIATIONS

19-OH PGE	19-hydroxy prostaglandin E
APC	allophycocyanin
B6	C57Bl/6
BSA	Bovine serum albumin
CCL	C-C motif chemokine ligand
CTLA-4	Cytotoxic T lymphocyte-associated protein 4
CXCL	C-X-C motif chemokine ligand
DC	Dendritic cell
DPC	Day postcoitum
E. coli	Escherichia Coli
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
Foxp3	Forkhead box P3
G-CSF	Granulocyte colony-stimulating factor
GFP	Green fluorescent protein
GM-CSF	Granulocyte macrophage colony-stimulating factor
HLA	Human leukocyte antigen
IP	Intra-peritoneal
IDO	Indoleamine 2,3-dioxygenase
IFN-γ	Interferon-γ
IL	Interleukin
LAG-3	Lymphocyte activation gene-3
LPS	Lipopolysaccharide
MHC	Major histocompatibility complex
miRNA	MicroRNA
min	Minute
mLN	Mesenteric lymph nodes
NK	Natural killer
PALN	Uterus draining lymph nodes
PBS	Phosphate buffered saline
PGE	prostaglandin E

PE	Phycoerythrin
PMA	Phorbol 12-myristate 13-acetate
pTreg	Peripheral T regulatory
qPCR	Quantitative polymerase chain reaction
RBC	Red blood cell
RT	Room temperature
s	Second
STAT	Signal transducer and activation of transcription
TCR	T cell receptor
tDC	Tolerogenic dendritic cells
TGF-β	Transforming growth factor-β
Th	T helper
TNF	Tumour necrosis factor
Treg	T regulatory
tTreg	Thymic T regulatory
WT	Wild-type

Chapter 1 Literature Review

This chapter has contributed to publications entitled “MicroRNA regulation of immune events at conception” and “miRNA Regulation of Immune Tolerance in Early Pregnancy”. For full detail of the publication, please see section 8.4.

1.1 Introduction

Preeclampsia and related hypertensive disorders of pregnancy are major contributors to fetal and maternal morbidity and mortality in the world (Steegers et al., 2010). Preeclampsia affects 5-8% of all pregnancies and leads to approximately 60,000 maternal deaths and 500,000 perinatal deaths annually (WHO, 2005). The pathophysiological origin of preeclampsia remains unclear, but it is suggested that the peri-conception phase of pregnancy is important, particularly for the early onset category of preeclampsia. Implantation is the time when maternal immune disturbances can contribute to disruption of placental development (Redman and Sargent, 2010). Compromised placental development is characterised by insufficient remodelling of spiral arteries and defects of maternal uterine blood flow (Huppertz, 2008, Redman and Sargent, 2010, Steegers et al., 2010). Inadequate immune adaptation for gestation also contributes to the inflammatory responses characteristic of preeclampsia (Redman and Sargent, 2010, Sibai et al., 2005).

As half of the fetal genes are derived from the father, the fetus is considered as semi-allogeneic and so can be recognised by maternal immune system as 'non-self'. The maternal immune system clearly recognises and responses to fetal antigens, and adaptive immune tolerance towards paternally-derived transplantation antigens must be induced for protection from gestational disorders (Aluvihare et al., 2004, Trowsdale and Betz, 2006). A key element of maternal immune adaptation for pregnancy is the T regulatory (Treg) cell population, which supports embryo implantation (Aluvihare et al., 2004, Robertson et al., 2009a). Treg cells prevent rejection of the fetus, suppress detrimental inflammation and facilitate matermal vessel remodelling to allow sufficient trophoblast invasion and access to maternal blood supply (Nevers et al., 2011). Therefore, it is critical to identify the factors that determine the quality of the Treg cell response, including activation and expansion of the Treg cell pool, and Treg cell stability and suppressive function, are these will be crucial for pregnancy success.

The mechanisms regulating the Treg cell responses are not fully understood. It is known that the cytokine milieu is critical for Treg cell activation, differentiation and proliferation. Various cytokines are implicated in promoting Treg cells and suppressing generation of Th1 immune responses to limit inflammation in the gestational tissues (Lin et al., 1993, Munoz-Suano et al., 2011, Szekeres-Bartho et al., 2009). Amongst these cytokines, interleukin 10 (IL10) has potent anti-inflammatory properties. IL10 is produced by a range of different types of cells, including T cells, B cells, macrophages, dendritic cells (DCs) as well as Treg cells (Annacker et al., 2001).

Previous studies in mice have demonstrated that the seminal fluid delivered at mating influences the immune environment to promote expansion of the Treg cell pool before implantation (Robertson et al., 2009a). It has been identified that seminal fluid provides antigens and cytokines that act to influence the phenotypes of antigen presenting cells, stimulate Treg cell populations, and to induce chemokines that

recruit Treg cells into the endometrium. Clinical observations have revealed that seminal fluid contact may protect women from developing preeclampsia. Studies by Dekker and Robillard (Dekker et al., 1998) and several others (Kho et al., 2009, Einarsson et al., 2003, Klonoff-Cohen et al., 1989) have reported that preeclampsia is more common in nulliparous women, women who have a new male partner, or when there has been lack of exposure to the conceiving partner's seminal fluid before conception. The increased incidence of preeclampsia is linked with short duration of sexual cohabitation with the conceiving partner, the use of barrier methods of contraception, or when donor gametes are used in *in vitro* fertilisation treatment (Dekker et al., 1998, Einarsson et al., 2003, Kho et al., 2009, Klonoff-Cohen et al., 1989, Salha et al., 1999). These observations raise the question of how the male partner contributes to preeclampsia and whether Treg cells and seminal fluid are involved.

There is growing evidence showing that microRNAs (miRNAs) play important roles in the immune response, which suggests they may contribute to immune adaptation to pregnancy. Two miRNAs that appear to regulate Treg cell response are miR-155 and miR-223. miR-155 has been shown to regulate Treg cell pool expansion (Kohlhaas et al., 2009, Stahl et al., 2009). While miRNAs act directly in Treg cells, they can also regulate the antigen-presenting cells that are required to generate Treg cells. In particular, miRNAs regulate the phenotype and function of DCs which play important roles in mediating tolerance through controlling Treg cell responses. miR-223 is also thought to be important in macrophage and DC function (Johnnidis et al., 2008). However, there are no studies focusing on the relationship between miR-155 or miR-223 and Treg cell responses during early pregnancy.

This chapter will discuss the critical factors that facilitate generation of stable Treg cells in order to establish and maintain immune tolerance for embryo implantation and ongoing pregnancy success. This chapter will also summarise current understanding of the factors and cytokines that regulate the abundance and stability of Treg cells in early pregnancy in both human and mice.

1.2 Maternal immune tolerance

In 1953, Sir Peter Medawar questioned the paradoxical immunological relationship between the mother and the fetus. He asked "How does the pregnant mother contrive to nourish within itself, for many weeks or months, a fetus that is an antigenically foreign body?" (Medawar, 1953). He proposed three possible mechanisms by which maternal immune tolerance may be established: 1) the fetus is anatomically separated from the mother; 2) the fetus is antigenically immature; and 3) the mother is immunologically indolent or inert. These ideas have guided research in reproductive immunology for more than 50 years (Billington, 2003).

Over more than 60 years of study, it has been revealed that Sir Medawar's hypotheses are incorrect. Rather than being suppressed, the maternal immune system is activated during pregnancy - it recognises the fetal antigens with T and B cell responses, and when behaving correctly, it facilitates

pregnancy success (Chaouat, 2007, Zhou and Mellor, 1998, Chaouat and Kolb, 1985, Kiger et al., 1985, Tafuri et al., 1995). Fetal cells are found in the maternal circulation during the first and second trimester (Herzenberg et al., 1979, Walknowska et al., 1969), indicating that the fetus is not anatomically separated from the mother. The antigenicity of placental trophoblast is reduced, with attenuated expression of major histocompatibility complex (MHC) genes, however transplantation antigens are clearly expressed (Fernandez et al., 1999). In addition, women can produce antibodies, as well as lymphocytes which react against fetal human leukocyte antigens (HLA) (Hunt et al., 2003, Van Rood et al., 1958, Tilburgs et al., 2009), indicating that the maternal immune system is not indolent or inert, but is actively responsive to the presence of fetal alloantigens.

Recent studies have confirmed that activated T cells reactive with fetal HLA can be found in the peripheral blood and decidua of pregnant women (Tilburgs et al., 2009, van Kampen et al., 2001). Studies in mice with transgenic T cells that specifically recognise fetal antigens also indicate that the maternal immune system is aware of conceptus antigens from the time of conception (Moldenhauer et al., 2010). It is clear that maternal T cell-mediated immune responses to the semi-allogeneic fetus are activated in pregnancy. Skewing the T cell response from effector-mediated rejection towards Treg cell-mediated tolerance, is critical for the fetus to survive. The quality of the T cell response is most important at implantation when the conceptus first encounters maternal immune cells at implantation (Trowsdale and Betz, 2006).

Seminal fluid is involved in the initial activation phase of the maternal immune adaptation for pregnancy, by providing paternal alloantigens and cytokines to drive the expansion of a T cell response that includes Treg cells (Robertson et al., 2009a, Robertson et al., 2009b) which are crucial in establishing maternal immune tolerance in readiness for implantation (Aluvihare et al., 2004, Shima et al., 2010). The importance of seminal fluid will be discussed in 1.3.

1.2.1 T helper cell paradigms

To address how T cell activation can support embryo implantation and maintain the progress of placental development, it is important to understand the different phenotypes of T cells. Generally, T helper cells (Th cells) are characterised as Th1, Th2, Th17 effector cells, or Treg cells. Th1 cells are identified by the transcription factor T-bet and signal transducer and activator of transcription (STAT) 4, and can produce pro-inflammatory cytokines including interferon-γ (IFN-γ) and tumour necrosis factor (TNF). In contrast, Th2 cells are characterised by the transcription factor GATA-3 and STAT6 and also the production of anti-inflammatory cytokines such as IL4, IL5 and IL13. Th17 cells are also pro-inflammatory and play a pivotal role in autoimmune disease pathogenesis and allograft rejection (Crome et al., 2010, Peck and Mellins, 2010).

Treg cells are generally defined as CD4+CD25+ T cells expressing the unique intracellular marker, Forkhead box P3 (Foxp3). Treg cells regulate the generation and effector function of Th1, Th2 and Th17 cells (Rudensky, 2011). The differentiation of Th1 and Th2 cells are generally considered as irreversible, but in contrast, Th17 and Treg cells exhibit phenotypic plasticity and can switch between the two phenotypes when micro-environmental conditions change (Zhou et al., 2009).

1.2.2 Regulatory T cells

Treg cells are 5-10% of peripheral blood CD4+ T cells in rodents and only 1-3% of CD4+ T cells in humans (Sakaguchi, 2000, Shevach, 2002). Sakaguchi and colleagues were the first to identify Treg cells as a unique subtype of T lymphocytes (Sakaguchi et al., 1995). Treg cells suppress autoreactive immune responses to prevent autoimmune diseases and are involved in suppression of allograft rejection (Sakaguchi et al., 1995). Null mutation in the master Treg cell transcription factor Foxp3 results in a fatal lymphoproliferative disorder in mice, which indicates the pivotal role of Treg cells in the regulation of inflammation and immune tolerance (Brunkow et al., 2001). In the last 10 years, Treg cells have been shown to be crucial in the establishment and maintenance of maternal immune tolerance in both human and mice (Aluvihare et al., 2004, Zenclussen et al., 2005, Heikkinen et al., 2004, Saito et al., 2005).

There are two distinct pathways by which Treg cells are generated. Treg cells can differentiate in the thymus in response to self-antigens, or in the periphery in response to various antigens including foreign, exogenously-derived antigens, or developmental or tissue-specific antigens not expressed in the thymus (See section 1.2.2.1).

1.2.2.1 Treg cell ontogeny

Two distinct origins and pathways of generation of Treg cells exist, based on the tissue in which they originate, their target antigens and their suppressive functions. Both are crucial in mediating tolerance towards endogenous non-pathogenic antigens and foreign but benign antigens normally in contact with the body. Firstly thymic Treg cells (tTreg cells) differentiate in the thymus via a selective process based on expression of specific T cell receptors (TCRs) (Fontenot et al., 2005). Secondly, Treg cells can be induced from the peripheral T cell pool (so-called ‘peripheral’ or ‘inducible’ Treg cells) which are generated in the periphery (Lee et al., 2011).

1.2.2.1.1 Treg cell differentiation in the thymus

The development of T cells in the thymus is determined by different avidities for self-antigens of TCRs. The thymus is the organ that ‘educates’ immature T cell populations before their release into the periphery. A serial selection sequence involving neglect, positive and negative selection and processes based on interactions between naïve T cells and the thymic epithelial cells, results in a pool of surviving

T cells. Failure to interact with self-antigens presented by MHC molecules on the thymic epithelial cells results in apoptosis of T cells (neglect). T cells with a high avidity between their TCR and MHC are positively selected and these T cells with excess reactivity to self-antigens undergo apoptosis (negative selection) (McCaughtry and Hogquist, 2008, Palmer and Naeher, 2009, Siggs et al., 2006).

The generation of tTreg cells is believed to be the result of T cells that 'escape' from negative selection. When the TCR/MHC affinity of the naive T cells is just weaker than the threshold to induce negative selection and also with a relatively high affinity for self-antigen amongst cells that pass positive selection, Treg cells differentiate (Jordan et al., 2001). As tTreg cells are generated largely based on their reactivity to self-antigen, tTreg cells are considered to exert protection from a range of autoimmune diseases (Sakaguchi, 2005).

1.2.2.1.2 Treg cell generation in the periphery

Treg cells can also differentiate from pathways independent of the thymus. Studies have revealed that peripheral Treg cells (pTreg cells) are generated in the periphery to maintain the Treg cell population (Cozzo et al., 2005, Seddon and Mason, 1999). Recent studies clearly demonstrate the requirement of pTreg cells in maintaining immune tolerance. By using a TCR transgenic mouse model, oral administration of antigens results in induction of antigen specific Treg cells, which protect mice from experimental asthma (Mucida et al., 2005). These pTreg cells are anergic and suppressive both *in vivo* and *in vitro* (Mucida et al., 2005). Neonatal adoptive transfer of splenocytes containing tTreg cells can successfully rescue Foxp3-deficient mice (Haribhai et al., 2011). Supplementation by either tTreg cells or pTreg cells alone fails to rescue Foxp3 deficient mice, however, Foxp3-deficient mice receiving both tTreg and pTreg cells in combination exhibit a less severe inflammatory bowel disease and lower serum concentrations of TNF-α, IFN-γ and IL17 (Haribhai et al., 2011). Acute depletion of pTreg cells in the rescued mice results in inflammation and weight loss (Haribhai et al., 2011). These data suggest that pTreg cells are necessary to establish tolerance to the full range of self-antigens in the periphery.

pTreg cells generated with affinities for antigens not expressed in the thymus expand TCR diversity specific for peripheral tissue antigens. Thus, the immune system can regulate responses against antigens that are foreign to self, and protect the body from aggressive or inappropriate responses. In the reproductive setting, pTreg cells with affinities for paternal alloantigens allow the maternal immune system to tolerate antigens absent from the thymus but associated with the developing fetus. It is also possible that tTreg cells contribute, as many T cells generated in the thymus have responsiveness to alloantigens (Sakaguchi et al., 2008)

1.2.2.2 Interactions between dendritic cells and Treg cells

Like other types of T cells, Treg cells require interaction with antigens to achieve proliferation and full suppressive activity (Samy et al., 2006). Antigen presenting cells, such as DCs, are required to convert

naïve CD4+ T cells to Treg cells and are also involved in the regulation of expansion and activation of resting Treg cells (Rutella et al., 2006). This is also the case in pregnancy. Local depletion of uterine DCs on gestational day 3.5 in mice results in a reduced implantation rate, suggesting that dendritic cell functions taking place before implantation are crucial to enable successful placental development (Plaks et al., 2008).

The outcome of T cell and DC interaction is determined by the functional phenotype of DCs, reflected in the surface expression of CD80/86 and the specific DC cytokine profile. To convert naïve CD4+ T cells and expand pre-existing Treg cells, antigens must be presented by specific subsets of dendritic cells with tolerogenic properties (tDCs) (Manicassamy and Pulendran, 2011, Sharma et al., 2007b, Steinman et al., 2003). tDCs arise when DCs are differentiated in a cytokine milieu dominated by granulocyte macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), transforming growth factor-β (TGF-β), IL10 and IL4 (Rutella et al., 2006, Steinman et al., 2003). tDCs are characterized as immature or semi-mature DC which do not express IL12 and express limited levels of co-stimulatory molecules CD80 and CD86 as well as pro-tolerogenic molecules programmed death ligand-1, immunoglobulin-like transcript 3, immunoglobulin-like transcript 4 and indoleamine 2,3-dioxygenase (IDO) (Cella et al., 1997, Probst et al., 2005, Smits et al., 2005, Steinman et al., 2003). Treg cell generation relies on the presentation of cognate antigens by tDCs, and therefore, Treg cell generation, proliferation and differentiation is clonally specific to the antigens presented (Yamazaki et al., 2006). These antigens may include paternal alloantigens, or minor histocompatibility antigens from the fetus or placenta.

Treg cells can be converted from naïve CD4+ T cells, or activated from pre-existing Treg cells by tDCs (Munn et al., 2002, Steinman et al., 2003). It has been shown that IDO activates mature Treg cells in tumour-draining lymph nodes in mice (Sharma et al., 2007b). After adoptive transfer of IDO+ DCs into non-tumour bearing mice, resting Treg cells in the host were activated, in an IDO-dependent manner (Sharma et al., 2007b). Further evidence for the importance of IDO can be seen with an 8-fold increase in Foxp3 mRNA in murine CD4+ T cells co-cultured with IDO+ DCs at 3-6 days (Fallarino et al., 2006), indicating that IDO+ DCs are capable of converting CD4+ T cells into Treg cells. TGF-β is anti-inflammatory (Gorelik and Flavell, 2002, Sakaguchi, 2000, Shevach, 2002) and appears to be essential for the generation of Treg cells via indirect effects on DCs (Ghiringhelli et al., 2005b) and direct effect on expression of the signature transcription factor *Foxp3* in Treg cells (Chen et al., 2003).

Anti-inflammatory cytokine conditions are essential in converting naive T cells into Treg cells, rather than Th1, Th2 or Th17 cells. The activated Treg cells secrete TGF-β and IL10, which maintain the tolerogenic properties of tDCs (Sharma et al., 2007b), and suppress detrimental immune responses from being induced. This reactive loop between Treg cells and tDCs can be altered by infection or

inflammation, when pro-inflammatory cytokines, bacterial lipopolysaccharide (LPS) or other Toll-like receptor (TLR) ligands induce DC maturation, skewing the phenotypes of Treg cells into Th1 and Th17 immunity (Manicassamy and Pulendran, 2011).

1.2.2.3 Mechanisms of Treg cell-mediated suppression

There are a number of ways that Treg cells act to suppress the immune system. Treg cell-mediated suppression can be via cell-cell contact (direct) or modulating the maturation and function of DCs (indirect), or in a contact-independent manner (Shevach, 2009).

The best-defined mechanism by which Treg cells exert suppression is to inhibit IL2 production by other types of T lymphocytes. Once CD4+ T effector cells are activated via TCR, IL2 and other pro-inflammatory cytokines are rapidly produced. The proliferation activity of CD4+CD25- T cells is significantly reduced after co-culture with CD4+CD25+ T cells via inhibiting IL2 production in CD4+CD25- T cells (Takahashi et al., 1998, Thornton and Shevach, 1998). As IL2 is critical for survival and activation of T cells, by limiting production of IL2, Treg cells can suppress the proliferation of T effector cells.

Other studies have revealed that IL2 is also critical in maintaining Treg cell function, as IL2 deficient and IL2 receptor deficient mice develop systemic and lethal autoimmune disease (Sadlack et al., 1993, Schorle et al., 1991, Suzuki et al., 1997, Willerford et al., 1995). As Treg cells constitutively express CD25 (IL2 receptor), therefore, another proposed mechanism of Treg cell suppression is to compete with other T cells for IL2 and limit the availability of the cytokine (Pandiyan et al., 2007). The T effector cells are deprived of IL2 and undergo apoptosis in a Bim-dependent manner (Pandiyan et al., 2007). Whether Treg cells in gestational tissues inhibit IL2 production of T effector cells or compete with local T cells for IL2 requires more studies to clarify.

Treg cells also modify the function of antigen presenting cells, including macrophages and DCs (Misra et al., 2004, Taams et al., 2005). The core Treg cell suppressive function is mediated via expression of cytotoxic T lymphocyte–associated molecule 4 (CTLA-4). The critical role of CTLA-4 in Treg cell suppressive function has been demonstrated using CTLA-4 specific blocking antibody and Treg-specific *Ctla-4* deficient mice (Liu et al., 2001, Wing et al., 2008). *Ctla-4* deficient mice show spontaneous lymphoproliferation including an elevated Treg cell proportion amongst CD4+ T cells, increased Treg cell numbers. Despite this, fetal autoimmune disease was observed (Wing et al., 2008). Binding of CTLA-4 results in the down-regulation of costimulatory molecules CD80 and CD86 on antigen presenting cells (Cederbom et al., 2000, Sakaguchi et al., 2009b, Oderup et al., 2006), which remain down-regulated even in the presence of stimuli which would normally elevate CD80/86 expression (Cederbom et al., 2000). In addition, binding of CTLA-4 to CD80/86 results in upregulation of IDO (Belladonna et al., 2007), which provokes the development of Treg cells.

In addition to modification of CD80/86 expression on antigen presenting cells, Treg cells may modify the capacity of DC to induce pro-inflammatory responses. Lymphocyte activation gene-3 (LAG-3) expressed by Treg cells binds to MHCII on antigen presenting cells which inhibits DC activation during Treg:DC interactions (Liang et al., 2008), and therefore suppresses pro-inflammatory responses. LAG-3 depletion by antibody administration diminishes the suppressive function of Treg cells *in vitro* and *in vivo,* and Treg cells from LAG-3 deficient mice exhibit reduced suppressive capacity (Huang et al., 2004). Taken together, these observations show a reciprocal interaction between tDCs and Treg cells; Specific tDC surface markers regulate Treg cell generation, while Treg cells can suppress pro-inflammatory immune responses via regulating antigen presenting cell phenotypes and maturation.

As well as the cell-cell contact mechanisms mediating suppression, a contact-independent suppression can be achieved by Treg cell release of inhibitory cytokines such as TGF-β and IL10 as well as IL35 (Collison et al., 2009, Collison et al., 2007, Sakaguchi et al., 2009a). TGF-β deficient mice develop autoimmune disease and also have a diminished number of Treg cells (Huber et al., 2004, Kulkarni et al., 1993, Shull et al., 1992). Administration of neutralising antibody against TGF-β results in attenuated suppressive activity in both human and mice (Strauss et al., 2007, Fahlen et al., 2005). TGF-β is expressed in a transmembrane form that is important for Treg cell suppressive activity in both human and mice (Ghiringhelli et al., 2005a, Nakamura et al., 2004). The transmembrane form of TGF-β may be involved in the regulation of differentiation of other T cells into Treg-like cells (Fahlen et al., 2005, Andersson et al., 2008).

The immune-inhibitory function of IL10 has been well studied. IL10 deficient mice develop autoimmune disease, in particular colitis (Berg et al., 1996, Kuhn et al., 1993a). In an adoptive transfer model, administration of neutralising antibody against IL10 resulted in rejection of allografts (Hara et al., 2001, Kingsley et al., 2002). The relationship between IL10 and Treg cells is discussed further in section 1.6.

IL35, the new member of the IL12 family, has also been shown to be inhibitory and required for the maximal suppressive activity (Collison et al., 2007). Ectopic expression of IL35 induced regulatory activity on naïve T cells, and recombinant IL35 treatment suppresses T-cell proliferation *in vitro (Collison et al., 2007)*.

1.2.3 Treg cell response during normal pregnancy

1.2.3.1 Treg cell responses in human pregnancy

Several studies have focused on lymphocyte subpopulations during pregnancy in humans. Sasaki and colleagues have shown that CD4+CD25+ Treg cells are increased in both decidua and peripheral blood during early pregnancy (Sasaki et al., 2004). Other studies also demonstrate this systemic elevation of Treg cells in the first trimester, with a peak in the second trimester followed by a reduction in the third

trimester and further decline after successful labour in normal pregnant women (Saito et al., 2005, Heikkinen et al., 2004, Tilburgs et al., 2006, Xiong et al., 2013).

In addition, the suppressive function of the Treg cell population in pregnancy has been demonstrated *in vitro*. Isolated peripheral CD4+CD25+ T cells from normal pregnant women are enriched with a higher expression of *Foxp3* mRNA, compared with CD4+CD25- T cells (Somerset et al., 2004). These Treg cells suppress the proliferation of autologous CD4+CD25- T cells in response to dendritic cells (Somerset et al., 2004). Moreover, the densities of Treg cells were greater in the decidua compared with peripheral blood, indicating the importance of not only expansion of Treg cells in the periphery, but also their recruitment to the uterus (Sasaki et al., 2004, Tilburgs et al., 2006).

Furthermore, the reduction of Treg cells in the third trimester suggests that Treg cell loss might be involved in the process of parturition at term. Women who undergo a spontaneous vaginal delivery have a lower proportion of decidual CD4+CD25+ T cells, compared with those who undergo a caesarean section (Sindram-Trujillo et al., 2004). In women, the percentage of peripheral Treg cells declines towards the end of pregnancy, accompanied by an elevation of activated T cells, and a sharp increase of activated T cells in term labour (Zhao et al., 2007). A recent study also demonstrated that the suppressive activity of Treg cells is diminished towards the end of pregnancy in both term and pre-term labour (Kisielewicz et al., 2010), indicating that Treg cell demise or conversion to T effector cells may be involved in the initiation of labour.

1.2.3.2 Treg cell responses in rodent pregnancy

Similar changes in the immune cell profile are observed in rodent pregnancies with system expansion of Treg cells (Aluvihare et al., 2004, Zhao et al., 2007). Treg cells are expanded in spleen, inguinal lymph nodes, iliac lymph nodes and blood of female mice on gestational day 10.5 after allogeneic mating (female mated to MHC disparate males) (Aluvihare et al., 2004). Treg cells increase systemically on day 7.5 postcoitum (DPC), and in the peripheral blood reached a peak on DPC 7.5, followed by a reduction to non-pregnant levels just before labour (Zhao et al., 2007). Expansion of the Treg cell compartment has been observed on DPC 3.5 in the uterus draining lymph nodes (PALN) of female mice after allogeneic mating (Robertson et al., 2009a). An increase Treg cell number is also seen in the mouse uterus on DPC 3.5 where Treg cells are required to exert suppressive function at implantation (Guerin et al., 2011).

Using rodent models, the importance of Treg cells in regulating the maternal immune environment in pregnancy has been addressed. By using an adoptive transfer model, the crucial role of Treg cells was identified in mice. T cells depleted of CD4+CD25+ Treg cells or complete T cells from normal pregnant mice were adoptively transferred into pregnant T cell deficient mice. The allogeneic fetuses were completely rejected in the absence of Treg cells amongst the transferred population, however, no fetal

rejection was found in syngeneic pregnancies (no MHC disparity between male and female mice) (Aluvihare et al., 2004). *In vivo* depletion of CD25+ cells resulted in a similar fetal rejection in mice (Darrasse-Jeze et al., 2006). When anti-CD25 monoclonal antibody PC61 was administrated to deplete CD25+ cells from female mice after allogeneic mating, fewer fetuses survived to term, while in syngeneic mating, there was no change in reduced fetal survival rate (Darrasse-Jeze et al., 2006).

Other studies have utilised anti-CD25 monoclonal antibody to deplete CD25+ cells at or prior to implantation, and confirm that this results in a pregnancy failure in allogeneic pregnancy but not syngeneic pregnancy in mice (Zenclussen et al., 2005, Shima et al., 2010), indicating that paternal alloantigen specific Treg cells are pivotal for successful implantation. In the abortion-prone CBA/J x DBA/2 mouse model, adoptive transfer of Treg cells from CBA/J females mated with Balb/c males into CBA/J female mice mated with DBA/2 males before implantation (between DPC 0 to DPC 2) caused a reduction in fetal loss as well as increased expression of decidual Foxp3. Treg cells must be previously exposed to antigen to exert suppressive function, as donor Treg cells from virgin mice are ineffective. Moreover, Treg cells transfer after the day of implantation is ineffective (Zenclussen et al., 2005), indicating that sufficient Treg cells must be present at the time of implantation in order to protect the embryo as trophoblast cells first commence invasion (Zenclussen et al., 2005), indicating that sufficient Treg cells are most important prior to and at the time of implantation. Depletion of Treg cells in the pre-implantation period caused failure of implantation in all allogeneically-mated female mice, but did not interfere with pregnancy when treatment occurred in mid- or late-gestation (Shima et al., 2010). Taken together, these studies indicate that paternal antigen-specific Treg cells are crucial in supporting fetal implantation. Therefore, it is essential to define the factors that regulate the size of the Treg cell population or Treg cell suppressive function or stability during pregnancy.

1.3 Role of seminal fluid in immune priming for pregnancy

1.3.1 Composition of seminal fluid

Seminal fluid is a complex biological fluid that contains spermatozoa, seminal plasma, male alloantigens and soluble factors with immune-regulatory functions. Male alloantigens, such as MHC are expressed on human sperm cell precursors and mature gametes, and also can be found in seminal plasma in soluble form. Experiments in mice show male alloantigens can be presented by antigen presenting cells recruited to the female uterine endometrium after insemination (Robertson et al., 1996).

One of the soluble factors in seminal fluid is TGF-β. High amounts of TGF-β1 and TGF-β3 are found in seminal plasma of mouse, human and many mammalian species, with a lower amount of TGF-β2 (Lokeshwar and Block, 1992, Nocera and Chu, 1993, O'Leary et al., 2011, Srivastava et al., 1996, Tremellen et al., 1998). Another highly abundant immune-regulatory factor, prostaglandin, is detected in

humans (Templeton et al., 1978), other primates (Kelly et al., 1976) and some mammalian species (Claus et al., 1992, Oliw et al., 1986, Rego et al., 2014) but not in rodents and pigs (Schjenken and Robertson, 2014). Among these prostaglandins, 19-hydroxy prostaglandin E (19-OH PGE) with immunosuppressive activity which regulates female tract responses to seminal fluid (James and Hargreave, 1984). In addition, in the presence of TGF-β and PGE2, macrophages and DCs are differentiated to mediate tolerogenic immune responses (Blois et al., 2007, Jaiswal et al., 2012).

1.3.2 Seminal fluid and the inflammatory response

A pregnant mother is initially exposed to paternal alloantigens in seminal fluid at or prior to conception (Hutter and Dohr, 1998), and secondly when trophoblast cells invade during implantation (Holland et al., 2012). Both MHCI and MHCII molecules are carried in the ejaculate, either on sperm or within seminal plasma (Fernandez et al., 1999, Hutter and Dohr, 1998), to provide the first exposure of the female reproductive tract to paternal antigens. In human, this will generally also be a regular exposure to the conceiving partner before conception occurs. TGF-β, and 19-OH PGE2 are two abundant factors implicated as immune regulatory agents acting to initiate the Treg cell response (Kelly and Critchley, 1997a, Robertson et al., 1997), and these may interact with additional, less well defined pro-tolerance factors in seminal fluid. Exposure to seminal fluid elicits maternal tolerance to paternal alloantigen by increasing the CD4+CD25+ T cell population (Robertson et al., 2009a) and CD4+ Treg cells (Guerin et al., 2011) in the PALN on DPC 3.5, as well as in the mouse uterus (Guerin et al., 2011). In mouse models where either the plasma or sperm fractions of the ejaculate are removed by surgical excision of the male seminal vesicles or vasectomy respectively, the expected increase in the Treg cell pool is diminished, showing both the sperm and/or epididymal secretions, as well as secretions from the seminal vesicles, are importance in female Treg cell induction (Guerin et al., 2011, Robertson et al., 2009a). The induction of Treg cells, is associated with a state of transient, antigen specific hypo-responsiveness to male partner transplantation antigens, as demonstrated in a tumour challenge model (Robertson et al., 2009a).

1.3.2.1 Seminal fluid induced immune response

At coitus, seminal fluid interacts with epithelial cells lining the cervix and female reproductive tract to induce several cytokine and chemokines. In mice, cytokines and chemokines including GM-CSF, G-CSF, IL6, C-X-C motif chemokine ligand 1(CXCL-1) and CXCL-2 and C-C motif chemokine ligand-3 (CCL-3) are elevated in the endometrial tissues after insemination (Johansson et al., 2004, Pollard et al., 1998, Sanford et al., 1992, Schjenken et al., 2015, Robertson et al., 1998, Robertson et al., 1996). The requirement for seminal plasma for this female cytokine response is demonstrated in mice where males where the seminal vesicles are surgically excised, which results in no or only low induction of cytokine expression after mating (Schjenken et al., 2015).

In humans, similar cytokine responses are observed in the cervix after coitus. *In vitro* and *in vivo* studies have revealed an induction of GM-CSF, IL1A, IL6, IL8, CCL-2 and CCL-20 in the cervix after seminal fluid contact (Sharkey et al., 2012a, Sharkey et al., 2012b, Sharkey et al., 2007). When barrier contraception (a condom) is used to prevent seminal fluid contact, no cytokine response is seen in cervix tissue, showing seminal fluid plays a pivotal role in initiating this inflammation-like response (Sharkey et al., 2012b). Significant cytokine induction can also be demonstrated in human ectocervical epithelial cells exposed to seminal fluid *in vitro* (Sharkey et al., 2007).

The change in cytokines and chemokines induced by seminal fluid in the female reproductive tract results in an influx of immune cells including macrophages, granulocytes, neutrophils and DCs into subepithelial stromal region in mice (De et al., 1991, McMaster et al., 1992, Robertson et al., 1998, Robertson et al., 1996, Robertson et al., 1992). Similar leukocyte recruitment can be observed in the human cervix, where macrophages, DCs and memory T cells are found to accumulate in the epithelial layer and deeper stromal tissues (Sharkey et al., 2012b).

1.3.2.2 Male alloantigen recognition and presentation

Female macrophages and DCs recruited to the endometrium, and possibly male DCs carried in the ejaculate, are potentially responsible for presenting male alloantigens deposited by seminal fluid at coitus (Robertson et al., 1996, McMaster et al., 1992). These antigen presenting cells pick up male alloantigens and traffic to the PALN to drive the proliferation and differentiation of Treg cells or interact locally with uterine residing T cells (Robertson et al., 2009a).

The presentation of male alloantigens could thus be mediated either by maternal antigen presenting cells or paternal antigen presenting cells. The appearance of male alloantigens has been demonstrated by mating wild-type (WT) female mice to male mice expressing green fluorescent protein (GFP) ubiquitously under the beta actin promoter. GFP+ cells (paternal cells) could be found in the uterus, decidua and lymph nodes on DPC 0.5 (Zenclussen et al., 2010) showing that male alloantigens might be presented by paternal antigen presenting cells immediately after insemination. In addition, some of the GFP+ cells were positive for the DC marker CD11C and also expressed MHCII, indicating that DCs in seminal fluid may be recognised by maternal T cells (Zenclussen et al., 2010).

In addition, Moldenhauer et al demonstrated that male alloantigens are cross-presented by maternal antigen presenting cells (Moldenhauer et al., 2009). Using an OT-I and OT-II transgenic mouse model, antigen presentation pathways were addressed. Female mice were administered ovalbumin-specific OT-I T cells after mating to male mice expressing ovalbumin. Ovalbumin-specific T cells were found locally in the PALN with high proliferative activity. When ovalbumin was presented by maternal antigen presenting cells, the CD8+ T cells were activated, but male partner antigen presenting cells presenting ovalbumin failed to induce similar responses (Moldenhauer et al., 2009).

1.3.3 Seminal fluid contributes to Treg cell responses

There are several studies demonstrating the contribution of seminal fluid to the Treg cell response in pregnancy. Both seminal plasma and sperm are required for expansion of the Treg cell pool in the peri-implantation period as demonstrated by experiments investigating effects of mating with seminal vesicle deficient and vasectomised mice. The Treg cells in both the PALN and the uterus were increased after exposure to intact seminal fluid, compared with virgin oestrus female mice, but exposure to sperm alone in the absence of seminal plasma failed to induce any elevation (Guerin et al., 2011, Robertson et al., 2009a). However, exposure to seminal plasma of vasectomised males induced similar expansion of Treg cells in both PALN and uterus, compared with females exposed to intact seminal fluid (Guerin et al., 2011). However, the absence of either sperm or seminal plasma caused the expression of Foxp3 mRNA in the uterus to be reduced, compared with females exposed to intact seminal fluid, suggesting both elements are needed for the full Foxp3 response (Guerin et al., 2011). Mating to seminal vesicle deficient and vasectomised male mice also failed to expand the uterine Treg cell pool and the elevation of Foxp3 mRNA level (Guerin et al., 2011). Mechanical stimulation failed to alter Treg cell proliferation, which demonstrates hormonal changes elicited by cervical stimulation are not sufficient, and reinforces the importance of seminal fluid exposure (Schumacher et al., 2007). More recent studies also confirm the expansion of Treg cells in PALN by exposure to intact seminal fluid, as well as the presence of seminal plasma (Teles et al., 2013, Shima et al., 2015). In addition, *in vitro* exposure to different concentrations of seminal plasma induced Treg cell populations to expand, but not conventional T cells (Teles et al., 2013). Taken together, these studies confirm that seminal fluid provokes the expansion of Treg cells in lymph nodes and uterus the early pregnancy.

Seminal fluid may be involved in expanding Treg cells indirectly via regulation of the phenotype of uterine antigen presenting cells. Seminal plasma has been shown to promote a tolerogenic phenotype in DCs *in vitro* (Remes Lenicov et al., 2012). Human monocytes co-cultured with a high dilution of seminal plasma were differentiated to DCs but failed to develop a mature phenotype in the presence of LPS (Remes Lenicov et al., 2012). Once these DCs were activated, they secreted high levels of anti-inflammatory cytokines, such as IL10 and TGF-β, and low level of pro-inflammatory cytokines, and a tolerogenic phenotype in DCs is found which indicates that seminal plasma may be involved in the regulation of Treg cell responses by promoting tolerogenic DCs. (Remes Lenicov et al., 2012),

1.3.4 microRNAs in the seminal fluid

As detailed above, seminal fluid regulates the female tract immune environment and contributes to the establishment of maternal immune tolerance through expansion of the Treg cell pool. Recently, non-coding RNAs, including miRNAs have been identified to be involved in the regulation of immunity

(Baltimore et al., 2008). Thus, we postulate that miRNA carried by seminal fluid or induced in females by seminal fluid are involved in the female immune response following coitus.

miRNAs are short non-coding RNAs, usually 22-25 nucleotides long, which undergo a series of maturation steps using endonucleases (Dorsha and Dicer), to attain functional competence. Once miRNAs are mature, they are incorporated into the RNA-induced silencing complex and then transport to the target site (Taganov et al., 2007). The most well-studied functions of miRNAs are the degradation of target mRNA (Dong et al., 2013) or the inhibition of translation process of target transcripts (Olsen and Ambros, 1999).

Interestingly, seminal fluid contains a large number of miRNAs (Krawetz et al., 2011). These are carried by sperm and also are present in exosomes in the plasma fraction of seminal fluid (Vojtech et al., 2014). Sperm miRNAs are delivered to the oocyte at fertilisation where they impact on embryo development and transgenerational inheritance of paternal epigenetic exposures (Fullston et al., 2013, Liu et al., 2012, Rodgers et al., 2015). Some immune-regulating miRNAs have been identified to be carried by sperm, such as miR-17, miR-19, miR-23b, miR-146a, miR-155, miR-223 and let-7c (Amanai et al., 2006, Nixon et al., 2015). This raises the question of whether miRNAs carried by sperm may target female immune pathways in the peri-conception period. The role of miRNAs in the regulation of immune responses and key miRNAs will be discussed in section 1.5.

1.4 miRNAs involved in the regulation of immune cells in the peri-conception period

There is growing evidence that specific miRNAs are involved in the regulation of functional capacity of immune cells (Baltimore et al., 2008). Using *Dicer* null mice, altered innate and adaptive immune responses could be observed when miRNAs are absent, with a systemic compromise in T cells (Taganov et al., 2007). Conditional depletion of Dicer in T cells causes reduced T cells in both the thymus and periphery (Cobb et al., 2006, Muljo et al., 2005). Ablation of Dicer in B cells resulted in a diminished B cell pool and compromised antibody-producing capacity (Koralov et al., 2008). Taken together, these observations show miRNAs are essential in various leukocytes that are crucial for regulation of the immune response.

There is evidence that endogenous miRNAs could be involved in the regulation of endometrial immune environment, but this has not been examined directly. Women who had altered immune-regulatory miRNA expression in the first trimester and pre-conception immune-regulatory miRNAs appear more likely to experience development of immune-associated pregnancy complications, such as preeclampsia and miscarriage (Winger et al., 2015).

miRNAs regulate genes and factors involved in the process of maternal immune tolerance (Schjenken et al., 2016). miRNAs such as miR-152 and miR-148a regulate HLA expression (Manaster et al., 2012).

Given the evidence showing that miRNAs differentially regulate immune responses, and since immune tolerance is critical in pregnancy success, it is possible that some specific miRNAs carried by seminal fluid or induced by seminal fluid in female tissues regulate the female immune environment in the peri-conception period, either directly in T cells or via regulating of antigen presenting cell phenotypes.

1.4.1 miRNAs and antigen presenting cells

As discussed in section 1.3.2.3, DCs are involved in the process of Treg cell proliferation and differentiation, and the phenotype of DCs is critical in the induction of Treg cell differentiation. miRNAs are known to regulate the function and phenotype of DCs and so could contribute to Treg cell pool expansion.

Specific miRNAs are involved in the differentiation of DCs from monocytes. Twenty miRNAs have been identified as differentially expressed in human monocyte-derived dendritic cells over a course of 5 days in culture (Hashimi et al., 2009). Amongst these 20 miRNAs, miR-21 and miR-34 were identified as the key to monocyte-derived dendritic cell differentiation via regulating the target gene *Wnt1* and *Jag1* (Hashimi et al., 2009). Inhibition of miR-21 and miR-34 or supplementation of exogenous Wnt-1 and Jagged-1 resulted in the dysregulation of DC-specific intercellular adhesion molecule-3 grabbing non-integrin /CD14 expression ratio, and disrupted endocytic function of immature DCs (Hashimi et al., 2009). Since endocytosis is critical in antigen presenting cell function (Burgdorf and Kurts, 2008), dysregulation of these miRNAs may disrupt the capacity of antigen presenting cells to take up male alloantigens and expression of co-stimulatory molecules which may in turn impact on the establishment of immune tolerance following coitus.

miR-155 is reported to play a critical role in maintaining optimal function of DCs (Rodriguez et al., 2007). miR-155 deficiency does not alter DC maturation as MHCII and co-stimulatory molecule expression was comparable in miR-155 deficient DCs, compared with WT DCs (Rodriguez et al., 2007). However, the antigen presenting capacity of miR-155 deficient DCs was approximately 2.5-fold lower compared to WT DCs, as the DC-specific intercellular adhesion molecule-3 grabbing non-integrin expression level was suppressed by PU.1, and these miR-155 deficient DCs failed to activate T cells effectively (Rodriguez et al., 2007). *In vitro* studies suggest miR-155 is also involved in DC maturation. miR-155 was consistently overexpressed in activated DCs after 6 hrs and 24 hrs of maturation (Stumpfova et al., 2014). Increased levels of miR-155 were correlated with accumulation of the key cell cycle inhibitor ($p27^{kip1}$) in DCs, and overexpression of miR-155 in DCs resulted in $p27^{kip1}$ elevation and DC apoptosis (Lu et al., 2011). In addition, miR-155 was induced in DCs after microbial stimulation, where it down-regulates the production of pro-inflammatory cytokines (Ceppi et al., 2009). Taken together, these data show that miR-155 plays an important role in the regulation of DC function in both innate and adaptive immunity.

miRNAs that promote a tolerogenic phenotype in DCs may also contribute to the establishment of maternal immune tolerance. Studies have identified specific miRNA profiles that distinguish immature, activated and tolerogenic DCs in humans, and show that miR-17, miR-133b, miR-203 and the miR-23b cluster are uniquely expressed in tDCs (Stumpfova et al., 2014). miR-23b has been demonstrated to function as a tolerogenic agent. miR-23b over-expressing mice showed delayed onset of collagen-induced arthritis and experimental autoimmune encephalomyelitis, as well as lower clinical score and incidence of collagen-induced arthritis and experimental autoimmune encephalomyelitis, compared with WT mice (Zhu et al., 2012). By suppressing IL17, TNF-α or IL1β-induced NF-κB activation and cytokine expression, miR-23b represses autoimmune inflammation (Zhu et al., 2012). In addition, an *in vitro* study revealed transfection of miR-23b into mouse DCs promoted a tolerogenic phenotype via targeting Notch 1 and the NF-κB pathway, with elevated IL10 production and the capacity to induce CD4+Foxp3+ Treg cell differentiation (Zheng et al., 2012).

miRNAs which regulate macrophage polarisation could be important for the success of pregnancy, as macrophage polarisation has a clear effect on inflammation (Liu and Abraham, 2013). Macrophages can be classified as M1-like macrophages (pro-inflammatory) or M2-like macrophages (anti-inflammatory). Between these two phenotypes, 109 miRNAs have been identified as being differentially expressed (Graff et al., 2012, Zhang et al., 2013). miR-155 expression is upregulated in M1 macrophages compared to M2 (Graff et al., 2012, Zhang et al., 2013), and promotes M1-like polarisation via suppressing suppressor of cytokine signalling 1 (Wang et al., 2010a). miR-223 deficient mice exhibit the pro-inflammatory M1 phenotype in macrophages (Zhang et al., 2013) while M2 polarisation can be promoted by let-7c (Zhuang et al., 2012), indicating that miR-223 and let-7c may promote M2 polarisation.

Considering the critical role of antigen presenting cell throughout gestation to support the success of pregnancy, it is clear that miRNAs with potential to induce an appropriate phenotype and polarisation of antigen presenting cells in early pregnancy need further investigation.

1.4.2 miRNA and Treg cells

To identify miRNAs that contribute to Treg cells that are critical in supporting gestation, the miRNA profile of Treg cells and conventional CD4+ T cells has been investigated (Cobb et al., 2006). Treg cells appear to have a miRNA profile distinct to conventional CD4+ T cells, with 35 miRNAs (including miR-146a, miR-155 and miR-223) being preferentially expressed and 33 miRNAs being down-regulated in Treg cells (Cobb et al., 2006). Interestingly, miR-223 was identified as the most upregulated miRNA in Treg cells, compared with conventional CD4+ T cells (Cobb et al., 2006). The relationship between miR-223 and Treg cells remains to be investigated as only one study has examined miR-223 expression

in Treg cells and showed a correlation with a reduced Treg cell population in pregnant women (Herberth et al., 2014).

One of the most well-studied miRNAs in Treg cells is miR-155. It has been revealed that miR-155 is a target of Foxp3, as Foxp3 binds to an intron in the DNA sequence encoding the miR-155 precursor mRNA BIC (Marson et al., 2007). During Foxp3+ thymocyte differentiation, the expression level of miR-155 is upregulated by Foxp3 (Lu et al., 2009b). In isolated Foxp3+ and Foxp3- thymocytes, miR-155 expression levels were found to be approximately 20-fold higher in Foxp3+ CD4+ single positive thymocytes compared to Foxp3- thymocytes. Additionally, disruption of Foxp3 expression in peripheral Treg cells leads to down-regulation of miR-155 as well as BIC mRNA (Lu et al., 2009b). Compared to the miR-155 sufficient controls, absence of miR-155 caused a decrease of Treg cell proliferative activity in the periphery (Lu et al., 2009b). When T cell depleted bone marrow was transferred into miR-155-deficient mice, a reduction in the proportion of Treg cells was observed on day 100 post bone marrow transfer, suggesting miR-155 is crucial in maintaining competitive fitness in lympho-replete mice (Lu et al., 2009b).

Unlike miR-155, miR-146a is reported to be indispensable for Treg cell-mediated suppression. In order to evaluate the role of miR-146a in Treg cell suppressive function, *Mirn-146a*$^{-/-}$/Foxp3KO mice with miR-146a null mutation specific to Foxp3+ Treg cells were generated. These mice developed immune-mediated pathologies, such as conjunctivitis, blepharitis and dermatitis. Interestingly, the Treg cell populations in these *Mirn-146a*$^{-/-}$/Foxp3KO mice were increased compared to *Mirn-146a*$^{+/+}$/Foxp3KO mice (Lu et al., 2010). Moreover, an increased Treg cell population was also observed in *Mirn-146a*$^{-/-}$/B6 mice, suggesting that miR-146a deficiency increases the Treg cell population. However, miR-146a deficient Treg cells failed to restrain activation of miR-146a sufficient T effector cells (Lu et al., 2010). Taken together, these data indicate miR-146a is important for Treg cells to maintain their suppressive function. Moreover, in human monocytes, it has been shown that miR-146a and miR-146b were both upregulated in a NF-κB dependent manner after challenge with LPS (Taganov et al., 2006).

Considering the essential role of seminal fluid in expanding the Treg cell pool in early gestation, more studies are required to address how specific miRNAs contribute to Treg cell responses during the peri-conception period.

1.5 IL10

Cytokines secreted by placental trophoblast and uterine cells are involved in the regulation of Th1 immune responses and act to limit inflammation in local tissues (Lin et al., 1993, Munoz-Suano et al., 2011, Szekeres-Bartho et al., 2009). Amongst these cytokines, IL10 has potent anti-inflammatory

properties. IL10 is produced by a range of different types of cells, including T cells, B cells, macrophages, DCs and Treg cells (Annacker et al., 2001).

IL10 stimulates and regulates a diverse range of cells, including DCs, natural killer (NK) cells, macrophages and T cells (Fiorentino et al., 1991a, Fiorentino et al., 1991b, Maynard and Weaver, 2008). IL10 inhibits antigen presentation, as well as the expression of MHCII and costimulatory molecules CD80/CD86 in DCs and the modification of DC phenotype ultimately regulates the activation of T cells (Mosser and Zhang, 2008). In addition, IL10 can induce the differentiation of Treg cells from naïve T cells (Nevers et al., 2011). IL10 may then be one important factor that regulates the response and phenotype of Treg cells during early pregnancy.

1.5.1 IL10 and Treg cells

IL10 is highly expressed in CD4+CD25+ T cells from 7-day-old mice, indicating that CD4+CD25+ T cells can secret IL10 (Asano et al., 1996). IL10 inhibited the production of pro-inflammatory cytokines such as IL17, IFN-γ, IL2 and TNF-α (Gu et al., 2008, Lochner et al., 2008), and also promoted T cell Foxp3 expression *in vitro* (Heo et al., 2010).

The feedback loop between IL10 and Treg cells is crucial in maintaining the Treg cell population. After repetitive stimulation with immature DCs, naïve CD4+ T cells differentiated to non-proliferating IL10 producing T cells, with upregulated expression of CTLA-4 (Jonuleit et al., 2000). When colitis was induced in IL10 and recombination-activating gene 1 (Rag1) double deficient mice, Treg cells failed to maintain their regulatory activity due to loss of Foxp3 expression (Murai et al., 2009), indicating IL10 is required in the presence of inflammation. In addition, IL10 receptor deficient mice also failed to maintain Foxp3 expression in Treg cells, indicating that IL10 acts directly on the Treg cells (Murai et al., 2009). Treg cells can partially develop pro-tolerance functions independently of IL10 (Maynard et al., 2007, Pillai et al., 2011, Rowe et al., 2011), indicating other tolerogenic mechanisms exist in addition to IL10 secretion. The effect of IL10 deficiency on Treg cells in pregnancy therefore warrants further investigation.

1.5.2 IL10 and pregnancy

The importance of IL10 during pregnancy has been addressed in several studies using murine models. Studies have revealed that absence of maternal IL10 did not impact on either syngeneic or allogeneic pregnancy progression (Rowe et al., 2011, Svensson et al., 2001, White et al., 2004). However, administration of low dose of LPS or other TLR ligands to IL10 null mice caused a greater degree of fetal loss, indicating that IL10 null mice are highly sensitive to inflammatory challenge (Murphy et al., 2005, Robertson et al., 2007). In addition, the importance of IL10 in gestation was addressed by the CBA/J x DBA/2 abortion-prone murine model. After transferring protective Treg cells into abortion-prone

mice, administration of neutralising IL10 antibody resulted in abrogating the protection from Treg cells, again indicating the pivotal role of IL10 for pregnancy tolerance (Schumacher et al., 2007).

As discussed earlier, an inappropriate amount or type of leukocytes and pro-inflammatory cytokines in the tissues at the maternal-fetal interface may result in preeclampsia. IL10, as a pivotal anti-inflammatory cytokine, has been studied for its role in normal pregnancy and preeclampsia. In IL10 null mice, exposure to 9.5% oxygen from DPC 7.5 until DPC 17 in a hypoxia-induced preeclampsia model resulted in preeclampsia-like symptoms, including hypertension, proteinuria and reduced fetal weights, compared with WT mice (Lai et al., 2011). In the hypoxia-induced preeclampsia model, IL10 null mice also appeared to have elevated soluble fms-like tyrosine kinase 1, compared with WT mice (Lai et al., 2011). Administration of recombinant IL10 from DPC 8 to DPC 16 resulted increased fetal weight, elevated blood pressure and restored proteinuria to normal levels, as well as reducing excess levels of soluble fms-like tyrosine kinase 1 (Lai et al., 2011). TLR-3 induced preeclampsia-like features were exacerbated in IL10 null mice and administration of exogenous IL10 showed beneficial effects on endothelial function (Chatterjee et al., 2011). Similar observations are found in pregnant hypertensive rats, where administration of IL10 can reverse hypertension and endothelial dysfunction (Tinsley et al., 2010). Taken together these studies suggest that IL10 could be one key target in preeclampsia diagnostics or for therapeutic treatment.

A reduced level of IL10 has been observed in preeclamptic women. Women experiencing preeclampsia showed a significant skewing from type 2 cytokines towards type 1 cytokines, and in particular a significant reduction in IL10 was observed in serum at the time of delivery (Borekci et al., 2007, Sharma et al., 2007a).

1.6 Peri-conception origin of preeclampsia

Altered maternal immune responses are associated with several pregnancy complications, such as recurrent pregnancy loss, infertility and preeclampsia. In particular, the origin of preeclampsia is not fully understood, and different types of preeclampsia may be caused by different factors. It is hypothesised that the peri-conception phase is critical in the origin of early onset preeclampsia (the most severe form of the syndrome) as compromised placental development is associated with preeclampsia development (Huppertz, 2008, Redman and Sargent, 2010, Steegers et al., 2010).

1.6.1 Immune responses during trophoblast cell invasion in normal pregnancies

In human pregnancy, appropriate placentation requires appropriate maternal decidual adaptation including adequate remodelling of uterine spiral arteries. Cells of the maternal immune system operate in a delicate balance to regulate the events of placental trophoblast invasion and uterine vascular remodelling (Lala and Chakraborty, 2003, Wells, 2007). T cells, Treg cells, macrophages, DCs, uterine

NK cells and other leukocytes presented in the decidua facilitate trophoblast cell migration and invasion (King, 2000, Trundley and Moffett, 2004, Williams et al., 2009). By controlling removal of native cells from the spiral artery, these leukocytes allow trophoblast cells to reach the endometrium (Lash et al., 2010, Nagamatsu and Schust, 2010). Treg cells and their secreted cytokines promote maternal immune adaption towards the semi-allogeneic fetus and prevent development of detrimental Th1 and Th17 immunity (Robertson, 2010). Treg cells also suppress pro-inflammatory cells to ensure the proper invasion (Williams et al., 2009, Somerset et al., 2004, Tilburgs et al., 2008). Together, the immune cells present in the decidua during early pregnancy regulate the immune response to ensure proper vascular remodelling (Amsalem et al., 2014, Jasper et al., 2011, Klauber et al., 1997, Woidacki et al., 2015) and trophoblast invasion (Lala and Chakraborty, 2003, Wells, 2007). The appropriate type and proper amount of facilitating immune cells are therefore key to healthy pregnancy, and disturbances in the immune response may result in compromised placental development. Compromised or incomplete placental development is believed to result in pregnancy complications, such as preeclampsia or miscarriage (Redman and Sargent, 2010, Veenstra van Nieuwenhoven et al., 2003).

1.6.2 Altered immune responses in women with preeclampsia

Disruption of placenta formation is generally characterised by inadequate remodel spiral arteries and failure to achieve high capacity maternal blood flow (Huppertz, 2008, Redman and Sargent, 2010, Steegers et al., 2010). In addition, placentas from women experiencing preeclampsia showed altered vasculature and excess inflammation, compared with placentas from healthy individuals (Roberts and Post, 2008, Roberts and Escudero, 2012). Some serum makers, such as placenta protein 13, long pentraxin 3 and pregnancy associated plasma protein-A, have been reported to be different in the first trimester of pregnancy (Burger et al., 2004, Cetin et al., 2006, Rovere-Querini et al., 2006, Spencer et al., 2007), and altered serum placenta protein 13 level was found as early as 7 to 8 weeks of gestation in women experiencing preeclampsia (Huppertz et al., 2008), which suggests preeclampsia (at least the early onset form) may develop during the earliest steps of development of the placenta.

Unlike normal pregnancy, women with preeclampsia show evidence of inappropriate immune responses and alternatively activated immune cells and cytokines (Freeman et al., 2004, LaMarca et al., 2013). An emerging consensus view is that insufficient trophoblast invasion resulting in placental ischemia is accompanied by, and probably secondary to imbalanced immune cell activation, in turn resulting in placental damage and excessive and chronic inflammation (Redman and Sargent, 2010, Cornelius et al., 2013, Irani et al., 2010). The chronic inflammation is caused by increased pro-inflammatory responses and cytokines, as well as diminished Treg cells and anti-inflammatory cytokines (LaMarca et al., 2013, Santner-Nanan et al., 2009, Prins et al., 2009, Wallace et al., 2011). These altered immune responses are increasingly viewed as causal, at least in a proportion of preeclampsia and related pregnancy

complications. Therefore, in order to understand the mechanisms of how preeclampsia is developed, it is important to understand how these immune cells are regulated in normal gestation and particularly the factors that regulate their origin and proliferation at the outset of pregnancy.

The role of prior contact with the conceiving partner's seminal fluid in reducing the incidence of preeclampsia has been observed in several clinical cohort studies. Preeclampsia is generally regarded as a disease of primiparous women, with second and subsequent pregnancies less susceptible than first pregnancies, while women who have a new conceiving partner have increased risk of pregnancy-induced hypertensive disorders, indicating that the benefit from prior seminal fluid contact is partner-specific (Dekker et al., 1998). In addition, the incidence of pregnancy-induced hypertension is inversely related to the duration of exposure to partner's seminal fluid (Dekker et al., 1998), which suggests that a sufficient peroid of sexual cohabitation and seminal fluid contact may be required to protect women from developing PE. An increased incidence of preeclampsia was also found in women who received donor gametes where prior exposure to gamete antigens in the context of seminal fluid did not occur (Salha et al., 1999), again consistent with seminal fluid exposure potentially providing protection in women from preeclampsia development. In the mouse model, seminal fluid is prominent in expanding Treg cell populations in early pregnancy, however, the benefit from repeated seminal fluid exposure and the mechanisms of partner-specificity have not yet been well investigated in mouse models.

1.6.3 Treg cells are deficient in preeclampsia

In women with preeclampsia, the Treg cell population is reduced in the peripheral blood and decidual tissues as compared to normal pregnant women, when evaluated before the onset of labour or caesarean section (Quinn et al., 2011, Santner-Nanan et al., 2009, Sasaki et al., 2007, Prins et al., 2009). These reduced Treg cells appeared to also have altered suppressive activities and phenotypes (Steinborn et al., 2008, Steinborn et al., 2012). The decidual Treg cell population is reduced in late-onset severe preeclampsia and further decreased in patients with early-onset severe preeclampsia (Quinn et al., 2011). In preeclamptic women, the Treg cell population in peripheral blood is reported to be even lower than non-pregnant women, and with a shift towards IL17 expressing phenotypes (Santner-Nanan et al., 2009, Tian et al., 2016). In addition, decreased decidual Treg cells were found to promote apoptosis in trophoblast cells which resulted in compromised trophoblast cell invasion into the decidua, which may increase the incidence of preeclampsia (Munoz-Suano et al., 2011).

The imbalance between Treg and Th17 cells associated with preeclampsia has been investigated. T cell dysfunction can trigger the development of preeclampsia, as the reduced placental blood flow, inadequate uterine spiral arterial modification and compromised fetal growth is observed in T cell deficient mice (Croy et al., 2011). In addition, in the reduced uterine perfusion pressure rat model of

preeclampsia, adoptive transfer of Treg cells from normal pregnant rats reversed preeclampsia-like symptoms via attenuating circulating pro-inflammatory cytokines IL17 and TNF (Cornelius et al., 2015).

1.7 Summary

There is growing evidence showing the importance of Treg cells in the establishment and maintenance of maternal immune tolerance towards the semi-allogeneic fetus to support pregnancy success. Clinical studies as well as experimental mouse models have demonstrated that an insufficient number of stable functional Treg cells is associated with pregnancy complications, such as preeclampsia. However, factors that govern the appropriate Treg cell responses remain poorly understood. In particular, how male alloantigens, the major anti-inflammatory cytokine IL10, and key microRNAs miR-155 and miR-223 impact on Treg cells, is of considerable interest.

The hypothesis to be investigated in this research thesis is that male alloantigens, miR-155, miR-223, and IL10 all regulate Treg cell expansion and stability directly or via regulating the functions and phenotypes of antigen presenting cells in the peri-conception period (Figure 1.1).

The experiments described in this thesis will address the involvement of male alloantigens and miR-155 and miR-223 in the regulation of Treg cell expansion and stability in early gestation. The role of IL10 in regulating Treg cell pool expansion, phenotype and pregnancy outcomes will be addressed in mid and late gestation.

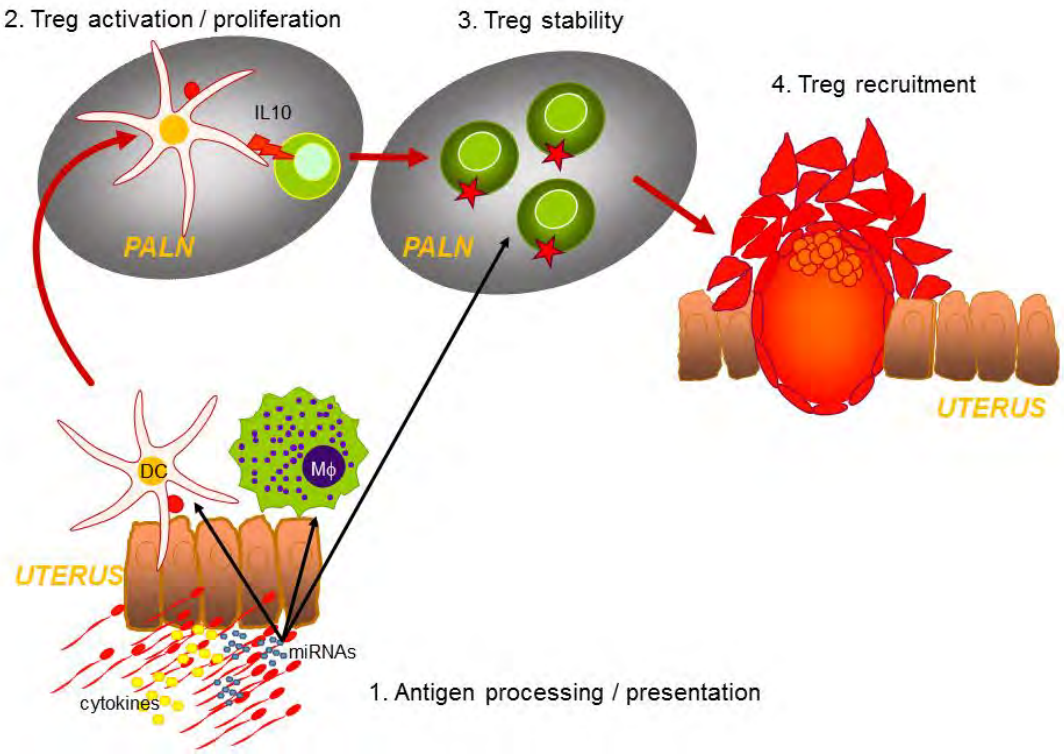

Figure 1.1 Current working model of Treg cell expansion post coitus. 1. Residential antigen presenting cells process male alloantigens carried by seminal fluid, and present antigens to resting Treg cells or naïve CD4+ T cells in the PALN. miRNAs carried by seminal fluid or induced by seminal fluid in the female reproductive tract may regulate the phenotype of antigen presenting cells or directly regulate Treg cell population. 2. Treg cells are activated and undergo proliferation and differentiation in the PALN, in the appropriate cytokine and miRNA environment. 3. The key cytokine IL10 is involved in regulating the size of the Treg cell pool, and Treg cell stability and phenotype. 4. The activated functional Treg cells are recruited to the uterus to inhibit Th1 and Th17 effector cells, to suppress excess inflammatory responses, and to support embryo implantation and placental development.

1.8 Hypotheses

This study will address the following hypotheses:

1. IL10 is critical for regulating the Treg cell pool and absence of IL10 can alter Treg cell phenotype in pregnancy;
2. Repeated exposure to seminal fluid acts to boost the Treg cell pool, progressively strengthening immune competence to sustain pregnancy, and
3. miR-155 and miR-223 induced by seminal fluid are additional regulators of the quality and strength of the Treg cell response in early pregnancy.

1.9 Research aims

The experiments in this study will utilise mouse models to address the following Aims:

1. To investigate the effect of IL10 on Treg cell number and phenotype in mid-gestation, using *Il10* null mutant mice;
2. To investigate whether repeated prior contact with the same male transplantation antigens impacts on the Treg cell population and stability in early pregnancy, and
3. To determine whether miR-155 and miR-223 influence Treg cell number and phenotype in early pregnancy, using *miR-155* and *miR-223* null mutant mice.

Chapter 2 Materials and Methods

2.1 Animals and treatments

2.1.1 Mice

All animal experiments were approved by the University of Adelaide Animal Ethics Committee and were conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (7th ed., 2004). All mice were maintained in specific pathogen-free conditions with controlled light (12 hours (hr) light, 12 hr dark cycle) and constant temperature (24 ^{o}C) at the Laboratory Animal Services Medical School facility. Food and water were provided ad libitum. All female mice utilised in these studies were 10-20 weeks old, and the male mice utilised as studs were between 10 weeks and 24 months old.

2.1.1.1 C57Bl/6 mice

For chapter 3 and 4, C57Bl/6 (B6) female mice (Harlan sub-strain) were purchased from Laboratory Animal Services at the University of Adelaide, South Australia. For chapter 5 and 6, B6 mice (Jackson sub-strain) were purchased from Animal Resource Centre, Western Australia.

2.1.1.2 Il10 null mice

Mice with genetic deficiency in IL10 were previously generated by targeted mutation of the *Il10* gene in 129/Ola embryonic stem cells, and then propagated on a B6 background and injection into B6 blastocysts (Kuhn et al., 1993b). *Il10* null mice were bred in-house at Laboratory Animal Services as homozygous breeding pairs and received broad-spectrum antibiotics (Oxymav 100: 100 g/kg oxytetracycline hydrochloride; Mavlab, Queensland, Australia) in autoclaved drinking water twice weekly at a concentration of 2 mg/mL to prevent auto-immune colitis.

2.1.1.3 miR-155 null mice

miR-155 null mutant mice were purchased from Jackson Laboratory (Bar Habour, ME, USA, Stock No: 007745 | bic/miR-155) and bred in-house at Laboratory Animal Services as homozygous breeding pairs. These mice were previously generated by replacing *bic/mir-155* gene with an in-frame β-galactosidase reporter gene (with polyA sequence and followed by a *loxP*-flanked neomycin resistance cassette) in F1H4 embryonic stem cells (derived from B6:129 hybrid mice), and chimeras were bred to B6 to establish *miR-155* null mice (Thai et al., 2007). *LoxP*-flanked selection cassettes were removed by crossing these mice with a cre-deleter strain (congenic B6 background), resulting in *bic/mir-155* mutant mice. These *miR-155* mutant mice were backcrossed to B6 for at least five generations.

2.1.1.4 miR-223 null mice

miR-223 null mice were purchased from Jackson Laboratory (Bar Habour, ME, USA, Stock No: 013198 | miR-223) and bred in-house at Laboratory Animal Services as homozygous breeding pairs. These mice were originally generated using a targeting vector designed to replace the entire coding region of the microRNA-223 (*mir-223*) gene with an *frt*-flanked neomycin resistance cassette. The construct was electroporated into (B6 x 129S4Sv/Jae) F1-derived V6. ES cells. Correctly targeted ES cells were injected into B6 blastocysts and the resulting chimeric males were bred to C57Bl/6 females to generate a colony of *miR-223* mice. These mice were backcrossed for at least 5 generations to B6. SJL-*Ptprc*^{a} *Pepc*^{b}/BoyJ and thus also harbor the CD45.1 (Ly5.1 or Ptprc^{a}) allele.

2.1.1.5 Balb/c mice

For chapter 3, 4, 5 and 6, Balb/c male mice were purchased from Laboratory Animal Services at The University of Adelaide, South Australia.

2.1.1.6 Balb/b mice

For chapter 4, Balb/b male mice were kindly provided by Rachel Kuns, QIMR Berghofer Medical Research Institute, Queensland.

2.1.2 Mating

Adult naturally cycling female mice were housed with a proven-fertile stud male. Female mice were checked each morning between 0900 h and 1100 h for the presence of a vaginal copulatory plug. The day a plug was observed was considered day 0.5 postcoitum (DPC), and female mice were separated from the studs.

2.1.3 RU486 treatment

Plugged B6 mice were given RU486 (Mifepristone, Sigma-Aldrich, Missouri, USA) to prevent embryo implantation in the multiple-mating study (Chapter 4). RU486 stock (8 mg/kg of RU486) was prepared in methanol and stored for a maximum of 1 week at -20 ^{o}C. On DPC 3.5, between 0900 h and 1100 h, female mice were injected subcutaneously with 8 mg/kg RU486 in sesame oil. The female mice were rested for at least 2 days after RU486 injection before being housed with stud males for further mating.

2.1.4 Estrus Cycle Tracking

In some experimental mice, the stage of the oestrus cycle was determined by analysis of wet mounts of vaginal lavage cells ('vaginal smears') as described previously (Byers et al., 2012). Vaginal smears were performed between 0830h and 1100h by lavage of the vagina with 20 µl sterile phosphate buffered saline (PBS). Smears were deposited on SuperFrost glass slides (HD Scientific Supplies Pty Ltd, NSW, Australia) and coverslipped. The cellular contents were analysed under a phase contrast

microscope (Olympus, Edwardstown, SA, Australia) to determine the stage of the oestrus cycle (Table 2.1). The oestrous cycle of all female mice was tracked for 7 days before mice were utilised in experiments.

Table 2.1 Oestrus stage determination by vaginal smears

Stage of the oestrus cycle	Cytology of cells in vaginal smears	Length of each stage
Proestrus	E or EC	24 h
Oestrus	EC+ or C+	12 h
Metestrus	C++ clumps or C+E L++	36 to 48 h
Diestrus	Low number of cells C-- L--	36 h

E= epithelial cells, C= cornified epithelial cells, L= leukocytes, + indicates normal level of cells, ++ indicates many cells

2.2 Microarray and real time PCR

2.2.1 Microarray

2.2.1.1 RNA Extraction

The gene expression profile of Treg cells from *Il10*+/+ or *Il10*-/- mice was determined by microarray. Treg cells from DPC 8.5-10.5 female mice were previously isolated from para-aortic lymph nodes (PALN) by MACS CD4+CD25+ Regulatory T cell Isolation Kit for mice (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instruction. Then cells were spun down to harvest cell pellets and then stored at -80 ^{o}C.

Treg cell RNA extraction was performed by using miRNeasy Mini Kits (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's instruction with minor amendments. After adding QiAzol lysis regent (700 μl), tubes were shaken vigorously for 10 seconds (s) to homogenise CD4+CD25+ T lymphocytes. The tubes containing homogenate were left on the bench top for 5 minutes (min) at room temperature (RT). Then chloroform (0.2 volumes) was added to the homogenate and shaken vigorously for 15 s. The tubes were incubated for 3 min at RT before centrifuging at 12,000 x g for 15 min at 4 ^{o}C. The upper aqueous phase was collected and 1.5 volume of 100% ethanol was added and samples were mixed thoroughly. The sample was then transferred into an RNeasy Mini spin column, centrifuged at 10,000 x g for 15 s, and the flow-through was discarded. Buffer RWT (500 μl) was added into the spin column, centrifuged at 10,000 x g for 15 s and the flow-through was discarded. This step was then repeated before the spin column was dried by centrifugation at 10,000 x g for 2 min. Treg cell RNA was then eluted with 30 μl of RNase free water.

The concentration and purity of each RNA sample was determined using a Nanodrop Spectrophotometer (ND-1000, Nanodrop Technologies Inc., Wilmington, DE). RNA quality was determined using an RNA 6000 Pico Total RNA Kit (Agilent Technologies, Santa Clara CA) at Adelaide Microarray Centre prior to use in microarray experiments. RNA with a RNA Integrity Number (RIN) >7 was used in this study.

2.2.1.2 Microarray analysis

For microarray analysis, RNA was pooled (from 2-4 mice per pool) resulting in four biological replicates of CD4+CD25+ T cells from both *Il10*-/- and *Il10*+/+ mice. Microarray analysis was performed using Affymetrix Mouse Gene 2.0 ST Arrays at the Adelaide Microarray Centre. Total RNA (1 ng) was amplified using the Ovation PicoSL WTA System V2 (Nugen Inc., San Carlos, CA, USA) and MinElute Reaction Cleanup Kit (Qiagen Inc., Valencia, CA, USA), according to the manufacturer's instructions, to provide 5 µg of cRNA for each microarray.

The microarray data were normalised and analysed using Partek Genomics Suite (Partek, Inc.). Raw data from the Affymetrix platform (cel files) were imported and normalised using RMA background correction, Partek's own guanine-cytosine content correction, and mean probe summarization. A threshold of either a greater than 1.4-fold change (low stringency) or a greater than 2.0-fold change (high stringency) was used to identify differentially expressed genes, with a false-discovery rate of $p<0.05$.

2.2.1.3 Reverse Transcription

Total cellular RNA (25 ng to 327 ng) was reverse transcribed from 125 ng random hexamer primed RNA from each of 8-12 individual *Il10*-/- and *Il10*+/+ mice employing a Superscript-III Reverse Transcriptase kit (Invitrogen, Scoresby, VIC, Australia) following the manufacturer's instructions (https://www.thermofisher.com/au/en/home/references/protocols/nucleic-acid-amplification-and-expression-profiling/pcr-protocol/superscript-3-one-step-rt-pcr-system-with-platinum-taq-high-fidelity.html).

2.2.1.4 Quantitative Real-Time PCR

Quantitative real-time PCR (qPCR) was used to confirm microarray data. qPCR was performed using 1 ng of cDNA, supplemented with 0.1-0.5 µM 5' and 3' primers (Table 2.2) and 1 x Power SYBR Green PCR Master Mix (Life Technologies, Applied Biosystems, CA, USA). The negative control included in each reaction contained H_2O substituted for cDNA. qPCR amplification was performed using an ABI Prism 7000 Sequence Detection System (Life Technologies, Applied Biosystems, CA, USA) using reaction conditions of 95 ^oC for 10 min, then 40 cycles of 95 ^oC for 15 s and 60 ^oC for 1 min. Target gene abundance was calculated using the Delta C(t) method normalizing target gene expression to the

Gmpr reference gene (Livak and Schmittgen, 2001). *Gmpr* was selected from a list of candidate reference genes (*Gmpr, Gapdh, Gm6578*) that were shown to be stable based on microarray data. These candidates were then assessed for their suitability using Normfinder (MOMA, Aarhus N, Denmark). From this, *Gmpr* was selected as the most stable reference gene.

2.2.1.5 Primer Design

2.2.1.5.1 Primers designed by Primer Express

Primer pairs specific for mRNA expression were designed using Primer Express version 2 software (Life Technologies, Applied Biosystems, CA, USA). Messenger RNA sequences were downloaded from the Entrez nucleotide database, accessible from the National Centre for Biotechnology Information website (http://www.ncbi.nlm.nih.gov). All primers were purchased from Geneworks (Adelaide, SA, Australia). A list of all primer sequences for target genes analysed, including product size and Genbank accession number are provided in Table 2.2.

2.2.1.5.2 Primer specificity and efficiency

Prior to using the primers to confirm microarray data, primer specificity was determined by gel electrophoresis (2% agarose gel; Promega, Madison, USA) of products generated by qPCR (see 2.2.1.4) and pUC 19 DNA/Hpa II DNA ladder (500 ng/μL, GeneWorks, Thebarton, SA, Australia) to confirm the correct product size. Analysis of the dissociation curve and gel electrophoresis was used to exclude the formation of primer dimers or non-specific products. PCR products were excised from the gel and purified using UltraClean® 15 DNA Purification Kit (MoBio, Carlsbad, CA, USA) according to the manufacturer's instruction (https://mobio.com/media/wysiwyg/pdfs/protocols/12100-300.pdf). The purified DNA samples were then sent to Australian Genome Research Facility for Sanger Sequencing (Adelaide, SA, Australia). The returned sequence result of each primer pair was confirmed with NCBI BLAST database.

Once it was determined that the primers were specific for the gene of interest, assay optimisation and validation experiments were performed to determine the amplification efficiency of each primer pair. Primer efficiency was determined by performing qPCR with a serial dilution (neat, 1/8, 1/64, 1/512 and 1/4096) of cDNA from murine PALN tissue. Then the amplification efficiency was calculated based on the equation: E = (10^ (-1/slope)-1) *100, where the slope was the standard curve of log10 of dilutions and corresponding CT value from each qPCR result. All primers were determined to have a correlation coefficient of >0.95 and an efficiency of between 90-110%.

Table 2.2 Primers designed by Primer Express for qRT-PCR

Gene	5'-3' Primer sequence	Concentration (μM)	Product size (bp)	Genebank Accession number
Ctla2a	5' CAGGAAGGAAAAGGCGGAG	0.25	51	NM_007796
	3' GGCTGGTGGTACTTGAGGAGAG			
Ctse	5' TGACCGTGGAAATAACCAAGTG	0.10	51	NM_007799
	3' CCTCTTTAGGGAACTGCGGG			
Gmpr	5' CAAAGTGGGAGTCGGACCAG	0.50	51	NM_025508
	3' CCTGTCTTGGTTCGGGTGG			
Ifng	5' GCGTCATTGAATCACACCTG	0.25	129	NM_008337.3
	3' TGAGCTCATTGAATGCTTGG			
Il1r1	5' CCCTGGCTTGTGTTACAGCA	0.25	52	NM_008362
	3' AATGTGGAGCCGCTGTGG			
Il12rb2	5' CTCTTTCCATTTTTGCATCAAGTTC	0.10	51	NM_008354
	3' CACCACCGAAGATGAGTGGG			
Il17a	5' CTGAGAGCTGCCCCTTCACT	0.25	51	NM_010552
	3' CCACACCCACCAGCATCTTC			
Gapdh	5' AGAGGCCCTATCCCAACTCG	0.25	91	XM_017321385
	3' TCCCTAGGCCCCTCCTGTTA			
Gm6578	5' AAAGGGCCTTCCTCCATGTG	0.25	122	NR_003631
	3' TGGTTCCTTGCCTAGGGCTT			

2.3 Fetal outcome assessment

Mated *Il10*+/+ and *Il10*-/- (chapter 3), B6 (chapter 4), *miR-155*+/+ and *miR-155*-/- (chapter 5) and *miR-223*+/+ and *miR-223*-/- (chapter 6) female mice were killed between 1100 h and 1400 h on DPC 17.5 by cervical dislocation. Uteri were examined for the presence of viable fetuses and females were classified as having 'viable pregnancy' (at least one viable fetus), or not. The whole uterus of each female was dissected and the total number, and number of viable and resorbing implantation sites were recorded. Each viable fetus was dissected from the amniotic sac and umbilical cord and fetuses and placentae were weighed.

2.3.1 Lipopolysaccharide (LPS) treatment

Female mice were weighed on DPC 0.5 and DPC 9.5 to determine whether they were pregnant. Females that had gained more than 2 grams on DPC 9.5, compared with DPC 0.5 weight, were

considered to be pregnant. *Il10*-/- and *Il10*+/+ females were administered with LPS (*Salmonella typhimurium*; 0.25 µg in 200 µl PBS with 0.1% bovine serum albumin (BSA); intra-peritoneally (i.p), Sigma-Aldrich) on DPC 9.5 to evaluate the impact of inflammatory challenge on pregnancy outcomes. *miR-155*-/-, *miR-155*+/+, *miR-223*-/- and *miR-223*+/+ females were administered with LPS from *E. coli* (*Escherichia coli*; 1.0 µg in 200 µl PBS with 0.1% BSA; i.p, Sigma-Aldrich) on DPC 9.5. For all experiments, the vehicle control was an equivalent volume of PBS with 0.1% BSA.

2.4 Flow Cytometry Analysis

Mesenteric lymph nodes (mLN), spleen, blood, PALN were collected from B6 (chapter 4), and mLN, spleen, blood, PALN and uterus were collected from *miR-155*+/+ and *miR-155*-/- (chapter 5) or *miR-223*+/+ and *miR-223*-/- (chapter 6) female mice on DPC 3.5 or at oestrus for flow cytometry analysis.

2.4.1 Blood collection

For anaesthesia, mice received an i.p. injection of 15 µl/g body weight of 2% Avertin (tribromoethanol; Sigma-Aldrich, St. Louis, USA). Blood was collected by cardiac puncture from the female mice after anaesthesia, and then lymphocytes were separated using Lympholyte®-Mammal Separation media (Cedarlane, Ontario, Canada). Blood was diluted with 1.5 mL RPMI 1640 (no phenol red) (Gibco®, Life Technologies, Scoresby, VIC, Australia), supplemented with 10% fetal bovine serum (FBS) (Gibco®, Life Technologies) and 1% penicillin/streptomycin (10,000 U/mL) (Gibco®, Life Technologies) (cRPMI) prior to underlying Lympholyte separation media (3 mL). Blood was centrifuged at 800 x g for 20 min at RT without brake. The well-defined lymphocyte layer (middle layer) was collected after centrifugation, and then washed in cRPMI for 800 x g for 10 min before flow cytometry staining.

2.4.2 Spleen collection

Spleens were harvested and homogenised by gently pressing the splenic tissue through a 70 µm nylon cell strainer (BD Falcon, Bedford, USA) using a 5 mL syringe plunger (BD, Bedford, USA). Cells were then filtered through the 70 µm nylon cell strainer and washed in cRPMI. Red blood cells (RBC) were lysed in 1x RBC lysis buffer (2 mL, 0.155 M NH_4Cl, 10 mM $KHCO_3$, 99.2 µM EDTA disodium salt in RO water) at RT for 15 s, and washed in cRPMI at 400 x g for 5 min before flow cytometry staining.

2.4.3 mLN and PALN collection

mLN and PALN were collected and homogenised by gently grinding the tissues between the frosted ends of two SuperFrost glass slides (HD Scientific Supplies Pty Ltd, NSW, Australia) in a 35 mm petri dish (BD Falcon, Bedford, USA). Cells were then filtered through a 70 µm nylon cell strainer and washed in cRPMI at 400 x g for 5 min before flow cytometry staining.

2.4.4 Uterus collection

Uterine tissue was harvested, placed in PBS and trimmed of fat, mesentery, and blood vessels under a dissection microscope (Olympus, New York, NY). Uterine horns were then finely chopped in 2 mL of RPMI 1640 supplemented with 2% FBS (Gibco, Life Technologies), 1 mg/mL collagenase *(Clostridium histolyticum,* Sigma-Aldrich) and amplification grade DNase I (4 unit/mL). Fragmented uterine tissues were incubated with regular shaking for 30 min at 37 ^{o}C. Digested cells were passed through a 70 μm cell strainer. Undigested tissues were ground between the frosted ends of two SuperFrosted glass slides (HD Scientific Supplies Pty Ltd, NSW, Australia), washed with RPMI 1640 supplemented with 10% FBS and passed through a 70 μm nylon cell strainer. To remove non-viable cells, FBS (2 mL) was carefully deposited in the bottom of the tube, and viable cells were collected as the pellet after centrifugation at 400 x g for 5 min without brake, before flow cytometry staining.

2.4.5 Cell count

For chapter 4, the total cell numbers from PALN, mLN spleen and blood were determined by using CountBright™ Absolute Counting Beads, for flow cytometry according to the manufacturer's instruction (https://tools.thermofisher.com/content/sfs/manuals/mp36950.pdf). For chapter 5 and 6, the total cell numbers from PALN, uterus, mLN, spleen and blood were determined by using a haemocytometer.

2.4.6 Cytokine stimulation assay

Half of the cells isolated from mLN, spleen, blood and PALN for experiments in chapter 4 were used for *in vitro* cytokine stimulation assays. The other half of the cells were stained for flow cytometry (described in 2.4.7). Cells for *in vitro* cytokine stimulation assays were initially resuspended in cRPMI, diluted in 0.4% trypan blue (Sigma-Aldrich, MO, USA) and counted using a haemocytometer. Viable cells were resuspended at 1.0 x 10^{7} cells/mL, and incubated with stimulation media (cRPMI with phornp; 12-myristate 13-acetate (PMA) (final concentration 0.1 μg/mL), ionomycin (final concentration 1.0 μg/mL), and monensin (eBioscience, final concentration 2 μM) at 37 ^{o}C with 5% CO_2 for 4 h. Then cells were harvested and washed in cRPMI at 400 x g for 5 min before flow cytometry staining.

2.4.7 Labelling of Single Cell Suspensions for Flow Cytometry

In this section, all centrifuge steps were at 300 x g for 5 mins at 4 ^{o}C unless otherwise stated. Single cell suspensions from mLN, spleen, blood, PALN and uterus were washed with 2 mL of fluorescence-activating cell sorting (FACS) buffer (0.1% BSA/0.5% Sodium Azide in 1 x PBS) and centrifuged. Cell viability was determined by adding 100 μL of 1/1000 fixable viability dye FSV620 (BD Pharmingen™, BD Biosciences, San Diego, USA) for 15 min at RT. Cells were then washed in 3 mL of PBS. Fc Receptors were blocked by resuspending cells in 50 μl of 1/100 anti-Fc-γIIR antibody (FcBlock, BD Pharmingen™) in FACS buffer for 10 min at 4 ^{o}C.

For Treg cell labelling, surface markers were labelled by adding 50 µl of an antibody master mix (Fluorescein isothiocyanate (FITC) conjugated anti-CD3 antibody (BD Pharmingen™); Phycoerythrin (PE) conjugated anti-CD4 antibody (BD Pharmingen™) and PE-Cyanine 7 conjugated anti-CD25 antibody (eBioscience, San Diego, USA)) (all antibodies diluted 1/100 in FACS buffer) for 15 min at RT (Table 2.3). Cells were then washed with 3 mL of FACS buffer and PBS followed by centrifugation. Following surface staining, the intracellular Treg cell marker Foxp3 was stained using the Foxp3/Transcription Factor Staining Buffer Set (eBioscience). Cells were initially permeabilised with 500 µl permeabilisation solution at 4 ^{o}C for 30 min and washed in permeabilisation buffer. To detect Treg cells, the cells were then incubated with Allophycocyanin (APC) conjugated anti-Foxp3 antibody (eBioscience) at 1/100 dilution in permeabilisation buffer for 30 min (Table 2.3). Cells were then washed in permeabilisation buffer (3 mL) and resuspended in PBS (300 mL) before FACS analysis.

For APC labelling, surface markers were labelled by adding 50 µl of an antibody master mix (APC-Cy7 conjugated anti-CD11b antibody (BD Pharmingen™); BV510 conjugated anti-CD11c antibody (BD Pharmingen™); PE conjugated anti-F4/80 antibody (BD Pharmingen™); Alex488 conjugated anti-I-A/I-E antibody (BD Pharmingen™); PE-Cyanine 7 conjugated anti-CD80 antibody (eBiosciences) and APC conjugated anti-CD86 antibody (eBiosciences)) (all antibodies diluted 1/100 in FACS buffer, except 1/50 dilution for F4/80) for 15 min at RT (Table 2.4). Cells were then washed in FACS buffer (3 mL) and resuspend in PBS (300 µL) before FACS analysis.

For cytokine stimulation assay, surface and intracellular markers for both unstimulated cells and stimulated cells were shown in Table 2.5. Surface markers were labelled by adding 50 µl of an antibody master mix (FITC conjugated anti-CD4 antibody (BD Pharmingen™) and PE-Cyanine 7 conjugated anti-CD25 antibody (eBioscience, San Diego, USA)) (all antibodies diluted 1/100 in FACS buffer) for 15 min at RT (Table 2.5). Cells were then washed with 3 mL of FACS buffer and PBS followed by centrifugation. For unstimulated cells, following surface staining, the intracellular Treg cell marker Foxp3 was stained using the Foxp3/Transcription Factor Staining Buffer Set (eBioscience). Cells were initially permeabilised with 500 µl permeabilisation solution at 4 ^{o}C for 30 min and washed in permeabilisation buffer. To detect Treg cells, the cells were then incubated with APC conjugated anti-Foxp3 antibody (eBioscience) at 1/100 dilution in permeabilisation buffer for 30 min (Table 2.5). Cells were then washed in permeabilisation buffer (3 mL) and resuspended in PBS (300 mL) before FACS analysis. For stimulated cells, following surface staining, cells were incubated in 50 µl of an antibody master mix to detect intracellular cell marker Foxp3 and Il17 (APC conjugated anti-Foxp3 antibody (eBioscience) and PE conjugated anti-IL17A antibody) (all antibodies diluted 1/100 in permeabilisation buffer). Then cells were washed and resuspended in PBS (300 µl) before FACS analysis.

Data were analysed using a FACS Canto II analyser (BD Biosciences, San Jose, USA) and FACS Diva software (version 6.0, BD Biosciences). Gates were applied to the forward scatter/side scatter dot plots to exclude cell debris from analysis. The FACS gating strategies are described in each results chapter.

Table 2.3 Monoclonal antibodies used in flow cytometric analysis of Treg cells

mAb	Conjugate	Clone	Source
CD3	FITC	17A2	BD Pharmingen™
CD4	PE	H129.19	BD Pharmingen™
CD25	PE-Cy7	PC61.5	eBioscience
FOXP3	APC	FJK-16S	eBioscience

Table 2.4 Monoclonal antibodies used in flow cytometric analysis of antigen presenting cells

mAb	Conjugate	Clone	Source
CD11b	APC-Cy7	M1/70	BD Pharmingen™
CD11c	BV510	HL3	BD Pharmingen™
F4/80	PE	T45-2342	BD Pharmingen™
I-A I-E (MHC II)	ALEX488	M5/144.15.2	BD Pharmingen™
CD80	PE-CY7	16-10A1	eBioscience
CD86	APC	GL1	eBioscience

Table 2.5 Monoclonal antibodies used before and after *in vitro* stimulation in flow cytometric analysis

mAb	Conjugate	Clone	Source
CD4	FITC	GK 1.5	BD Pharmingen™
CD25	PE-Cyanine7	PC 61.5	BD Pharmingen™
FOXP3	APC	FJK-16S	eBioscience
IL17A	PE	eBio17B7	eBioscience

2.5 Statistical Analysis

Data were assessed for normal distribution with a Shapiro-Wilk normality test using GraphPad Prism v6 (GraphPad software Inc, San Diego, USA) or SPSS Statistics Version 20.0 (IBM Corporation, Armonk, NY, USA). The effect of maternal genotype on % viable pregnancy (number of females classified as 'viable pregnancy', as percentage of total mated mice) was analysed by Chi-square analysis. The effect of maternal genotype on resorption rate and viable fetus per litter in chapter 3, 5 and 6 was analysed by unpaired T-test, or one-way ANOVA and Sidak T test. The effect of maternal genotype on fetal weight, placental weight and fetal:placental weight ratio in chapter 5 and 6 was analysed by mixed model analysis, with mother as subject, and litter size (total number of implantations) as covariate when identified as having a significant effect (as specified in Figure legends). If data were not normally distributed, data were analysed using Kruskal-Wallis H test and Mann-Whitney U test. Data are presented as the mean ± SEM (standard error of mean). The difference between groups was considered statistically significant if $p<0.05$. In some cases, superscript letters (a, b and c) were used to indicate differences between treatment groups. In other cases, an asterisk (*) identifies a treatment or genotype that differs significantly from the control.

Chapter 3 An Altered T Regulatory Cell Gene Expression Profile is Associated with Lipopolysaccharide Induced Fetal Loss in Pregnant IL10 Deficient Mice

The data in this chapter has contributed to a publication entitled “Unstable Foxp3^{+} regulatory T cells and altered dendritic cells are associated with lipopolysaccharide-induced fetal loss in pregnant interleukin 10-deficient mice”. For full detail of the publication, please see section 8.4.

3.1 Abstract

Maternal immune tolerance of the semi-allogeneic fetus requires CD4+Foxp3+ T Regulatory (Treg) cells, which suppress inflammation and anti-fetal immunity. Prominent among the regulatory cytokines that contribute to the establishment of tolerance is interleukin-10 (IL10). IL10 deficiency is known to elevate susceptibility to fetal loss in syngeneic and allogeneic pregnancies, but the mechanisms are not well elucidated. In this study, we assessed the specific contribution of maternal IL10 in the maintenance of a tolerogenic immune environment and examined the impact of IL10 deficiency on the Treg cell transcriptome. To evaluate the impact of maternal IL10 deficiency on susceptibility to fetal loss, *Il10*-/- or *Il10*+/+ females were mated with Balb/c males, pregnant females were administered LPS (0.25 μg) or PBS control on day 9.5 postcoitum (DPC), and pregnancy outcomes were determined on DPC 17.5. *Il10*-/- mice mated to Balb/c were more susceptible to fetal loss when challenged with LPS, with a lower proportion of *Il10*-/- mice carrying viable fetuses (33% in *Il10*-/- and 91% in *Il10*+/+ mice, p=0.019) and a higher rate of fetal resorption in *Il10*-/- mothers (68% in *Il10*-/- and 20% in *Il10*+/+ mice, p=0.03). In a separate cohort, *Il10*-/- or *Il10*+/+ CD4+CD25+ T cells were isolated from para-aortic lymph nodes (PALN) of pregnant mice on DPC 9.5-11.5, total RNA was extracted, and Affymetrix microarray and qPCR were conducted to evaluate the Treg cell gene expression profile. Affymetrix microarray revealed an altered transcriptional profile in Treg cells from pregnant *Il10*-/- mice, with elevated expression of the immune associated genes, *cathepsin E* (*Ctse*), *Il1r1*, *Il12rb2* and *Ifng*. In conclusion, the absence of maternal IL10 plays a crucial role in protecting against inflammation induced fetal loss in pregnancy and further impacts the gene expression profile in Treg cells. These findings suggest a pivotal role for IL10 in facilitating robust immune protection of the fetus from inflammatory challenge and suggest IL10 deficiency could contribute to human gestational disorders where altered T cell responses are implicated.

3.2 Introduction

Inheritance by the conceptus of maternal and paternal transplantation antigens results in the fetus being a semi-allogeneic challenge to the mother. Substantial adaptations in the maternal immune system are required to tolerate the fetus and suppress deleterious inflammatory responses that cause gestational disorders and fetal demise. Secretion of immune-regulatory cytokines and hormones by placental trophoblasts and uterine cells (Lin et al., 1993, Munoz-Suano et al., 2011, Szekeres-Bartho et al., 2009) constrain inflammation and limit type 1 immunity, particularly in the gestational tissues and local lymph nodes. Prominent amongst these cytokines is IL10 (Lin et al., 1993, Thaxton and Sharma, 2010), a cytokine that stimulates and reinforces modifications to the innate and adaptive immune system, including induction of regulatory phenotypes in the dendritic cell (DC), macrophage and T lymphocyte compartments (Fiorentino et al., 1991a, Fiorentino et al., 1991b, Maynard and Weaver, 2008).

Experiments in *Il10* null mutant (*Il10*-/-) mice demonstrate that in the absence of maternal IL10, neither allogeneic nor syngeneic pregnancies are compromised (Rowe et al., 2011, Svensson et al., 2001, White et al., 2004). However pregnant *Il10*-/- mice are highly vulnerable to inflammatory challenge, with administration of low dose LPS or other Toll-like receptor (TLR) ligands causing elevated rates of fetal resorption (miscarriage) (Murphy et al., 2005, Robertson et al., 2007) or preterm delivery (Robertson et al., 2006, Thaxton et al., 2009) in syngeneic matings, depending on gestational timing of the insult.

The mechanisms through which IL10 controls inflammatory mediators have not been defined, although uterine natural killer (uNK) cells and tumour necrosis factor (TNF) appear to be involved in promoting fetal loss when pregnant *Il10*-/- mice are challenged (Murphy et al., 2005). We postulate that in the absence of IL10, anti-inflammatory mechanisms protecting the fetus are compromised. One of the key leukocyte populations likely to be affected by absence of IL10 is T regulatory (Treg) cells, a subset of anti-inflammatory and immune suppressive CD4+ T lymphocytes, defined by their expression of the transcription factor fork-head box P3 (Foxp3) (Fontenot et al., 2005, Fontenot et al., 2003). The critical role of Treg cells in limiting inflammation and mediating immune tolerance is demonstrated in *Foxp3* null mutant mice (*Scurfy* mice) that develop a lethal multi-organ lymphoproliferative disorder (Brunkow et al., 2001). IL10 is implicated in the generation of Treg cells and is prominent in aspects of their suppressive function (Maynard and Weaver, 2008, Shevach, 2009), including in pregnancy where neutralising IL10 abrogates the protective effects of Treg cells in a murine abortion model (Schumacher et al., 2007). IL10 also influences macrophages and DCs, which have important roles in sustaining pregnancy independently of T cells (Erlebacher, 2013) as well as through specific antigen-presenting and immune-regulatory functions including control of the generation of induced Treg cells from naïve T lymphocyte precursors (Maynard and Weaver, 2008). During murine pregnancy, Treg cells are elevated in the gestational tissues and systemic circulation by approximately 50% in mid-gestation (Kallikourdis et al., 2007, Rowe et al., 2011) and similar changes are evident in pregnant women (Saito et al., 2005, Santner-Nanan et al., 2009). Experiments in Treg cell depleted mice (Aluvihare et al., 2004, Zhao et al., 2007) and abortion-prone mice (Zenclussen et al., 2005) show these cells are essential for establishing allogeneic pregnancy. Their immune regulatory actions are most crucial around the time of embryo implantation, when their abundance is a limiting factor in implantation and placental development (Shima et al., 2010). Experiments using tetramers demonstrate the majority of the maternal Treg cells which expand in pregnancy are fetal antigen-specific (Rowe et al., 2011), and dependence on the CNS1 regulatory region in the Foxp3 gene confirms this is at least partly the result of extrathymic generation of inducible (peripheral) Treg cells responding to fetal alloantigen (Samstein et al., 2012) after initial priming in response to paternal seminal fluid at conception (Robertson et al., 2009a). Treg cells have recently been shown to protect the fetus from preterm delivery induced by the Toll-like receptor (TLR) 4

ligand bacterial LPS (Bizargity and Bonney, 2009) and fetal loss induced by the TLR9 ligand DNA motif CpG (Lin et al., 2014). Although IL10 is important in Treg cell suppressive function, Treg cells can develop and execute at least some pro-tolerance functions independently of IL10 (Maynard et al., 2007, Pillai et al., 2011, Rowe et al., 2011).

We have recently demonstrated that *Il10*-/- mice exhibit altered local T cells responses, with a pronounced hyperplasia in PALN draining the uterus and a greater than 6-fold increase in CD4+ and CD8+ T cells observed (Prins et al., 2015). Amongst the increased CD4+ T cells, Treg cells were found to be substantially enriched, with 11-fold higher numbers at DPC 9.5 in *Il10*-/- mice (Prins et al., 2015). Moreover, more activated phenotypes of DCs and macrophages were found in the *Il10*-/- mice during pregnancy (Prins et al., 2015). Despite the increased numbers of IL10-/- Treg cells, these cells expressed low Foxp3 and had impaired suppressive capacity (Prins et al., 2015). Given the significance of Foxp3 as a master regulator of Treg cell function (Yagi et al., 2004, Fontenot et al., 2003), this raises the question of whether IL10 deficiency may impact on the molecular composition of the Treg cells during pregnancy and contribute to facilitating robust immune protection of the fetus. In this study we utilised *Il10* null mutated mice to address the role of IL10 in the protecting fetus from inflammatory insults and the gene expression profile in the Treg cell population.

3.3 Results

3.3.1 Effect Maternal IL10 Deficiency Elevates LPS-Induced Fetal Loss

Previously, we reported that the *Il10* null mutation causes elevated fetal loss when pregnant mice carrying syngeneic IL10-deficient fetuses are administered low-dose LPS (Robertson et al., 2007). To investigate the contribution of maternal IL10 to this increased susceptibility, *Il10*-/- and *Il10*+/+ B6 females were mated with *Il10*+/+ Balb/c males to generate allogeneic pregnancies with maternal, but not fetal IL10 deficiency. On DPC 9.5, pregnant females were administered LPS at a low dose (0.25 μg/mouse) identified previously to induce fetal loss in pregnant *Il10*-/- mice but not in *Il10*+/+ controls (Robertson et al., 2007). In mice examined just before term on DPC 17.5, *Il10*-/- mice were more severely affected by LPS treatment than control mice, with a lower proportion of *Il10*-/- mice carrying viable fetuses at DPC 17.5 (33% in *Il10*-/- vs 91% in *Il10*+/+ mice, $p=0.019$) (Figure 3.1A). Furthermore, LPS caused a higher rate of fetal resorption in *Il10*-/- mothers (68% in *Il10*-/- vs. 20% in *Il10*+/+ mice, $p=0.030$) (Figure 3.1B), resulting in significantly fewer viable fetuses per mated females (2.57 viable fetuses in *Il10*-/- vs 5.91 fetuses in *Il10*+/+, $p<0.05$) (Figure 3.1C). No effects of genotype or LPS treatment on fetal weight, placental weight, or fetal weight: placental weight ratio were seen (Figure 3.1D, E, and F). Thus, elevated fetal loss in *Il10*-/- mothers can be largely attributed to maternal, as

opposed to fetal, IL10 deficiency, implicating a defect in the maternal immune adaptation protecting pregnancy.

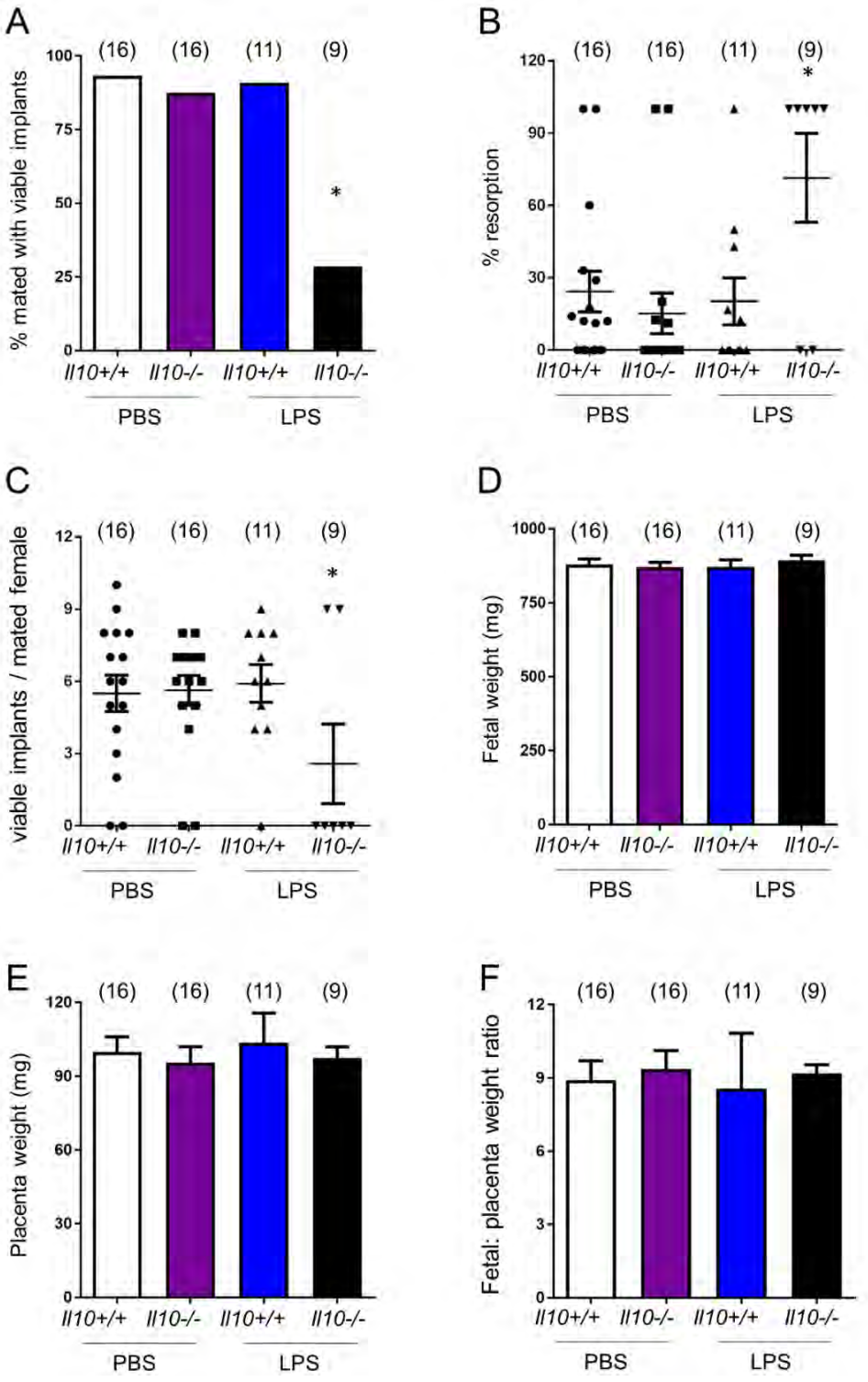

Figure 3.1 The effect of *Il10* null mutation on pregnancy parameters after low-dose LPS challenge. *Il10*+/+ and *Il10*-/- B6 mice were mated with Balb/c males and injected i.p. with LPS (1μg) or control (PBS) on DPC 9.5, then autopsied on DPC 17.5. Data are the percentage of mated females pregnant with viable fetuses (A); the

percentage of total implantation sites per pregnant female undergoing resorption (individual data points with mean ± SEM; B), the number (mean ± SEM) of viable implantation sites per mated mouse (individual data points with mean value shown; C), the fetal weight (estimated marginal mean ± SEM; D), the placental weight (estimated marginal mean ± SEM; E), and the fetal: placental weight ratio (estimated marginal mean ± SEM; F). Numbers of mated mice are shown in parentheses. The effect of genotype was evaluated in (A) by Chi-square analysis and in (B) by ANOVA and Sidak t-test. The effect of genotype was evaluated in (D-F) by mixed model analysis (*P<0.05 compared with *Il10*+/+ group at same LPS dose).

3.3.2 IL10 Deficiency Alters Gene Expression Profile in Treg Cells

Treg cells from *Il10*-/- mice exhibited reduced Foxp3 expression and functional capacity (Prins et al., 2015) which raised the question of whether Treg cells have an altered genotype in pregnant *Il10*-/- mice. To examine this, we performed a microarray experiment using Affymetrix microarrays on CD4+CD25+ T cells isolated by magnetic cell sorting from PALN of *Il10*-/- and *Il10*+/+ mice on DPC 9.5. Four biological replicates of CD4+CD25+ T cell RNA (each pooled from two or three different mice) from each genotype were reverse transcribed into cDNA and hybridised to Affymetrix Mouse Gene 2.0 ST Arrays. Principal component analysis showed clustering of cDNAs according to genotype (Figure 3.3A). A total of 52 probe sets were classified as highly differentially expressed with high-stringency criteria (fold change >2.0, P<0.05) between genotypes, with 45 genes upregulated and 7 genes downregulated (Figure 3.3B). Of the genes upregulated in Treg cells from *Il10*-/- mice, seven have potential relevance to Treg cell function, identified by Ingenuity Pathway Analysis and literature searches as linked with cytokine-cytokine receptor interactions (*Il10*; upregulated 2.1-fold), maintenance of Treg cell function (*Ctse*, *Lilrb4*, and *Slpi*; upregulated 7.2-, 2.1-, and 2.7-fold, respectively), and loss of Treg cell function (*Cd24a*, *Ighm*, and *Igj*; upregulated 2.0-, 10.1-, and 3.1-fold, respectively) (Table 3.1). Using a low-stringency analysis (fold-change >1.4, P<0.1), a total of 299 genes were classified as moderately differentially regulated by IL10 deficiency, including 247 that were upregulated and 52 that were downregulated in *Il10*-/- compared to *Il10*+/+ Treg cells. Of these, 47 were identified as associated with cytokine signalling or Treg cell stability and function, with a total of 42 upregulated and 5 downregulated in *Il10*-/- compared to *Il10*+/+ CD4+CD25+ T cells (Table 3.2). Several genes of interest were independently quantified by qPCR in CD4+CD25+ T cells from PALN of pregnant *Il10*+/+ and *Il10*-/- mice on DPC 9.5. In the absence of IL10, increases were detected in expression of *Ctse* encoding the intracellular proteinase cathepsin E (155-fold, P<0.0001) (Figure 3.3A), *Ctla2* encoding the cytotoxic T lymphocyte associated protein 2 complex (CTLA2) (4.4-fold, P<0.01) (Figure 3.3B), *Il1r1* encoding the IL1 receptor type 1 (10.4-fold, P<0.05) (Figure 3.3D), and *Ifng* encoding the Th1 cytokine IFNG (6.3-fold, P<0.05) (Figure 3.3E). A trend to increased expression was seen in Il12rb2 encoding the IL12 receptor beta 2 subunits (8.3-fold, P=0.076) (Figure 3.3F). No consistent change was seen in expression of other genes identified as moderately differentially regulated in the microarray, including Il17a (Figure 3.3C).

Table 3.1 Genes of interest identified as highly differentially expressed using high-stringency criteria in Affymetrix microarray analysis of mRNA expression in purified Treg cells from *Il10*+/+ or *Il10*-/- mice.

Accession #	Gene Symbol‡	WT mean	KO mean	Difference	Fold-change	P value
Cytokine - Cytokine Receptor Interactions						
NM_010548	*Il10**	275.21	575.51	300.30	2.09	0.0087
Maintenance of Treg function (immune tolerance)						
NM_007799	*Ctse*	454.62	3256.71	2802.09	7.16	0.0000
NM_013532	*Lilrb4*	396.62	849.90	453.28	2.14	0.0483
NM_011414	*Slpi*	248.11	680.08	431.97	2.74	0.0463
Loss of Treg function (inflammation and apoptosis)						
NM_009846	*Cd24a*	614.44	1772.60	1158.16	2.88	0.0302
BC053409	*Ighm*	26.46	266.33	239.87	10.06	0.0239
NM_152839	*Igj*	151.28	471.01	319.73	3.11	0.0191

*High stringency criteria: Fold-change >2.0, p<0.05, Difference between means >100.

‡ All genes listed are upregulated in *Il10*-/- mice compared to *Il10*+/+ mice.

Table 3.2 Genes of interest identified as moderately differentially expressed using low stringency criteria in Affymetrix microarray analysis of mRNA expression in purified Treg cells from Il10 +/+ or Il10 -/- mice.

Accession #	Gene Symbol	WT mean	KO mean	Difference	Fold-change	P value
Upregulated in *Il10*-/- compared to *Il10*+/+						
Cytokine - Cytokine Receptor Interactions						
ENSMUST00000168841	*Ccr2*	1140.44	1733.71	593.28	1.52	0.0027
NM_008337	*Ifng*	161.95	228.54	66.59	1.41	0.0017
NM_010548	*Il10*	275.21	575.51	300.30	2.09	0.0087
NM_008354	*Il12rb2*	155.63	235.86	80.22	1.52	0.0049
NM_010552	*Il17a*	112.80	167.19	54.39	1.48	0.0699
NM_008362	*Il1r1*	445.15	834.33	389.18	1.87	0.0475
NM_010555	*Il1r2*	616.30	865.09	248.79	1.40	0.0912
Maintenance of Treg function (immune tolerance)						
NM_007796	*Ctla2a*	566.15	984.66	418.51	1.74	0.0065
NM_001145801	*Ctla2b*	75.99	162.47	86.49	2.14	0.0114
NM_007799	*Ctse*	454.62	3256.71	2802.09	7.16	0.0000
ENSMUST00000102894	*Gp49a*	62.06	199.23	137.17	3.21	0.0727

NM_013532	*Lilrb4*	396.62	849.90	453.28	2.14	0.0483
NM_134250	*Havcr2*	89.47	162.57	73.10	1.82	0.0460
ENSMUST00000112063	*Klrd1*	134.36	226.54	92.18	1.69	0.0538
ENSMUST00000040250	*Kmo*	125.79	192.92	67.13	1.53	0.0361
NM_001168392	*Ramp1*	254.87	361.58	106.71	1.42	0.0047
NM_011414	*Slpi*	248.11	680.08	431.97	2.74	0.0463
Loss of Treg function (Inflammation and Apoptosis)						
ENSMUST00000071130	*Alox5ap*	103.56	162.98	59.42	1.57	0.0524
NM_019735	*Apip*	197.34	300.70	103.36	1.52	0.0952
NM_001081001	*Brca2*	150.40	212.02	61.62	1.41	0.0185
NM_001167949	*Atp2b4*	346.07	521.67	175.60	1.51	0.0082
NM_001113179	*Bub1*	217.26	359.51	142.25	1.65	0.0306
ENSMUST00000040182	*Ccdc88a*	183.33	258.63	75.30	1.41	0.0388
NM_009846	*Cd24a*	614.44	1772.60	1158.16	2.88	0.0302
NM_023223	*Cdc20*	257.37	369.84	112.47	1.44	0.0792
NM_007793	*Cstb*	800.50	1149.87	349.37	1.44	0.0099
ENSMUST00000113480	*Cysltr1*	156.41	255.65	99.24	1.63	0.0132
NM_133720	*Cysltr2*	442.39	630.91	188.52	1.43	0.0779
NM_001172092	*Depdc1a*	87.18	181.70	94.52	2.08	0.0314

NM_013542	*Gzmb*	275.27	679.82	404.55	2.47	0.0955
NM_008252	*Hmgb2*	1046.23	1474.18	427.95	1.41	0.0202
NM_013552	*Hmmr*	165.16	265.01	99.85	1.60	0.0564
NM_008287	*Hrsp12*	265.76	459.97	194.21	1.73	0.0516
NM_008297	*Hsf2*	265.12	375.02	109.90	1.41	0.0011
NM_028680	*Ift57*	253.93	392.46	138.53	1.55	0.0030
BC053409	*Ighm*	26.46	266.33	239.87	10.06	0.0239
NM_152839	*Igj*	151.28	471.01	319.73	3.11	0.0191
NR_029853	*Mir363*	18.77	69.03	50.26	3.68	0.0669
ENSMUST00000034065	*Nek1*	206.78	291.49	84.71	1.41	0.0304
NM_016764	*Prdx4*	207.89	332.82	124.93	1.60	0.0140
NM_009185	*Stil*	122.78	192.02	69.24	1.56	0.0369
NM_011623	*Top2a*	994.79	1517.60	522.81	1.53	0.0166

Downregulated in *Il10-/-* compared to *Il10+/+*

Cytokine - Cytokine Receptor Interactions

NM_009835	*Ccr6*	738.72	517.30	-221.42	-1.43	0.0039
NM_008348	*Il10ra*	579.48	391.00	-188.48	-1.48	0.0775
NM_019583	*Il17rb*	508.26	317.06	-191.20	-1.60	0.0377

Maintenance of Treg function (immune tolerance)						
NM_011267	*Rgs16*	770.78	516.25	-254.53	-1.49	0.0749
AK042280	*Slamf6*	428.06	266.81	-161.25	-1.60	0.0686

Stringency criteria: Fold-change >1.4, p<0.1, Difference between means >50.

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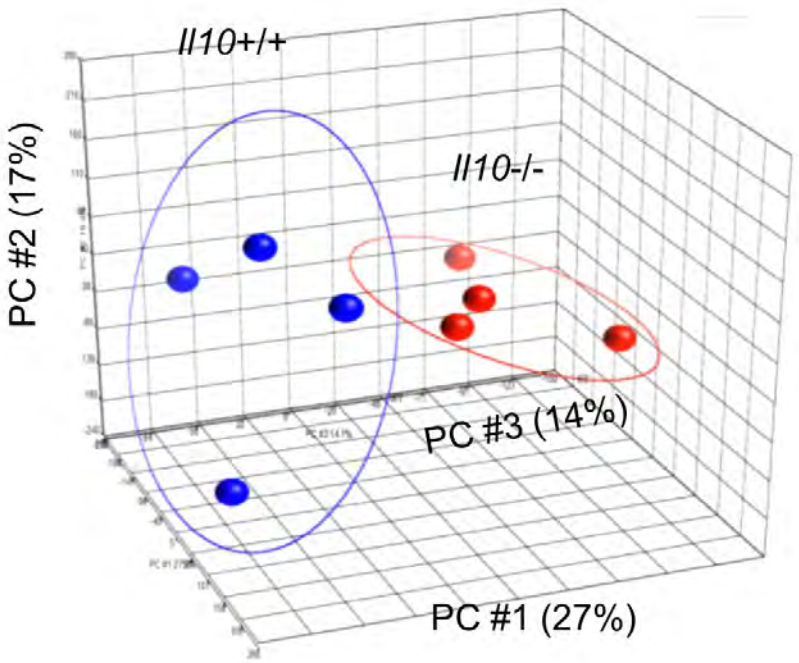


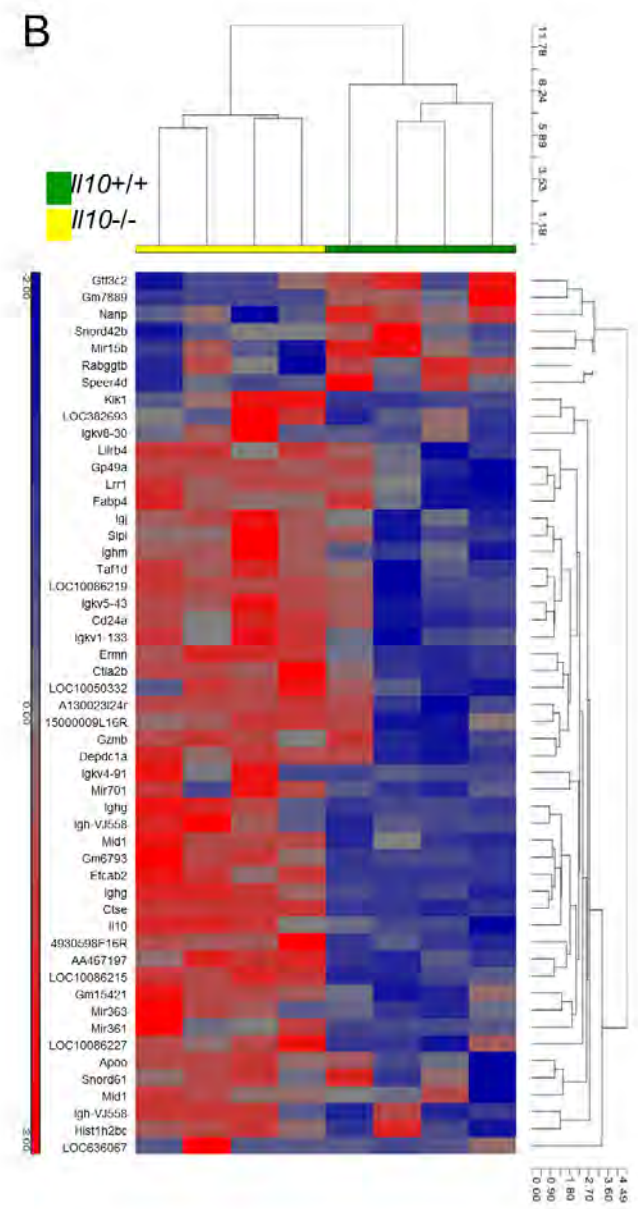


Figure 3.2 Microarray analysis showing the effect of *Il10* null mutation on the gene expression profile in CD4+CD25+ Treg cells. Treg cells were isolated from PALN on day 9.5 pc from *Il10*+/+ and *Il10*-/- mice mated with Balb/c males. RNA was extracted and gene expression measured by Affymetrix Mouse Gene 2.0 ST arrays (n = 4 biological replicated pooled from n=2-4 mice from a total of 12 samples per genotype). Microarray data was analysed by Partek Genomics Suite to generate (A) principal component analysis, blue dots represents *Il10*-/- and red dots represent *Il10*+/+ cells, and (B) heat map data for a total of 52 probe sets classified as highly differentially expressed with high stringency criteria (fold change >2.0, p<0.05) between genotypes.

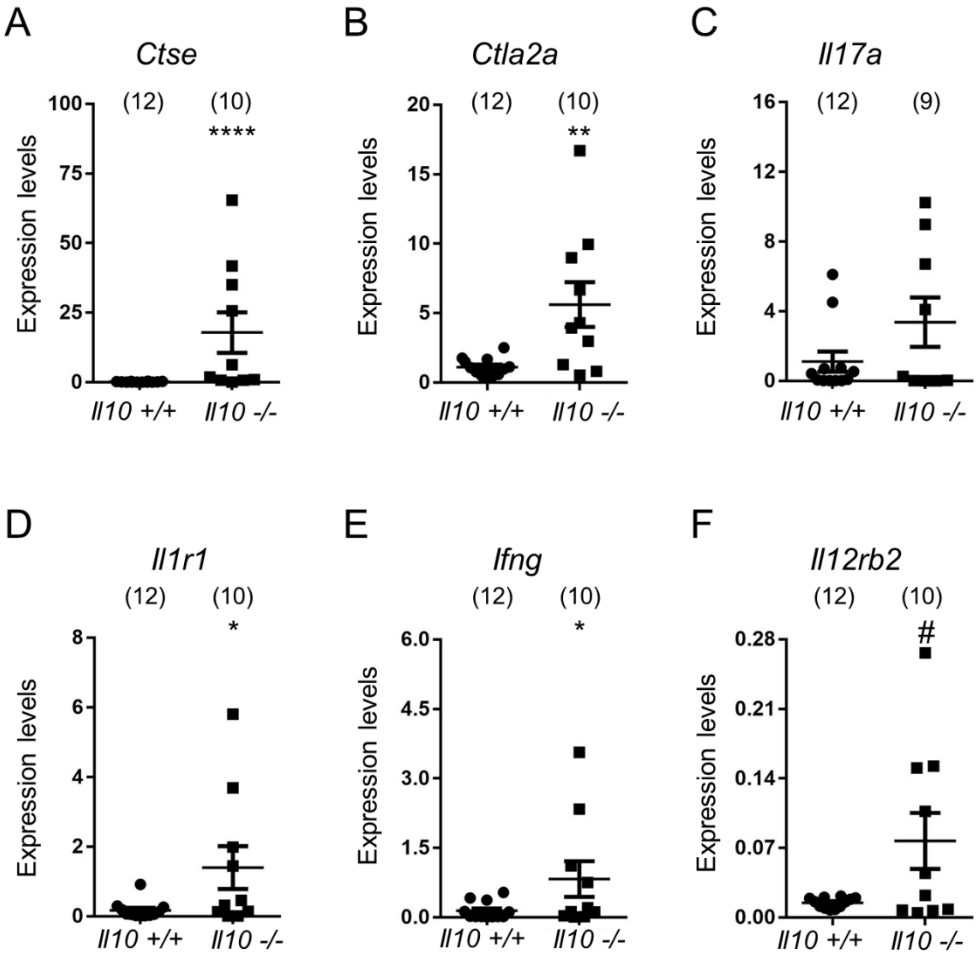


Figure 3.3 The effect of *Il10* null mutation on gene expression in Treg cells. CD4+CD25+lymphocytes were recovered from PALNs of *Il10*+/+ and *Il10*-/- B6 mice on DPC 9.5 after mating with Balb/c males. Gene expression was assessed by microarray and qPCR was used to confirm the expression of *Ctse* (A), *Ctla2a* (B), *Il17a* (C), *Il1r1* (D), *Ifng* (E) and *Il12rb2* (F) which were identified as being differentially regulated. Data are the expression (mean ± SEM), normalised to reference gene *Gmpr*, in Treg cells from *Il10*+/+ mice (n = 12) and *Il10*-/- mice (n = 9 or 10). The effect of genotype was evaluated using Mann-Whitney U-test (*p<0.05, **p<0.01, ****p<0.0001, #p<0.1).

3.4 Discussion

Appropriate control of maternal inflammation is essential for optimal fetal growth and on-time birth. Treg cells are paramount among the immune-regulatory leukocytes operating to suppress inflammation and promote tolerance at the fetal-maternal interface in pregnant females (Aluvihare et al., 2004, Guerin et al., 2009). Currently, a lack of understanding of the maternal and fetal factors that control the generation and suppressive function of Treg cells is a limitation in our capacity to tackle human gestational disorders, including preeclampsia and preterm birth. These conditions result from excessive maternal inflammation and are associated with disturbances in the Treg cell populations (Guerin et al., 2009, Santner-Nanan et al., 2009).

In the present study, we demonstrated that *Il10*-/- mice are more susceptible to fetal loss when challenged with LPS in allogeneic pregnancy, even when the foetuses are *Il10*+/- as a result of mating *Il10*-/- females with *Il10*+/+ males, and then demonstrated the Treg cells generated in PALN of pregnant *Il10*-/- mice display altered expression of *Ctse* and other genes linked with Treg function. This, coupled with the finding that IL10 deficient Treg cells have altered functional features indicating decreased stability (Prins et al., 2015) are likely to contribute to the elevated susceptibility of *Il10*-/- mice to inflammatory challenge and fetal loss (Murphy et al., 2005, Robertson et al., 2007, Robertson et al., 2006).

Altered T cell immunity in IL10-deficient mice appears not to affect tolerance of fetal alloantigens, because steady-state pregnancy progresses normally irrespective of the alloantigenic status of the fetus (White et al., 2004). The functional requirement for IL10 becomes apparent when pregnant *Il10*-/- mice are administered low-dose LPS to induce a systemic inflammatory response, revealing a key role for IL10 in protecting the fetal-placental tissues from uncontrolled inflammatory cytokines and natural killer cell cytotoxicity causing fetal death and/or preterm labour (Murphy et al., 2005, Robertson et al., 2007, Robertson et al., 2006). Thus, it seems that a major function of the copious IL10 produced by leukocytes, placental trophoblasts, and other cell lineages in gestational tissues (Thaxton and Sharma, 2010) is to buffer pregnancy in the event of an inflammatory insult.

These experiments show that IL10 in the maternal compartment, as opposed to the fetal or placental tissues, is most crucial for protecting pregnancy. This implies a pivotal role for endogenous IL10 in supporting anti-inflammatory and protolerance mechanisms in the mother. IL10 has key actions in Treg

cell biology (Asseman et al., 1999, Rubtsov et al., 2008) with our studies demonstrating that when maternal IL10 synthesis is disrupted, the generation of Treg cells is disturbed (Prins et al., 2015).

It is important to consider that in the present study, because fetuses were heterozygous for the *Il10* gene, the maternal compartment is not entirely IL10 deficient. IL10 derived from the fetus and placenta can enter the maternal system. This fetal-derived IL10 would be highest through the second half of gestation, when maternal blood enters the placental labyrinth. Paracrine effects of fetal IL10 on maternal immune cells could reasonably explain the resolution of lymph node hypertrophy by DPC 12.5 observed in Prins et al., 2015 (Prins et al., 2015). This may also explain why the fetal growth impairment seen previously after mid-gestation LPS administration when IL10 is absent from both maternal and fetal tissues (Robertson et al., 2007) was not recapitulated in the Prins study (Prins et al., 2015).

Affymetrix microarray analysis indicated an altered pattern of gene expression in Treg cells in pregnant *Il10-/-* mice. This may be a compensatory response to lack of autocrine IL10 signalling. Production of IL10 by Treg cells is one of the central pathways mediating suppressive function, and IL10 deficiency constrains Treg cell function to differing extents depending on the tissue (Izcue et al., 2006, Rubtsov et al., 2008). This fits with an emerging picture of unique, nonredundant, and specialized roles for individual suppressive mediators in Treg cells that are exploited in different physiological and pathophysiological settings (Rubtsov et al., 2008). Cell lineage-restricted *Il10* null mutation in Foxp3+ cells shows a dominant, nonredundant role for Treg cell-derived IL10 in maintaining immune homeostasis at environmental surfaces, including the colon, lung, and skin (Rubtsov et al., 2008). T cell-specific blockade of IL10 signalling shows that IL10 production from CD4+Foxp3+ Treg cells is important for Treg cell suppression of Th17 and Th1 cells (Huber et al., 2011).

Among the most strongly upregulated genes in Treg cells from *Il10-/-* mice was *Ctse*, encoding the endolysosomal aspartic proteinase, cathepsin E. Cathepsin E is implicated as a key factor contributing to adaptive Treg suppressive mechanisms that are independent of IL10. Elevated *Ctse* was previously reported in Treg cells engineered for deficiency in both IL10 and IL35, where it promotes TNF-related apoptosis inducing ligand (TRAIL)-mediated suppression, to compensate for loss of IL10 and IL35 (Pillai et al., 2011). Cathepsin E is also associated with increased turnover of IL1B and IL18 through degradation of the protein sequestering molecule a2-macroglobulin (Shibata et al., 2003). *Ctse* is induced in inflammation (Nakanishi et al., 1993) in response to cytokines, including IL17 (McAllister et

al., 2014) and IFNG (Tsukuba et al., 2003). A previous study found elevated *Ctse* expression in *Il10*-/- mice after ozone-induced inflammation in the lung (Backus et al., 2010).

Other differentially regulated genes were detected in Treg cells from *Il10*-/- mice, with upregulated *Ifng* and a trend to elevated *Il17* expression. This shift in disposition to Th1 and Th17 gene expression may contribute to the instability of *Il10*-/- Treg cells we observed after pro-inflammatory stimulation. Genes encoding cytokine receptors IL1r1 and IL12rb2 were elevated, as was the T cell marker Ctla2. IL12rb2 is required for TGFB-dependent stimulation of Treg cell development, and signalling via this receptor is thought to regulate the number and functional maturity of Treg cells (Zhao et al., 2008).

In summary, we have demonstrated that in pregnancy, maternal IL10 is a key determinant of protection from inflammatory challenges. Treg cells generated in the absence of IL10 remain competent to sustain allogeneic pregnancy under steady-state conditions, but in the event of inflammatory challenge their compromised stability would be a factor in the uNK cell activation and shift to Th1 immunity that causes fetal loss. These findings are relevant to understanding the role of IL10 in the immune response to pregnancy and may ultimately contribute to elucidating the pathology of preeclampsia and related complications of human pregnancy, where a less robust Treg cell response (Santner-Nanan et al., 2009) and altered regulation of placental IL10 (Hennessy et al., 1999) are both implicated in the underlying inflammatory aetiology.

Chapter 4 Effect of Repeated Exposure to Seminal Fluid on the Expansion and Stability of the Treg Cell Pool in Early Pregnancy

4.1 Abstract

Immune adaptation to accommodate pregnancy requires sufficient T regulatory (Treg) cells in the endometrium to suppress inflammation, prevent maternal immune rejection and promote maternal vascular changes during the critical peri-implantation period. Previously we have demonstrated that seminal fluid exposure stimulates the proliferation of Treg cells in mouse lymph nodes and reproductive tract tissues. In this study, we aimed to determine the contribution of male major histocompatibility complex (MHC) molecules and repeated exposure to seminal fluid in determining the strength of the Treg cell response.

Female C57Bl/6 (B6) female mice were mated either once or four times to syngeneic males (Balb/b males; H-2^b, no MHC disparity) or allogeneic males (Balb/c males; H-2^d, MHC disparity), or three times to syngeneic Balb/b then one time to allogeneic Balb/c males (partner-switching model). Progression to pregnancy between matings was prevented by administration of RU486 on day 3.5 postcoitum (DPC). Virgin oestrous females were used as non-mated controls when required. The population characteristics and stability of para-aortic lymph node (PALN) Treg cells were determined on DPC 3.5 after the final mating using flow cytometry and cell culture methods. In an additional cohort, pregnancy outcomes were assessed through administration of LPS (1.0 μg) or vehicle to pregnant B6 females on DPC 9.5 of the final mating. Females were then sacrificed on DPC 17.5 to assess pregnancy outcome parameters including percentage of viable pups, resorption rate, fetal weight, placental weight and fetal: placental weight ratio.

A significant expansion in the size of the Treg cell population in the PALN was observed in female mice mated four times to Balb/c (19.6-fold, $p<0.001$), compared with females mated only once to Balb/c. Although increases were also seen in females mated four times to Balb/b (7.4-fold, $p<0.001$) or three times to Balb/b and one time to Balb/c (2.6-fold, $p<0.01$) compared to oestrus controls, the population was not greater than in females mated only once, and was less than that in females mated four times to Balb/c ($p<0.01$).

Repeated mating with Balb/c males also resulted in a more stable Treg cell population in the PALN, compared with females mated four times with Balb/b (45% decrease in the Th17/Treg ratio, $p<0.05$). In the steady state, females mated either syngeneically or allogeneically were similarly capable of sustaining pregnancy, as no significant difference in the number of viable pups or resorption rate was seen. However, LPS injection resulted in a reduced number of viable pups per litter (47%, $p<0.05$) and

an elevated resorption rate (1.72-fold, p=0.07) in repeated syngeneic mating compared to repeated allogeneic mating groups.

Collectively, these data provide evidence that repeated exposure to seminal fluid acts to expand the Treg cell pool and increase the stability of Treg cells in the female reproductive tract during early pregnancy. These data also demonstrate that MHC alloantigens play a crucial role in the expansion of a stable Treg cell population, as repeated mating with syngeneic males was not capable of eliciting the same Treg cell response. Interestingly, the robust response observed after repeated mating in allogeneic males led to increased protection against inflammatory challenge in mid-gestation. These findings may provide a mechanistic explanation linking clinical observations that prior contact with the conceiving partner's seminal fluid is associated with reduced pregnancy complications in women.

4.2 Introduction

A variety of adaptions within the maternal immune system are required to establish a tolerogenic immune environment during the peri-conception period and to maintain it through gestation (Trowsdale and Betz, 2006). Key to these adaptions is the expansion of regulatory CD4+CD25+ Treg cells (Guerin et al., 2009, Shima et al., 2010, Shevach, 2009). These cells compromise 5-10% of CD4+ T cells in rodents and 1-3% of CD4+ T cells in humans (Sakaguchi, 2000, Shevach, 2002) and are defined as having constitutive expression of CD25 (IL-2 receptor α chain) (Sakaguchi et al., 1995) and expression of the unique marker Foxp3 (Fontenot et al., 2005, Fontenot et al., 2003).

In pregnancy, Treg cells support implantation and subsequent fetal development by suppressing proliferation and function of CD4+ and CD8+ T cells, B cell and NK cells, as well as impacting DC and macrophage maturation and function (Guerin et al., 2009, Shevach, 2002). The expansion of the Treg cell pool plays an important role in preparing the uterus for implantation as Treg cell depletion prior to implantation results in pregnancy loss in allogeneic matings (Shima et al., 2010, Aluvihare et al., 2004). Treg cells can be generated within the thymus (Shevach, 2002) or in the periphery, where local naïve CD4+ T cells convert to Treg cells (Akbar et al., 2003). Like other T cells, their suppressive and proliferative capacity depends on interaction with cognate antigen presented by antigen presenting cells (Thornton and Shevach, 1998, Walker et al., 2003). In the context of pregnancy, the relevant antigens are paternal MHC antigens, which are present in seminal fluid and also expressed by conceptus tissue.

There is strong clinical and experimental evidence demonstrating that Treg cells contribute to maintenance of normal pregnancy in humans. In pregnant women, Treg cells are increased in both peripheral blood and decidua in the first and second trimester, followed by a subsequent decline in the third trimester and postpartum (Saito et al., 2005, Heikkinen et al., 2004, Tilburgs et al., 2006, Xiong et al., 2013). Altered Treg cell populations and compromised Treg cell function are associated with multiple pregnancy complications, such as preeclampsia, and recurrent miscarriage. Elevated Th17 and reduced Treg cell populations are found in the peripheral blood from preeclamptic women, compared with normal pregnant women (Santner-Nanan et al., 2009). In addition, low expression of the Treg cell marker *Foxp3* in the endometrium in non-pregnant cycles is also associated with unexplained infertility (Jasper et al., 2006).

Over recent years, studies have begun to highlight that T-cell subsets are not as stable as previously assumed and under certain conditions can exhibit plasticity and covert into another T-cell subtype. Treg cells can lose Foxp3 expression and convert to IL-17 producing or Th17 cells in a pro-inflammatory environment (Osorio et al., 2008, Yang et al., 2008). Treg cells can switch to a Th17 phenotype following co-culture with DCs activated by the fungal recognition receptor, dectin-1 (Osorio et al., 2008). In addition, TGF-β producing thymic derived Treg cells activated by CD4+CD24- T cells can differentiation to Th17 cells in the presence of IL-6 *in vitro* (Xu et al., 2007). Given the plasticity between Treg cells and Th17 cells, studies are required to explore the importance of Treg cell stability in supporting pregnancy success.

Seminal fluid plays an important role in the expansion of Treg cells at the outset of pregnancy (Robertson et al., 2009a). Initially, seminal fluid exposure leads to a transient inflammatory response coupled with an infiltration of inflammatory cells. This occurs in the ectocervix in humans (Sharkey et al., 2007, Pandya and Cohen, 1985) and the uterus in mice (Johansson et al., 2004). The presence of tolerogenic molecules in seminal fluid, including HLA-G, TGF-β and 19-OH PGE are required to drive Treg cell expansion through converting DCs into tolerogenic DCs (Kelly and Critchley, 1997b, Hutter and Dohr, 1998, Robertson et al., 2002). The pivotal role of seminal fluid in the expansion of Treg cells has been elegantly demonstrated in mice, where the absence of seminal fluid in a vasectomised and surgical excision of seminal vesicle male mice failed to expansion Treg cell population in the PALN and uterus (Guerin et al., 2011).

In addition to the provision of pro-tolerogenic immune regulatory molecules, seminal fluid also provides paternal antigens which are an important component for eliciting the female response (Holland et al., 2012). Paternal alloantigens, particularly MHC, are expressed at low or undetectable levels on sperm cells but are present in seminal plasma in a soluble form. These MHC and also minor histocompatibility complex antigens are also expressed by the conceptus, including subsets of placental trophoblasts, on specific lineages that differ between mice and human. Paternal antigens have been demonstrated to activate CD4+ and CD8+ T cells following seminal fluid exposure at conception (Moldenhauer et al., 2009) indicating that paternal antigens presented by maternal antigen presenting cells may be involved in the regulation of Treg cells and their function. Additionally, elevated paternal-specific Treg cells can be found in the PALN in female mice on DPC 3.5 and 5.5 following allogeneic matings compared with unmated oestrus females (Shima et al., 2015).

Given the link between Treg cells and pregnancy pathologies, it is interesting that the duration of prior exposure to the conceiving partner's seminal fluid is associated with some pregnancy pathologies in humans. Clinical studies demonstrate that women who have had limited exposure to a partner's seminal fluid through use of barrier contraception or short period of cohabitation (<6 months) are more likely to develop preeclampsia (Kho et al., 2009, Einarsson et al., 2003, Klonoff-Cohen et al., 1989) than individuals who have had a longer period of seminal fluid exposure (Kho et al., 2009), indicating that longer term exposure to a specific partner's seminal fluid exposure may be protective for women from preeclampsia development. These findings suggest that the immunological tolerance developed following seminal fluid exposure is partner specific and longer periods of exposure to seminal fluid strengthens the tolerogenic response leading to better outcomes in pregnancy. The question beyond this observation is whether repeated exposure to partner's seminal fluid strengthens the Treg cell pool, and whether this benefit is partner-specific; and whether partner alloantigens, in particular MHC antigens are important in Treg cell expansion. Given the pivotal role Treg cells play in establishing maternal immune adaption and the contribution of seminal fluid to this response, we hypothesised that male alloantigens strengthen the Treg cell response in a partner-specific manner in early pregnancy.

The experiments described in this chapter aim to assess the influence of repeated exposure to male alloantigens on Treg cell expansion in the local PALN, distal secondary lymph tissues and in the peripheral blood. In these experiments, a mouse model was utilised where female mice were exposed to either syngeneic (Balb/b males) or allogeneic (Balb/c males) male ejaculates once or four times, or

exposed to syngeneic ejaculate three times followed by allogeneic ejaculate once. Treg cell number and stability were analysed by FACS and pregnancy outcomes were analysed on DPC 17.5.

4.3 Results

4.3.1 Expansion of Treg cells pool after repeated exposure to seminal fluid

Previously, we demonstrated that exposure to male seminal fluid drives the expansion of the Treg cell pool on DPC 3.5 in the mouse uterus and PALN (Guerin et al., 2011). To investigate whether repeated exposure to seminal fluid further expands the Treg cell population, and whether this expansion is in a partner-specific manner, female C57Bl/6 (B6) female mice were mated either once or four times with Balb/c (H-2d; allogeneic mating - MHC disparate), Balb/b (H-2b; syngeneic mating - same MHC), or three times with Balb/b and one time with Balb/c males (partner switching model). As a mating control, oestrous B6 females were utilised. Between each mating, pregnancies were prevented by RU486 as described in 2.1.3 and PALN were collected on DPC 3.5. The percentage and total number of Treg cells were quantified by FACS. mLN, spleen and blood were also assessed with the data presented in the Appendix (see chapter 8, section 8.1.1).

In order to understand the impact of repeated mating on the Treg cell pool, we compared Treg cell populations between repeated matings and a single mating. Despite a 1.1-fold and a 2.9-fold increase in Treg cell numbers in the PALN following 1 x mating with Balb/c males and 1x mating with Balb/b males, compared to the unmated oestrus control (Figure 4.1A), these differences were not statistically significant. No significant change in Treg cell numbers were observed following 1 x mating with Balb/b males compared to the 1 x Balb/c mating group.

We then explored the impact of repeated mating (4 x mating) on the expansion of Treg cells on DPC 3.5 in the PALN. Repeated mating with allogenic Balb/c males resulted in a significant increase in Treg cell numbers compared to both the oestrus control (19.6-fold, $p<0.001$) and the 1 x Balb/c mating group (17.2-fold, $p<0.001$) (Figure 4.1A). Interestingly, while repeated mating with syngeneic Balb/b males did result in an increased Treg cell population compared to the oestrus control (7.5-fold, $p<0.001$), there was no significant expansion observed between females mated 1x with Balb/b males and 4x with Balb/b males (Figure 4.1A). Most notably, repeated mating (4 x mating) to Balb/c males resulted in a significant increase in the number of Treg cells compared to 4 x Balb/b mating (2.6-fold increase,

p<0.01) showing that repeated exposure to MHC disparate seminal fluid resulted in a more robust Treg cell response (Figure 4.1A).

We then assessed the impact of repeated mating using the partner switching model (3 x Balb/b matings and 1 x Balb/c mating) and demonstrated that while switching partners following repeated mating also resulted in a significant increase in Treg cell number compared to oestrous females (6.6-fold, p<0.01), these numbers were significantly lower than repeated mating with Balb/c males (82%, p<0.0001) and equivalent to a single mating with a Balb/b male. Further, while not significant, switching partners resulted in a lower number of Treg cells compared to repeated mating to Balb/b males (Figure 4.1A).

4.3.2 Stability of Treg cells

In addition to the number of Treg cells, the stability and function of these cells also play an important role in regulating the female immune environment during gestation. In this study, Treg cell stability was assessed in cells collected from the PALN by flow cytometry following 4h *in vitro* stimulation using PMA (final concentration 0.1 µg/mL) and ionomycin (final concentration 1.0 µg/mL). In all groups, stimulation of these cells resulted in a significant reduction in the proportion of Treg cells (Figure 4.1B) and an increase in the proportion of Th17 T cells (Figure 4.1C).

We then compared the impact of stimulation on the ratio of CD4+IL17A+ cells to CD4+CD25+Foxp3+ cells in all groups. An increase in the ratio of Th17/Treg cells indicates lower stability in the Treg cell phenotype. Stimulation of total cells did not significantly alter the Th17/Treg ratio in most comparisons with the exception of a significant decrease in the Th17/Treg ratio in the 4x Balb/c repeated mating group compared to the 4x Balb/b mating group (45%, p<0.05, Figure 4.1D). Further, a trend towards an increase in the Th17/Treg ratio was observed in females mated 4x with Balb/b, compared with females mated 1x with Balb/b (p=0.057, Figure 4.1D). This increase was not observed after repeated mating in Balb/c males. Therefore, optimal Treg cell stability requires repeated exposure to seminal fluid from the same MHC dissimilar male haplotype.

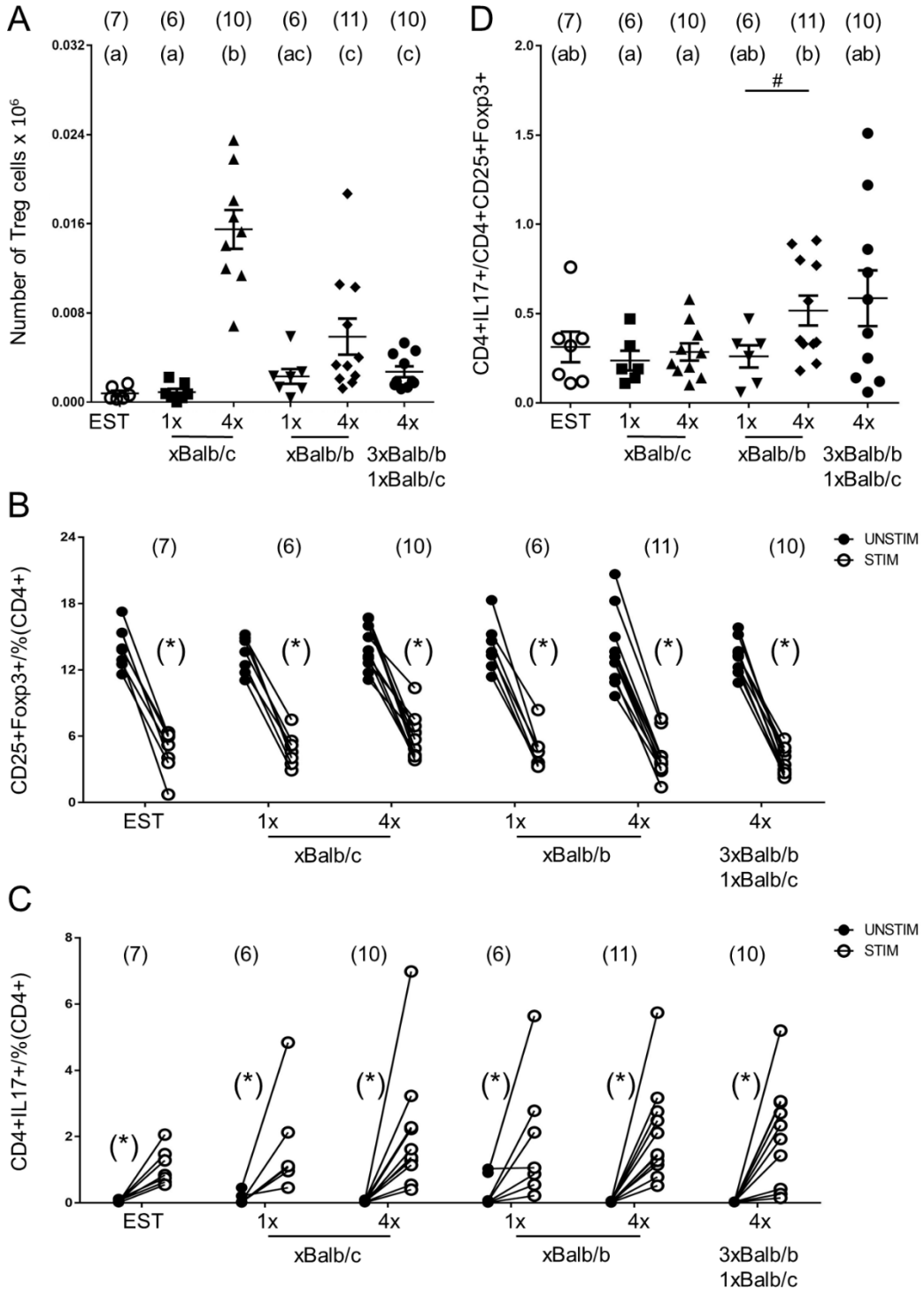


Figure 4.1 The effect of repeated exposure to male alloantigens on the number of Treg cells and stability of Foxp3 expression in CD4+ T cells from PALN. B6 females were left unmated, mated 1x with Balb/c, 4x with Balb/c, 1x with Balb/b, 4x with Balb/b or 3x with Balb/b and 1x with Balb/c, and on DPC 3.5, PALN was analysed by flow cytometry for Treg cell parameters. Data presented as mean ± SEM and are the total cell number of CD4+CD25+ Foxp3+Treg cells in the PALN (A), the expression of Foxp3 immediately after cell collection or 4h after strong polyclonal PMA/ionomycin stimulation in vitro (B), the expression of IL17A immediately after cell

collection or 4h after strong polyclonal PMA/ionomycin stimulation in vitro (C), and the ratio of CD4+IL17+ to CD4+CD25+Foxp3+ cells from PALN after stimulation (D). The effect of PMA stimulation was evaluated using a paired T test in B and C, and the effect of mating was evaluated using Kruskall-Wallis and Mann-Whitney test in A and D (#p = 0.057).

4.3.3 Repeated exposure to male alloantigens reduced susceptibility of fetal loss post LPS challenge in mid-gestation

Clinical observations suggest that shorter sexual cohabitation is associated with elevated risk of pregnancy pathologies such as preeclampsia (Kho et al., 2009). In addition, women who have a new conceiving partner are more likely to develop preeclampsia (Klonoff-Cohen et al., 1989, Einarsson et al., 2003) suggesting that the protective effects following longer periods of cohabitation are partner specific. To determine whether the alterations in Treg cell number and stability following repeated mating with allogeneic or syngeneic males, or partner switching in males have an impact on pregnancy outcomes a cohort of B6 females were assessed for pregnancy outcomes Females were mated either once or four times with Balb/c males, Balb/b males or three times with Balb/b and one time with Balb/c males. As before, RU486 was administered on DPC 3.5 between each mating to prevent pregnancy, and on DPC 9.5 of the final mating, a low dose inflammatory challenge (LPS; 1.0 μg) or vehicle control (PBS) was administered to pregnant females, then outcomes were assessed on DPC 17.5.

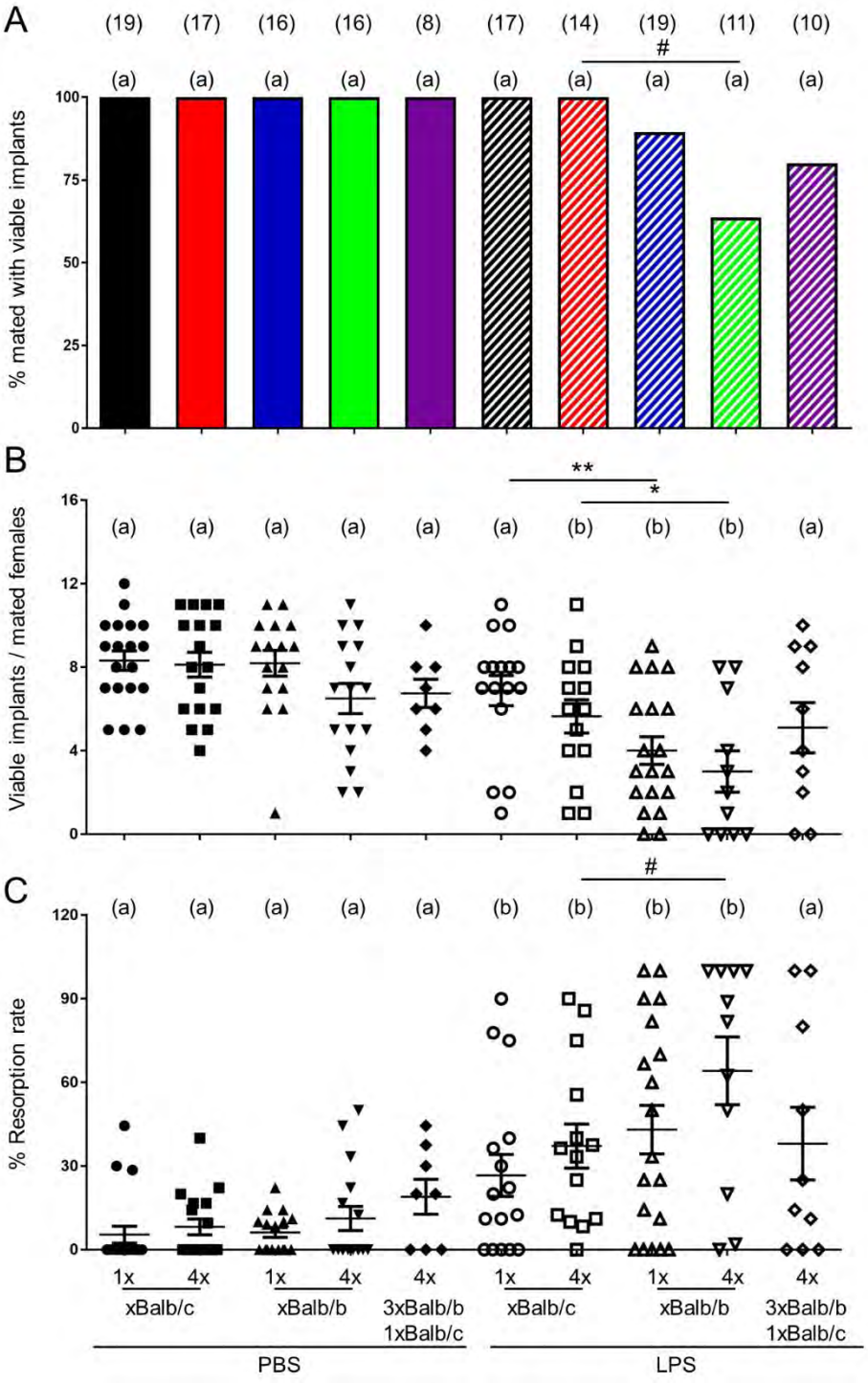
Using a low dose inflammatory challenge, there was no impact of LPS on the proportion of mated females with a viable pregnancy in all groups compared to the equivalent vehicle control. However, females mated 1x or 4x to Balb/b males or mated 3 x Balb/b and 1x Balb/c had a lower proportion of viable implants compared to the vehicle control, although the difference failed to reach significance (Figure 4.2A). Of those groups, consistent with the Treg cell profiles, females given LPS after mating 4x to Balb/b males also had an 36% reduction in females carrying viable implants compared to the 4x Balb/c group ($p=0.08$, Figure 4.2A).

LPS injection resulted in a reduction in the number of viable pups per mother in all groups except 4x matings to Balb/c, and an elevation in the resorption rate in all groups except 3x to Balb/b and 1x to Balb/c, compared to mating matched PBS controls (Figure 4.2B and C). Interestingly, following LPS treatment, a significantly lower number of viable fetuses was observed in females mated 1x (42%, $p<0.01$, Figure 4.2B) and 4x to Balb/b (47%, $p<0.05$, Figure 4.2B), compared to females mated 1x or 4x to Balb/c males on DPC 17.5. Associated with the reduction in viable fetuses, LPS administration also resulted in a trend towards a higher rate of fetal resorption in females mated 4x to Balb/b (1.7-fold, $p=0.07$, Figure 4.2C).

We then examined the impact of LPS exposure following repeated mating with Balb/c, Balb/b or Balb/c + Balb/b on fetal weight, placental weight and fetal: placental weight ratios. As can be seen in

Figure 4.2D-F, LPS treatment in different mating groups had no major impact on these parameters, although there was a 4.8% decrease in fetal weight in the 1x Balb/c group compared to the equivalent PBS control (Fig 4.2D, p=0.042).

Overall, these data suggest that repeated exposure to seminal fluid from allogeneic males results in a greater expansion and more stable population of Treg cells in the uterine draining lymph nodes in early pregnancy in a partner specific manner. Further, this mating protocol resulted in greater protection from inflammatory challenge in mid-gestation, suggesting that the more robust population of Treg cells provided by repeated seminal fluid exposure contributes to the mechanism for better protection against inflammatory insult in later gestation.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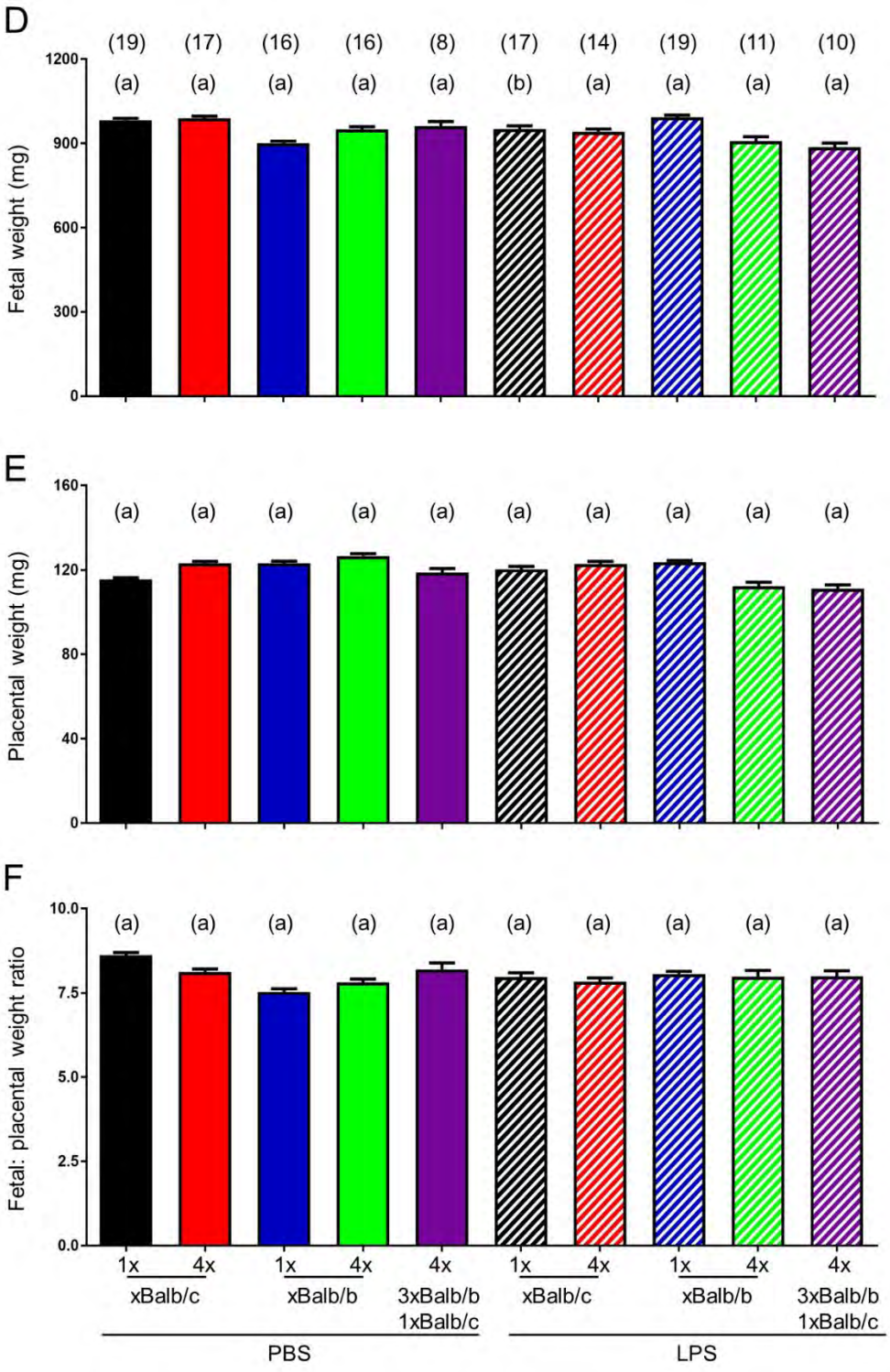


Figure 4.2 The effect of repeated exposure to male syngeneic or allogeneic antigens on pregnancy parameters after low-dose LPS challenge. B6 mice were mated either once or four times to Balb/c, Balb/b or mated three time to Balb/b and one time to Balb/c and injected i.p. with LPS (1μg) or control (PBS) on DPC 9.5, then autopsied on DPC 17.5. Data are the percentage of mated females pregnant with viable fetuses (A), the number (mean ± SEM) of viable implantation sites per mated mouse (individual data points with mean value

shown; B), the percentage of total implantation sites per pregnant female undergoing resorption (individual data points with mean ± SEM; C), the fetal weight (estimated marginal mean ± SEM; D), the placental weight (estimated marginal mean ± SEM; E), and the fetal: placental weight ratio (estimated marginal mean ± SEM; F). The effect of genotype was evaluated in A by chi-square analysis and in B by ANOVA and Sidak t-test. The effect of genotype was evaluated in D-F by mixed model analysis. *$p<0.05$, #$p<0.1$, compared with mating groups at same LPS dose; a, b, c, d indicates $p<0.05$, comparing between LPS or vehicle control administration within the mating group.

4.4 Discussion

A sufficient number and function of Treg cells is crucial to establishing maternal immune tolerance for successful pregnancy. Treg cells suppress inflammation and promote immune adaption at the maternal-fetal interface (Aluvihare et al., 2004, Guerin et al., 2009). Understanding the regulation and function of Treg cells is crucial to develop treatments for human gestational complications, such as preeclampsia and recurrent miscarriage (Guerin et al., 2009), where altered Treg cells are implicated. Seminal fluid contributes to Treg cell expansion in early gestation, with contributions from both sperm and seminal plasma (Guerin et al., 2011, Robertson et al., 2009a). The role of previous seminal fluid contact in protecting women from developing preeclampsia has been observed in clinical studies, and is the most reasonable explanation for observations that nulliparous women, women who have a new conceiving partner and pregnancies initiated with donor gametes are associated with higher incidence of preeclampsia (Salha et al., 1999, Dekker et al., 1998). These observations also suggest that the benefit of prior seminal fluid contact is partner-specific. However, to date there has not been direct evidence to demonstrate that this protection comes from a more robust and suppressive Treg cell pool.

The current study was undertaken to evaluate the impact of repeated female reproductive tract contact with seminal fluid on Treg cell number and function in the PALN, other distal lymphoid organs and also peripheral blood during the peri-implantation period of pregnancy. Our data demonstrates that the Treg cell pool is further expanded after repeated mating particularly in the uterine draining lymph nodes, and to a greater extent after mating with allogeneic males. In addition to an increase in the Treg cell population, this study also demonstrates that repeated allogeneic mating leads to a more stable Treg cell population compared to mating with syngeneic males. The changes to the Treg cell population have a functional consequence, leading to an increased resilience to inflammatory insult in mid-gestation following repeated mating to allogeneic males. This result is consistent with an important role for Treg cells in protecting fetuses from inflammatory insults throughout gestation. Strikingly, these changes are not observed following repeated mating with syngeneic males or following male partner switching, highlighting the importance of consistent exposure to the same disparate MHC antigens in establishing a tolerogenic maternal immune environment.

Changes in the Treg cell population in the peri-implantation period has previously been examined in mice, where an increase in the percentage of CD4+CD25+ T cells was observed on DPC 3.5 in a range of lymphoid organs (Aluvihare et al., 2004, Guerin et al., 2011). This Treg cell expansion has been

attributed to seminal fluid as females mated to seminal plasma deficient and vasectomised males fail to expand the Treg cell pool (Guerin et al., 2011). Surprisingly, in the current study unlike previous studies the number of Treg cells in the PALN after a single mating to Balb/c was increased compared to oestrus females, but the increase did not reach statistical significance. This deviance from previous studies is likely to be due to the smaller number of mice in the current study. In addition, the methodology for quantifying absolute Treg cell populations was not identical between the previous and current study, which may potentially resulted in this deviance.

Treg cells are known to play a crucial role in maintaining an appropriate immune environment in pregnancy. In the mouse model, T cells depleted of CD4+CD25+ Treg cells or unsorted T cells from normal pregnant mice were adoptively transferred into pregnant T cell deficient mice. The allogeneic fetuses were completely rejected in the absence of Treg cells amongst the transferred population, however, no fetal rejection was found in syngeneic pregnancies (Aluvihare et al., 2004). *In vivo* depletion of CD25+ cells resulted in a similar fetal rejection in mice (Darrasse-Jeze et al., 2006). When anti-CD25 monoclonal antibody PC61 was administrated to deplete CD25+ cells from female mice after allogeneic mating, fewer fetuses survived to term, while in syngeneic mating, there was no change in reduced fetal survival rate (Darrasse-Jeze et al., 2006). Clinically, changes in the maternal immune environment in pregnancy, including increases in pro-inflammatory responses and decreases in the Treg cell population are associated with pregnancy complications such as preeclampsia, and an increased length of exposure to a specific partner's seminal fluid reduces our risk (Kho et al., 2009). Altogether, these studies suggest that repeat exposure to seminal fluid may strengthen the Treg cell pool. In the current study, we demonstrate that repeated exposure to male alloantigens expands the Treg cell population in the PALN, compared with oestrus controls and females mated once to allogeneic males, indicating a pivotal role of repeated seminal fluid exposure in progressively expanding Treg cells in the peri-conception period. It is notable that Treg cell expansion is less apparent following mating with syngeneic males, with no change in the Treg cell pool following repeated exposure to syngeneic seminal fluid. Taken together, these data suggest that both male major and minor antigens can initiate Treg cell proliferation, but major antigens are more capable to stimulate Treg cell expansion.

The findings presented in this chapter provide a mechanism to explain why women who have a short period of cohabitation prior to falling pregnant, or primiparous women, have greater susceptibility to obstetric disorders associated with inflammation and dysfunction of the maternal immune response

(Dekker et al., 1998). In patients with preeclampsia, reduced Treg cell population (Sasaki et al., 2007, Prins et al., 2009, Santner-Nanan et al., 2009, Quinn et al., 2011) and a shift towards Th17 phenotypes in Treg cells are observed (Santner-Nanan et al., 2009, Tian et al., 2016), indicating that the importance of robust Treg cell population in protecting women from developing preeclampsia. In the current study, the importance of repeated exposure to male alloantigens in the induction of stable Treg cells on DPC 3.5 was demonstrated in mice, as a female mice mated 4x to Balb/c exhibit the largest expansion of Treg cells. In this study, we also assessed the stability of these cells following polycloncal stimulation *in vitro*. Strikingly, Treg cells in PALN from females repeatedly mated to allogeneic males exhibited increased stability compared to females repeatedly mated to syngeneic males or females in the partner switching group, indicating that exposure to MHC disparate seminal fluid can progressively strengthen as well as expand the Treg cell pool. This is the first mouse study which has demonstrated that the benefit from seminal fluid contacting benefits from an extended period of seminal fluid exposure and is partner-specific, and repeated exposure to male alloantigens can further expand Treg cell population and also stabilise Treg cell phenotype. Taken together, the data reinforce growing evidence that male alloantigens are essential for the expansion of a robust Treg cell population in early pregnancy.

Apart from the number of Treg cells and their stability, the suppressive activity of Treg cells is also associated with normal pregnancy (Somerset et al., 2004). Treg cells suppress autoreactive immune responses to prevent autoimmune diseases, inhibit inflammation and are involved in suppression of allograft rejection (Sakaguchi et al., 1995). In order to accommodate the semi-allogeneic fetus, Treg cells with appropriate suppressive activity are required. In women experiencing recurrent miscarriage, the suppressive activity of Treg cell are diminished, as an increased number of Treg cells are required to exert suppression when compared to fertile women (Arruvito et al., 2007, Wang et al., 2010b). While the current study does not quantify Treg cell suppressive activity, our unpublished data demonstrates that repeated exposure to allogeneic male seminal fluid enhances suppressive activity in Treg cells, compared to the Treg cells in females that have single seminal fluid exposure (Moldenhauer, unpublished).

It is interesting to note that despite the clear differences in Treg cell number and stability following mating with allogeneic Balb/c compared to syngeneic Balb/b males, there is no change in their capacity to sustain pregnancy under normal physiological conditions. This can be observed in PBS injected females where no change in the capacity to sustain pregnancy, the number of viable pups or the

resorption rate is observed. However, the importance of these changes became apparent when pregnant females were administrated a low dose of LPS to result in inflammatory challenge, where a reduction in viable fetuses and an elevated resorption rate were observed in females mated 4x to Balb/b, compared to females mated 4x to Balb/c. The adverse outcome after LPS treatment is likely to be due to the reduced Treg cell population and impaired stability observed in early pregnancy, resulting in a Treg population unable to suppress the systemic inflammation. These findings suggest that the decrease in Treg cell number, stability and potentially suppressive function in females mated 4x to Balb/b compared to 4 x Balb/c may impact their capacity to withstand an inflammatory challenge and reinforces the importance of seminal fluid exposure in the induction of protective Treg cells prior to implantation.

Further studies are required to better quantify the mechanisms underlying the adverse pregnancy outcomes after inflammatory challenge in these repeated mated female mice. The Treg cell proportion and number, as well as stability and suppression could be addressed after the inflammatory challenge and also in the late gestation period to further investigate how impaired Treg cell population leads to adverse pregnancy outcomes. It would also be of value to consider Treg cell transfer experiments, to determine whether the increased susceptibility to inflammatory challenge can be mitigated by supplementation of paternal antigen-reactive Treg cells.

Taken together, this study demonstrates that repeated allogeneic matings results in further expanded and more stable Treg cell population in the PALN in mice on DPC 3.5. This elevation is not observed in repeated syngeneic matings, highlighting the pivotal role of MHC disparity in the expansion of Treg cells. In addition, this study also reveals that Treg cells following multiple matings with allogeneic males (4x Balb/c) are more stable than those Treg cells from females mated repeatedly to syngeneic males (4x Balb/b) and while females are able to sustain pregnancy, they are more susceptible to inflammatory challenge in the mid-gestation. These findings provide compelling proof-of-concept in the mouse model of a benefit of long term seminal fluid exposure and the importance of male alloantigens for the expansion of stable Treg cells. The observations may be ultimately beneficial in understanding the factors contributing to immune-associated pregnancy complications, such as recurrent miscarriage and preeclampsia in women.

Chapter 5 Altered Treg Cell and Antigen Presenting Cell Populations are Associated with LPS-Induced Fetal Loss in Pregnant miR-155 Deficient Mice

5.1 Abstract

Maternal immune tolerance of the semi-allogeneic fetus requires CD4+Foxp3+ T-regulatory (Treg) cells, which suppress inflammation and anti-fetal immunity. In pregnant mice, expansion of the Treg cell pool is initiated by seminal fluid contact at coitus. Recent studies have demonstrated that microRNAs (miRNA) play a role in Treg cell generation, with miR-155 established as a key Treg cell miRNA. Seminal fluid has recently been found to induce female reproductive tract miRNAs following coitus. However, the contribution of miR-155 to in early pregnancy is yet to be assessed. To assess the contribution of miR-155 to Treg expansion in early pregnancy, miR-155-/- or miR-155+/+ (C57Bl/6) females (n=10-15) were mated with Balb/c males. T cell and antigen presenting cell profiles from the uterus and para-aortic lymph nodes (PALN) which drain the uterus were assessed using flow cytometry 3.5 day postcoitum (DPC). Virgin oestrous miR-155-/- and miR-155+/+ females were used as no mating controls. To investigate the impact of miR-155 deficiency on susceptibility to a pro-inflammatory challenge, a second cohort of pregnant females was administered LPS (1.0 μg) or PBS control on DPC 9.5, and pregnancy outcomes were determined on DPC 17.5.

miR-155 deficiency resulted in significant alterations to the Treg cell profile in early pregnancy with the most striking changes being observed in the PALN on DPC 3.5, where a 53%, a 69% and a 35% reduction was observed in Treg proportion, Treg number and Foxp3 expression, respectively.

In the absence of miR-155, fewer macrophages in the PALN expressed CD80 and CD86 (reduced by 31% and 43% respectively). Fewer DCs expressed MHCII+ in the PALN on DPC 3.5 (reduced by 69%) in the absence of miR-155. miR-155 deficiency also resulted in a 69% reduction in the number of CD86+ DCs in the PALN.

miR-155 deficiency altered the outcomes of pregnancy challenged with LPS, with an 8.3% reduction in fetal weight and a 14% reduction in the fetal: placental ratio in late gestation. A 19-fold higher resorption rate was observed in miR-155-/- females after LPS treatment, compared with miR-155+/+ females treated with LPS.

In conclusion, the absence of miR-155 alters the maternal immune profile in early pregnancy and this may be a determinant of increased susceptibility to inflammation-induced fetal loss later in gestation. These findings suggest that miR-155 has a pivotal role in establishing the appropriate maternal immune environment during the peri-conception period that facilitates appropriate protection against

inflammatory challenge and may be relevant to understanding how Treg-associated pregnancy pathologies arise in women.

5.2 Introduction

The fetus inherits paternal alloantigens, which results in a semi-allogeneic challenge to the maternal immune system. Immune adaptions towards the semi-allogeneic fetus are required to establish a tolerogenic immune milieu and suppress excessive inflammatory responses that can contribute to gestational disorders. Maternal immune tolerance is medicated by Treg (CD4+ CD25+ Foxp3+) cells (Aluvihare et al., 2004), which are potent suppressors of the generation and function of Th1 and Th17 immune responses. Treg cells are a unique subtype of T cells, compromising approximately 5-10% of CD4+ T cells in mice. Treg cells also influence the function and maturation of DCs and macrophages (Shevach, 2009, Guerin et al., 2009), and conversely altered presenting capacity and maturation of antigen presenting cells can lead to impaired T cell responses. Depletion of Treg cells results in loss of the ability to maintain allogeneic pregnancy in mice (Aluvihare et al., 2004, Shima et al., 2010) and impaired Treg function is implicated in human gestational disorders (Jasper et al., 2006).

Seminal fluid deposition in the female reproductive tract results in a transient inflammatory response and initiates immune changes in human (Pandiyan et al., 2007, Sharkey et al., 2007), mice (Johansson et al., 2004) and all other mammalian species studied thus far (Schjenken and Robertson, 2014). At coitus, interactions between seminal fluid and epithelial cells in the reproductive tract induce elevated synthesis of cytokines and chemokines (Sharkey et al., 2012a, Sharkey et al., 2012b, Sharkey et al., 2007) which results in an influx of antigen presenting cells in both human (Sharkey et al., 2012b) and mice (De et al., 1991, McMaster et al., 1992, Robertson et al., 1998, Robertson et al., 1996, Robertson et al., 1992).

In pregnancy, Treg cells are initially expanded following seminal fluid contact at coitus (Guerin et al., 2011, Robertson et al., 2009a). There is accumulating evidence to suggest that seminal fluid is involved in facilitating establishment of immune tolerance (Robertson, 2005). An elevated Treg cell population was observed in mice exposed to intact seminal fluid (Guerin et al., 2011), but exposure to sperm alone (Guerin et al., 2011) or mechanical stimuli to mimic copulation (Schumacher et al., 2007) failed to induce Treg cell population. In addition, a recent study revealed that uterine DCs cross-present male alloantigens to activate maternal T cells (Moldenhauer et al., 2009) to contribute to Treg cell expansion.

Thus, to address how Treg cells are regulated, it is essential to investigate both DCs and Treg cell responses.

A reduced number of Treg cells with less suppressive activity has been observed in abortion-prone murine models (Zenclussen et al., 2005, Zenclussen, 2005). Depletion of CD4+CD25+ T cells lead to gestation failure in mice, indicating that Treg cells are crucial to sustain pregnancy. In addition, reduced Treg cells with less suppressive capacity and with a shift towards an IL-17 expressing phenotype were observed in preeclampsia (Quinn et al., 2011, Santner-Nanan et al., 2009, Sasaki et al., 2007, Prins et al., 2009, Tian et al., 2016). Amongst the factors that contribute to Treg cell responses and functions, miRNAs are well established immunological regulators. As the functions of individual miRNAs are highly redundant, and each miRNA can target more than 100 genes (Lu and Clark, 2012), miRNAs are involved in nearly all developmental, homeostatic and pathological processes (Griffiths-Jones et al., 2008) and act to regulate immune cells (Mehta and Baltimore, 2016) and pregnancy (Bidarimath et al., 2014).

miR-155 is a key immune regulatory miRNA. It regulates multiple immune cells, including B cells, T cells, and antigen presenting cells. Several studies have shown that miR-155 regulates T cell differentiation into Th1, Th2 and Th17 subtypes (O'Connell et al., 2007, Rodriguez et al., 2007, Vigorito et al., 2007), as well as controlling Treg cell development (Lu et al., 2009a, Kohlhaas et al., 2009). In addition, miR-155 is induced in DC after inflammatory stimulation (Baltimore et al., 2008), and also regulates antigen presenting capacity in DCs (Rodriguez et al., 2007). In addition, miR-155 expression is altered in women with recurrent pregnancy loss (Tang et al., 2016, Winger et al., 2015) and also preeclampsia (Pineles et al., 2007). However, the role of miR-155 in early pregnancy is yet to be addressed.

In the experiments described in this chapter, we utilised miR-155-/- mice to investigate the role of miR-155 in the regulation of the peri-conception immune milieu and the role of miR-155 in Treg cell, macrophages and DC regulation.

5.3 Results

5.3.1 miR-155 deficiency results in a systemic change to the maternal T cell profile in early pregnancy

To determine the impact of miR-155 deficiency on the T cell profile in early pregnancy, miR-155-/- and miR-155+/+ female mice were mated to Balb/c males. The PALN, mesenteric lymph nodes (mLN),

spleen and blood were collected from females on DPC 3.5 for T cell quantification. Virgin oestrous females were used as an unmated control. In this chapter, the T cell population was characterised using antibodies against CD3, CD4, CD25 and Foxp3. Figure 5.1 depicts the flow cytometry gating strategy, where lymphocytes were initially gated among total cells in each tissue. CD4+ T cells were then gated within the lymphocyte population by CD3 and CD4 positive staining. Finally, Treg cells were gated by assessing the CD3+CD4+ T cell population for CD25 and Foxp3 expression. Data for PALN are described in detail in this chapter, while the results for mLN, spleen and blood are presented in chapter 8, section 8.2.1.

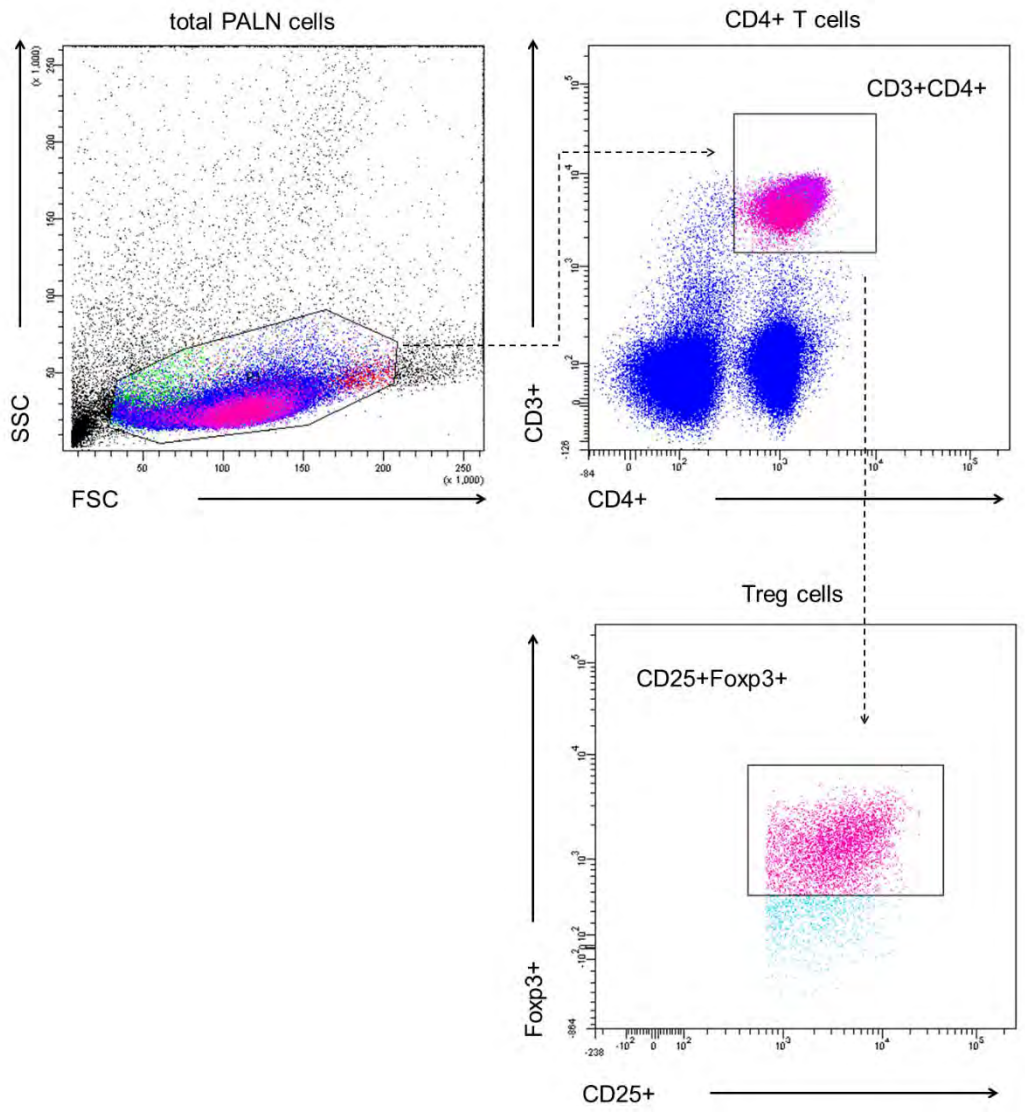


Figure 5.1 T cell flow cytometry gating strategy. Total lymphocytes were initially gated using SSC and FSC. From within the lymphocyte population, the expression of firstly CD3 and then CD4 (from within the CD3+ population) was utilized to define the CD4+ T cell population (CD3+CD4+ T cells). The Treg cell population was defined on the basis of expression of CD25 and then Foxp3 (from within the CD25 population) (CD25+Foxp3+ Treg cells).

5.3.1.1 Total cell expansion in female reproductive tissues

To assess the impact of miR-155 deficiency on immune cell population expansion in early pregnancy, we firstly examined the total cell number in the PALN on DPC 3.5. While the total cell number in the PALN increased by 1.3-fold ($p<0.05$) in the miR-155+/+ females after mating, this elevation was not observed in the absence of miR-155 (Figure 5.2), and miR-155 mice were also relatively lymphocytopaenic at oestrus. miR-155 deficiency resulted in a 31% and 42% reduction in the total cell number at oestrus and on DPC 3.5, compared to WT controls (Figure 5.2).

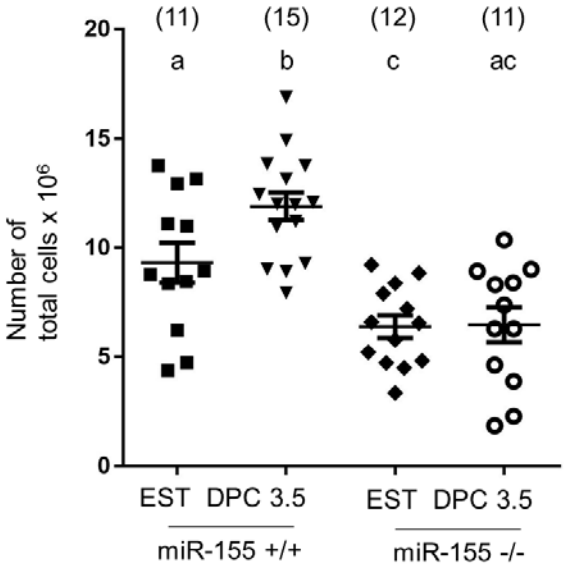


Figure 5.2 The effect of miR-155 deficiency on populations of total cells in the PALN. miR-155+/+ and miR-155-/- mice were mated with Balb/c males, and on DPC 3.5, total cells from the PALN were quantified by counting using a haemocytometer. Unmated virgin oestrus mice were used as non-mated controls. Data are presented as mean ± SEM and are number of total cells $x10^6$ in the PALN. Differences between groups were evaluated using Kruskal-Wallis test and Mann-Whitney U test. (a,b,c,d indicates $p<0.05$).

5.3.1.2 T cell profile in female reproductive tract lymph nodes

To assess the impact of miR-155 deficiency on the establishment of a tolerogenic immune environment in early pregnancy, we firstly examined the T cell profile in the PALN. Within the CD3+ population, while no change was observed in the proportion of CD4+ T cells amongst total lymph node cells (Figure 5.3A), the increase in total CD4+ cell number seen following mating in miR-155+/+ mice (2.0-fold, $p<0.0001$) was not observed in miR-155-/- females. In addition, miR-155 deficiency resulted in a reduction in the number of T cells both at oestrus (39%, $p<0.05$) and on DPC 3.5 (53%, $p<0.0001$), compared to miR-155+/+ females (Figure 5.3B). Interestingly, miR-155 deficiency also altered the level of CD4 expression within T cells, with the increase in CD4 MFI observed after mating in miR-155+/+ females (1.6-fold, $p<0.0001$) not observed in miR-155-/- females. Furthermore, miR-155 deficiency resulted in a reduction in the CD4 MFI both at oestrus (19%, $p<0.001$) and on DPC 3.5 (46%, $p<0.0001$), compared to WT controls (Figure 5.3C). This data indicates that in early pregnancy, miR-155 deficiency alters CD4+ T cell populations.

Given the importance of Treg cells in the establishment of a tolerogenic immune environment in early pregnancy, we then assessed the Treg cell population within the PALNs. No change in the proportion of Treg cells within the T cell population was observed following mating in miR-155+/+ or miR-155-/- mice, compared to the genotype-matched oestrus controls. miR-155 deficiency resulted in a lower proportion of Treg cells, both at oestrus (52%, $p<0.0001$) and on DPC 3.5 (53%, $p<0.0001$), compared to WT mice (Figure 5.3D). Consistent with our previous observations (Guerin et al., 2011) the total number of Treg cells was increased in miR-155+/+ following mating (2.1-fold, $p<0.0001$). Treg cell number was also expanded (1.7-fold, $p<0.05$) following mating in miR-155 deficient mice, but not to the same extent as WT controls. Overall, miR-155 deficiency resulted in a reduction in PALN Treg cells at oestrus (69%, $p<0.05$) and DPC 3.5 (76%, $p<0.0001$) (Figure 5.3E).

The level of Foxp3 expression as measured by MFI in Treg cells is associated with Treg suppressive capacity (Chauhan et al., 2009). Foxp3 MFI is induced after mating in miR-155+/+ mice (1.3-fold, $p<0.05$). In contrast, no change in Foxp3 MFI was observed following mating in miR-155-/- mice, with miR-155-/- mice having a significantly lower Foxp3 MFI on DPC 3.5 compared to miR-155+/+ mice (35%, $p<0.0001$) (Figure 5.3F).

Altogether, these data show that the absence of miR-155 alters the maternal immune response in early pregnancy, reducing total CD4+ T cells and in particular reducing the CD4+ Treg cell population in the

PALN, therefore potentially impacting capacity to generate immune tolerance to support implantation and pregnancy.

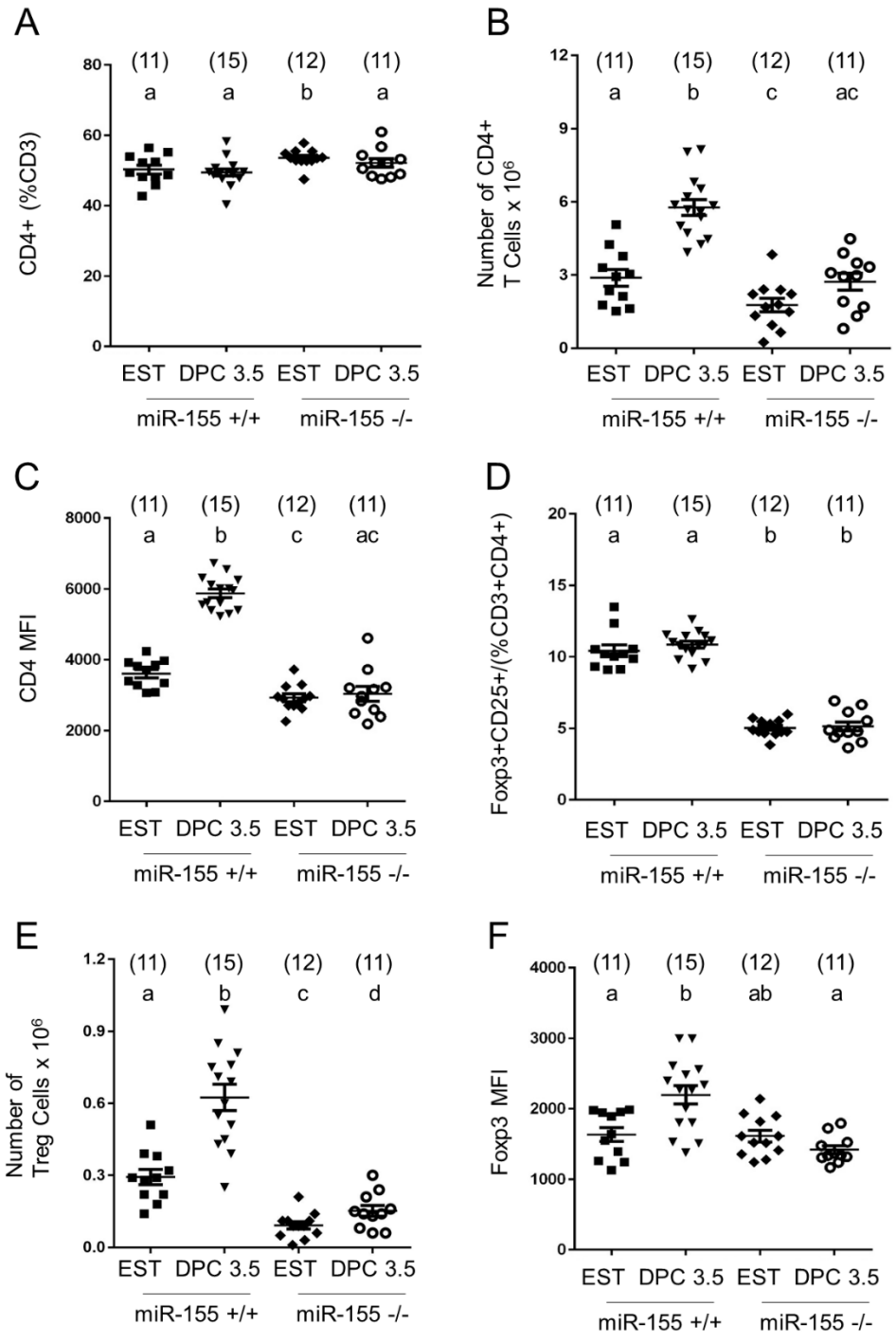


Figure 5.3 The effect of miR-155 deficiency on populations of T cells in the PALN. miR-155+/+ and miR-155-/- mice were mated with Balb/c males, and on DPC 3.5, CD3+, CD4+, CD25+ and Foxp3+ cells from the PALN were analysed by flow cytometry to quantify T cells. Virgin oestrus mice were used as non-mated controls. Data are presented as mean ± SEM and are the percentage of CD4+ T cells within the CD3+ population (A), the total number of CD4+ T cells x10^6 (B),the CD4 MFI (C), the percentage of Treg cells (D), the total number of Treg cells x10^6 (E), and the MFI of Foxp3 (F). Differences between groups were evaluated using Kruskal-Wallis test and Mann-Whitney U test. (a,b,c,d indicates $p<0.05$).

5.3.2 Effect of miR-155 deficiency on the macrophage and dendritic cell profile during early pregnancy

Given the importance of antigen presenting cells in the peri-conception immune environment and in generating the T cell response, we then assessed the impact of miR-155 deficiency on the profile of macrophages and DCs in early pregnancy. At oestrus and on DPC 3.5, cells from the mLN, spleen, PALN and uterus were collected for analysis by flow cytometry.

Macrophages were assessed by examining populations of total macrophages (CD11B+ F4/80+ cells), activated macrophages (MHCII+ CD11B+ F4/80+ cells), and macrophages expressing the co-stimulatory molecules CD80 (CD80+ CD11B+ F4/80+ cells) and CD86 (CD86+ CD11B+ F4/80+ cells). Figure 5.4 depicts the flow cytometry gating strategy, where leukocytes were initially gated based on size and complexity among total cells in each tissue. Macrophages were then identified on the basis of F4/80 and CD11B positive staining. Specific subsets of macrophages were then determined based on MHCII, CD80 and CD86 expression from within the CD11B+F4/80+ cell population (Figure 5.4).

DCs were assessed by examining populations of total dendritic cells (CD11C+ F4/80- cells), activated DCs (MHCII+ CD11C+ F4/80- cells), and DCs expressing the co-stimulatory molecules CD80 (CD80+ CD11C+ F4/80- cells) and CD86 (CD86+ CD11C+ F4/80- cells). Figure 5.5 depicts the flow cytometry gating strategy, where leukocytes were initially gated based on size and complexity among total cells in each tissue. DCs were then identified on the basis of F4/80 negative staining and CD11C+ positive staining. Specific subsets of DCs were then determined based on MHCII, CD80 and CD86 expression from within the CD11C+ F4/80- cell population (Figure 5.5). Data for uterus and PALN are described in detail in this chapter, while the results of mLN and spleen are presented in chapter 8, section 8.2.2 and section 8.2.3.

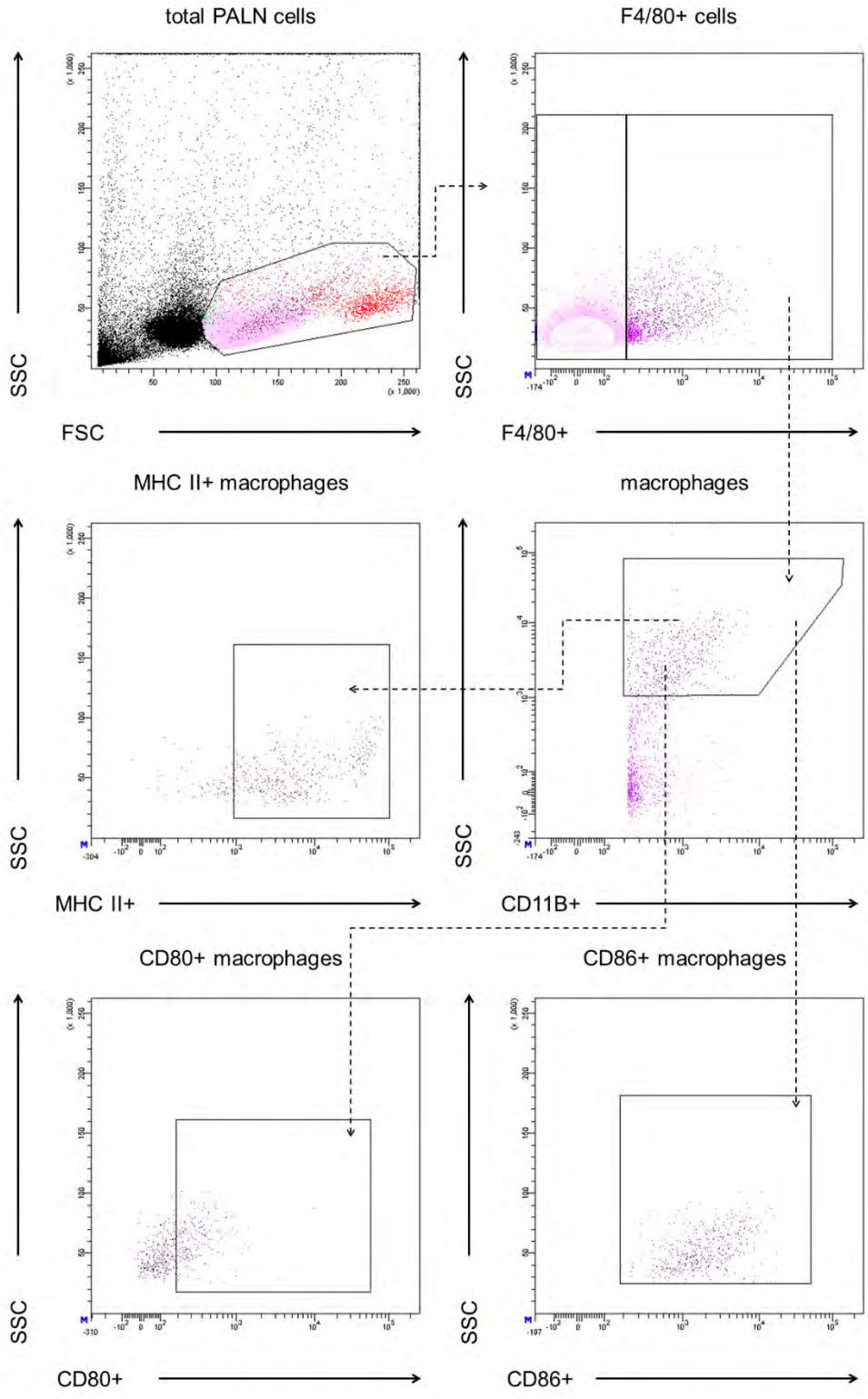


Figure 5.4 Macrophage flow cytometry gating strategy. Total leukocytes were initially gated using SSC and FSC. From within the leukocyte population, cells were assessed for the expression of firstly F4/80+, and then CD11B+ (from within the F4/80+ population) to define the total macrophage population (CD11B+ F4/80+ cells). Activated macrophages were then defined by examining MHCII+ cells from within the macrophage population

(MHCII+ CD11B+ F4/80+ cells). Finally, macrophages expressing the co-stimulatory molecules CD80 (CD80+ CD11B+ F4/80+ cells) and CD86 (CD86+ CD11B+ F4/80+ cells) were defined from within the macrophage population.

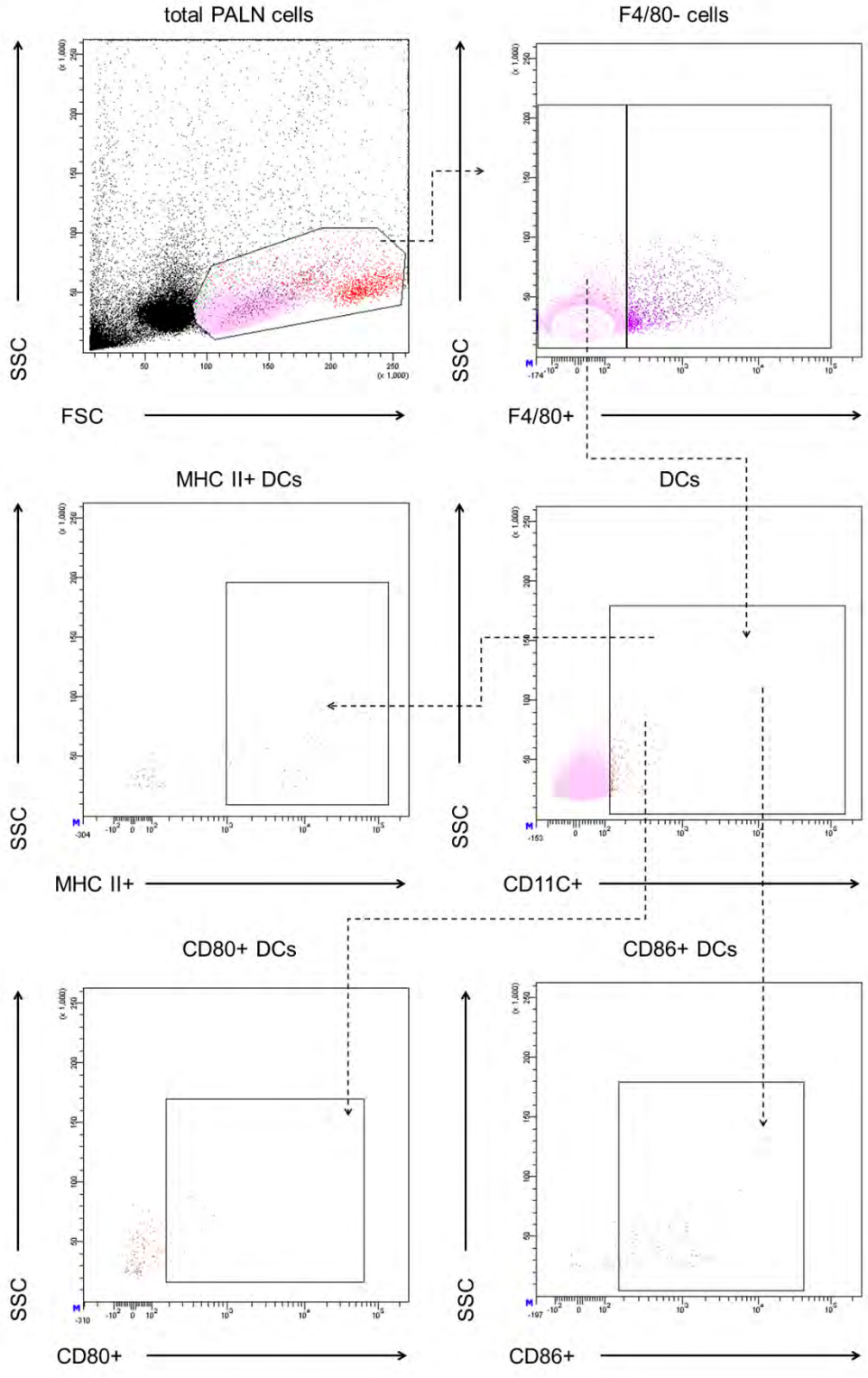


Figure 5.5 Dendritic cell flow cytometry gating strategy. Total leukocytes were initially gated using SSC and FSC. From within the leukocyte population, cells were assessed for expression of CD11C+ (from within the F4/80- population) to define the total dendritic cell population (CD11C+ F4/80- cells). Activated dendritic cells were then defined by examining MHCII+ cells from within the dendritic cell population (MHCII+ CD11C+ F4/80-

cells). Finally, DCs expressing the co-stimulatory molecules CD80 (CD80+ CD11C+ F4/80- cells) and CD86 (CD86+ CD11C+ F4/80- cells) were defined from within the dendritic cell population.

5.3.2.1 Macrophage profile in female reproductive tract tissues and associated lymph nodes

To assess the impact of miR-155 deficiency on macrophage phenotype and numbers in early pregnancy, we first collected the uterus and PALN from oestrus or DPC 3.5 miR-155+/+ or miR-155-/- females. In these experiments, we were able to assess the uterus in addition to the lymph nodes as substantial numbers of macrophages and DC can be recovered from the uterus.

5.3.2.1.1 PALN

Within the PALN, mating resulted in a significant increase in the proportion of macrophages amongst total viable cells in miR-155+/+ (1.4-fold, $p<0.05$) but not in miR-155-/- females compared to the genotype-matched oestrus controls (Figure 5.6A). No change in the proportion of macrophages was observed at oestrus or on DPC 3.5 in miR-155-/- mice compared with WT (Figure 5.6A). Mating resulted in an increase in the total CD11B+ F4/80+ macrophage number in miR-155+/+ (1.8-fold, $p<0.01$) but this was not evident in miR-155-/- females. Indeed, miR-155 deficiency was associated with a reduction in CD11B+ F4/80+ macrophages on DPC 3.5 (38%, $p<0.01$) (Figure 5.6B). Thus, in the absence of miR-155, the population of macrophages is impacted on DPC 3.5 but not at oestrus. These results show fewer macrophages were present in the PALN in the absence of miR-155, and this would be expected to adversely impact the antigen-presenting role that macrophages perform.

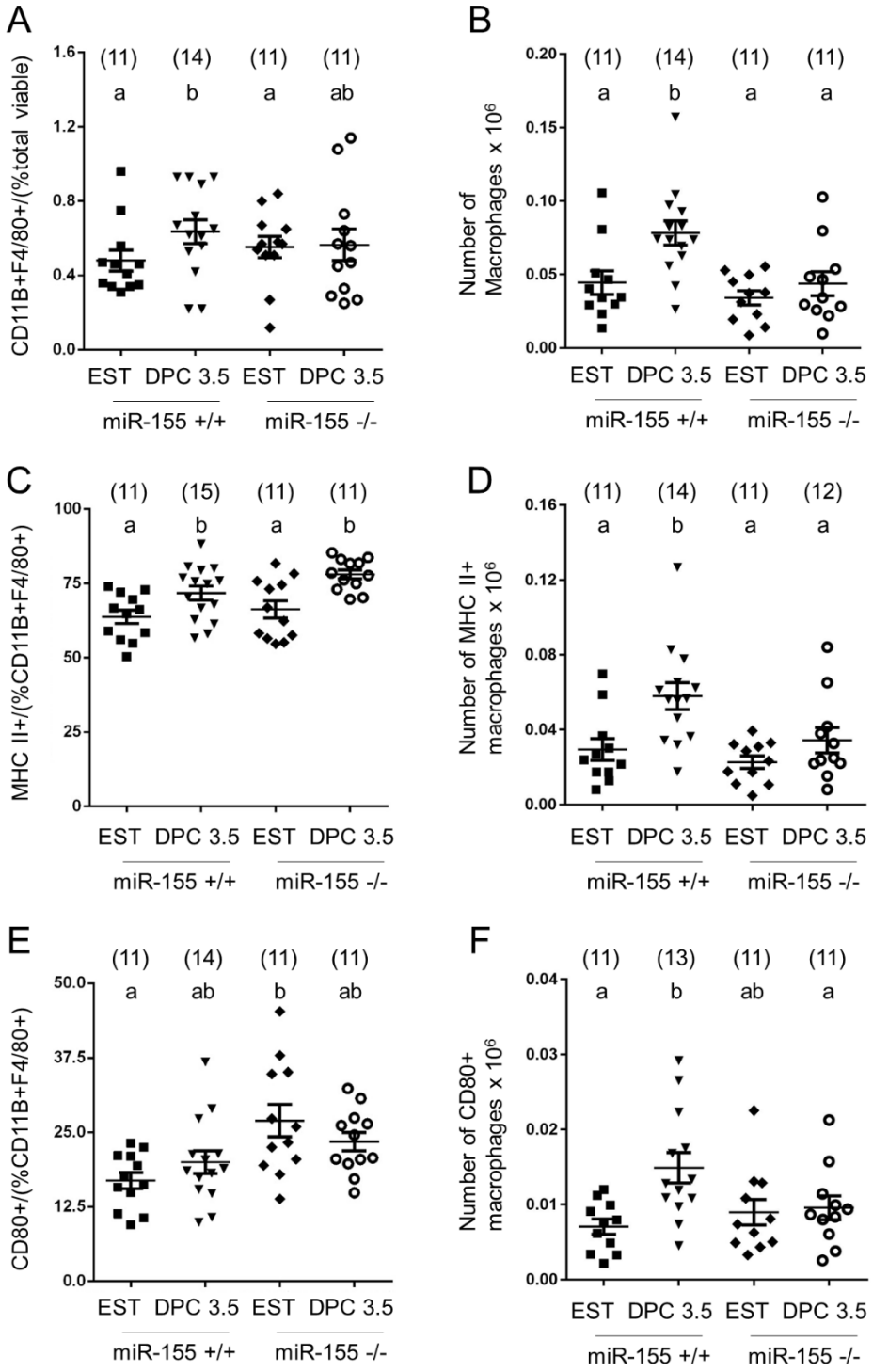
We then further examined whether miR-155 deficiency impacted the subpopulation of activated macrophages (MHCII+ CD11B+ F4/80+ macrophages) in the PALN. Mating resulted in a significant elevation in the proportion of activated macrophages in both miR-155+/+ (1.1-fold, $p<0.05$) and miR-155-/- females (1.2-fold, $p<0.05$), compared to genotype-matched oestrus controls (Figure 5.6C). However, this did not translate to changes between genotypes in percent of macrophages that exhibited activation markers either at oestrus or on DPC 3.5 (Figure 5.6C). In contrast, the total number of activated macrophages was affected by genotype, with an increase observed following mating in miR-155+/+ mice (2.0-fold, $p<0.01$) (Figure 5.6D). This did not occur in miR-155-/- females, where miR-155 deficiency was associated with fewer activated macrophages compared with WT on DPC 3.5 (41%, $p<0.05$) (Figure 5.6D).

Expression of the co-stimulatory molecules CD80 and CD86 within the macrophage population was then examined to evaluate capacity for co-stimulation, which is necessary for T cell activation and survival. No change in the proportion of CD80 positive macrophages was observed following mating in either

miR-155+/+ or miR-155-/- females, with 17-27% of the macrophage population expressing CD80. Furthermore, miR-155 deficiency did not impact the CD80+ macrophage population on DPC 3.5 but did result in an increase in the proportion of CD80+ macrophages at oestrus (1.6-fold, $p<0.05$) (Figure 5.6E). In contrast, while the total number of CD80+ macrophages was increased following mating in miR-155+/+ females (1.8-fold, $p<0.01$), no change was observed in miR-155-/- females (Figure 5.6F). As a consequence, miR-155 deficiency caused a significant reduction in the total number of CD80+ macrophages compared with WT females on DPC 3.5 (31%, $p<0.05$) but not at oestrus (Figure 5.6F).

Within the macrophage population, no change in the proportion of CD86+ macrophages was observed following mating or between genotypes (Figure 5.6G) with CD86+ macrophages making up >80% of the total macrophage population. However, changes were observed in the total number of CD86+ macrophages, with a significant increase observed following mating in miR-155+/+ females (1.8-fold, $p<0.01$) but this did not occur in miR-155-/- females (Figure 5.6H). As a consequence, miR-155 deficiency resulted in a significant decrease in the number of CD86+ macrophages compared to WT females on DPC 3.5 (43%, $p<0.05$), but not at oestrus (Figure 5.6H).

These data indicate that as well as reduced numbers, the activation status and antigen presentation capabilities of macrophages are compromised on DPC 3.5 in miR-155-/- mice.


A
CD11B+F4/80+/(%total viable)
(11) (14) (11) (11)
a b a ab
1.6
1.2
0.8
0.4
0.0
EST DPC 3.5 EST DPC 3.5
miR-155 +/+ miR-155 -/-
B
Number of Macrophages x 10^6
(11) (14) (11) (11)
a b a a
0.20
0.15
0.10
0.05
0.00
EST DPC 3.5 EST DPC 3.5
miR-155 +/+ miR-155 -/-
C
MHC II+/(%CD11B+F4/80+)
(11) (15) (11) (11)
a b a b
100
75
50
25
0
EST DPC 3.5 EST DPC 3.5
miR-155 +/+ miR-155 -/-
D
Number of MHC II+ macrophages x 10^6
(11) (14) (11) (12)
a b a a
0.16
0.12
0.08
0.04
0.00
EST DPC 3.5 EST DPC 3.5
miR-155 +/+ miR-155 -/-
E
CD80+/(%CD11B+F4/80+)
(11) (14) (11) (11)
a ab b ab
50.0
37.5
25.0
12.5
0.0
EST DPC 3.5 EST DPC 3.5
miR-155 +/+ miR-155 -/-
F
Number of CD80+ macrophages x 10^6
(11) (13) (11) (11)
a b ab a
0.04
0.03
0.02
0.01
0.00
EST DPC 3.5 EST DPC 3.5
miR-155 +/+ miR-155 -/-

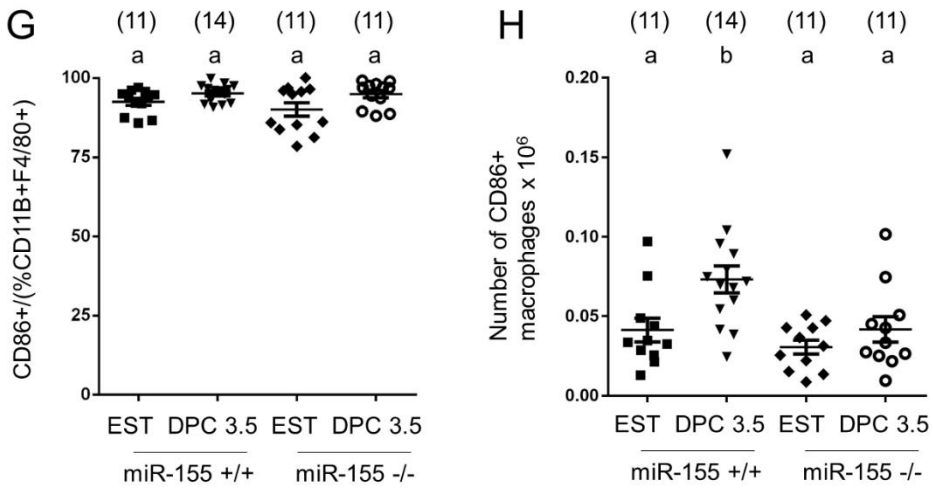
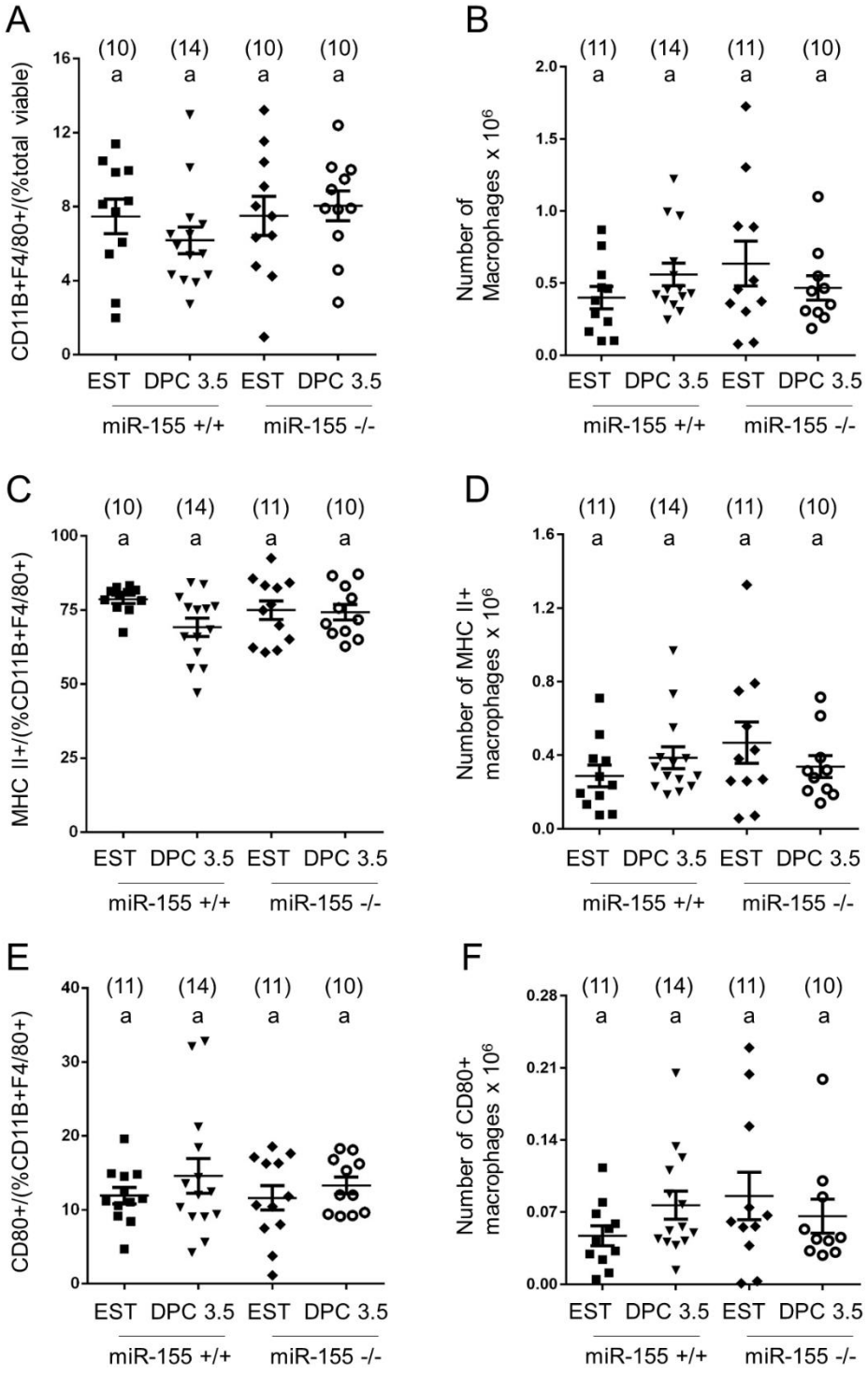


Figure 5.6 The effect of miR-155 deficiency on population of macrophages and the expression of MHCII, CD80, and CD86 by F4/80+ macrophages in PALN. miR-155+/+ and miR-155-/- B6 mice were mated with Balb/c males, and on DPC 3.5, CD11B+ F4/80+ cells from the PALN were analysed by flow cytometry to quantify proportion of macrophages and the expression of MHCII, CD80, and CD86. Virgin oestrus mice were used as non-mated controls. Data are presented as mean ± SEM and are the percentage of F4/80+ macrophages within viable cells (A), the total number of macrophages x 10^6 (B), the percentage of activated macrophages (MHCII+ macrophages) (C), the total number of activated macrophages x 10^6 (D), the percentage of CD80+ macrophages (E), the total number of CD80+ macrophages x 10^6 (F), the percentage of CD86+ macrophages (G) and the total number of CD86+ macrophages x 10^6 (H). Differences between groups were evaluated using Kruskal-Wallis test and Mann-Whitney U test. (a,b,c,d indicates $p<0.05$).

5.3.2.1.2 Uterus

In addition to the PALN, we also assessed the macrophage population in the uterus. Unlike the PALN, there was no change in the proportion or the total number of macrophages, activated macrophages, CD80+ macrophages or CD86+ macrophages between oestrus and mated females, or between genotypes, in miR-155+/+ or miR-155-/- mice. (Figure 5.7, A-H). It is notable that the variance within data sets is higher for the uterus compared with PALN, and this makes it more difficult to detect significant effects of miR-155 deficiency.


A
(10) (14) (10) (10)
a a a a
CD11B+F4/80+/(%total viable)
16
12
8
4
0
EST DPC 3.5 EST DPC 3.5
miR-155 +/+ miR-155 -/-
B
(11) (14) (11) (10)
a a a a
Number of Macrophages x 10^6
2.0
1.5
1.0
0.5
0.0
EST DPC 3.5 EST DPC 3.5
miR-155 +/+ miR-155 -/-
C
(10) (14) (11) (10)
a a a a
MHC II+/(%CD11B+F4/80+)
100
75
50
25
0
EST DPC 3.5 EST DPC 3.5
miR-155 +/+ miR-155 -/-
D
(11) (14) (11) (10)
a a a a
Number of MHC II+ macrophages x 10^6
1.6
1.2
0.8
0.4
0.0
EST DPC 3.5 EST DPC 3.5
miR-155 +/+ miR-155 -/-
E
(11) (14) (11) (10)
a a a a
CD80+/(%CD11B+F4/80+)
40
30
20
10
0
EST DPC 3.5 EST DPC 3.5
miR-155 +/+ miR-155 -/-
F
(11) (14) (11) (10)
a a a a
Number of CD80+ macrophages x 10^6
0.28
0.21
0.14
0.07
0.00
EST DPC 3.5 EST DPC 3.5
miR-155 +/+ miR-155 -/-

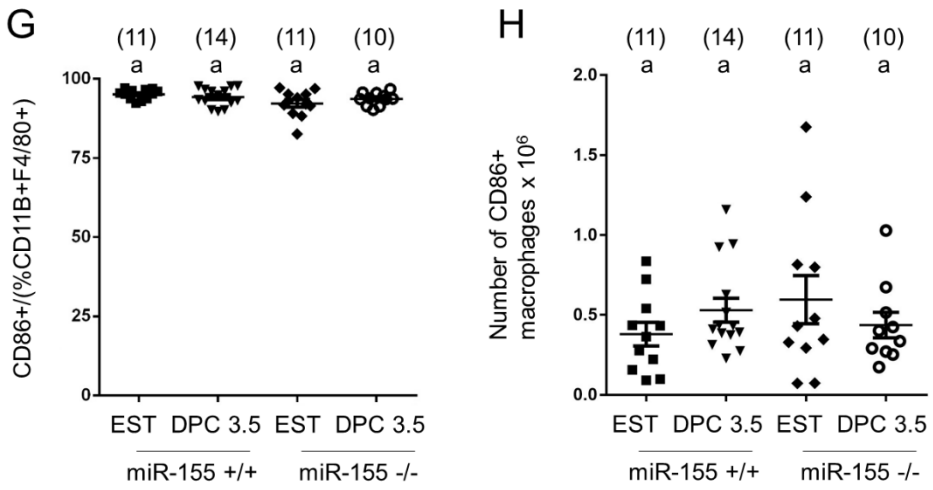


Figure 5.7 The effect of miR-155 deficiency on the population of macrophages and expression of MHCII, CD80, and CD86 by F4/80+ macrophages in uterine tissues. miR-155+/+ and miR-155-/- B6 mice were mated with Balb/c males, and on DPC 3.5, CD11B+ F4/80+ cells from the uterine tissue were analysed by flow cytometry to quantify proportion of macrophages and the expression of MHCII, CD80, and CD86. Virgin oestrus mice were used as non-mated controls. Data are presented as mean ± SEM and are the percentage of F4/80+ macrophages within viable cells (A), the total number of macrophages x 10^6 (B), the percentage of activated macrophages (MHCII+ macrophages) (C), the total number of activated macrophages x 10^6 (D), the percentage of CD80+ macrophages (E), the total number of CD80+ macrophages x 10^6 (F), the percentage of CD86+ macrophages (G) and the total number of CD86+ macrophages x 10^6 (H). Differences between groups were evaluated using Kruskal-Wallis test and Mann-Whitney U test. (a,b,c,d indicates $p<0.05$).

5.3.2.2 Dendritic cell profile in female reproductive tract tissues and associated lymph nodes

To assess the impact of miR-155 deficiency on dendritic cell numbers and phenotype in early pregnancy, uterus and PALN were collected from oestrus or DPC 3.5 miR-155+/+ or miR-155-/- females.

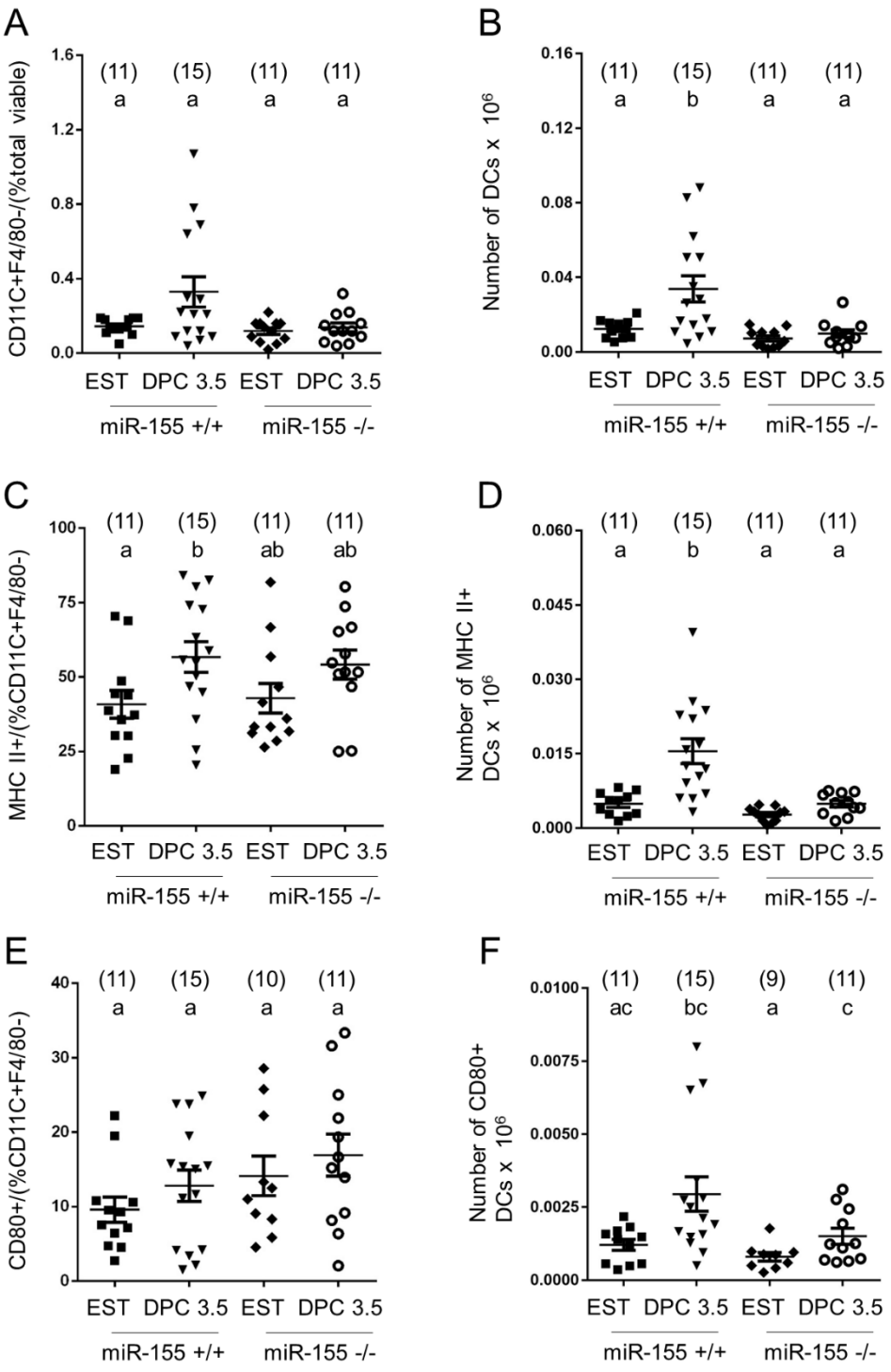
5.3.2.2.1 PALN

Within the population of viable cells, there was no change in the proportion of F4/80-CD11C+ DCs in miR-155+/+ or miR-155-/- mice following mating or between genotypes (Figure 5.8A). However, a 2.8-fold ($p<0.05$) increase in the total number of F4/80- CD11C+ DCs following mating in miR-155+/+ females was observed but this was not evident in miR-155-/- females (Figure 5.8B). miR-155 deficiency resulted in a significant reduction in the total number of F4/80- CD11C+ DCs on DPC 3.5 (71%, $p<0.01$) but there was no difference between genotypes at oestrus (Figure 5.7B).

The DC population was then further examined to explore the impact of miR-155 deficiency on MHCII+ mature DC in the PALN. The proportion of MHCII+ DCs was increased following mating in miR-155+/+ (1.8-fold, $p<0.05$) but not in miR-155-/- mice, and no change was observed between genotypes at oestrus or on DPC 3.5 (Figure 5.8C). Similar to macrophages, a reduced DC population in the PALN of miR-155-/- mice on DPC 3.5 may indicate less capability for antigen presentation to activate T cells after mating.

Examination of MHCII+ DC numbers showed that the increase in MHCII+ DCs observed following mating in miR-155+/+ females (3.2-fold, $p<0.05$), did not occur in miR-155-/- females. As a consequence, miR-155 deficiency resulted in a significant reduction in MHCII+ DCs on DPC 3.5 (69%, $p<0.01$) but there was no difference at oestrus (Figure 5.8D).

Expression of the co-stimulatory factors CD80 and CD86 were then examined within the DC population in the PALN. No change was observed in the percentage of CD80+ DCs within the DC population following mating or between genotypes (Figure 5.8E). Mating resulted in a significant elevation in the CD80+ DC number in miR-155+/+ (2.4-fold, $p<0.05$) and in miR-155-/- females (1.9-fold, $p<0.05$). Similar results were observed for CD86+ DCs with no change in proportions following mating or between genotypes (Figure 5.8G). Mating resulted in a significant elevation in the CD80+ DC number in miR-155+/+ mice (2.9-fold, $p<0.01$). As a consequence, miR-155 deficiency resulted in a significantly lower number of CD86+ DCs on DPC 3.5 (69.1%, $p<0.05$) but not at oestrus (Figure 5.7H). The less activated DCs and reduced costimulatory factor expression on DPC 3.5 may indicate that DCs in miR-155-/- mice are less capable of activating T cell responses.


A
CD11C+F4/80-/(%total viable)
(11) (15) (11) (11)
a a a a
EST DPC 3.5 EST DPC 3.5
miR-155 +/+ miR-155 -/-
B
Number of DCs x 10^6
(11) (15) (11) (11)
a b a a
EST DPC 3.5 EST DPC 3.5
miR-155 +/+ miR-155 -/-
C
MHC II+/(%CD11C+F4/80-)
(11) (15) (11) (11)
a b ab ab
EST DPC 3.5 EST DPC 3.5
miR-155 +/+ miR-155 -/-
D
Number of MHC II+ DCs x 10^6
(11) (15) (11) (11)
a b a a
EST DPC 3.5 EST DPC 3.5
miR-155 +/+ miR-155 -/-
E
CD80+/(%CD11C+F4/80-)
(11) (15) (10) (11)
a a a a
EST DPC 3.5 EST DPC 3.5
miR-155 +/+ miR-155 -/-
F
Number of CD80+ DCs x 10^6
(11) (15) (9) (11)
ac bc a c
EST DPC 3.5 EST DPC 3.5
miR-155 +/+ miR-155 -/-

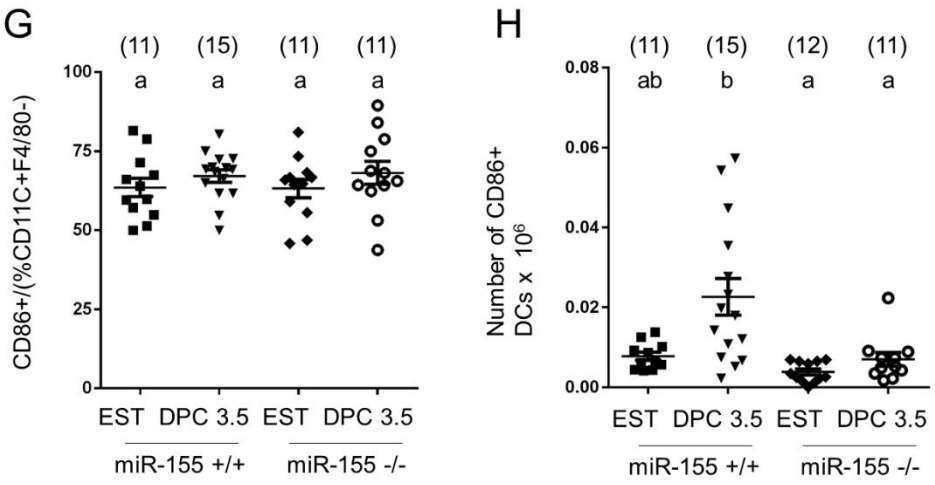


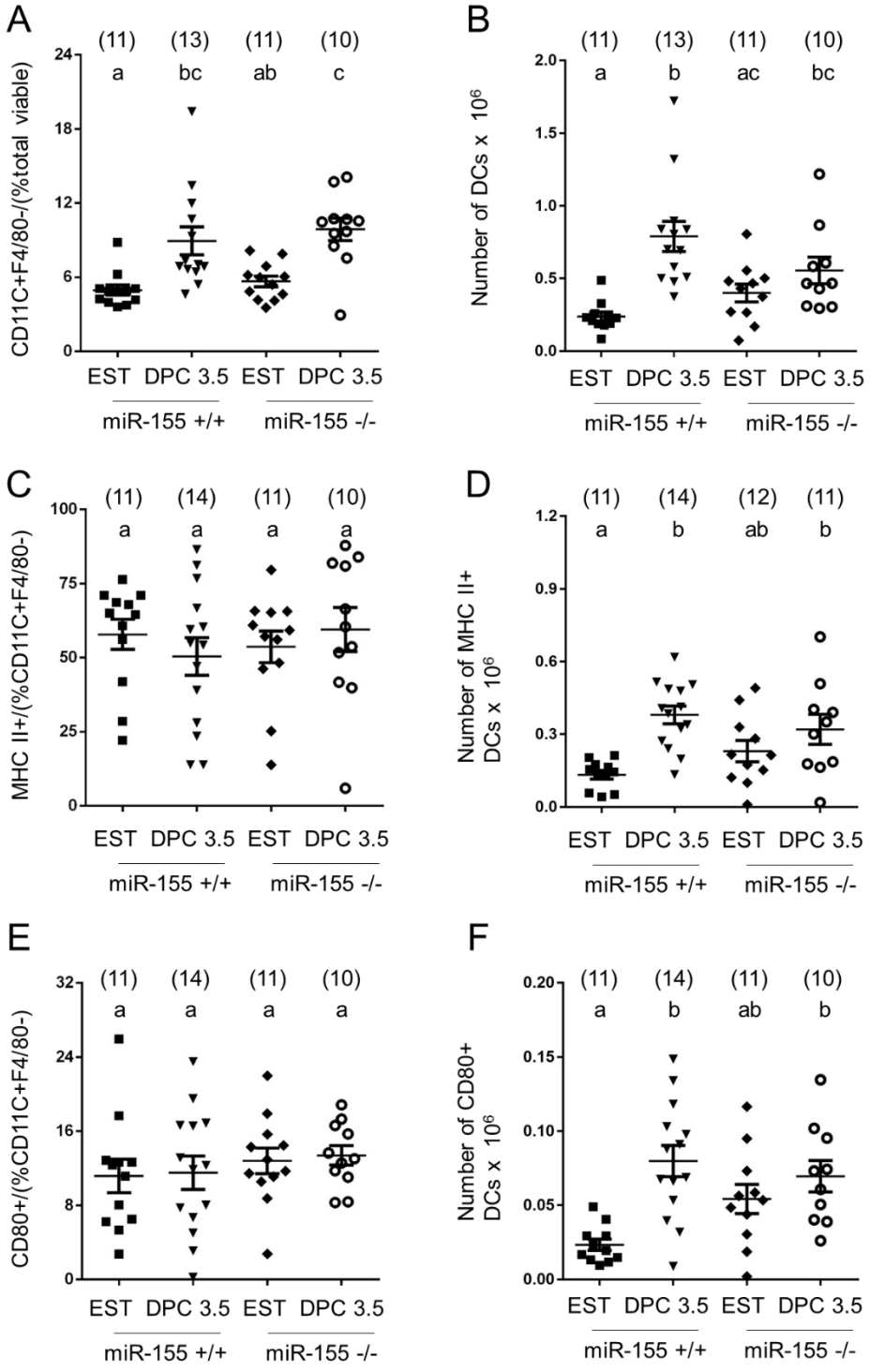
Figure 5.8 The effect of miR-155 deficiency on the population of DCs and expression of MHCII, CD80, and CD86 by CD11C+ DCs in PALN. miR-155+/+ and miR-155-/- B6 mice were mated with Balb/c males, and on DPC 3.5, CD11C+ F4/80- cells from the PALN were analysed by flow cytometry to quantify proportion of DCs and the expression of MHCII, CD80, and CD86. Virgin oestrus mice were used as non-mated controls. Data are presented as mean ± SEM and are the percentage of CD11C+ DCs within viable cells (A), the total number of DCs x 10^6 (B), the percentage of activated DCs (MHCII+ DCs) (C), the total number of activated DCs x 10^6 (D), the percentage of CD80+ DCs (E), the total number of CD80+ DCs x 10^6 (F), the percentage of CD86+ DCs (G) and the total number of CD86+ DCs x 10^6 (H). Differences between groups were evaluated using Kruskal-Wallis test and Mann-Whitney U test. (a,b,c,d indicates $p<0.05$).

5.3.2.2.2 Uterus

Within the uterus, the proportion of CD11C+ F4/80- DCs amongst the total viable cells was increased following mating in both miR-155+/+ (1.8-fold, $p<0.01$) and miR-155-/- (1.7-fold, $p<0.05$) mice (Figure 5.9A). miR-155 deficiency did not alter in the proportion of CD11C+ F4/80- DCs at oestrus or on DPC 3.5 (Figure 5.9A). The absolute number of CD11C+ F4/80- DCs increased with mating in miR-155+/+ females (3.3-fold, $p<0.0001$). Mating in miR-155-/- females resulted in a similar shift in mean number of DCs, but this did not reach statistical significance. miR-155 deficiency did not impact the number of CD11C+ F4/80- DCs at oestrus or on DPC 3.5 compared to WT mice (Figure 5.9B).

The DC population was then further examined to explore the impact of miR-155 deficiency on expression of MHCII amongst DC in the uterus. The proportion of MHCII+ DCs was high at 50-90% and was not altered following mating or between genotypes in miR-155+/+ or miR-155-/- mice (Figure 5.9C). However, MHCII+ DC number was increased following mating in miR-155+/+ females (3.0-fold, $p<0.001$) with only a trend towards significance in miR-155-/- females. miR-155 deficiency did not impact MHCII+ DC numbers at oestrus or on DPC 3.5 (Figure 5.9D).

Expression of the co-stimulatory factors CD80 and CD86 were then explored within the DC population in the uterus. No change in the proportion of CD80+ DCs was observed following mating in either miR-155+/+ or miR-155-/- females, and these was no difference between genotypes (Figure 5.9E). However, CD80+ DC numbers were increased following mating in miR-155+/+ females (3.4-fold, $p<0.001$) but not in miR-155-/- females (Figure 5.9F). No changes were seen in the total number of CD80+ DCs in the absence of miR-155 at oestrus or on DPC 3.5. Similar results were observed in the CD86+ DC population with no changes in the proportion of DCs following mating or between genotypes in both miR-155+/+ or miR-155-/- females, and an increase in the number of CD86+ DCs following mating in miR-155+/+ (3.7-fold, $p<0.0001$) but not miR-155-/- females (Figure 5.8H).


A
(11) (13) (11) (10)
a bc ab c
CD11C+F4/80-/(%total viable)
24
18
12
6
0
EST DPC 3.5 EST DPC 3.5
miR-155 +/+ miR-155 -/-
B
(11) (13) (11) (10)
a b ac bc
Number of DCs x 10^6
2.0
1.5
1.0
0.5
0.0
EST DPC 3.5 EST DPC 3.5
miR-155 +/+ miR-155 -/-
C
(11) (14) (11) (10)
a a a a
MHC II+/(%CD11C+F4/80-)
100
75
50
25
0
EST DPC 3.5 EST DPC 3.5
miR-155 +/+ miR-155 -/-
D
(11) (14) (12) (11)
a b ab b
Number of MHC II+ DCs x 10^6
1.2
0.9
0.6
0.3
0.0
EST DPC 3.5 EST DPC 3.5
miR-155 +/+ miR-155 -/-
E
(11) (14) (11) (10)
a a a a
CD80+/(%CD11C+F4/80-)
32
24
16
8
0
EST DPC 3.5 EST DPC 3.5
miR-155 +/+ miR-155 -/-
F
(11) (14) (11) (10)
a b ab b
Number of CD80+ DCs x 10^6
0.20
0.15
0.10
0.05
0.00
EST DPC 3.5 EST DPC 3.5
miR-155 +/+ miR-155 -/-

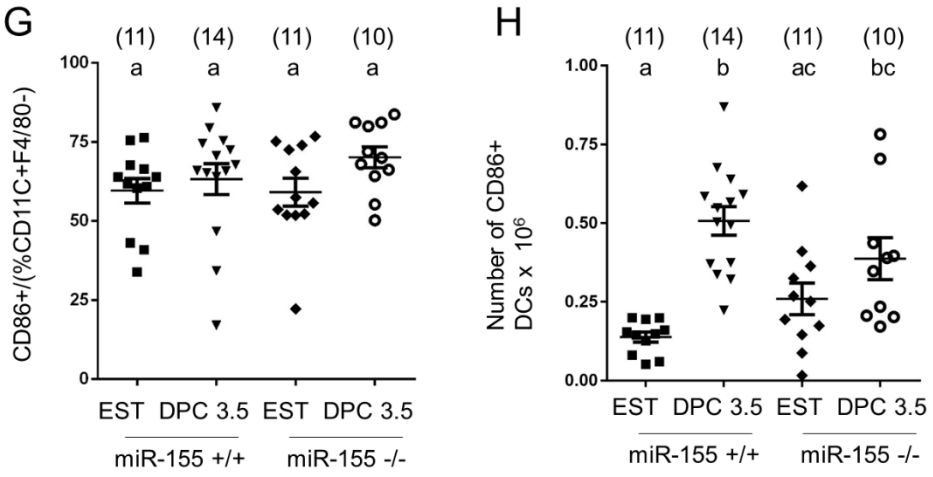


Figure 5.9 The effect of miR-155 deficiency on the population of DCs and expression of MHCII, CD80, and CD86 by CD11C+ DCs in uterine tissues. miR-155+/+ and miR-155-/- B6 mice were mated with Balb/c males, and on DPC 3.5, CD11C+ F4/80- cells from the uterine tissue were analysed by flow cytometry to quantify proportion of DCs and the expression of MHCII, CD80, and CD86. Virgin oestrus mice were used as non-mated controls. Data are presented as mean ± SEM and are the percentage of CD11C+ DCs within viable cells (A), the total number of DCs x 10^6 (B), the percentage of activated DCs (MHCII+ DCs) (C), the total number of activated DCs x 10^6 (D), the percentage of CD80+ DCs (E), the total number of CD80+ DCs x 10^6 (F), the percentage of CD86+ DCs (G) and the total number of CD86+ DCs x 10^6 (H). Differences between groups were evaluated using Kruskal-Wallis test and Mann-Whitney U test. (a,b,c,d indicates $p<0.05$).

5.3.3 Systemic impact of miR-155 deficiency on DPC 3.5

To evaluate the extent to which the impact of miR-155 deficiency was associated with local as opposed to systemic changes to the immune system, the spleen, peripheral blood and mLN were also evaluated at oestrus and on DPC 3.5. At oestrus, a reduced number of CD4+ T cells was observed in the mLN and spleen, compared to the WT oestrous control. Consistent with literature (Lu et al., 2009a), miR-155 deficiency resulted in a reduction in CD4+ Treg cells, measured as proportion of CD4+ T cells, or total numbers. As well, Foxp3 MFI was reduced in the mLN and spleen (Figure 8.4 to 8.6, appendix section 8.2.1), compared to the WT controls.

miR-155 deficiency was not associated with any change in CD4+ T cell proportion or numbers in the spleen, mLN or in peripheral blood on DPC 3.5. As in oestrus mice, the proportion and number of Treg cells was reduced in all three tissues. However, the extent of reduction in the Treg cells population was not as great as that observed in the PALN, indicating that miR-155 deficiency had a greater local impact on the Treg cell expansion in this site.

On DPC 3.5, miR-155 deficiency did not alter macrophage population and phenotype in the spleen, mLN or peripheral blood, but a systemically reduced proportion and number of DC was observed in the mLN and spleen. In addition, the number of MHCII+ DC was decreased in the mLN, and a diminished number of CD86+ DC was observed in both mLN and spleen in the absence of miR-155 on DPC 3.5. These data suggest that the antigen-presenting capability of macrophages and DCs is systemically altered in the absence of miR-155, and therefore can impact on T cell activation.

Taken together, the studies in other tissue sites indicate that miR-155 deficiency causes a systemic reduction in Treg cell populations and alters DC phenotypes, however the impact on Treg cells in the reproductive tissues on DPC 3.5 is greater in magnitude than that seen in other sites.

5.3.4 Maternal miR-155 deficiency elevates LPS-induced fetal loss

As detailed above, miR-155 deficiency results in an altered maternal immune environment in early pregnancy. This altered environment appeared not to overtly affect fertility in our breeding colony, where miR-155-/- females were mated with syngeneic miR-155-/- males to yield normal litter sizes at regular intervals, indicating steady state pregnancy progresses relatively unperturbed (Zhang, data not shown). As detailed in chapter 3, the functional requirements for immune mediators can become apparent following inflammatory challenge. Therefore, to formally investigate the impact that miR-155 deficiency may have on the generation and maintenance of a tolerogenic immune environment, miR-155-/- and

miR-155+/+ female mice were mated to allogeneic Balb/c males to produce pregnancies with maternal, but not fetal, miR-155 deficiency. On DPC 9.5, pregnant females were administrated a low-dose inflammatory challenge of 1.0 μg LPS, or PBS control. This dose of LPS was chosen as it was determined to be just below the threshold for impact on pregnancy loss in miR-155+/+ mice

There was no detectable impact of miR-155 deficiency on fertility or fecundity evident in mice administered PBS and examined just before term on DPC 17.5. The incidence of progression from mating to pregnancy, total and viable implantation sites, and fetal and placental weights were not different between genotypes (Figure 5.10A-E).

A major impact of miR-155 deficiency was revealed in the response to LPS challenge. miR-155-/- females were more severely affected than miR-155+/+ mice, with a lower proportion of miR-155-/- mice carrying viable fetuses ($p<0.05$, Figure 5.10A) and a lower number of viable fetuses per mated female ($p<0.0001$, Figure 5.10B) on DPC 17.5. The change in viable fetuses was associated with a substantially higher rate of fetal resorption in miR-155-/- dams following LPS treatment (19.2-fold, $p<0.0001$, Figure 5.10C). Additionally, LPS treatment impacted fetal outcomes in miR-155-/- females with an 8.3% reduction in fetal weights ($p<0.05$, Figure 5.10D) and a 14% reduction in the fetal: placental weight ratio ($p<0.0001$, Figure 5.10F). No effect of LPS treatment on placental weight was observed in either genotype (Figure 5.10E). Thus, maternal miR-155 deficiency appears to impact the maternal immune environment in pregnancy, such that miR-155 deficient mice exhibited greater susceptibility to fetal loss after inflammatory challenge in mid-gestation.

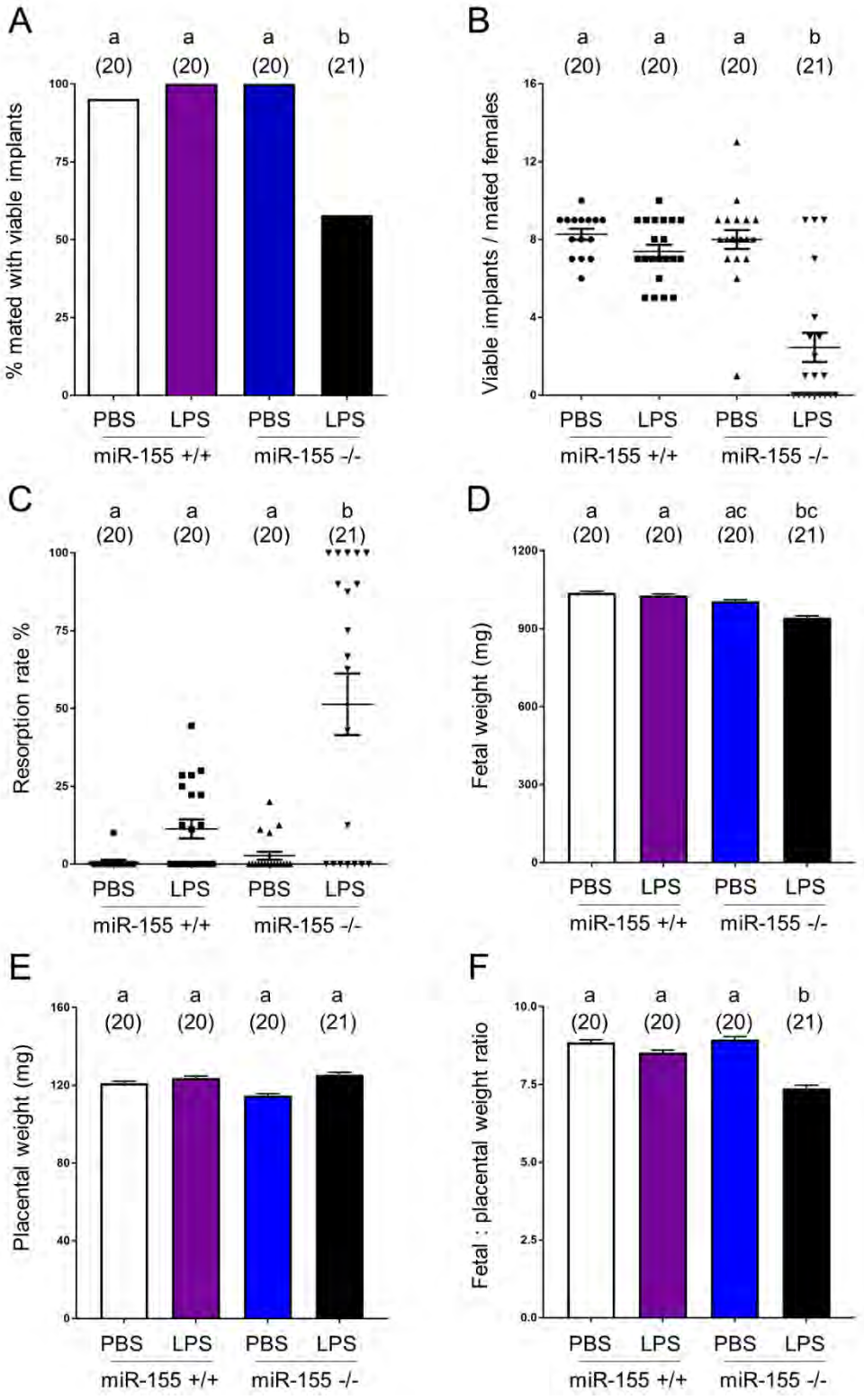


Figure 5.10 The effect of miR-155 deficiency on pregnancy parameters after low-dose LPS challenge. miR-155+/+ and miR-155-/- mice were mated with Balb/c males and injected i.p. with LPS (1μg) or control (PBS) on DPC 9.5, then autopsied on DPC 17.5. Data are the percentage of mated females pregnant with viable fetus (A), the number (mean ± SEM) of viable implantation sites per mated mouse (individual data points with mean

value shown; B), the percentage of total implantation sites per pregnant female undergoing resorption (individual data points with mean ± SEM; C), the fetal weight (estimated marginal mean ± SEM; D), the placental weight (marginal mean ± SEM; E), and the fetal: placental weight ratio (estimated marginal mean ± SEM; F). Numbers of mated mice are shown in parentheses. The effect of genotype was evaluated in A by Chi-square analysis and in B by ANOVA and Sidak t-test. The effect of genotype was evaluated in D-F by mixed model analysis, using mother as subject and litter size as covariate (a,b,c,d indicates $p<0.05$).

5.4 Discussion

A sufficient number and functional competence in Treg cells is critical for maternal immune tolerance in pregnancy. As detailed in chapter 1, Treg cells suppress inflammation and promote immune adaptation at the maternal-fetal interface (Aluvihare et al., 2004, Guerin et al., 2009). Understanding the function of Treg cells is crucial for developing treatment for human gestational complications, such as preeclampsia and recurrent miscarriage (Guerin et al., 2009), where altered Treg cells are implicated. In order to develop this knowledge, it is important to understand how Treg cells are normally regulated during pregnancy. miR-155 is one of the most well studied microRNAs in the immune system. It is involved in the regulation of multiple cell types including antigen presenting cells and also Treg cells, therefore it may impact on the immune response required for pregnancy success. miR-155 is induced after inflammatory challenge in DCs (Dunand-Sauthier et al., 2014), and has been reported to regulate the antigen presentation capacity of DCs (Rodriguez et al., 2007). miR-155 appeared to have conserved function in both human and mice and is associated with phenotype regulation in macrophages (Zhang et al., 2013, Graff et al., 2012). miR-155 deficiency resulted a systemic reduction in Treg cells in mice (Cobb et al., 2006, Lu et al., 2009a) and it's expression is regulated by Foxp3 (Lu et al., 2009a, Marson et al., 2007). However, no study has previously investigated the role of miR-155 in antigen presenting cells and Treg cells in early pregnancy.

Altered miR-155 expression patterns are found in pregnancy complications. In recurrent pregnancy loss patients, miR-155 expression is higher in the peripheral blood as well as in placenta (Tang et al., 2016, Winger et al., 2015). Altered immune regulatory microRNA expression in the peripheral blood mononuclear cells including miR-155 is associated with preeclampsia (Winger et al., 2015). To understand how aberrant miR-155 expression patterns may contribute to pregnancy complications, the role of miR-155 in normal pregnancy needs to be addressed.

In the studies described in this chapter, miR-155 deficiency in mice was found to result in an overall diminished total cell number in PALN. This was evident at oestrus and on DPC 3.5, and is associated with the total CD4+ T cell number being diminished in the PALN at oestrus and also on DPC 3.5, when the cell proliferation induced with the immune response to conception is clearly evident in WT mice (Johansson et al., 2004). In the PALN, the smaller CD4+ T cell population is associated with a reduction in Treg cells. This manifests as a reduction in the proportion of Treg cells amongst CD4+ T cells as well as reduced Foxp3+ CD4+ T cell number. It is notable that the proportion of CD4+ T cells are elevated in

the PALN in miR-155-/- females at oestrus but not on DPC 3.5, indicating that miR-155 deficiency impacts on the CD4+ T cell expansion, and affects the Foxp3+ Treg cells to an even greater extent than CD4+ effector cells.

In the current study, miR-155 deficiency did not impact on the total CD4+ T cells on DPC 3.5 in the spleen, blood and also mLN which is consistent with a previous study demonstrating that under steady-state circumstances (e.g. without antigen challenge) the number of CD4+ T cells in thymus and spleen are not reduced in the absence of miR-155 (Lu et al., 2009a). However, in the reproductive setting, paternal MHC alloantigens are present in seminal fluid at conception and are released by the placenta and can be found in the maternal circulation from mid-gestation (Herberth et al., 2014, Walknowska et al., 1969) and the maternal immune system can interact with fetal MHC (Hunt et al., 2003, Van Rood et al., 1958, Tilburgs et al., 2009). In mated miR-155 deficient mice, the paternal alloantigen challenge can be seen to induce expansion in the PALN CD4+, CD8+ and Treg cell pool by DPC 3.5. However absence of miR-155 is associated with a less robust T cell response, resulting in a diminished CD4+ T cell population on DPC 3.5. There is a selective adverse impact on the Treg cells, such that the absence of miR-155 appears likely to have a substantial impact on establishment of maternal immune tolerance.

The absence of miR-155 appears to cause Treg cells to be diminished in the PALN partly because of a smaller resident population at oestrus before mating, and mainly because of failure of T cell proliferation to be activated after mating. With reduced total lymph node cell numbers at oestrus on DPC 3.5, and a particularly diminished Treg cell proportion (Figure 5.11). This reduction in the number of Treg cells in the absence of miR-155 was largely specific to the PALN, with a much smaller systemic reduction observed at oestrus and on DPC 3.5. This is consistent with previous studies which have demonstrated that the Treg cell population was diminished systemically (Cobb et al., 2006, Lu et al., 2009a). This study is the first one which addressed that miR-155 deficiency resulted in Treg cell reduction in the early pregnancy, indicating that the miR-155 impact on the capability of Treg cell expansion and proliferation.

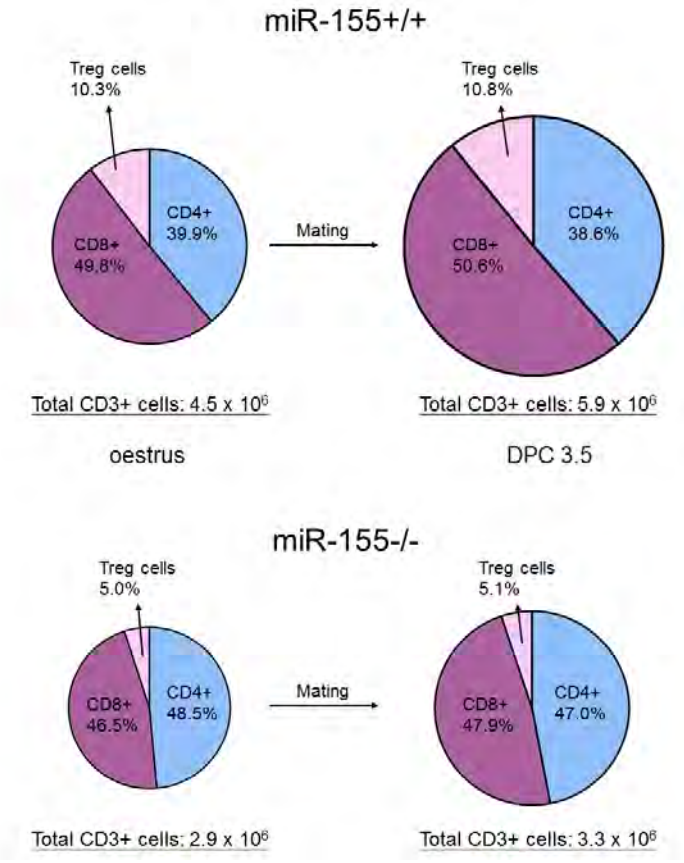


Figure 5.11 Proportion of CD4+, CD8+ and CD4+ CD25+ Foxp3+ Treg cells in the PALN in miR-155+/+ and miR-155-/- mice at oestrus and on DPC 3.5. Fewer Treg cells are present in PALN of the miR-155-/- mice at oestrus, and expansion of the Treg cell population by DPC 3.5 is limited, compared to the WT controls.

In addition, the MFI of Foxp3 is reduced in PALN on DPC 3.5. As Foxp3 is the 'master switch' which is critical in Treg cell development and suppressive function (Fontenot et al., 2005, Fontenot et al., 2003), reduced MFI of Foxp3 indicates that Treg cells in miR-155-/- mice may have poorer commitment to the Treg cell lineage and potentially reduced functional competence. Notably, the reduced Foxp3 MFI was only observed on DPC 3.5 but not at oestrus in PALN, indicating that in the absence of miR-155, Treg cells in PALN may maintain normal suppressive activity. However, after mating the Treg cells in miR-155-/- may not be as suppressive as those from miR-155+/+ female mice. Although previous studies reported that miR-155 deficiency does not impact the suppressive capabilities in natural Treg cells (Stahl et al., 2009, Lu et al., 2009a), Chauhan et al have demonstrated that reduced expression of Foxp3 indicates the suppressive function of Treg cells is compromised (Chauhan et al., 2009).

The diminished Treg cell pool in early gestational period in miR-155-/- mice is accompanied by, and may be the consequence of altered phenotype of DCs. The DCs are differentially regulated in the absence of miR-155, and in particular, a more than 4-fold reduction in the number of DCs was found in the PALN. These DCs are less activated, with decreased expression of MHCII, CD80 and CD86. These observations are consistent with previous studies showing that miR-155 is upregulated in mature or activated DCs (Stumpfova et al., 2014, Ceppi et al., 2009). Less MHCII expression may suggest that DC presentation capability is impaired. The antigen presenting capability was not addressed in this study, however Rodriguez et al (Rodriguez et al., 2007) have demonstrated that antigen presentation capacity is impacted by miR-155 deficiency, such that miR-155 deficient DCs fail to activate T cells effectively. It has also been demonstrated that DCs isolated from miR-155-/- mice are incompetent to activate T cells *in vitro* due to repression of Arginase-2, which suggests that miR-155 activity is crucial for DCs to activate T cells (Dunand-Sauthier et al., 2014). In the current study, the reduced Treg cell population and diminished expression of MHCII, CD80 and CD86 are consistent with the literature. Therefore, the reduced Treg cell population in early pregnancy may be due to diminished capacity for DCs to prime and activate Treg cell proliferation.

In the mouse uterus, both mature macrophages and DCs are thought to be differentiated from undifferentiated macrophage precursors (Keenihan and Robertson, 2004), indicating that the possibilities of impaired reservoir of macrophages in miR-155 deficient mice for DC differentiation and could therefore impact on the DC population as well as the T cell population.

Macrophages are amongst the antigen presenting cell influx found in the cervix 12h post coitus in human (Sharkey et al., 2012b) and the day after mating in mice (Robertson et al., 1996, Robertson et al., 1998, Robertson et al., 1992). An altered phenotype of PALN macrophages with antigen presenting cell activity might also contribute to the reduced Treg cell population. In the current study, the expected increase in macrophages seen in WT mice on DPC 3.5 did not occur in miR-155 deficient mice. Similarly, fewer MHCII+ activated macrophages, and CD80+ and CD86+ macrophages, were found on DPC 3.5 in PALN. DPC 3.5 may not be the optimal time points to quantify DC and macrophage population and phenotype. It would be relevant to further investigate the impact of miR-155 deficiency on the inflammation-like response to seminal fluid induction in the 12-24 hours after coitus, where even greater differences might be expected to be seen.

miR-155 deficiency did not impact on the number or phenotype of macrophages in the mLN of the spleen, consistent with a specific effect in the context of the inflammatory response accompanying insemination. Also, there was no change in the uterus, but the greater variation between individual mice was likely a factor in this result.

Additionally miR-155 deficiency results in attenuated IL-2 signalling in lymphocytes in mice, and IL-2 is well known for its pivotal role in Treg cell homeostasis (Bayer et al., 2007). Therefore, it is also possible that limited IL-2 signalling contributes to reduced Treg cell proliferative activity during early pregnancy. Altered T cell immunity in miR-155 deficient mice does not affect tolerance of fetal alloantigen, as miR-155 deficient female mice mated to Balb/c males are capable of maintaining pregnancy with generally comparable outcomes to WT control mice. However, the importance of miR-155 becomes apparent when pregnant miR-155-/- female mice were administered low dose LPS to induce a systemic inflammatory response in mid-gestation. When miR-155-/- female mice were challenged with inflammatory stimuli, they had a reduced capacity to sustain pregnancy with a 51.4% resorption rate, compared to WT mice in which pregnancy was not impacted by the same LPS dose. miR-155 is known to down-regulate inflammatory cytokine production in response to bacterial stimuli (Ceppi et al., 2009). In the current study, inflammatory cytokines may not be regulated appropriately in miR-155 deficient mice after administration of LPS in mid-gestation. In part, this may be attributed to the insufficient Treg cell response, as Treg cells are important for suppressing and controlling inflammatory cytokine responses (Collison et al., 2009, Collison et al., 2007, Sakaguchi et al., 2009a, Liang et al., 2008). In miR-155 deficient mice challenged with LPS, the limited population of Treg cells may not be able to

control the excess inflammatory responses after inflammatory insults. Taken together, these data suggest that miR-155 is essential in protecting the fetus from excessive inflammation via regulating Treg cell development and antigen presenting cell function.

The current study does not quantify the Treg cell and antigen presenting cell population during mid and late gestation. Moreover, the dynamics of Treg cell population and antigen presenting cells were not mapped with LPS-induced systemic inflammation. Further studies are required to address the mechanisms of altered suppressive activity of Treg cells in the absence of miR-155 during gestation. In addition, this antigen presentation capability and phenotypes in antigen presenting cells and polarisation of macrophages in miR-155-/- mice has not been addressed. Further studies are required to address these questions in order to better understand how Treg cell responses are impacted in the absence of miR-155 over the course of gestation. Furthermore, adoptive transfer of Treg cells from pregnant WT mice into miR-155 deficient females prior to inflammatory challenge could be performed to investigate the protective role of Treg cells in the inflammation-induced fetal loss, and to confirm that the elevated fetal loss can indeed be attributed to these cells.

In summary, the current study reinforces a pivotal role for miR-155 in both innate and adaptive immunity and expands current knowledge to show a key role for miR-155 in pregnancy. Both Treg cell and antigen presenting cell populations are dysregulated and likely to be functionally impaired in the absence of miR-155. In particular, the expansion in Treg cells associated with seminal fluid contact at conception is impaired, presumably as a consequence of the compromised activation and antigen presentation capability of DCs. This leads to a diminished Treg cell population in the PALN at the time of implantation on DPC 3.5. Treg cells generated in the absence of miR-155 are competent to sustain the allogeneic fetus under steady-state conditions. However, when pregnant mice with miR-155 deficiency are challenged with a mid-gestation inflammatory insult, the diminished Treg cell population is unable to control excess inflammation and this leads to elevated fetal loss.

Chapter 6 Altered Treg Cell and Antigen Presenting Cell Populations are Associated with LPS Induced Fetal Loss in Pregnant miR-223 Deficient Mice

6.1 Abstract

Immune tolerance of the semi-allogeneic fetus requires CD4+Foxp3+ T-regulatory (Treg) cells, which suppress inflammation and anti-fetal immunity. In mice, Treg expand at the outset of pregnancy in response to signals in seminal fluid. Recent studies have demonstrated that microRNAs (miRNA) play a role in the regulation of immune responses. In particular, miR-223 is known to be a pivotal modulator of macrophage and neutrophil differentiation, and a negative regulator of the inflammatory response. In addition, miR-223 is expressed by T cells and is highly enriched in the CD4+ T cell to Treg cell transition. miR-223 expression is induced in endometrial tissue by seminal fluid in the mouse uterus after mating, however, the contribution of miR-223 to the maternal immune environment in early pregnancy is yet to be assessed. To investigate the contribution of miR-223 to Treg cell activation and proliferation in early pregnancy, miR-223-/- or miR-223+/+ C57Bl/6 females (n=10-15) were mated to Balb/c males. T cell and antigen presenting cells from the uterus and the uterus draining lymph nodes (PALN) were assessed using flow cytometry on day 3.5 postcoitum (DPC). Virgin oestrous miR-223-/- and miR-223+/+ females were used as non-mated controls. To investigate the impact of miR-223 deficiency on susceptibility to a pro-inflammatory challenge, a separate cohort of pregnant females on DPC 9.5 were administered lipopolysaccharide (LPS) (1.0 μg) or phosphate saline buffer (PBS) control, and pregnancy outcomes were determined on DPC 17.5.

miR-223 deficiency resulted in a significant alteration in the Treg cell profile in early pregnancy with the most striking changes being observed in the PALN following mating where reductions were observed in the proportion of Treg cells amongst the CD4+ T cell pool (22%), Treg number (28%) and Foxp3 mean fluorescent intensity (MFI) (1.3-fold) compared to mated miR-223+/+ females. In the absence of miR-223, a reduction in macrophages as a proportion of total cells (58%) and in the number of activated macrophages (15%) was observed in the PALN on DPC 3.5 compared to mated miR-223+/+ mice.

Finally, the absence of miR-223 led to altered outcomes in pregnancy following LPS inflammatory challenge, with a 10% reduction in fetal weight and a 19% reduction in the fetal: placental weight ratio in late gestation. LPS administration also significantly increased the resorption rate (8.78-fold) in miR-223-/- females compared to miR-223+/+ females.

Collectively, these data show that the absence of miR-223 alters the maternal immune profile in early pregnancy and this may cause increased susceptibility to inflammation-induced fetal loss later in

gestation. These findings suggest that miR-223 has a pivotal role establishing the appropriate maternal immune environment during the peri-conception period that activates immune tolerance to facilitate appropriate protection against inflammatory challenge in later gestation. These findings may be relevant to understanding how Treg-associated pregnancy pathologies such as preeclampsia arise in women where reduced miR-223 has been noted.

6.2 Introduction

Maternal immune tolerance is required to prevent rejection and accommodate the semi-allogeneic fetus. Immune adaptions towards the conceptus are required to establish a tolerogenic immune milieu and suppress excessive inflammatory responses which can contribute to gestational disorders. The immunological process to establish maternal immune tolerance requires Treg cells (Aluvihare et al., 2004) which are potent suppressors of the generation and function of Th1- and Th17-mediated immune responses.

Seminal fluid plays an important role in the expansion of Treg cell numbers at the outset of pregnancy (Robertson et al., 2009a). Initially, seminal fluid deposition in the female reproductive tract results in a transient inflammatory response that initiates immune changes. An influence of seminal fluid on the female tract immune response has been reported in humans (Pandiyan et al., 2007, Sharkey et al., 2007), mice (Johansson et al., 2004) and all other mammalian species studied thus far (Schjenken and Robertson, 2014). Interactions between seminal fluid and epithelial cells in the reproductive tract induce elevated synthesis of cytokines and chemokines (Sharkey et al., 2012a, Sharkey et al., 2012b, Sharkey et al., 2007, Schjenken et al., 2015) which results in an influx of antigen presenting cells, as described in both humans (Sharkey et al., 2012b) and mice (De et al., 1991, McMaster et al., 1992, Robertson et al., 1998, Robertson et al., 1996, Robertson et al., 1992). These changes help to prepare the female reproductive tract for pregnancy by promoting the development of the pre-implantation embryo (Bromfield et al., 2014, Chin et al., 2009, Robertson et al., 2001) preparing for embryo implantation (Plaks et al., 2008, Jasper et al., 2011) and clearing the uterine cavity of microorganisms introduced at mating (Robertson et al., 1999).

There is evidence that both the cellular and acellular components of the ejaculate contribute to the expansion of Treg cells. Uterine DCs cross-present male alloantigens to activate maternal T cells (Moldenhauer et al., 2009), and initiate activation and proliferation of the Treg cell subset. Examination

of the Treg cell population in the uterus and PALN of mice highlights the role of seminal plasma, as a Treg cell expansion is only observed following exposure to complete seminal fluid and seminal plasma, but not sperm or the mechanical stimulation to mimic copulation (Guerin et al., 2011, Schumacher et al., 2007). Interestingly, the induction of *Foxp3* expression observed following mating requires the complete ejaculate as exposure to seminal plasma or sperm alone failed to induce a comparable Treg cell response (Guerin et al., 2011). Perturbations to Treg cells and their function are associated with human gestation disorders, such as pregnancy loss (Winger and Reed, 2011), preeclampsia (Quinn et al., 2011, Santner-Nanan et al., 2009, Sasaki et al., 2007, Prins et al., 2009, Tian et al., 2016). Therefore, to address how Treg cells are regulated, it is essential to understand the contribution of both DCs and Treg cell responses.

In addition to Treg cells and DCs, macrophages are thought to have roles in antigen presentation, immune regulation, as well as tissue remodelling, particularly changes to the uterine vasculature (Ma et al., 2001, Aplin, 2002) to support placental development. Macrophages secrete vascular endothelial growth factor (VEGF) and other angiogenic factors which are regulated in the oestrous cycle and early pregnancy (Ma et al., 2001). Seminal fluid is involved in the regulation of angiogenesis as hamster females mated to accessory gland-deficient males exhibited reduced expression of *Vegf* (Chow et al., 2003).

Amongst the factors that contribute to Treg cell number and function, miRNAs are well studied but not in the context of pregnancy. miRNAs are involved in nearly all developmental, homeostatic and pathological processes (Griffiths-Jones et al., 2008) and are known to regulate immune cells (Mehta and Baltimore, 2016) and are involved in establishing pregnancy (Bidarimath et al., 2014).

We have recently demonstrated that immune-regulatory miRNAs are induced in the endometrium following seminal fluid exposure at coitus. One of the most highly regulated miRNAs is miR-223 which is induced in the endometrium after mating (Schjenken, unpublished observations). This miRNA has been identified as a marker for pregnancy complications such as miscarriage, preeclampsia and recurrent pregnancy loss (Winger et al., 2015, Tang et al., 2016) and an elevated expression of *miR-223* is associated with membrane with chorioamnionitis in advancing gestation (Montenegro et al., 2007).

miR-223 is involved in the regulation of multiple leukocytes, including neutrophils, macrophages, DCs and also Treg cells. miR-223 negatively regulates differentiation and activation in neutrophils, and mice with *miR-223* null mutation exhibit increased number of circulating neutrophils, and these neutrophils

exhibit an unusual hyper-maturation (Johnnidis et al., 2008). M2 macrophages are immuno-suppressive and miR-223 is known to contribute to M2 polarisation in macrophages (Zhou et al., 2015, Zhuang et al., 2012, Zhang et al., 2013). In the intestine, elevated secretion of pro-inflammatory cytokines IL1β and tumour necrosis factor, and reduced production of the anti-inflammatory cytokine TGF-β are found in macrophages in the miR-223 deficient mice (Zhou et al., 2015). A dysregulated population of DCs are also observed, such that intestinal DCs produce more pro-inflammatory cytokines upon activation (Zhou et al., 2015).

The pattern of seminal fluid induction of miR-223 expression in the endometrial lining of the female reproductive tract after coitus in mice (Schjenken, unpublished), is consistent with an influence on induction of tolerogenic DCs and Treg cells to establish tolerance for pregnancy. Given the role of miR-223 in immune tolerance in other tissues, the roles of miR-223 in the regulation of Treg cells and antigen presenting cells in early pregnancy are essential to elucidate.

In the experiments described in this chapter, we utilised miR-223-/- mice to investigate the role of miR-223 in the regulation of the peri-conception immune environment and the role of miR-223 in regulation of Treg cells, macrophages and DC in early pregnancy.

6.3 Result

6.3.1 miR-223 deficiency results in a systemic change to the maternal T cell profile in early pregnancy

To determine the impact of miR-223 deficiency on the T cell profile in early pregnancy, miR-223-/- and miR-223+/+ female mice were mated to Balb/c males. The PALN, mesenteric lymph nodes (mLN), spleen and blood were collected from females on DPC 3.5 for T cell quantification. Virgin oestrous females were used as an unmated control. Treg cells were identified as CD3+ CD4+ CD25+ Foxp3+ T cells, and the gating strategies were the same as detailed in chapter 5 (Figure 5.1). Data for PALN are described in detail in this chapter, while the results for mLN, spleen and blood are presented in chapter 8, section 8.3.1

6.3.1.1 Total cell expansion in the PALN

Hypertrophy in the PALN draining the uterus within the days after mating is an indication of the female immune response required to establish pregnancy. To assess the impact of miR-223 deficiency on

lymph node cellularity in early pregnancy, we firstly examined the total cell number in the PALN on DPC 3.5. While the total number in the PALN increased by 1.3-fold ($p<0.05$, Figure 6.1) in miR-223+/+ females after mating, this elevation was not observed in the absence of miR-223 (Figure 6.1). No change was observed at oestrus or on DPC 3.5 between genotypes (Figure 6.1).

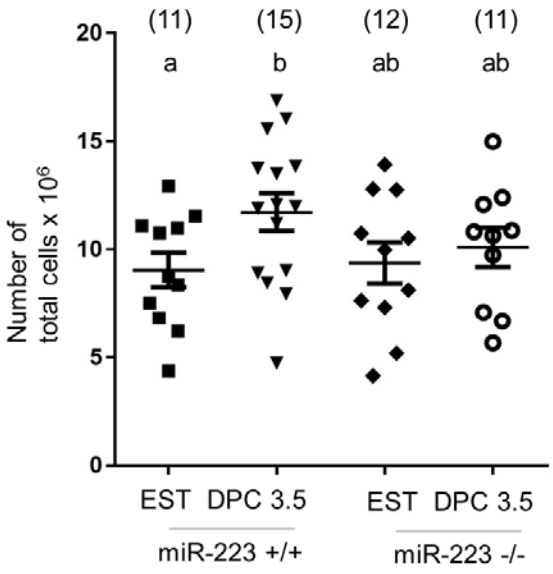


Figure 6.1 The effect of miR-223 deficiency on populations of total cells in the PALN. miR-223+/+ and miR-223-/- mice were mated with Balb/c males, and on DPC 3.5, total cells from the PALN were quantified by a haemocytometer. Unmated virgin oestrus mice were used as non-mated controls. Data are presented as mean ± SEM and are number of total cells $x10^6$ in the PALN. Differences between groups were evaluated using Kruskal-Wallis test and Mann-Whitney U test. (a,b,c,d indicates $p<0.05$).

6.3.1.2 T cell profile in the PALN

To determine whether miR-223 deficiency affects the Treg cell population on DPC 3.5 in the PALN, we firstly defined the population of T cells expressing CD4. Within the CD3 population, the proportion of T cells expressing CD4 was not changed (Figure 6.2A) after mating or between genotypes. However, while mating resulted in an elevation in the number of CD4+ T cells in miR-223+/+ mice (2.3-fold, $p<0.0001$), no change was observed following mating in miR-223-/- females. While miR-223 deficiency did not alter the number of CD4+ T cells at oestrus, a significant decrease (22%, $p<0.05$) was observed on DPC 3.5 (Figure 6.2B). Similarly, while mating resulted in an elevation in the MFI of CD4 in miR-223+/+ females (1.6-fold, $p<0.01$), no change was observed in miR-223-/- females (Figure 6.2C). In reference to CD4 MFI, miR-223 deficiency resulted in a significant reduction at both oestrus (16%, $p<0.001$) and DPC 3.5 (52%, $p<0.001$) (Figure 6.2C).

To understand the impact of miR-223 deficiency on Treg cells, we then assessed the CD4+CD25+ Foxp3+ Treg cell profile within the PALNs. miR-223 deficiency resulted in a significantly lower proportion of Treg cells amongst CD4+ T cells, both at oestrus (43%, $p<0.0001$) and on DPC 3.5 (28%, $p<0.0001$) (Figure 6.2D). No change was observed in the proportion of Treg cells following mating in miR-223+/+ females, but a significant increase (1.3-fold, $p<0.01$) was observed in miR-223-/- females (Figure 6.2D). Consistent with our previous observations (Guerin et al., 2011) the total number of Treg cells was increased following mating in miR-223+/+ females (2.1-fold, $p<0.001$). A similar increase was observed in miR-223-/- females following mating (1.4-fold, $p<0.01$) but not to the same extent. Further, while miR-223 deficiency did not impact Treg cell numbers at oestrus, a significant reduction was observed on DPC 3.5 (42%, $p<0.01$) (Figure 6.2E). Similar results were observed for Foxp3 MFI with a significant increase (1.3-fold, $p<0.01$) in Foxp3 MFI on DPC 3.5 in miR-223+/+ mice, but not in miR-223-/- mice (Figure 6.2F). While miR-223 deficiency did not impact Foxp3 MFI at oestrus, a significant reduction (24%, $p<0.001$) was seen on DPC 3.5 (Figure 6.2F). Taken together, miR-223 deficiency acted to reduce total CD4+ T cells and CD4+ Treg cells and suppressed the capacity of mating to induce the expected increase in the number and Foxp3 MFI of Treg cells. This reduction would be expected to potentially impact on the quality of immune tolerance in pregnancy.

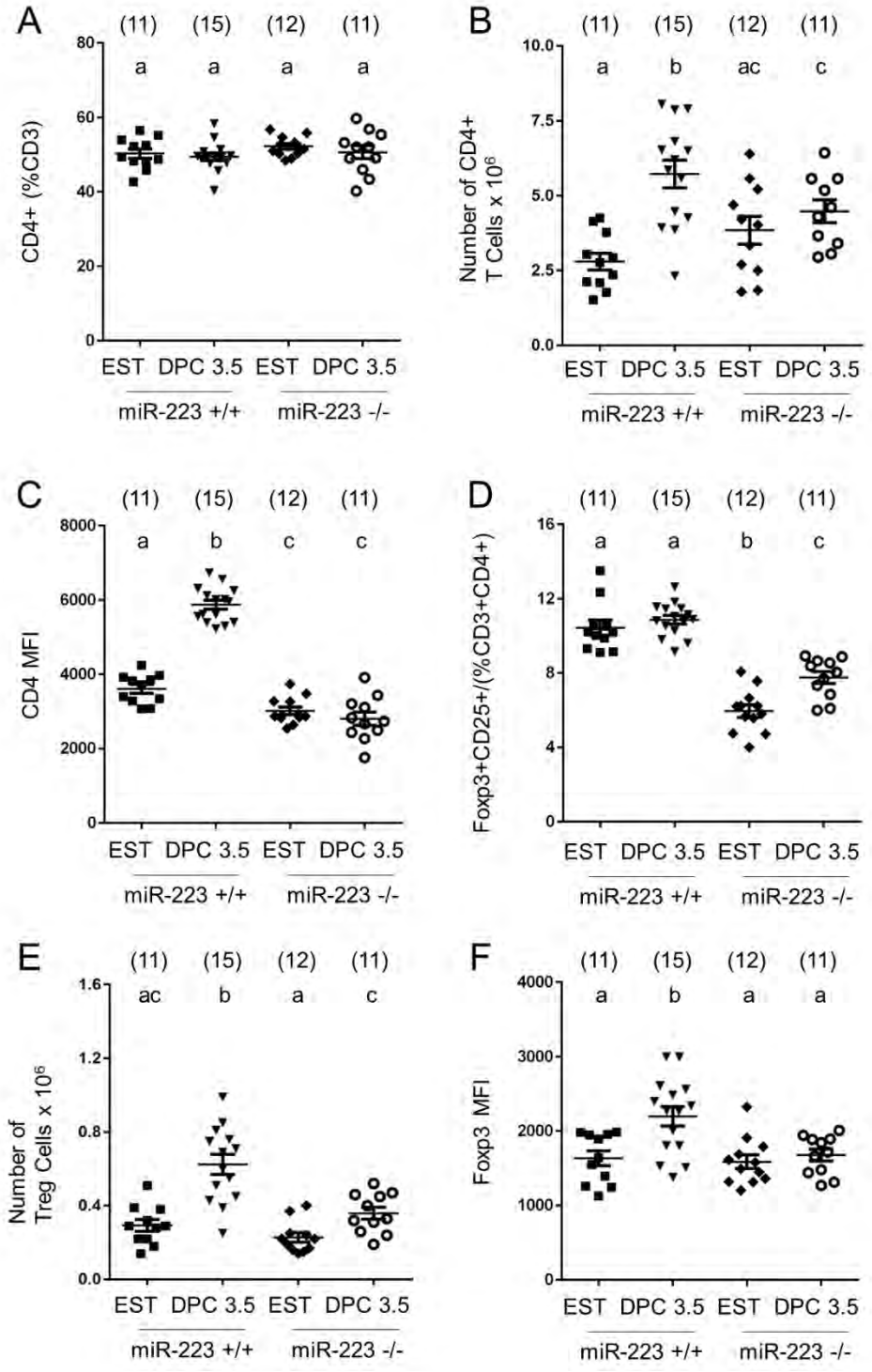


Figure 6.2 The effect of miR-223 deficiency on T cell populations in the PALN. miR-223+/+ and miR-223-/- mice were mated with Balb/c males, and on DPC 3.5, CD3+, CD4+, CD25+ and Foxp3+ cells from the PALN were analysed by flow cytometry to quantify T cells. Virgin oestrus mice were used as non-mated controls. Data are presented as mean ± SEM and are the percentage of CD4+ T cells within the CD3+ population (A), the total number of CD4+ T cells x10^6 (B), the CD4 MFI (C), the percentage of CD3+ CD4+ CD25+ Foxp3+Treg cells (D), the total number of CD3+ CD4+ CD25+ Foxp3+ Treg cells x10^6 (E), and the MFI of Foxp3 (F). Differences between groups were evaluated using Kruskal-Wallis test and Mann-Whitney U test. (a,b,c,d indicates $p<0.05$).

6.3.2 Effect of miR-223 deficiency on the macrophage and dendritic cell profile during early pregnancy

Given the importance of antigen presenting cells in the peri-conception immune environment and generating the T cell response, we then assessed the impact of miR-223 deficiency on the profile of macrophages and DCs in early pregnancy. At oestrus and on DPC 3.5, cells from mLN, spleen, PALN and uterus were collected for analysis of the antigen presenting cell profile using flow cytometry.

Macrophages were assessed by examining the population of total macrophages (CD11B+ F4/80+ cells), activated macrophages (MHCII+ CD11B+ F4/80+ cells), and macrophages expressing the co-stimulatory molecules CD80 (CD80+ CD11B+ F4/80+ cells) and CD86 (CD86+ CD11B+ F4/80+ cells). DCs were assessed by examining the total population of dendritic cells (CD11C+ F4/80- cells), activated DCs (MHCII+ CD11C+ F4/80- cells), as well as DCs expressing the co-stimulatory molecules CD80 (CD80+ CD11C+ F4/80- cells) and CD86 (CD86+ CD11C+ F4/80- cells). Macrophages and DCs were identified using the flow cytometry gating strategy described in chapter 5 (Figure 5.3 and 5.4).

Data for uterus and PALN are described in detail in this chapter, while the results of mLN and spleen are presented in chapter 8, section 8.3.2 and 8.3.3.

6.3.2.1 Macrophage profile in uterus and PALN

To assess the impact of miR-223 deficiency on the macrophage number and phenotype in early pregnancy, uterus and PALN were collected from oestrous or DPC 3.5 miR-223+/+ or miR-223-/- females. In these experiments, we were able to assess the uterus in addition to the lymph nodes as substantial numbers of macrophages and DC can be recovered from the uterus.

6.3.2.1.1 PALN

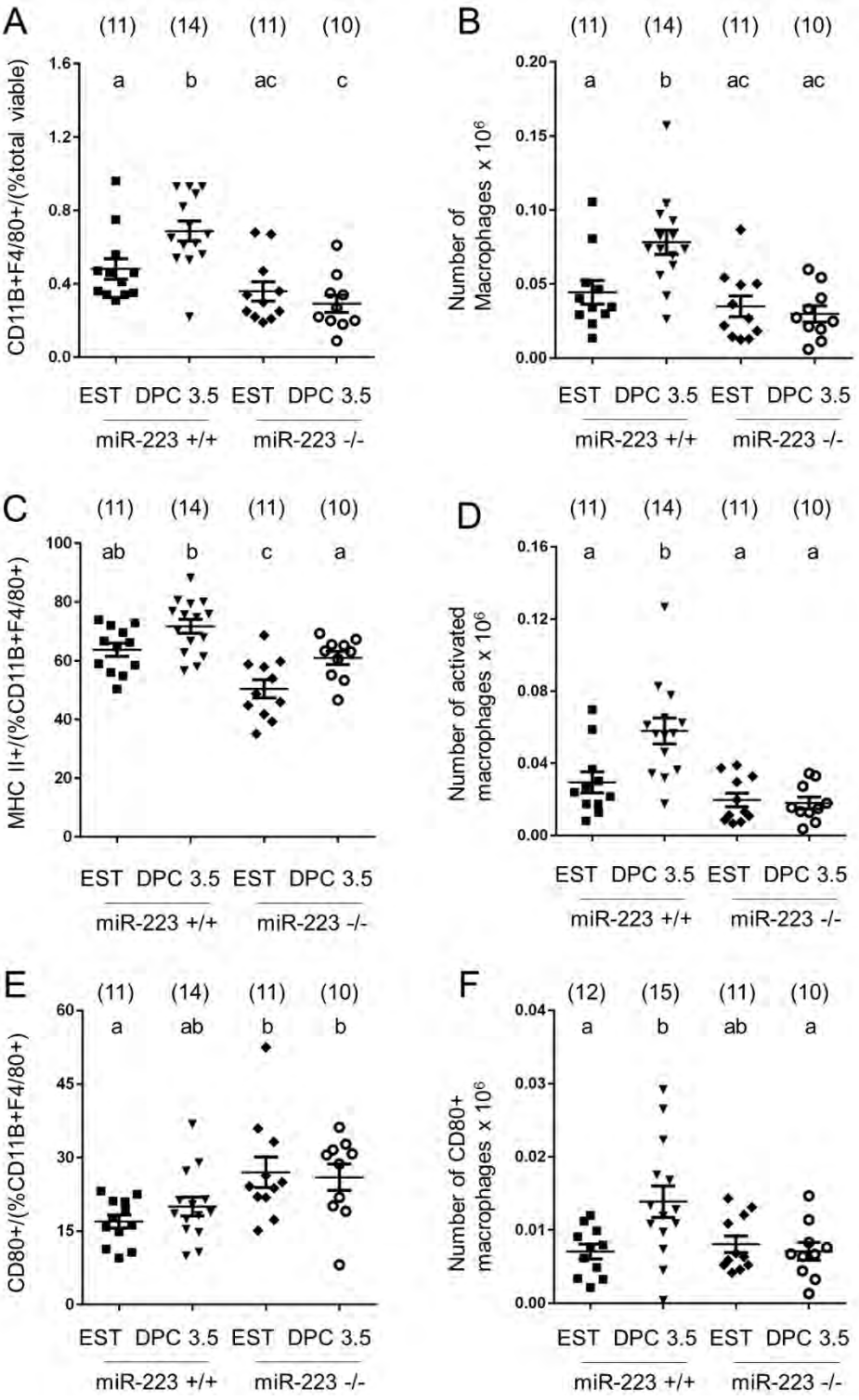
In the PALN, a significant increase in the proportion of macrophages in the viable leukocyte population (1.4-fold, $p<0.05$) was observed on DPC 3.5 in miR-223+/+ females, however this change was not evident in miR-223-/- females on DPC 3.5. While there was no difference at oestrus, miR-223 deficiency resulted in a significant reduction in the proportion of macrophages on DPC 3.5 (58%, $p<0.05$) (Figure 6.3A). A similar pattern was observed in the total number of macrophages in the PALN. A significant increase in macrophage numbers following mating (1.8-fold, $p<0.01$) was found in miR-223+/+ females but this did not occur in miR-223-/- females, compared to the genotype matched oestrus controls

(Figure 6.3B). Again, while there was no difference at oestrus, miR-223 deficiency resulted in a significant reduction (61.9%, $p<0.01$) in the number of macrophages on DPC 3.5 (Figure 6.3B).

We then further examined the macrophage population to identify whether miR-223 deficiency impacted the population of activated macrophages (MHCII+ CD11B+ F4/80+ macrophages) in the PALN. Mating resulted in a significant elevation in the proportion of activated macrophages, compared to genotype matched oestrus controls in both miR-223+/+ (1.1-fold, $p<0.05$) and miR-223-/- (1.2-fold, $p<0.05$) (Figure 6.3C). However, miR-223 deficiency resulted in a smaller proportion of activated macrophages at oestrus (21%, $p<0.01$) and on DPC 3.5 (15%, $p<0.01$), compared to genotype matched controls (Figure 6.3C). The total number of activated macrophages was significantly increased in miR-233+/+ females (2.0-fold, $p<0.01$) but not in miR-223-/- females, compared to oestrus genotype matched controls (Figure 6.3D). While there was no difference at oestrus, miR-223 deficiency led to a significant reduction (69%, $p<0.001$) in macrophage number on DPC 3.5 (Figure 6.3D).

Expression of the co-stimulatory molecules CD80 and CD86 within the macrophage population was then examined to evaluate co-stimulatory capacity, which is necessary for T cell activation and survival (June et al., 1987, Linsley et al., 1991). The percentage of CD80+ macrophages was not changed after seminal fluid exposure in either miR-223+/+ or miR-223-/- mice (Figure 6.3E). Interestingly, miR-223 deficiency resulted in a significant increase in the proportion of CD80+ macrophages at oestrus (1.6-fold, $p<0.05$), but not on DPC 3.5 (Figure 6.3E). In contrast, CD80+ macrophage numbers were significantly increased following mating in miR-223+/+ females (2.0-fold, $p<0.05$), but not in miR-223-/- females (Figure 6.3F). A 49% reduction ($p<0.05$) in the number of CD80+ macrophage was observed in miR-223-/- females on DPC 3.5, compared to WT controls. In regards to CD80+ macrophage numbers, there was no impact of miR-223 deficiency at either oestrus or on DPC 3.5 (Figure 6.3F).

The percentage of macrophages positive for CD86 was not changed after seminal fluid exposure or between genotypes (Figure 6.3G). However, CD86+ macrophage numbers were significantly increased following mating in miR-223+/+ mice (1.8-fold, $p<0.05$), but not in miR-223-/- mice (Figure 6.3H). While miR-223 deficiency did not affect CD80+ macrophage number at oestrus, a significant reduction (66%, $p<0.0001$) was observed on DPC 3.5 (Figure 6.3H). These data suggest that in the absence of miR-223, the population of macrophages is impacted on DPC 3.5 but not at oestrus.


A
(11) (14) (11) (10)
a b ac c
CD11B+F4/80+/(%total viable)
1.6
1.2
0.8
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EST DPC 3.5 EST DPC 3.5
miR-223 +/+ miR-223 -/-
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a b ac ac
Number of Macrophages x 10^6
0.20
0.15
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0.05
0.00
EST DPC 3.5 EST DPC 3.5
miR-223 +/+ miR-223 -/-
C
(11) (14) (11) (10)
ab b c a
MHC II+/(%CD11B+F4/80+)
100
80
60
40
20
0
EST DPC 3.5 EST DPC 3.5
miR-223 +/+ miR-223 -/-
D
(11) (14) (11) (10)
a b a a
Number of activated macrophages x 10^6
0.16
0.12
0.08
0.04
0.00
EST DPC 3.5 EST DPC 3.5
miR-223 +/+ miR-223 -/-
E
(11) (14) (11) (10)
a ab b b
CD80+/(%CD11B+F4/80+)
60
45
30
15
0
EST DPC 3.5 EST DPC 3.5
miR-223 +/+ miR-223 -/-
F
(12) (15) (11) (10)
a b ab a
Number of CD80+ macrophages x 10^6
0.04
0.03
0.02
0.01
0.00
EST DPC 3.5 EST DPC 3.5
miR-223 +/+ miR-223 -/-

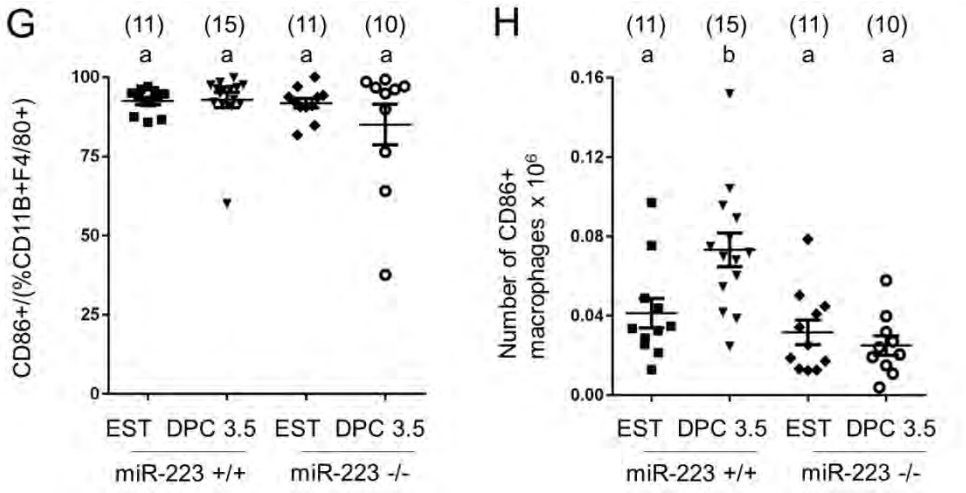


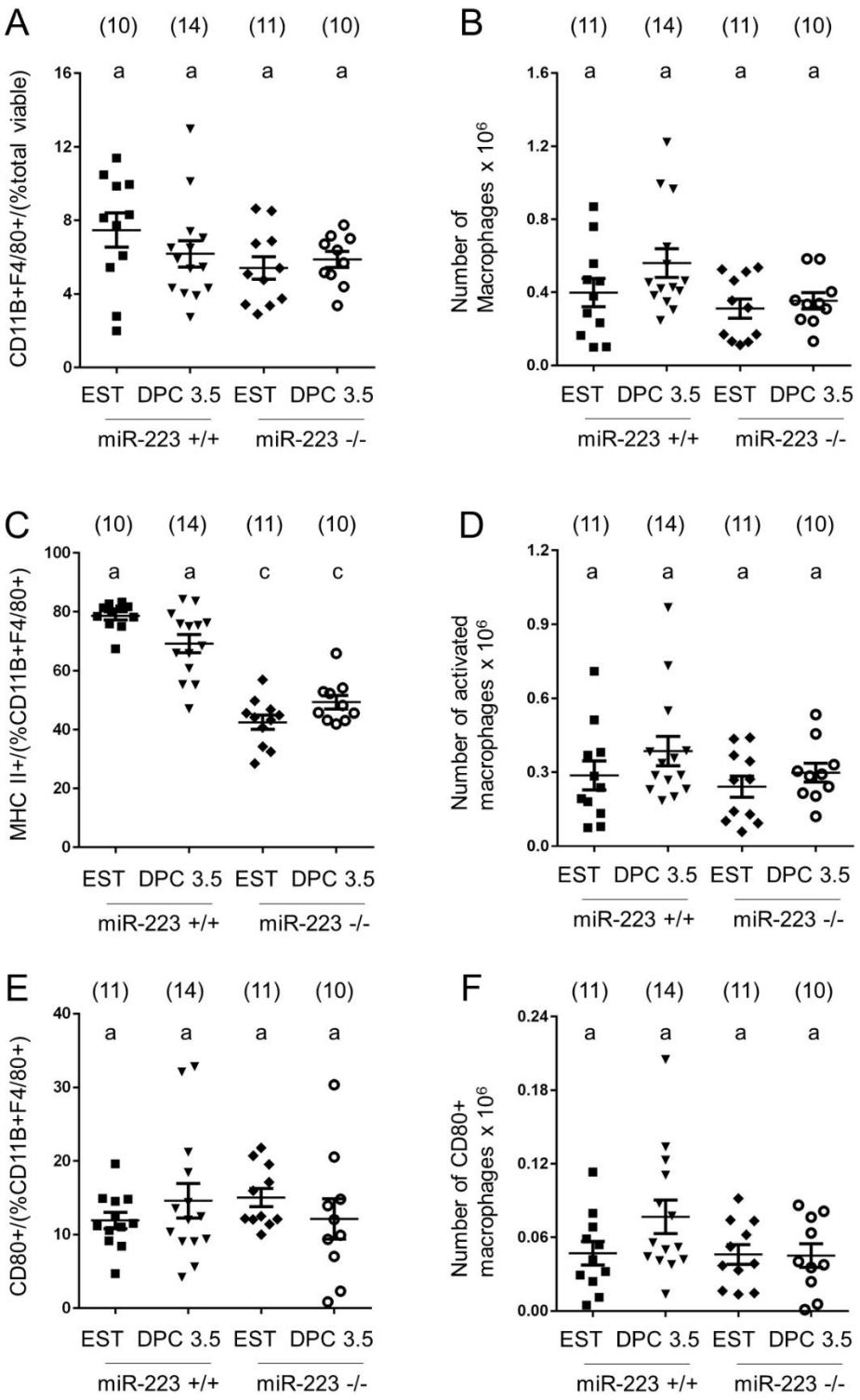
Figure 6.3 The effect of miR-223 deficiency on the macrophage population and the expression of MHCII, CD80, and CD86 by F4/80+ macrophages in the PALN. miR-223+/+ and miR-223-/- B6 female mice were mated with Balb/c males, and on DPC 3.5, CD11B+ F4/80+ cells from the PALN were analysed by flow cytometry to quantify the proportion of macrophages and the expression of MHCII, CD80, and CD86. Virgin oestrus mice were used as non-mated controls. Data are presented as mean ± SEM and are the percentage of F4/80+ macrophages within viable cells (A), the total number of macrophages x 10^6 (B), the percentage of activated macrophages (MHCII+ macrophages) (C), the total number of activated macrophages x 10^6 (D), the percentage of CD80+ macrophages (E), the total number of CD80+ macrophages x 10^6 (F), the percentage of CD86+ macrophages (G) and the total number of CD86+ macrophages x 10^6 (H). Differences between groups were evaluated using Kruskal-Wallis test and Mann-Whitney U test. (a,b,c,d indicates $p<0.05$)

6.3.2.1.2 Uterus

In the uterus, no change in the proportion of cells comprised by macrophages, or the total number of macrophages were observed following mating, or between genotypes at oestrus or on DPC 3.5. (Figure 6.4A-B).

We then further examined the macrophage population to identify whether miR-223 deficiency impacted the population of activated macrophages (MHCII+ CD11B+ F4/80+ macrophages) in the uterus. In contrast to what was observed in the PALN, a significant reduction in the proportion of activated macrophages was observed in miR-223-/- mice at oestrus (12%, $p<0.0001$) and on DPC 3.5 (29%, $p<0.05$), compared to the WT (Figure 6.4C). Irrespective of the changes observed in the proportion of activated macrophages, no change was observed in the number of activated macrophages following mating or between genotypes at oestrus or on DPC 3.5 (Figure 6.4D).

Analysis of the CD80+ and CD86+ macrophage population showed no change in the percentage or number of these macrophages after mating or between genotypes at oestrus or on DPC 3.5 (Figure 6.4E and F). These data suggest that miR-223 deficiency causes moderate changes to uterine macrophage activation status as measured by MHCII expression at oestrus and on DPC 3.5, but this does not substantially impact the number of activated macrophages present.


A
(10) (14) (11) (10)
a a a a
CD11B+F4/80+/(%total viable)
16
12
8
4
0
EST DPC 3.5 EST DPC 3.5
miR-223 +/+ miR-223 -/-
B
(11) (14) (11) (10)
a a a a
Number of Macrophages x 10^6
1.6
1.2
0.8
0.4
0.0
EST DPC 3.5 EST DPC 3.5
miR-223 +/+ miR-223 -/-
C
(10) (14) (11) (10)
a a c c
MHC II+/(%CD11B+F4/80+)
100
80
60
40
20
0
EST DPC 3.5 EST DPC 3.5
miR-223 +/+ miR-223 -/-
D
(11) (14) (11) (10)
a a a a
Number of activated macrophages x 10^6
1.2
0.9
0.6
0.3
0.0
EST DPC 3.5 EST DPC 3.5
miR-223 +/+ miR-223 -/-
E
(11) (14) (11) (10)
a a a a
CD80+/(%CD11B+F4/80+)
40
30
20
10
0
EST DPC 3.5 EST DPC 3.5
miR-223 +/+ miR-223 -/-
F
(11) (14) (11) (10)
a a a a
Number of CD80+ macrophages x 10^6
0.24
0.18
0.12
0.06
0.00
EST DPC 3.5 EST DPC 3.5
miR-223 +/+ miR-223 -/-

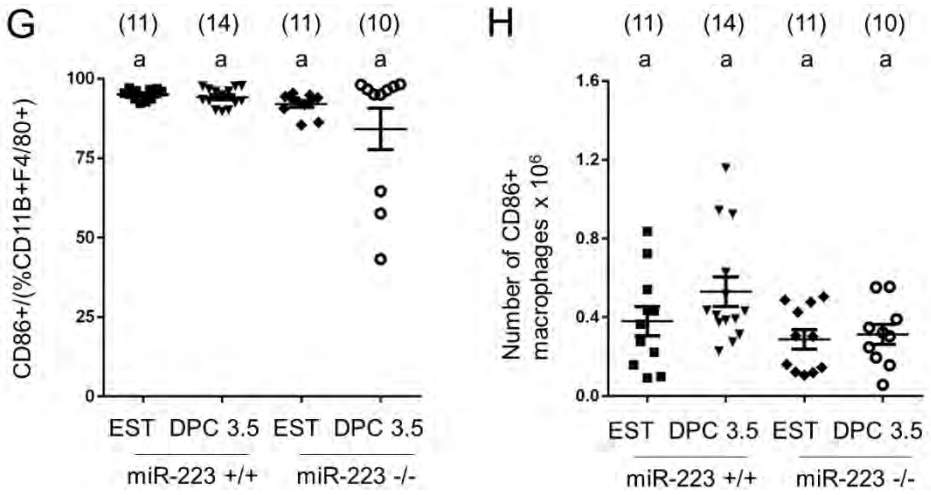


Figure 6.4 The effect of miR-223 deficiency on macrophage populations the uterine tissues. miR-223+/+ and miR-223-/- B6 female mice were mated with Balb/c males, and on DPC 3.5, CD11B+ F4/80+ cells from the uterine tissues were analysed by flow cytometry to quantify the proportion of macrophages and the expression of MHCII, CD80, and CD86. Virgin oestrus mice were used as non-mated controls. Data are presented as mean ± SEM and are the percentage of F4/80+ macrophages within viable cells (A), the total number of macrophages x 10^6 (B), the percentage of activated macrophages (MHCII+ macrophages) (C), the total number of activated macrophages x 10^6 (D), the percentage of CD80+ macrophages (E), the total number of CD80+ macrophages x 10^6 (F), the percentage of CD86+ macrophages (G) and the total number of CD86+ macrophages x 10^6 (H). Differences between groups were evaluated using Kruskal-Wallis test and Mann-Whitney U test. (a,b,c,d indicates $p<0.05$)

6.3.2.2 Dendritic cell profile in uterus and PALN

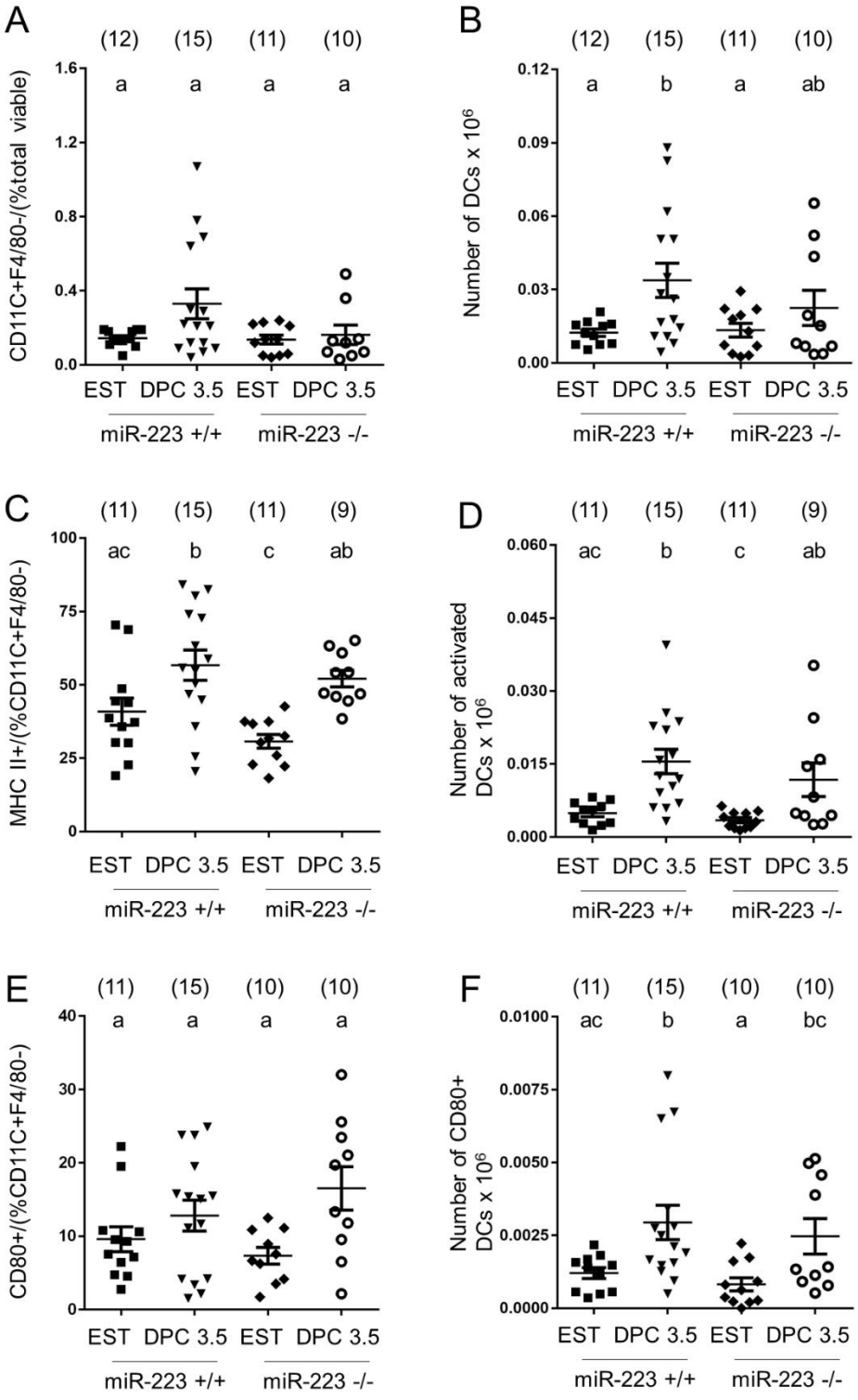
To assess the impact of miR-223 deficiency on the DC number and phenotype in early pregnancy, uterus and PALN were collected from miR-223+/+ and miR-223-/- females at oestrus or DPC 3.5. In these experiments, we were able to assess the uterus in addition to the lymph nodes as substantial numbers of macrophages and DC can be recovered from the uterus.

6.3.2.2.1 PALN

In the PALN, there was no change in the number of F4/80-CD11C+ DCs expressed as a proportion of viable cells in miR-223+/+ or miR-223-/- mice following mating or between genotypes (Figure 6.5A). A 2.8-fold ($p<0.01$) elevation in the number of DCs was observed on DPC 3.5 in miR-223+/+ females, however miR-223 deficiency led to failure to induce this elevation. No change was observed at oestrus or on DPC 3.5 between genotypes (Figure 6.5B).

We then further examined the DC population to identify whether miR-223 deficiency impacted the population of activated DCs (MHCII+ CD11C+ F4/80- DCs) in the PALN. Following mating, the proportion of activated DCs was significantly increased in both miR-223+/+ (1.8-fold, $p<0.05$) and miR-223-/- mice (1.6-fold, $p<0.05$) compared to the oestrus genotype matched controls. No change in the proportion of activated DCs was observed at oestrus or on DPC 3.5 between genotypes (Figure 6.5C). Seminal fluid exposure induced an increase in the number of activated DCs in both miR-223+/+ (3.2-fold, $p<0.001$) and miR-223-/- females (3.4-fold, $p<0.05$), compared to genotype matched oestrous controls (Figure 6.5D). The percentage of DCs positive for CD80 was not changed following mating, in either miR-223+/+ or miR-223-/- females, and no change in CD80+ DCs was observed between genotypes at oestrus or on DPC 3.5 (Figure 6.5E). In contrast, mating resulted in a significant increase in the number of CD80+ DCs in both miR-223+/+ (2.4-fold, $p<0.05$) and miR-223-/- females (2.7-fold, $p<0.05$), compared to genotype matched oestrus controls. No change in the number of CD80+ DCs was observed at oestrus or on DPC 3.5 between genotypes (Figure 6.5F).

The percentage and number of CD86+ DCs was not changed after seminal fluid exposure, in either miR-223+/+ or miR-223-/- females (Figure 6.5G). A 2.9-fold increase ($p<0.05$) in the number of CD86+ DCs was observed in miR-223+/+ females on DPC 3.5, however miR-223 deficiency failed to induce this elevation. No change was observed between genotypes at oestrus or on DPC 3.5 (Figure 6.5H).


A
(12) (15) (11) (10)
a a a a
CD11C+F4/80-/(%total viable)
1.6
1.2
0.8
0.4
0.0
EST DPC 3.5 EST DPC 3.5
miR-223 +/+ miR-223 -/-
B
(12) (15) (11) (10)
a b a ab
Number of DCs x 10^6
0.12
0.09
0.06
0.03
0.00
EST DPC 3.5 EST DPC 3.5
miR-223 +/+ miR-223 -/-
C
(11) (15) (11) (9)
ac b c ab
MHC II+/(%CD11C+F4/80-)
100
75
50
25
0
EST DPC 3.5 EST DPC 3.5
miR-223 +/+ miR-223 -/-
D
(11) (15) (11) (9)
ac b c ab
Number of activated DCs x 10^6
0.060
0.045
0.030
0.015
0.000
EST DPC 3.5 EST DPC 3.5
miR-223 +/+ miR-223 -/-
E
(11) (15) (10) (10)
a a a a
CD80+/(%CD11C+F4/80-)
40
30
20
10
0
EST DPC 3.5 EST DPC 3.5
miR-223 +/+ miR-223 -/-
F
(11) (15) (10) (10)
ac b a bc
Number of CD80+ DCs x 10^6
0.0100
0.0075
0.0050
0.0025
0.0000
EST DPC 3.5 EST DPC 3.5
miR-223 +/+ miR-223 -/-

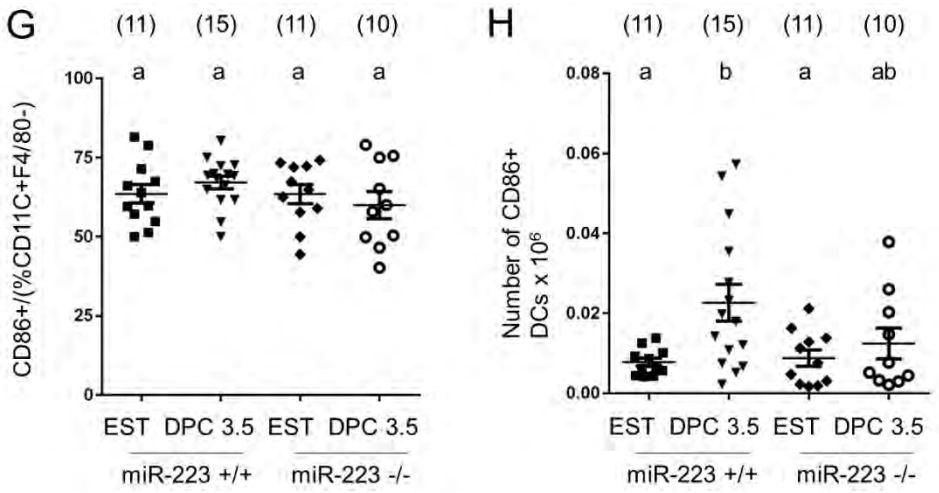


Figure 6.5 The effect of miR-223 deficiency on DC population and expression of MHCII, CD80, and CD86 by CD11C+ DCs in the PALN. miR-223+/+ and miR-223-/- B6 mice were mated with Balb/c males, and on DPC 3.5, CD11C+ F4/80- cells from the PALN were analysed by flow cytometry to quantify proportion of DCs and the expression of MHCII, CD80, and CD86. Virgin oestrus mice were used as non-mated controls. Data are presented as mean ± SEM and are the percentage of CD11C+ DCs within viable cells (A), the total number of DCs x 10^6 (B), the percentage of activated DCs (MHCII+ DCs) (C), the total number of activated DCs x 10^6 (D), the percentage of CD80+ DCs (E), the total number of CD80+ DCs x 10^6 (F), the percentage of CD86+ DCs (G) and the total number of CD86+ DCs x 10^6 (H). Differences between groups were evaluated using Kruskal-Wallis test and Mann-Whitney U test. (a,b,c,d indicates $p<0.05$).

6.3.2.2.2 Uterus

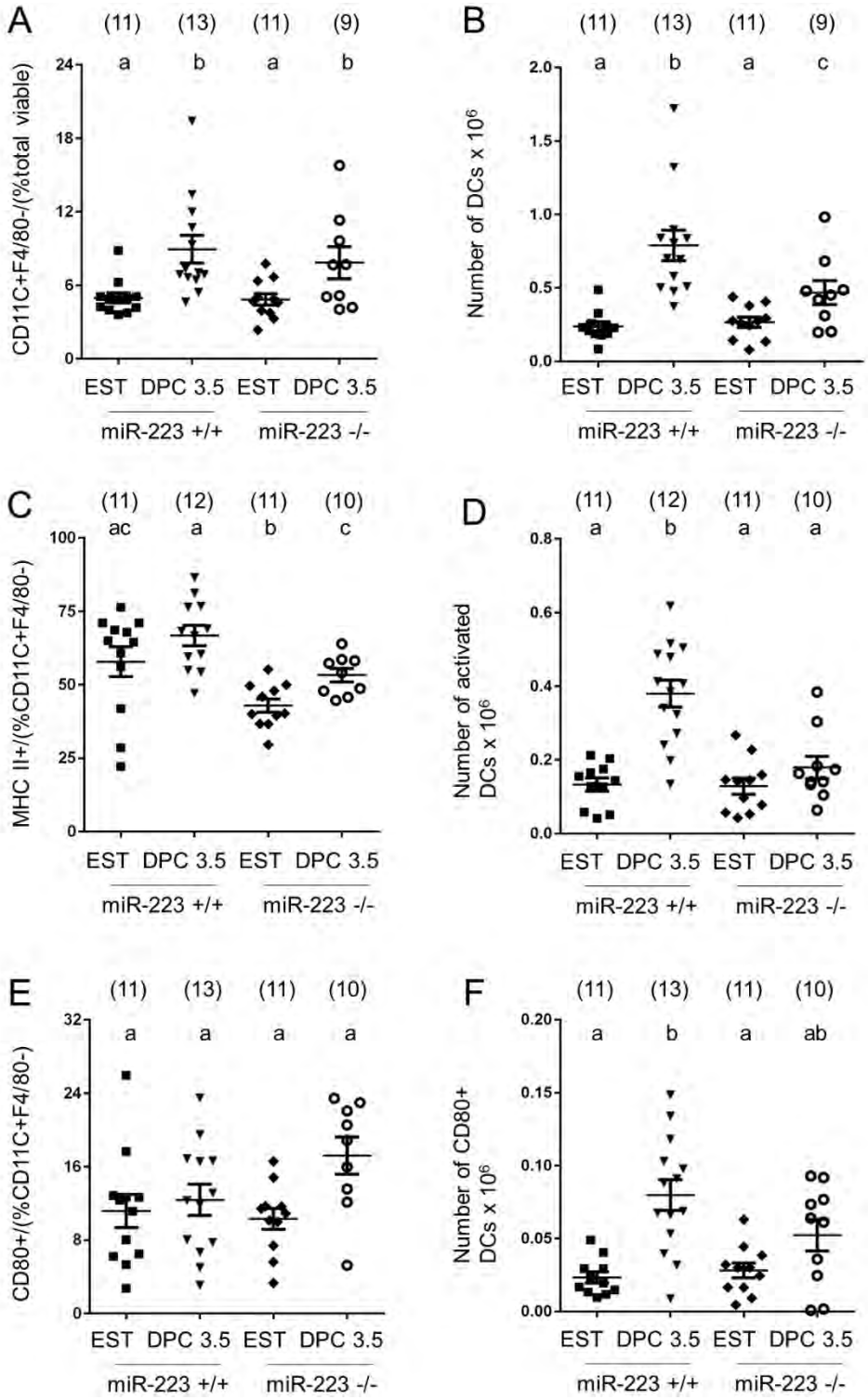
In the uterus, a significant increase in the proportion of cells comprised by DCs was observed following mating in both miR-223+/+ (1.8-fold, $p<0.01$) and miR-223-/- females (1.6-fold, $p<0.01$), compared to the genotype matched oestrus controls (Figure 6.6A). No changes in the proportion of DCs were observed at oestrus or on DPC 3.5 between genotype (Figure 6.6A). DC numbers followed a similar pattern after mating, with significant increases in miR-223+/+ (3.3-fold, $p<0.0001$) and miR-223-/- (1.8-fold, $p<0.05$) observed compared to genotype matched oestrus controls (Figure 6.6B). Further, miR-223 deficiency resulted in a significant reduction in DC number (41%, $p<0.05$) on DPC 3.5, compared to WT females, but this was not the case at oestrus (Figure 6.6B).

We then further examined the DC population to identify whether miR-223 deficiency impacted the population of activated DCs (MHCII+ CD11C+ F4/80- DCs) in the uterus. While no change was observed in the proportion of activated DCs in miR-223+/+ females following mating, a significant increase was observed in miR-223-/- females (1.2-fold, $p<0.05$) following mating, compared to genotype matched oestrus controls. Further, miR-223 deficiency resulted in overall significantly lower proportion of activated DCs at oestrus (26%, $p<0.05$) and on DPC 3.5 (20%, $p<0.05$), compared to genotype matched controls (Figure 6.5C). In contrast, a significant increase in activated DC number was observed following mating in miR-223+/+ (3.0-fold, $p<0.0001$), but not in miR-223-/- (Figure 6.6D). While there was no change at oestrus, miR-223 deficiency did result in a significant reduction (53%, $p<0.001$) in the number of activated DCs on DPC 3.5, compared to genotype matched controls (Figure 6.6D).

Analysis of the CD80+ and CD86+ DC population showed no change in the proportion of CD80+ DCs following mating in both miR-223+/+ or miR-223-/- females or at oestrus or on DPC 3.5 between genotypes (Figure 6.6E). However, a significant increase in CD80+ DC number was seen following mating in miR-223+/+ (3.4-fold, $p<0.05$) but not in miR-223-/- females compared to genotype matched oestrus controls (Figure 6.6F). At oestrus or on DPC 3.5, no change in the total number of CD80+ DCs was observed between genotypes (Figure 6.6F).

While no change in the proportion of CD86+ DCs was observed following mating in miR-223+/+ females, a significant increase was observed in miR-223-/- females (1.3-fold, $p<0.05$), compared to oestrous miR-223-/-females. No change in the proportion of CD86+ DCs was observed at oestrus or on DPC 3.5 between genotypes (Figure 6.6G). An increase in CD86+ DCs was observed in both miR-223+/+ (3.7-fold, $p<0.0001$) and miR-223-/- females (2.2-fold, $p<0.05$) following mating, compared to

genotype-matched oestrus controls (Figure 6.6H). However, no change was observed at oestrus or on DPC 3.5 between genotypes (Figure 6.6H). In summary, these data show that the number of DCs and activation status of DCs are reduced at oestrus and on DPC 3.5.


A
(11) (13) (11) (9)
a b a b
CD11C+F4/80-/(%total viable)
24
18
12
6
0
EST DPC 3.5 EST DPC 3.5
miR-223 +/+ miR-223 -/-
B
(11) (13) (11) (9)
a b a c
Number of DCs x 10^6
2.0
1.5
1.0
0.5
0.0
EST DPC 3.5 EST DPC 3.5
miR-223 +/+ miR-223 -/-
C
(11) (12) (11) (10)
ac a b c
MHC II+/(%CD11C+F4/80-)
100
75
50
25
0
EST DPC 3.5 EST DPC 3.5
miR-223 +/+ miR-223 -/-
D
(11) (12) (11) (10)
a b a a
Number of activated DCs x 10^6
0.8
0.6
0.4
0.2
0.0
EST DPC 3.5 EST DPC 3.5
miR-223 +/+ miR-223 -/-
E
(11) (13) (11) (10)
a a a a
CD80+/(%CD11C+F4/80-)
32
24
16
8
0
EST DPC 3.5 EST DPC 3.5
miR-223 +/+ miR-223 -/-
F
(11) (13) (11) (10)
a b a ab
Number of CD80+ DCs x 10^6
0.20
0.15
0.10
0.05
0.00
EST DPC 3.5 EST DPC 3.5
miR-223 +/+ miR-223 -/-

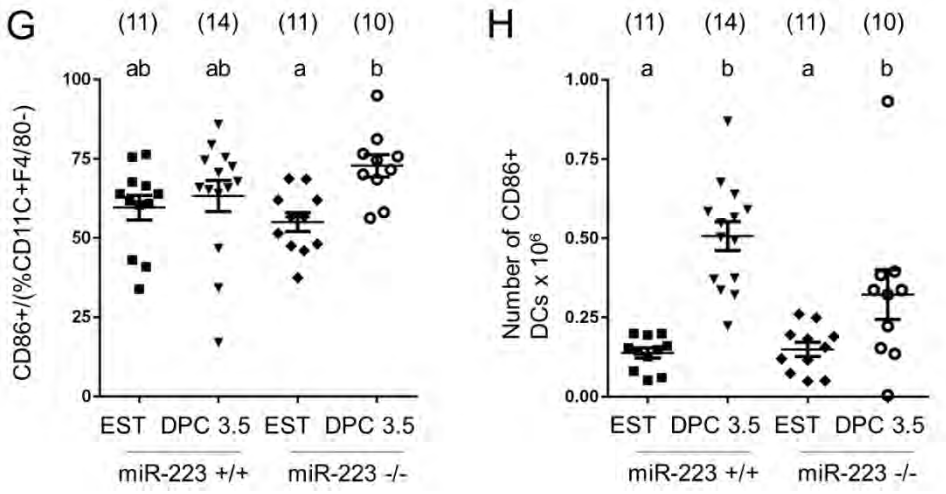


Figure 6.6 The effect of miR-223 deficiency on population of DCs and expression of MHCII, CD80, and CD86 by CD11C+ DCs in uterine tissues. miR-223+/+ and miR-223-/- B6 mice were mated with Balb/c males, and on DPC 3.5, CD11C+ F4/80- cells from the uterine tissues were analysed by flow cytometry to quantify proportion of DCs and the expression of MHCII, CD80, and CD86. Virgin oestrus mice were used as non-mated controls. Data are presented as mean ± SEM and are the percentage of CD11C+ DCs within viable cells (A), the total number of DCs x 10^6 (B), the percentage of activated DCs (MHCII+ DCs) (C), the total number of activated DCs x 10^6 (D), the percentage of CD80+ DCs (E), the total number of CD80+ DCs x 10^6 (F), the percentage of CD86+ DCs (G) and the total number of CD86+ DCs x 10^6 (H). Differences between groups were evaluated using Kruskal-Wallis test and Mann-Whitney U test. (a,b,c,d indicates $p<0.05$).

6.3.3 Maternal miR-223 deficiency elevates LPS-induced fetal loss

(The results in this section were generated and kindly provided by Dr. John Schjenken and are included here to allow a full discussion of the implication of immune cell changes in miR-223-/- mice).

As detailed above, miR-223 deficiency results in an altered maternal immune environment in early pregnancy. However, this altered environment appeared not to affect fertility, because in the breeding colony, where miR-223-/- females were mated with syngeneic miR-223-/- males, steady state pregnancy rates were comparable to controls (Schjenken, data not shown). As detailed in chapter 3, the functional requirements for immune mediators can become apparent following inflammatory challenge. Therefore, to investigate the impact of miR-223 deficiency on immune tolerance in pregnancy, miR-223-/- and miR-223+/+ female mice were mated to allogeneic Balb/c males to generate pregnancies with maternal, but not fetal, miR-223 deficiency. On DPC 9.5, pregnant females were administrated a low-dose inflammatory challenge of 1.0 μg LPS, or PBS control. This dose of LPS was chosen as it was determined in preliminary experiments to be just below the threshold for impact on pregnancy loss in miR-155+/+ mice.

In mice examined just before term on DPC 17.5, miR-223-/- females were more severely affected by LPS challenge than miR-223+/+ mice, with a lower proportion of miR-223-/- mice carrying viable fetuses (39% reduction, $p<0.05$, Figure 6.6A) and a lower number of viable fetuses per mated female (50% reduction, $p<0.001$, Figure 6.7B) on DPC 17.5. The change in viable fetuses was associated with a significantly higher rate of fetal resorption in miR-223-/- mothers (8.8-fold, $p<0.0001$) following LPS treatment (Figure 6.7C). The LPS treatment impacted fetal weights in both miR-223+/+ (11% reduction, $p<0.05$) and miR-223-/- (10% reduction, $p<0.05$) (Figure 6.7D). Strikingly, miR-223 deficiency impacted fetal weights even in the PBS treatment group with a significant reduction observed (9.3% reduction, $p<0.05$) (Figure 6.7D). No effects of genotype or LPS treatment on placental weight was observed (Figure 6.7E). However, the changes in fetal weights led to a significant reduction in the fetal: placental weight ratio in miR-223-/- following LPS (19% reduction, $p<0.0001$, Figure 6.7F), but not PBS treatment compared to miR-223+/+ females. Thus, maternal miR-223 deficiency appears to impact the establishment and maintenance of the appropriate maternal immune environment in pregnancy, such that miR-223 deficient mice show elevated susceptibility to fetal loss after inflammatory challenge.

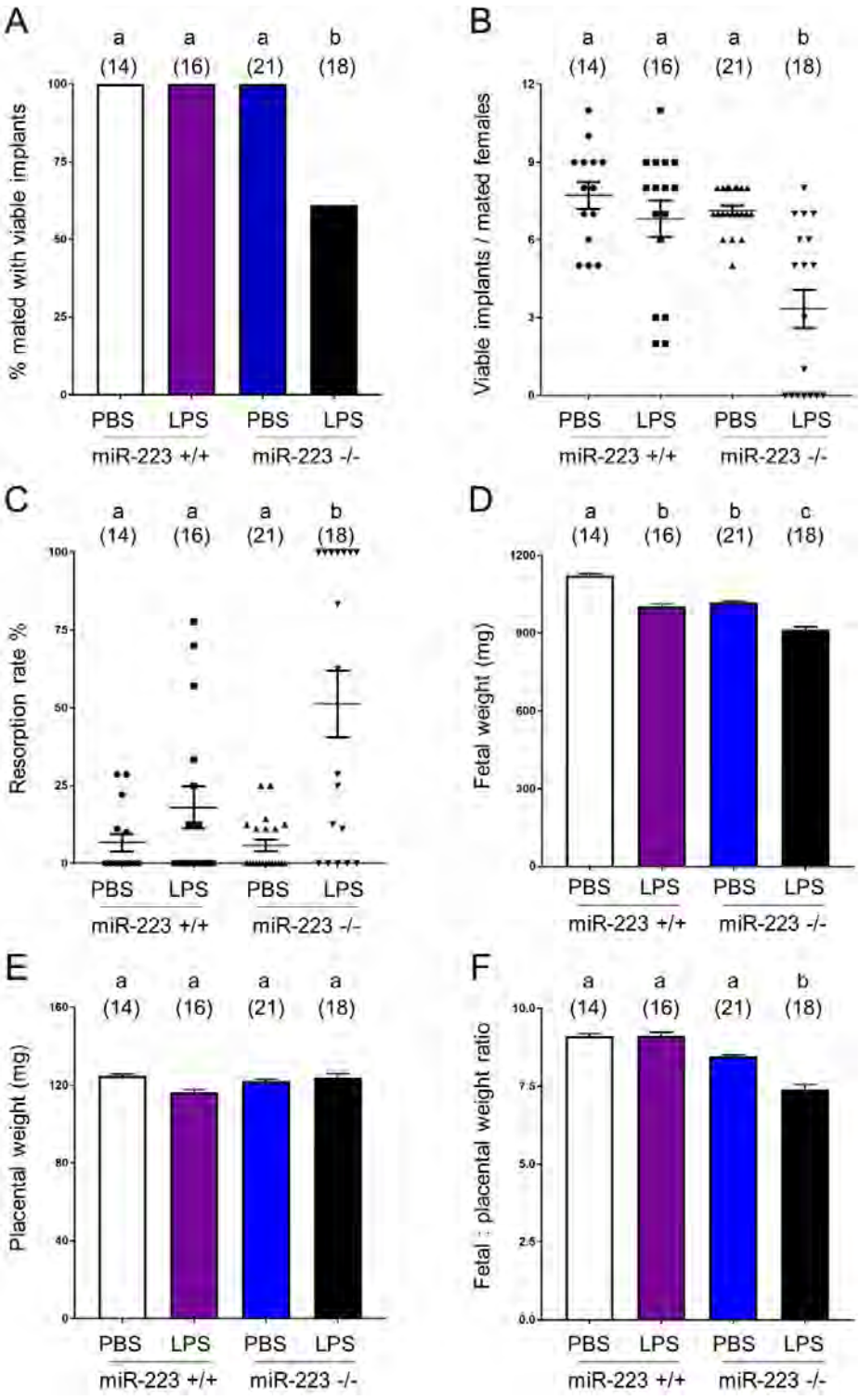


Figure 6.7 The effect of miR-223 deficiency on pregnancy parameters after low-dose LPS challenge. miR-223+/+ and miR-223-/- mice were mated with Balb/c males and injected i.p. with LPS (1μg) or control (PBS) on DPC 9.5, then autopsied on DPC 17.5. Data are the percentage of mated females pregnant with viable fetuses (A), the number (mean ± SEM) of viable implantation sites per mated mouse (individual data points with mean value shown; B), the percentage of total implantation sites per pregnant female undergoing resorption (individual

data points with mean ± SEM; C), the fetal weight (estimated marginal mean ± SEM; D), the placental weight (estimated marginal mean ± SEM; E), and the fetal: placental weight ratio (estimated marginal mean ± SEM; F). Numbers of mated mice are shown in parentheses. The effect of genotype was evaluated in A by chi-square analysis and in B by ANOVA and Sidak t-test. The effect of genotype was evaluated in D-F by mixed model analysis, using mother as subject and litter size as covariate (a,b,c,d indicates $p<0.05$).

6.3.4 Systemic impact of miR-223 deficiency on DPC 3.5

miR-223 deficiency resulted in a reduction in the proportion but not the absolute number of CD4+ T cells in the mLN, but no impact was observed in the proportion and number of CD4+ T cells in spleen or peripheral blood, indicating that miR-223 deficiency had minimal influence on the CD4+ T cell population at oestrus or on DPC 3.5 in these sites. A reduced proportion in Treg cells amongst CD4+ T cells was observed in the mLN and the spleen at oestrus and on DPC 3.5, but this did not occur in the peripheral blood. Together, these data indicate that miR-223 deficiency did not exert substantial systemic changes in the maternal immune response in oestrus or early pregnancy.

On DPC 3.5, miR-223 deficiency did not alter macrophage populations or phenotype systemically, with a small reduction in the proportion of macrophages observed at oestrus and on DPC 3.5 and a reduction in the proportion of CD80+ macrophages at oestrus in the mLN. In addition, miR-223 deficiency was associated with a small reduction in the proportion of activated macrophages in the spleen at oestrus. Furthermore, miR-223 deficiency failed to induce the same extent of activated DCs and CD80+ DCs expansion in the mLN on DPC 3.5. These data suggest that the miR-223 deficiency had minimal impact on the number and phenotype of Treg cells and antigen presenting cells in other distal lymphoid organs or in peripheral blood. Taken together, these data show less profound change due to miR-223 deficiency on the number and phenotype of Treg cell and antigen presenting cells in systemic tissues compared with the more substantial changes in the PALN.

6.4 Discussion

A sufficient number of functional Treg cells are essential for maternal immune tolerance in pregnancy. Treg cells suppress inflammation and promote immune adaptation at the maternal-fetal interface (Aluvihare et al., 2004, Guerin et al., 2009). Understanding the regulation and function of Treg cells is crucial for developing treatment for human gestational complications, such as preeclampsia and recurrent miscarriage where altered Treg cells are implicated (LaMarca et al., 2013, Santner-Nanan et al., 2009, Prins et al., 2009, Wallace et al., 2011). In order to develop this knowledge, it is crucial to understand how Treg cells are normally regulated during pregnancy.

There are growing studies focusing on the miRNAs in immune cells and in non-immune cells that influence immune response. Depletion of miRNA by *Dicer* null mutation results in a systemic

compromise in T cells (Taganov et al., 2007). The miRNA expression profile in conventional CD4+ T cells and Treg cells has been investigated in mouse.

Treg cells have a distinct miRNA expression profile, compared with CD4+ T cells, and miR-223 is highly enriched in the CD4+ T cell to Treg cell transition (Cobb et al., 2006). Treg cells have a distinctive miRNA expression profile with miR-146a, miR-155 and miR-223 and another 32 miRNAs preferentially expressed in Treg cells compared to conventional CD4+ T cells (Cobb et al., 2006). However, few studies have addressed the role of miR-223 in Treg cell development and function and little is known about the role of miR-223 in regulation of the Treg cell response.

A correlation between higher miR-223 and a lower Treg cell population has been observed in maternal blood (Herberth et al., 2014). High miR-223 level in the maternal blood is correlated with lower maternal Treg cell numbers in women who smoke (Herberth et al., 2014). Clinical studies have revealed that altered miR-223 expression patterns are found in pregnancy complications. In recurrent pregnancy loss patients, miR-223 expression is higher in the peripheral blood as well as placentas (Tang et al., 2016, Winger et al., 2015). In addition, seven miRNAs, including miR-223 in peripheral blood are identified as potential markers to screen pregnancy complications during first trimester or prior to conception, including miscarriage and preeclampsia (Winger et al., 2015). In recurrent pregnancy loss patients, miR-223 expression is higher in placental samples or peripheral blood (Tang et al., 2016).

To understand how aberrant miR-223 expression patterns may contribute to pregnancy complications, the role of miR-223 in normal pregnancy needs to be elucidated. The current study has demonstrated that miR-223 deficiency results in a local change to the response to male alloantigens in the PALN. Although the proportion of CD4+ T cells is not reduced in the absence of miR-223 in the PALN, the total number of CD4+ T cells are reduced on DPC 3.5, with a decreased CD4+ MFI at oestrus and on DPC 3.5. At oestrus (steady-state), the number of CD4+ T cells are not influenced by the absence of miR-233 in PALN, but a distinct reduction is seen on DPC 3.5 when male alloantigen challenge has occurred. This reduction seems to be due to limited total cell expansion in the PALN in the miR-223 deficient mice on DPC 3.5 (Figure 6.8).

Previous studies have focused on investigating how miR-223 regulates maturation, proliferation and differentiation of myeloid cells (Chen et al., 2004, Johnnidis et al., 2008). The current study has revealed that miR-223 may be also involved in CD4+ T cell activation and proliferation. In addition, the reduction pattern in the CD4+ T cell numbers are not observed in the mLN, spleen and peripheral blood at oestrus

or on DPC 3.5 in the absence of miR-223, indicating that miR-223 deficiency has a local impact in the reproductive setting, that becomes evident after the antigenic challenge of mating and early pregnancy.

In the present study, we firstly demonstrated that in the absence of miR-223, Treg cells are diminished in the PALN which drain the uterus. With limited total cell expansion, as well as reduced Treg cell proportion amongst the CD4+ T cells, the most striking reduction in the number of Treg cells is evident in the PALN, and a smaller reduction in the Treg cell proportion is also observed in mLN and spleen at oestrus and on DPC 3.5 in miR-223 deficient mice (see chapter 8, section 8.3.1). Currently only two studies have focused on miR-223 and Treg cells, one study linked reduced Treg cell numbers are associated with dysregulated macrophages and DCs in the intestine of miR-223 deficient mice (Zhou et al., 2015), and the other study reports that an elevation of miR-223 is correlated with lower Treg cell numbers in maternal blood in women who smoke (Herberth et al., 2014). This study has firstly addressed that of miR-223 is required in the Treg cell expansion in the early gestation period, which is consistent with the literature that miR-223 is highly expressed in Treg cells (Cobb et al., 2006).

The MFI of Foxp3 is reduced in PALN and other tissues on DPC 3.5 but not at oestrus. As Foxp3 is the 'master switch' which is critical in the Treg cell development and suppressive function (Fontenot et al., 2005, Fontenot et al., 2003), reduced MFI of Foxp3 indicates that Treg cells in miR-223-/- mice may be associated with reduced stability or impaired commitment to the Treg cell lineage. Notably, the reduced Foxp3 MFI was only observed on DPC 3.5 not at oestrus in the PALN. Since Foxp3 MFI is has been linked with suppressive competence (Wan and Flavell, 2007), this may indicate that in the absence of miR-223, Treg cells in PALN are impaired in achieving their optimal suppressive activity after priming to male alloantigens. Currently, no study has addressed the relationships between miR-223 and Treg cell suppressive activity.

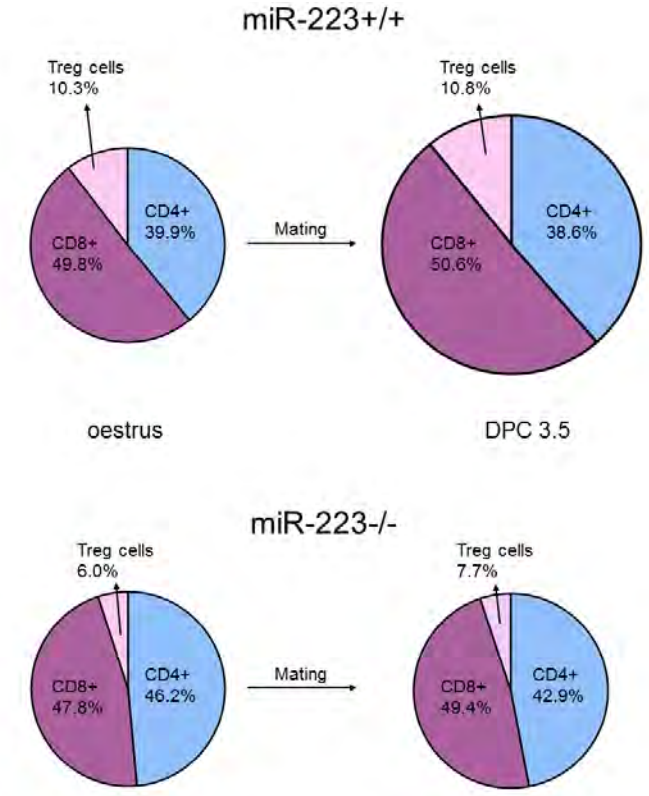


Figure 6.8 Proportion of CD4+, CD8+ and Treg cells in the uterus and PALN in the miR-223+/+ and miR-223-/- mice at oestrus and on DPC 3.5. Comparable amount of celles were discovered in the miR-223-/- mice at oestrus and on DPC 3.5, compared with WT controls.

After seminal fluid exposure, an influx of antigen presenting cells including macrophages and DCs are recruited to the uterus. These cells are important for priming T cells and inducing Treg cell population expansion (De et al., 1991, McMaster et al., 1992, Robertson et al., 1998, Robertson et al., 1996, Robertson et al., 1992). In addition, macrophages are involved in tissue remodelling and assist in preparing the uterus for fetal implantation, for example through effects on epithelial cell expression of embryo attachment ligands (Das et al., 1997, Feng et al., 1998, Jasper et al., 2011). Notably, in the absence of miR-223, a reduced proportion and number of macrophages and activated macrophages are observed in the uterus and PALN at oestrus and on DPC 3.5. This suggests that miR-223-/- females are less competent to respond to male alloantigens and the capacity to prepare for fetal implantation may be compromised. Previous studies show that absence of miR-223 reduces the number and proportion of macrophages and miR-223 is found to regulate the differentiation of macrophages and maintain their M2-like phenotype (Zhou et al., 2015, Zhuang et al., 2012). The current study also found a reduced proportion of macrophages in the mLN at oestrus and on DPC 3.5, and a reduced proportion of activated macrophages in the spleen on DPC 3.5, but the reduction is not to the same extent as in the uterus and PALN. Taken together, miR-223 deficiency has a greater impact on the macrophage population in reproductive tract, compared with systemic lymph nodes. This likely reflects a failure to respond to the activating signals that are present in the uterus following seminal fluid contact.

The diminished Treg cell pool in early gestational period in miR-223-/- mice is accompanied by, and may be the consequence of altered phenotype of DCs. A 1.68-fold reduction in numbers of DCs were found in PALN on DPC 3.5 in miR-223-/- females, suggesting that miR-223 is important in DC recruitment and/or proliferation. This contrasts with a previous study which demonstrated that CD11C+ DCs are not changed in the small intestine in the absence of miR-223 (Zhou et al., 2015). This may be due to the difference of tissues examined. In addition, miR-223 deficient mice did exhibit a decrease in myeloid DCs in the central nerve system in experimental autoimmune encephalomyelitis model (Ifergan et al., 2016), which is consistent with the current study.

As well as being fewer in number, these DCs appear less activated, with decreased expression of MHCII on DPC 3.5. Less MHCII expression suggests that antigen presenting cell presentation capability may be impaired which would contribute to explaining the reduced Treg cell population. Dysregulated macrophages and DCs have been linked with a reduced Treg cell population in the small intestine of

miR-223-/- mice (Zhou et al., 2015). This implies a similar mechanism exists in the PALN where less activated DCs in miR-223-/- mice are unable to induce Treg cell responses.

Antigen presenting cell influx is most obvious in the acute early phase of the inflammation-like response to seminal fluid, in the cervix 12 h post coitus in human (Sharkey et al., 2012b) and 8-12 hours after mating in mice (Robertson et al., 1998, Robertson et al., 1996, Robertson et al., 1992). In the current study, the antigen presenting cell population and phenotype were examined on DPC 3.5, which may not be the optimal time points to quantify differences in antigen presenting cell population and phenotype. In addition, the M1 vs M2 polarisation of macrophages was not determined in the current study. It would be relevant to further investigate the impact of miR-223 deficiency on the inflammation-like response to seminal fluid induction at 12-24 hours after coitus, and also to examine markers of M1 and M2 polarisation.

Despite all of the changes in the immune cells around peri-conception period, altered T cell immunity in miR-223 deficient mice does not overly affect tolerance of fetal alloantigen, as miR-223 deficient female mice mated to Balb/c males are capable of maintaining pregnancy with generally comparable outcomes to WT control mice. However, the importance of miR-223 becomes apparent when pregnant miR-223-/- female mice are administrated low dose LPS to induce a systemic inflammatory response on DPC 9.5 in mid-gestation. When miR-223-/- female mice are challenged with inflammatory stimuli, they have a reduced capacity to sustain pregnancy with a 51.3% in resorption rate, compared to WT mice in which pregnancy was not impacted by the same LPS dose.

miR-223 is known to promote M2 polarisation of macrophages which is anti-inflammatory (Zhou et al., 2015, Zhuang et al., 2012, Zhang et al., 2013), and macrophages and DCs in miR-223 deficient mice are highly pro-inflammatory (Zhou et al., 2015). Although we did not evaluate the macrophage response to LPS in this study, it can be assumed that LPS administration caused a similar pro-inflammatory activation. In part, this may be attributed to the insufficient Treg cell response, as Treg cells are important for suppressing and controlling inflammatory cytokine responses (Collison et al., 2009, Collison et al., 2007, Sakaguchi et al., 2009a, Liang et al., 2008). In miR-223 deficient mice challenged with LPS, the limited population of Treg cells may be insufficient to control the excessive inflammatory response induced by the LPS inflammatory insult.

The placenta hypertrophy in miR-223 deficient mice challenged with LPS may be a consequence of fewer macrophages observed in the uterus and PALN. Macrophages are known to be an important

source of VEGF and other angiogenic factors (Yoshida et al., 1997) in a pattern that tightly regulated by oestrous cycle stage and early pregnancy (Ma et al., 2001). When an inflammatory insult occurs in mid-gestation, these dysregulated macrophages may have compromised capability to support the last phases of placental development. Taken together, these data suggest that miR-223 is essential in the protecting foetus from excessive inflammation via regulating Treg cell development and antigen presenting cell phenotype and function.

Further functional studies are required to address the suppressive activity of Treg cells in the absence of miR-223 during gestation. In addition, the antigen presentation capability and phenotypes in antigen presenting cells and polarisation of macrophages in miR-223-/- mice has not yet been addressed. Assessment of the response to antigen or non-specific stimulation, and assay of suppressive function in Treg cells are required to address these questions, in order to better understand how Treg cell responses are impacted in the absence of miR-223. Furthermore, adoptive transfer of Treg cells from pregnant WT mice into miR-223 deficient females prior to inflammatory challenge could be performed to specifically investigate the protective role of Treg cells in the inflammation-induced fetal loss. It could be predicted that transfer of wild-type Treg cells from pregnant B6 donors would mitigate the elevated susceptibility to inflammatory challenge seen in miR223 null mutant mice.

In summary, the current study reinforces the pivotal role of miR-223 in both innate and adaptive immunity. Both Treg cell and antigen presenting cell populations are impaired in the absence of miR-223, in particular diminished Treg cell population are found in PALN. While Treg cells generated in the absence of miR-223 are competent to sustain the semi-allogeneic fetus under steady-state conditions, the absence of miR-223 and its impact on the maternal immune environment may contribute to fetal loss under inflammatory conditions. This may be relevant to understanding the significance of microRNAs in influencing Treg cell generation and suppressive competence in women, and their role in providing protection from gestational disorders.

Chapter 7 Final Discussion

7.1 Introduction

The peri-conception period, when the fertilised oocyte develops to the blastocyst stage and implantation commences, is a crucial phase in pregnancy. Disturbance in embryo development and/or uterine receptivity can cause infertility and impact fetal growth and placentation leading to pregnancy complications (Fowden et al., 2008), and is implicated as a key determinant in fetal programming of metabolic, immune and neurological issues in offspring (Hoet et al., 2000, Kwong et al., 2000, Sjoblom et al., 2005). Therefore, it is essential to determine factors that contribute to the regulation of the peri-conception environment, to address the causes of pregnancy complications.

There are many environmental factors and exposures which influence conception to impact on implantation and placental formation, fetal growth and infant health. Factors that contribute include but are not limited to nutrition intake and obesity of the mother and father prior to conception (Fleming et al., 2011), oocyte integrity and structure (Lane et al, Science 2015), epigenetic remodelling from fertilisation to implantation (Reik, 2007), changes to the sperm epigenome (Gannon et al., 2014, Yadav and Kotaja, 2014) and other paternal factors present in seminal plasma, the non-sperm fraction of seminal fluid (Bromfield et al., 2014).

Amongst the key local regulators of the maternal peri-conception environment are reproductive tract cytokines and immune cells. Appropriate regulation and adaption of the maternal immune environment is crucial to accommodate the semi-allogeneic fetus. The female tract microenvironment, local cytokine production and immune cells cooperate together and are tightly regulated to promote embryo implantation and developmental competence, to suppress inflammation and development of effector immunity to paternally-derived fetal antigens, as well to regulate the decidual response and vascular changes that support placentation (Schjenken and Robertson, 2014). Altered immune responses in the early pregnancy phase can impair placental development and change the trajectory of fetal growth, which explains why this early time is associated with pregnancy complications, such as preeclampsia, miscarriage and infertility (Guerin et al., 2009, Moldenhauer et al., 2017)

In addition to maternal contributions, the male partner also contributes to the adaptation of the female tract environment during the peri-conception period through effects mediated by seminal fluid. Seminal fluid contains cytokines, chemokines and antigens specific to individual males, including minor antigens and major histocompatibility complex (MHC) in human (Hutter and Dohr, 1998). These signalling factors interact with epithelial cells lining the cervix and trigger an influx of leukocytes,

including macrophages and dendritic cells (DCs) to initiate a transient inflammatory response. In broad terms, similar responses to seminal fluid are seen in humans (Sharkey et al., 2012b), mice (Robertson et al., 1998, Robertson et al., 1996), and all mammalian species examined to date (Schjenken and Robertson, 2014).

Antigens present within seminal fluid activate T cells response after being taken up, processed and presented by antigen presenting cells which are recruited to the endometrium following seminal fluid exposure (Robertson et al., 1996, McMaster et al., 1992). These antigen presenting cells mediate tolerogenic immune responses in the presence of prostaglandin E (PGE) and TGF-β, and capture paternal antigens before either emigrating from the uterus via the afferent lymphatics to activate T cells in the para-aortic lymph nodes (PALN) which drain the uterus, or interacting with resident T cells within tertiary lymphoid structures within the deep endometrium of the uterus (Robertson et al., 2009b). The net result of seminal fluid exposure is to drive a paternal antigen-specific T cell response (Moldenhauer et al., 2009). Amongst the activated T cells, expansion of the Treg cell subset is observed in the PALN and also in the uterus in the peri-conception period in mice (Robertson et al., 2009a, Guerin et al., 2011). In addition to mediating the transient inflammatory response required for T cell activation, these immune cells are also thought to be involved in tissue restructuring to promote the decidual response, facilitate uterine receptivity and prepare for implantation to support robust placental development (Plaks et al., 2008, Robertson, 2005).

A key consequence of seminal fluid exposure is the expansion of Treg cells which are crucial in establishing and maintaining the appropriate immune environment. Maternal inflammation needs to be controlled appropriately to achieve optimal fetal outcomes. Treg cells are pivotal in the regulation of suppressing inflammation and mediating maternal immune tolerance at the maternal-fetal interface in pregnant females (Aluvihare et al., 2004). These cells become activated to proliferate and are increased significantly during the peri-conception period in the PALN and uterus (Aluvihare et al., 2004, Zhao et al., 2007, Guerin et al., 2011).

In addition, the timing and presence of Treg cells are both crucial in mediating pregnancy success. The Treg cell population plays a particularly important role in MHC disparate allogeneic pregnancies. In syngeneic pregnancies, 50% of T cell deficient mice remain pregnant after receiving Treg cell-depleted T cells on day 4 postcoitum (DPC) (Aluvihare et al., 2004), indicating a lesser requirement for Treg cells in maintaining syngeneic pregnancies where there is no MHC disparity between the fetuses and the

mother. However, adoptive transfer of Treg cell-depleted T cell population into T cell deficient mice carrying allogeneic fetuses fails to rescue pregnancy (Aluvihare et al., 2004) and causes almost complete infertility, indicating that Treg cell are essential to maintain the allogeneic pregnancy.

The presence of a local Treg cell population in early pregnancy is crucial, as the transfer of Treg cells to abortion prone mice prior to, but not after implantation rescues pregnancy loss (Zenclussen et al., 2005). Adoptive transfer of T cells from normal pregnant mice into 0- to 2-day pregnant abortion-prone mice successfully rescues pregnancy, but Treg cell transfer on day 4 or 5 of pregnancy fails to prevent abortion, indicating that the presence of Treg cells in the peri-conception period is necessary for mediating the establishment of maternal immune tolerance for the genetically disparate fetuses.

Given the important role that Treg cells play in pregnancy, it is important to investigate how Treg cells are normally regulated. While Treg cell number is the top limiting factor in achieving a robust and appropriate Treg cell response, other factors including their stability and suppressive function contribute to their capacity to regulate the maternal immune environment. Currently, factors that regulate the generation, stability and suppressive activity of Treg cells are not fully understood, limiting our capacity to understand the biological basis of pregnancy disorders, including preeclampsia and recurrent miscarriage, and to develop effective treatments for these conditions.

In the present study, several factors postulated to play an important role in determining Treg cell expansion, stability and function were assessed in mice to better understand the regulation and role of Treg cells in the peri-conception period. In these studies, we demonstrated that IL10, miR-155 and miR-223 are all required to drive normal Treg cell expansion and stability. In each case, deficiency in these regulatory factors did not prevent pregnancy under steady state conditions, but caused elevated susceptibility to fetal loss following a mid-gestation inflammatory challenge. Furthermore, we demonstrate that while repeated exposure to MHC dissimilar alloantigens in male seminal fluid acts to further expand and strengthen the Treg cell response in early pregnancy, we were unable to demonstrate a major impact on susceptibility to inflammatory challenge in the model system evaluated herein.

7.2 Treg cell expansion

The requirement and importance of Treg cells during early pregnancy to maintain the semi-allogeneic fetus has been investigated in several studies (Aluvihare et al., 2004, Zenclussen et al., 2005). An

appropriate number of Treg cells are thought to be crucial to maintain pregnancy, as Treg cells are elevated in first and second trimester decidua and peripheral blood in humans (Heikkinen et al., 2004, Tilburgs et al., 2006, Xiong et al., 2013), and in the mouse uterus and PALN prior to embryo implantation on DPC 3.5 (Guerin et al., 2011, Robertson et al., 2009a). Experimental strategies that cause substantial reduction in the number of these Treg cells are inconsistent with normal implantation and progression of viable pregnancy (Aluvihare et al., 2004, Zenclussen et al., 2005, Shima et al., 2010). Consistent with numbers of Treg cells being a limiting factor in fertility, a diminished number of Treg cells is evident in women experiencing pregnancy complications such as preeclampsia and spontaneous miscarriage (Winger and Reed, 2011).

Despite the fact that most studies on the role of seminal fluid in pregnancy are conducted in mice, a number of clinical observations highlight the contribution of seminal fluid exposure in human pregnancy. Interestingly, in these studies, pregnancy complications such as preeclampsia are associated with a shorter period of sexual cohabitation with the conceiving partner, a change in sexual partners or the use of barrier contraceptive methods while longer term exposure to a partner's seminal fluid is linked with a reduction in the incidence of preeclampsia (Dekker et al., 1998, Einarsson et al., 2003, Kho et al., 2009, Klonoff-Cohen et al., 1989). In addition, a reduced Treg cell population in the peripheral blood (Prins et al., 2009, Santner-Nanan et al., 2009, Steinborn et al., 2008) is evident in clinical studies of preeclampsia. As detailed above, we and others have reported previously that seminal fluid contributes to the expansion of Treg cells in early pregnancy after a single mating (Robertson et al., 2009a, Guerin et al., 2011) and MHC dissimilar males more robustly expand the Treg cell pool (Tilburgs et al., 2009). In the current study, we used mouse models to examine the importance of regulatory factor operating at conception that alter Treg cell numbers and functional competence at implantation, including extent and nature of prior seminal fluid exposure, IL10, miR-155 and miR-223.

Given that seminal fluid expands Treg cells in early pregnancy (Guerin et al., 2011, Robertson et al., 2009a) and the association of Treg cells between preeclampsia, we postulated that repeated seminal fluid exposure leads to a stronger and more functionally competent Treg cell population in mice. To address this, we developed a mouse model to assess the impact on the Treg cell pool of repeated mating with allogeneic (MHC disparity) and syngeneic (identical MHC) males, or following partner switching at the most recent mating prior to conception. As expected based on previous studies, our results demonstrate that a single seminal fluid exposure commences a process of Treg cell expansion

evident in the PALN on DPC 3.5 (Figure 7.1). However, substantially greater expansion of the Treg cell population is seen if prior repeating mating to allogeneic males (Figure 7.1C). Notably, multiple matings with males of the same MHC haplotype resulted in a more robust Treg cell response (Figure 7.1), as Treg cell numbers from females mated four times to allogeneic males were significantly higher than Treg cells from females mated four times to syngeneic males has occurred. Similarly females in partner-switching groups also did not exhibit the same extent of Treg cell expansion, even though there was a significant expansion in both this group and the group mated with syngeneic males, compared with oestrus controls. Given the importance of MHC disparate matings in expanding the Treg cell population, we postulate that the number of Treg cells expanded in syngeneic and partner switching models may be less capable of facilitating robust placental formation than in females with repeated exposure to the same MHC disparate males. These results highlight the crucial role of MHC disparity in the expansion of the Treg cell pool and start to provide an explanation of why women who have had shorter sexual cohabitation or a new conceiving partner have increased incidence of preeclampsia (Dekker et al., 1998).

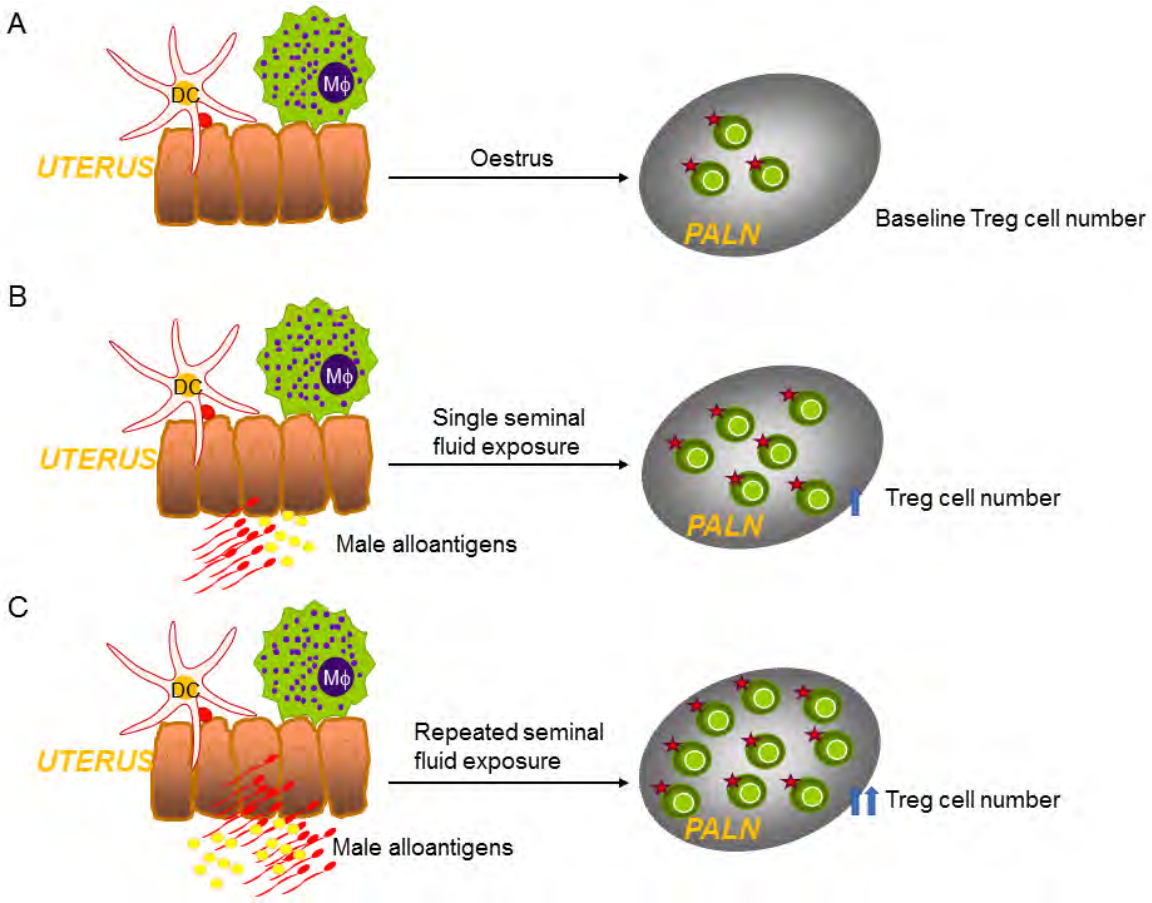


Figure 7.1 Repeated seminal fluid exposure drives Treg cell expansion and stability in the PALN on DPC 3.5. At oestrus, a baseline level of Treg cells located in the PALN (A), after single seminal fluid exposure, a moderate expansion of Treg cells is observed (B). Treg cell numbers are further augmented following repeated exposure to male alloantigens (C), compared to mice exposed to male minor antigens or in the male switching mating group.

While paternal factors associated with seminal fluid exposure and signalling capacity therefore are important in contributing to the expansion of the Treg cell population in early pregnancy, there is also evidence that intrinsic female factors affect Treg cell expansion and immune suppressive function. In particular, a large number of recent studies have focused on small non-coding microRNAs (miRNAs) and their contribution to the regulation of immune cell development and function (Baltimore et al., 2008). miRNAs are involved in the regulation of multiple immune cells, including macrophages, granulocytes, natural killer (NK) cells as well as B cell and T cell development and function (Mehta and Baltimore, 2016). The crucial role of miRNAs in immune function is highlighted by studies of mice with Dicer conditional mutation, where the T cell population is diminished in the thymus and periphery (Cobb et al., 2006, Muljo et al., 2005) and compromised antibody-producing capacity in B cells is also observed (Koralov et al., 2008). The critical role of miRNAs in Treg cells is demonstrated in mice with a Dicer null mutation in CD4+ T cells, which exhibit failure in Treg cell differentiation and reduced Foxp3 expression (Cobb et al., 2006, Cobb et al., 2005). In addition, miRNAs are also specifically implicated in the regulation of immune tolerance and immune response to conception (Bidarimath et al., 2014, Mehta and Baltimore, 2016).

There are numerous miRNAs associated with Treg cell function but one of the most well characterised miRNAs in Treg cells is miR-155, which is involved in development and function of DCs, macrophages, T cells and other immune cells. Within the Treg population, miR-155 deficiency results in a systemic decrease in Treg cell number but showed no impact on suppressive function (Lu et al., 2009a). As well as influencing antigen presenting capability in DCs (Rodriguez et al., 2007), miR-155 is also upregulated in M1 macrophages (pro-inflammatory) and promotes M1-polarisation in macrophages via suppressing suppressor of cytokine signalling 1 (Wang et al., 2010a). In addition, miR-155 over-expression is thought to contribute to preeclampsia via angiogenic regulating factor CYR61 (Zhang et al., 2010). Given these findings, we postulated that miR-155 may be a key miRNA involved in the regulation of the immune environment in early pregnancy.

Our data clearly demonstrate an impact of miR-155 on the Treg cell population with a systemic reduction in Treg cell numbers observed in miR-155-/- mice both at oestrus and on DPC 3.5. This study is consistent with previous studies showing that miR-155 contributes to Treg cell proliferation and competitive fitness (Lu et al., 2009a), and miR-155 deficient mice have a systemic impairment in their Treg cell population (Cobb et al., 2006). Interestingly, the greatest reduction was observed on DPC 3.5

which is the peri-conception period. The current study has also addressed the functional consequence of Treg cell deficiency in the absence of miR-155, and showed that the limited Treg cell population in these mice as unable to sustain pregnancy when inflammatory challenge occurs in mid-gestation (discussed in section 7.4).

Previous studies have demonstrated that miR-155 deficient mice exhibit reduced antigen presenting capability in DCs (Rodriguez et al., 2007, Dunand-Sauthier et al., 2014) and promotes the development of M1-like macrophages (Wang et al., 2010a). Given the link between Treg cell expansion and antigen presentation by antigen presenting cells, we assessed the impact of miR-155 deficiency on the antigen presenting cell population. In the current study, we demonstrate that in the absence of miR-155, a reduction in the number of DCs, activated DCs and also CD86+ DCs in the PALN on DPC 3.5 is observed. Previously, miR-155 has been identified to regulate the antigen presentation capability in DCs and

miR-155 deficient DCs are less capable to activate T cells effectively (Rodriguez et al., 2007). There we conclude that miR-155 can indirectly regulate Treg cell responses in early pregnancy, through effects on DC function.

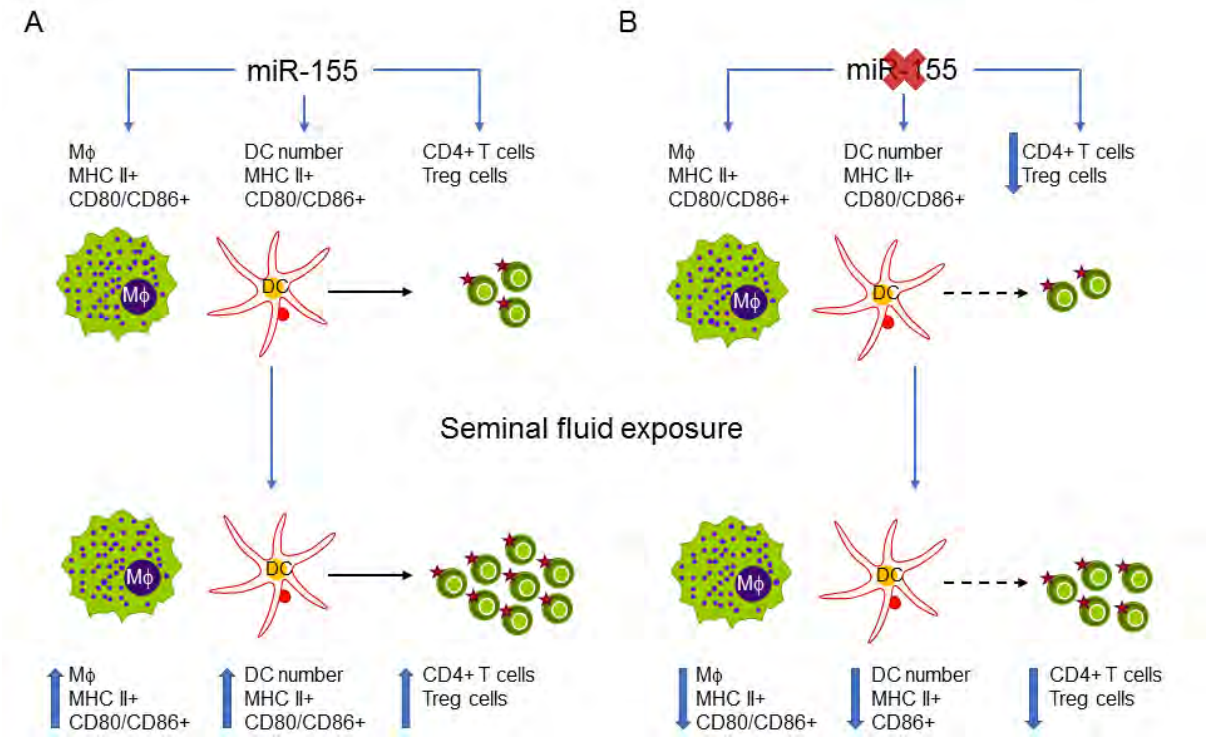


Figure 7.2 The impact of miR-155 deficiency on the female immune environment in the PALN during the peri-conception period. In wild-type miR-155+/+ mice, mating resulted in an elevation in the number of macrophages/DCs, activated macrophages/DCs as well as CD80/CD86+ macrophages/DCs in the PALN. An elevation of CD4+ T cells and Treg cells are also observed after mating in the PALN (A). In the absence of miR-155, a reduced CD4+ T cell and Treg cell population is evident in the PALN. Macrophages and DCs were reduced in number and developed a phenotype characterised by diminished expression of MHC II and co-stimulatory molecules (CD80/CD86) after mating. The reduced CD4+ and Treg cell population after mating may thus be the consequence of altered DC population and phenotype in the absence of miR-155 (B).

Similar to miR-155, miR-223 also contributes to regulation of immune responses. By targeting *Pknox1*, miR-223 is believed to contribute to M2 (anti-inflammatory) polarisation in macrophages (Zhuang et al., 2012), and mice with a *miR-223* null mutation exhibit M1-like macrophage phenotypes, including elevated pro-inflammatory cytokine IL-1β, IL-6 and tumour necrosis factor-α (Zhuang et al., 2012). Within the DC population, miR-223 deficiency results in an elevated population of DCs that have increased capacity to produce pro-inflammatory cytokines following stimulation by regulating C/EBPβ (Zhou et al., 2015). Little is known of miR-223 in the regulation of DC responses. In the current study, we explore the role of miR-223 in DC and macrophages and the impact on Treg cells in the peri-conception period.

In these studies, miR-223 deficiency resulted in a reduced proportion of activated macrophages at oestrus and on DPC 3.5 in the uterus, and reduced number of macrophages, activated macrophages, and CD80+/CD86+ macrophages on DPC 3.5 in the PALN as well as reduced number of DC and activated DCs in the PALN on DPC 3.5, indicating that miR-223 deficiency leads to an aberrant antigen presenting cell response when antigens are present in the inflammation-like response to seminal fluid. In addition, these reductions are not seen in other distal lymph organs at oestrus or on DPC 3.5, indicating that miR-223 deficiency impacts the local response to seminal fluid which is specific to the reproductive tract setting.

These data suggest that the reduced Treg cell population may be the consequence of an alteration in the capacity of DCs to respond to paternal antigens. Changes in the macrophage population may impact tissue remodelling and preparation of the uterus for fetal implantation, but any changes have minimal impact as normal implantation was observed (Das et al., 1997, Feng et al., 1998). Notably, in the absence of miR-223, the reduced proportion and number of macrophages and activated macrophages in the uterus and PALN was associated with reduced competence in miR-223-/- females to respond to male alloantigens (Figure 7.2). Intestinal macrophages and DCs appear to exhibit more pro-inflammatory phenotypes in the miR-223 deficient mice (Zhou et al., 2015), therefore it is consistent that macrophages and DCs presented in the uterus and PALN also exhibit a pro-inflammatory phenotype. However, tolerogenic phenotypes in DC and M2-like macrophages are more desirable in early pregnancy, as seminal fluid enhances DC differentiation into a tolerogenic phenotype in the uterus and PALN (Remes Lenicov et al., 2012), and M2-like macrophages are known to be anti-inflammatory.

Interestingly, miR-223 is highly enriched in the CD4+ T cell to Treg cell transition but there are few studies which have examined the function of this miRNA in Treg cells. Interestingly, one study has demonstrated that increased miR-223 correlates with reduced Treg cells in maternal blood in smoking women (Herberth et al., 2014) and another study has suggested that the altered macrophage population observed in miR-223 -/- mice can impact the Treg cell population (Zhou et al., 2015). miR-223 has been identified as a biomarker that can be utilised to predict adverse pregnancy outcomes including preeclampsia and miscarriage (Winger et al., 2015). Therefore, we postulated that miR-223 may impact the Treg cell population in pregnancy.

This study is the first to address whether miR-223 is required in the Treg cell expansion in the early gestation period. In the current study, we demonstrated that miR-223 can profoundly impact the Treg cell population in the PALN on DPC 3.5, and the data indicated that a greater local impact was induced compared with other peripheral lymph nodes. This result is consistent with the literature that miR-223 is highly expressed in Treg cells (Cobb et al., 2006). Insufficient numbers of Treg cells are associated with preeclampsia, recurrent miscarriage and other immune-based pregnancy complications (Nakabayashi et al., 2016, Prins et al., 2009), therefore miR-223 deficient may be a contributing factor in impaired pregnancy outcomes. It is notable that the Treg cell numbers are not changed at oestrus, indicating that miR-223 deficiency does not impact on the baseline Treg cell population. However, when Treg cells should be stimulated by the presentation of male alloantigens after mating, the proliferation of Treg cells is aberrant in the absence of miR-223.

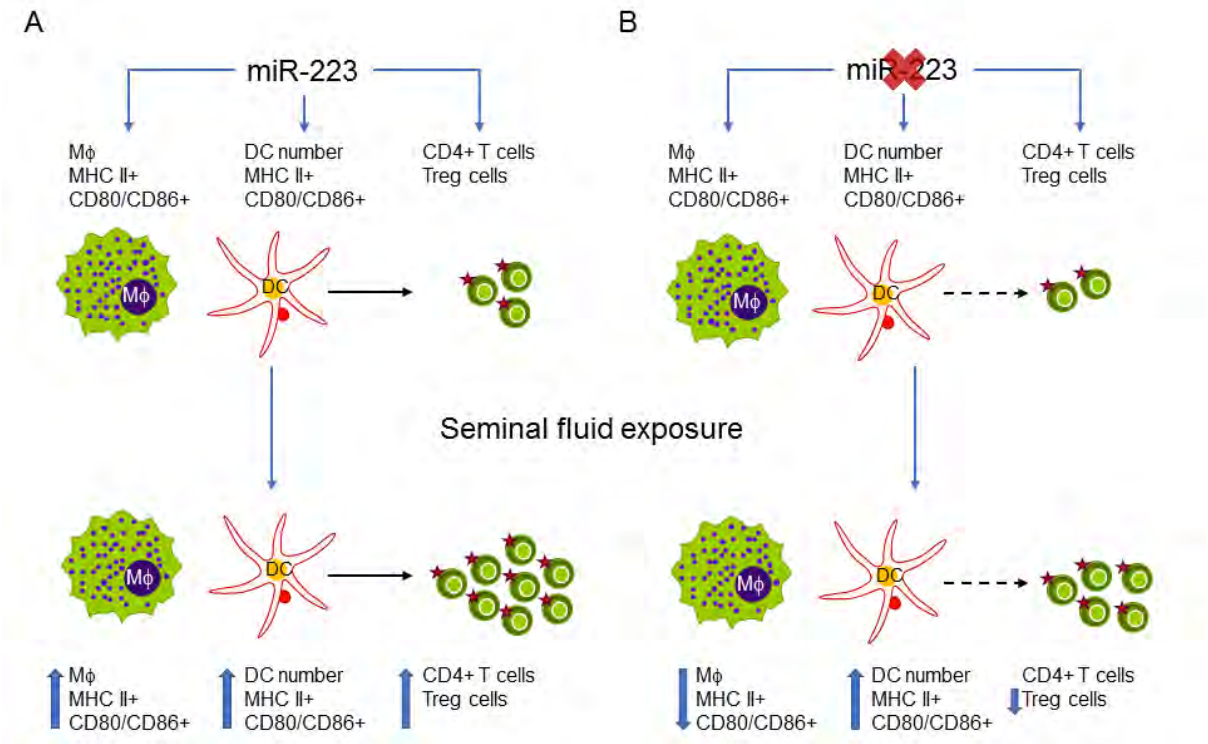


Figure 7.3 The impact of miR-223 deficiency on the female immune environment in the PALN during the peri-conception period. In wild-type miR-223+/+ mice, mating resulted in an elevation in the number of macrophages/DCs, activated macrophages/DCs as well as CD80/CD86+ macrophages/DCs in the PALN. An elevation of CD4+ T cells and Treg cells are also observed after mating in the PALN (A). In the absence of miR-223, a reduced Treg cell population is evident in the PALN. Macrophages are reduced in number and developed a phenotype characterised by diminished expression of MHC II and co-stimulatory molecules (CD80/CD86) after mating. The reduced Treg cell population after mating may be the consequence of altered macrophage and DC population and phenotype in the absence of miR-223 (B).

7.3 Treg cell stability

In addition to Treg cell number, the functional phenotype of Treg cells is also critical for maintaining immune tolerance, however there are few studies that have addressed the stability of Treg cells in pregnancy. In recent years, several studies have emerged to highlight that T-cell subsets are not as stable as previously assumed and under certain conditions, Treg cells can exhibit plasticity and convert into effector T-cell subtypes (Gao et al., 2012, Zhou et al., 2009). In particular, a pro-inflammatory environment can cause Treg cells to lose Foxp3 expression and convert to IL-17 producing or Th17 cells (Osorio et al., 2008, Yang et al., 2008). Treg cells can also switch to a Th17 phenotype following co-culture with DCs activated by the fungal recognition receptor, dectin-1 (Osorio et al., 2008). In addition, TGF-β producing thymic derived Treg cells can induce CD4+CD24- T cells to differentiate into Th17 cells in the presence of IL-6 *in vitro* (Xu et al., 2007).

Clinically, evidence of possible Treg cell instability has been linked with pregnancy complications. In women with preeclampsia, the Treg cell population in peripheral blood is reported to be even lower than non-pregnant women, and these cells have a shift towards IL-17 expressing phenotypes (Santner-Nanan et al., 2009, Tian et al., 2016). Given the plasticity between Treg cells and Th17 cells, studies are required to explore factors controlling Treg cell stability and their importance in supporting pregnancy success.

To understand factors influencing the stability of Treg cells in pregnancy, we initially examined the impact of seminal fluid exposure following mating in the multiple mating mouse model. While Treg stability was not changed after repeated exposure to allogeneic males, multiple mating with syngeneic males led to a decrease in the stability of Treg cells as evidenced by increased IL17 production within the Treg cell population following polyclonal activation. These results are consistent with evidence that MHC disparity between partners leads to better pregnancy outcomes and raise the question of whether poor pregnancy outcomes in MHC similar partners (Ober et al., 1998) is in part due to Treg cell instability as well as a reduced Treg cell population.

Intrinsic female factors are also linked with Treg cell stability. One such factor, IL10 is known to stimulate and reinforce modifications to the innate and adaptive immune system, including induction of regulatory phenotypes in the dendritic cell (DC), macrophage and T lymphocyte compartments (Fiorentino et al., 1991a, Fiorentino et al., 1991b, Maynard and Weaver, 2008). We have recently demonstrated that while IL10 deficient mice have increased proportions of Treg cells throughout early

and mid-pregnancy, these Treg cells are highly unstable after PMA/ionomycin stimulation (Prins et al., 2015), explaining why these mice have increased vulnerability to inflammatory insults in pregnancy and fetal loss (Murphy et al., 2005, Robertson et al., 2006, Robertson et al., 2007, Thaxton et al., 2009, Prins et al., 2015).

In an attempt to understand the impact of IL10 deficiency on the Treg population, we examined the Treg transcriptome in the presence or absence of IL10. Interestingly, while IL10 deficiency leads to an elevation of Treg cell numbers throughout early and mid-gestation in the PALN in mice (Prins et al., 2015), the Affymetrix microarray analysis demonstrated that IL10 deficiency altered the gene expression profile in Treg cells isolated from the PALN in mid-gestation. Amongst the genes differentially regulated, cathepsin E (*Ctse*) is a key factor contributing to Treg cell suppressive function independently of IL10. The upregulation of *Ctse* observed may reflect a compensatory effect to help sustain pregnancy, consistent with effects observed in IL10/IL35 double deficient mice where elevated *Ctse* rescues Treg cell suppressive activity (Pillai et al., 2011).

In addition to *Ctse*, upregulation of *Ifng* and a trend towards elevation in *Il17* expression may contribute to the instability of Treg cells in IL10-/- females observed after inflammatory stimulation (Prins et al., 2015). Cytokine receptors *Il1r1* and *Il12rb2* as well as T cell marker *Ctla2* were also elevated. IL12rb2 is required for TGFB-dependent stimulation of Treg cell development, and signalling via this receptor is thought to regulate the number and functional maturity of Treg cells (Zhao et al., 2008). These dysregulated pro-inflammatory genes in Treg cells in the absence of IL10 may contribute to the increased fetal loss observed following inflammatory challenge (Figure 7.3).

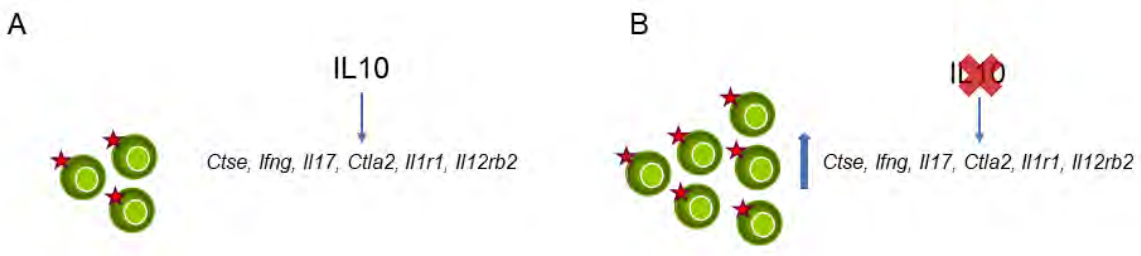


Figure 7.4. *Il10* null mutation alters the transcriptome in Treg cells, with an elevation in *Ctse* and other pro-inflammatory genes. In Il10+/+ mice, the expression of *Ctse* and the other pro-inflammatory genes are well-regulated in the Treg cell population (A). In the absence of IL10, an elevation of Treg cell population is evident in the PALN, with upregulated expression of *Ctse* and other pro-inflammatory genes (B).

7.4 The impact of altered Treg cells on pregnancy outcomes

Clinical studies have revealed that an altered immune environment impacts pregnancy quality. It has been demonstrated that shorter duration of sexual cohabitation is associated with elevated risk of pregnancy pathologies including preeclampsia and a small-for–gestational age fetus (Kho et al., 2009). In addition, women who have a new conceiving partner are more likely to develop preeclampsia (Klonoff-Cohen et al., 1989, Einarsson et al., 2003) suggesting that the protective effects following longer periods of cohabitation are partner specific. The serum IL10 level is diminished in women with preeclampsia, compared to normal pregnancy (Hennessy et al., 1999), and in mouse models, IL10 deficiency resulted in pre-term labour when mice were challenged with LPS (Robertson et al., 2006). miR-155 and miR-223 are identified as markers to predict adverse pregnancy outcomes (Winger et al., 2015). We postulated that the compromised immunological environment in the mouse models used in this study are likely to have impaired pregnancy quality. This was confirmed by elevated susceptibility to fetal loss after challenge with LPS, which is a model for the elevated inflammatory insult of preeclampsia. Previous studies have demonstrated that following LPS challenge in mid-gestation, *Il10* null mutation results in an elevated rate of fetal loss in syngeneic pregnancies (Robertson et al., 2007). In the current study, we have expanded the knowledge that IL10 in the maternal compartment is crucial in protecting allogeneically pregnant mice from excess inflammatory damage in mid-gestation. It is notable that the maternal compartment is not entirely IL10 deficient as the fetuses were heterozygous for the IL10 gene. Maternal IL10 deficiency does not impact the capacity to sustain pregnancy in steady-state conditions, as PBS injection does not cause fetal loss. The requirement of IL10 is more apparent when pregnant IL10 deficient females were challenged with low dose LPS, which results in fetal loss, indicating that IL10 has a key function in protecting fetuses from an excess inflammatory response (Murphy et al., 2005, Robertson et al., 2006, Robertson et al., 2007).

Similar to the IL10 mouse model, male alloantigens or number of copulation times showed no effect on fetal outcomes in steady-state, indicating that the number and stability of Treg cells, and whether Treg cells are induced by allogeneic mating or syngeneic mating does not impact on pregnancy competence. However, the number and stability of Treg cells become apparent as a limiting factor when pregnant females were challenged with a low dose of LPS. In particular there was a difference between females mated 4x to Balb/b and females mated 4x to Balb/c. Compared to females mated 4x to Balb/b, the further expanded Treg cell pool with more stabilised phenotype in females mated 4x to Balb/c, was

more likely to suppress the inflammatory challenge in mid-gestation. These data reinforce the importance of prior seminal fluid exposure in the induction of a sufficient Treg cell response prior to implantation, and also the critical role that robust Treg cells play in protecting the fetus from inflammatory insults.

As there was a diminished Treg cell population and altered number and phenotype of DCs and macrophages observed in miR-155 deficient and miR-223 deficient mice, we also examined the effect of miR-155 and miR-223 deficiency on the pregnancy outcomes after an inflammatory challenge in mid-gestation in these strains. Like the IL10 deficient mouse model and repeated mating mouse model, miR-155 and miR-223 deficiency had no impact on the maternal immune tolerance towards the semi-allogeneic fetuses in an unchallenged context, as no significant changes in fetal loss were observed in steady-state. However, a low dose LPS challenge led to greater fetal loss and reduced fetal to placental weight ratio, highlighting the importance of miR-155 and miR-223 in limiting excess inflammatory responses via the control of antigen presenting cell and Treg cell function. This is the first study addressing the impact of miRNA deficiency in pregnancy outcomes and further highlights that a consequence of changes to the immune population in the peri-conception period can impact on later fetal outcomes in pregnancy.

7.5 Clinical implications

Given that there is close similarity between mice and human in many immune regulatory factors, these findings are relevant to understanding which regulatory factors targeting Treg cells may be important in establishing maternal immune tolerance. Further studies are required to determine the extent to which repeated seminal fluid contact, IL10, and miRNAs are important in regulating the Treg cell response, including population expansion and phenotypic stability, in women. Defining the roles of these and other regulatory factors is likely to ultimately contribute to elucidating the pathology of preeclampsia and related complications of human pregnancy, where a less robust Treg cell response (Santner-Nanan et al., 2009) and altered regulation of placental IL10 (Hennessy et al., 1999) are both implicated in the underlying inflammatory aetiology.

Prior seminal fluid exposure contributes to Treg cell expansion, and this study is the first to demonstrate that the benefit from prior seminal fluid exposure requires both sperm and seminal plasma and is partner-specific, which can explain the clinical observation that women with a new conceiving partner

have a higher incidence of preeclampsia (Dekker et al., 1998), and why pregnancies conceived with donor eggs or sperm where no prior antigen priming has occurred, also have a higher risk of preeclampsia (Salha et al., 1999). Additionally, since miR-155 and miR-223 are involved in the regulation of human Treg cell expansion, and DC and macrophage expansion and maturation, our findings provide an explanation for how dysregulation in these miRNAs may contribute to pregnancy pathologies in women, including implantation failure, preeclampsia, recurrent miscarriage (Bidarimath et al., 2014).

7.6 Conclusion

The microenvironment and immune cells present in the peri-implantation period are crucial to successful pregnancy. The current study has identified events and regulators that play an important role in controlling this immune environment. IL10, male alloantigens, miR-155 and miR-223 are all critical factors in the generation of a robust Treg cell response, influencing antigen presentation, cell proliferation to drive population expansion, and imparting stability – all of which are crucial in the success of pregnancy. Further studies will expand on these findings and examine the function of these immune cell populations in the presence and absence of these factors. These findings provide evidence to demonstrate a key role for IL10, repeated seminal fluid exposure, miR-155 and miR-223 in the immune responses at peri-conception period. If similar roles are identified in women, this could eventually contribute to addressing the underlying reasons for why preeclampsia and other immune-related complications originate, and these regulators might even prove to be useful targets for improving the Treg cell response to protect women from these prevalent conditions.

Chapter 8 Appendix

Supplementary data

Publications arising from this thesis

8.1 Chapter 4

8.1.1 Treg cell expansion and stability in the mouse mLN, spleen and peripheral blood

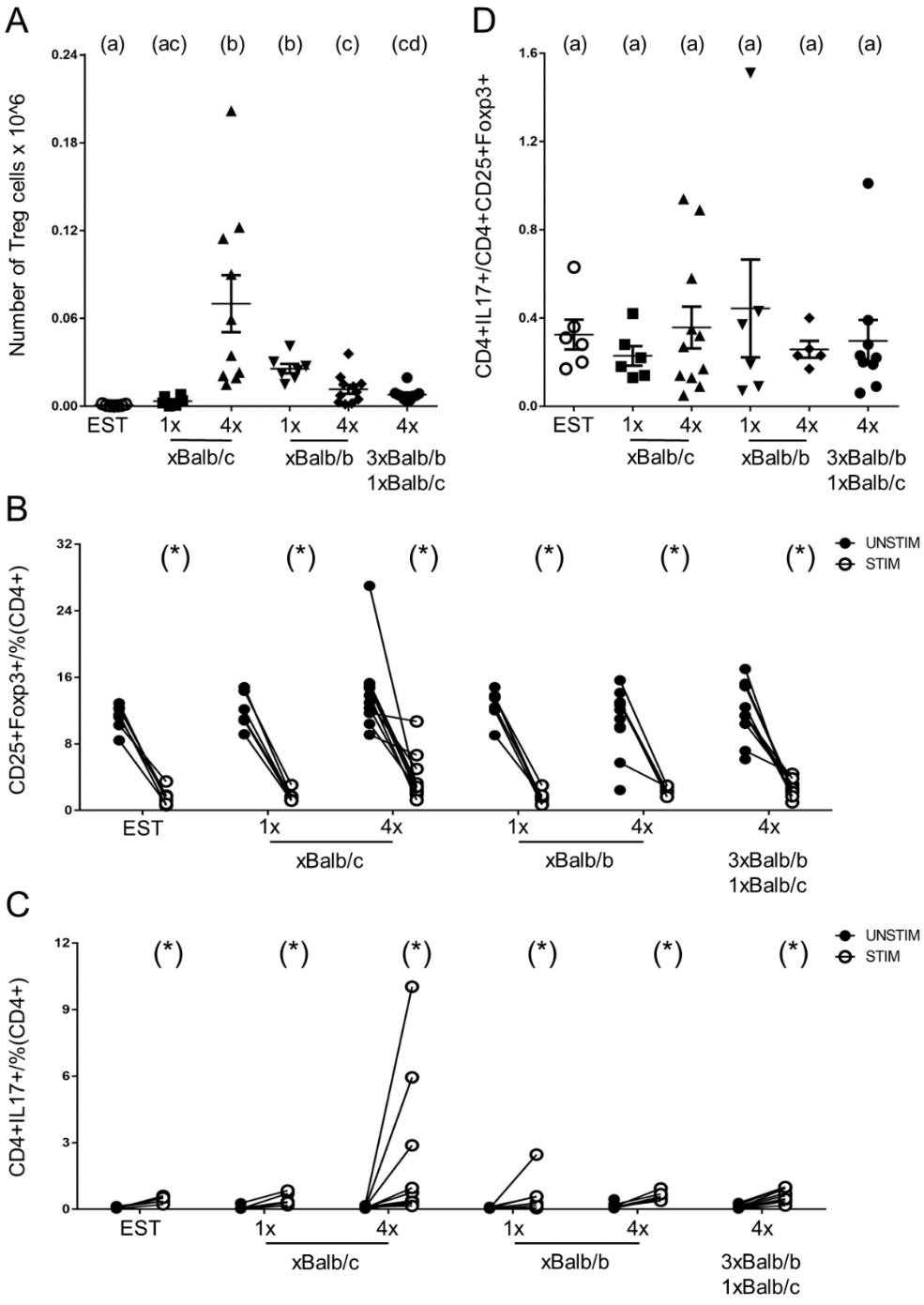


Figure 8.1 The effect of repeated exposure to male alloantigens on the number of Treg cells and stability of Foxp3 expression in CD4+ T cells in the mLN. B6 females were left unmated, mated 1x with Balb/c, 4x with Balb/c, 1x with Balb/b, 4x with Balb/b or 3x with Balb/b and 1x with Balb/c, and on DPC 3.5, mLN was analysed by flow cytometry for Treg cell parameters. Data presented as mean ± SEM and are the total cell number of

CD4+CD25+ Foxp3+Treg cells in the mLN (A), the expression of Foxp3 immediately after cell collection or 4h after strong polyclonal PMA/ionomycin stimulation *in vitro* (B), the expression of IL17A immediately after cell collection or 4h after strong polyclonal PMA/ionomycin stimulation *in vitro* (C), and the ratio of CD4+IL17+ to CD4+CD25+Foxp3+ cells from mLN after stimulation (D). The effect of PMA stimulation was evaluated using a paired T test in B and C, and the effect of mating was evaluated using Kruskall-Wallis and Mann-Whitney test in A and D.

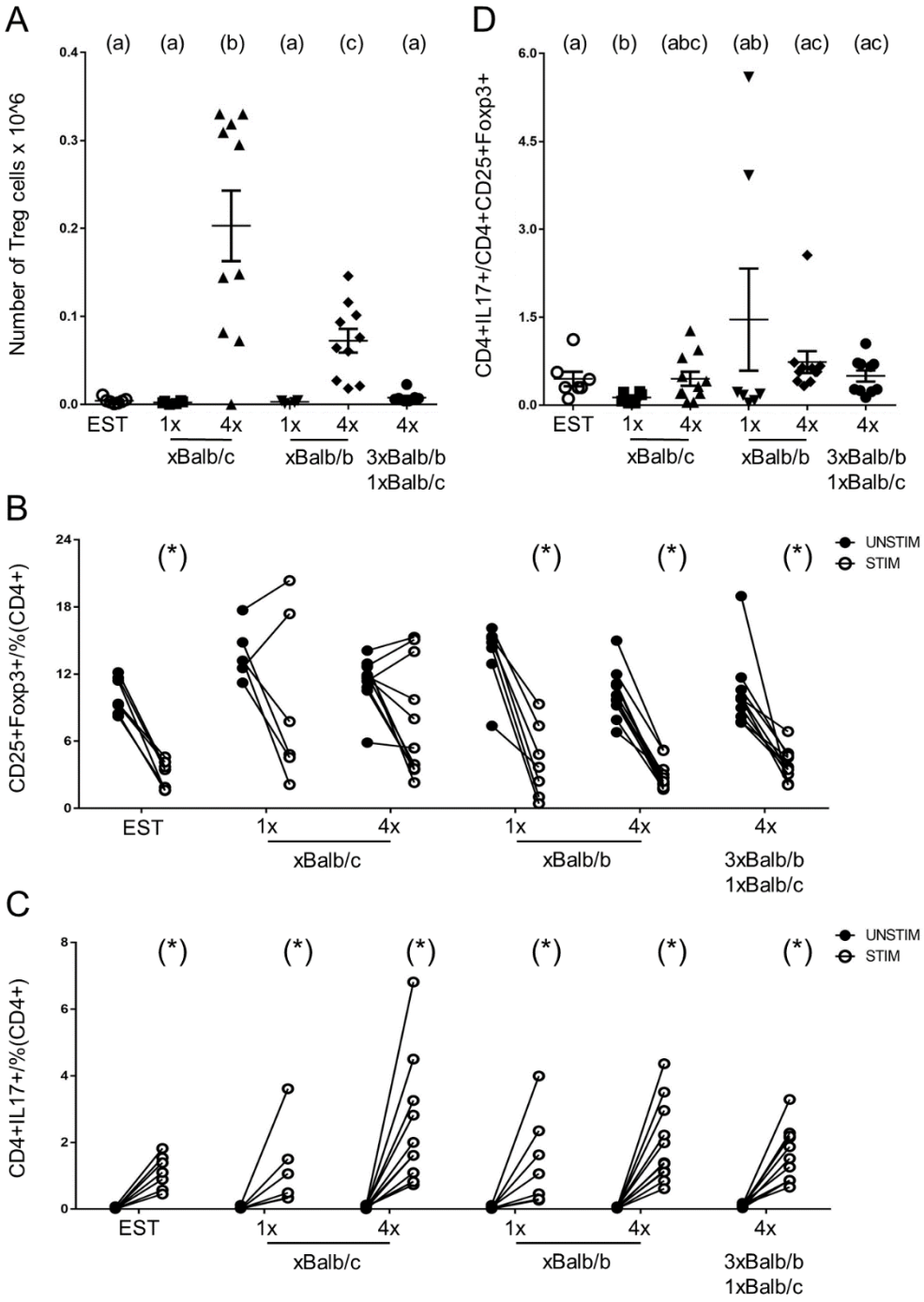


Figure 8.2 The effect of repeated exposure to male alloantigens on the number of Treg cells and stability of Foxp3 expression in CD4+ T cells in the spleen. B6 females were left unmated, mated 1x with Balb/c, 4x with Balb/c, 1x with Balb/b, 4x with Balb/b or 3x with Balb/b and 1x with Balb/c, and on DPC 3.5, spleen was analysed by flow cytometry for Treg cell parameters. Data are presented as the mean ± SEM are the total cell number of CD4+CD25+ Foxp3+Treg cells in the spleen (A), the expression of Foxp3 immediately after cell collection or 4h after strong polyclonal PMA/ionomycin stimulation *in vitro* (B), the expression of IL17A immediately after cell collection or 4h after strong polyclonal PMA/ionomycin stimulation *in vitro* (C), and the ratio of CD4+IL17+ to CD4+CD25+Foxp3+ cells from spleen after stimulation (D). Data are the mean ± SEM from

unmated or mated B6 mice. The effect of PMA stimulation was evaluated using a paired T test in B and C, and the effect of mating was evaluated using Kruskall-Wallis and Mann-Whitney test in A and D.

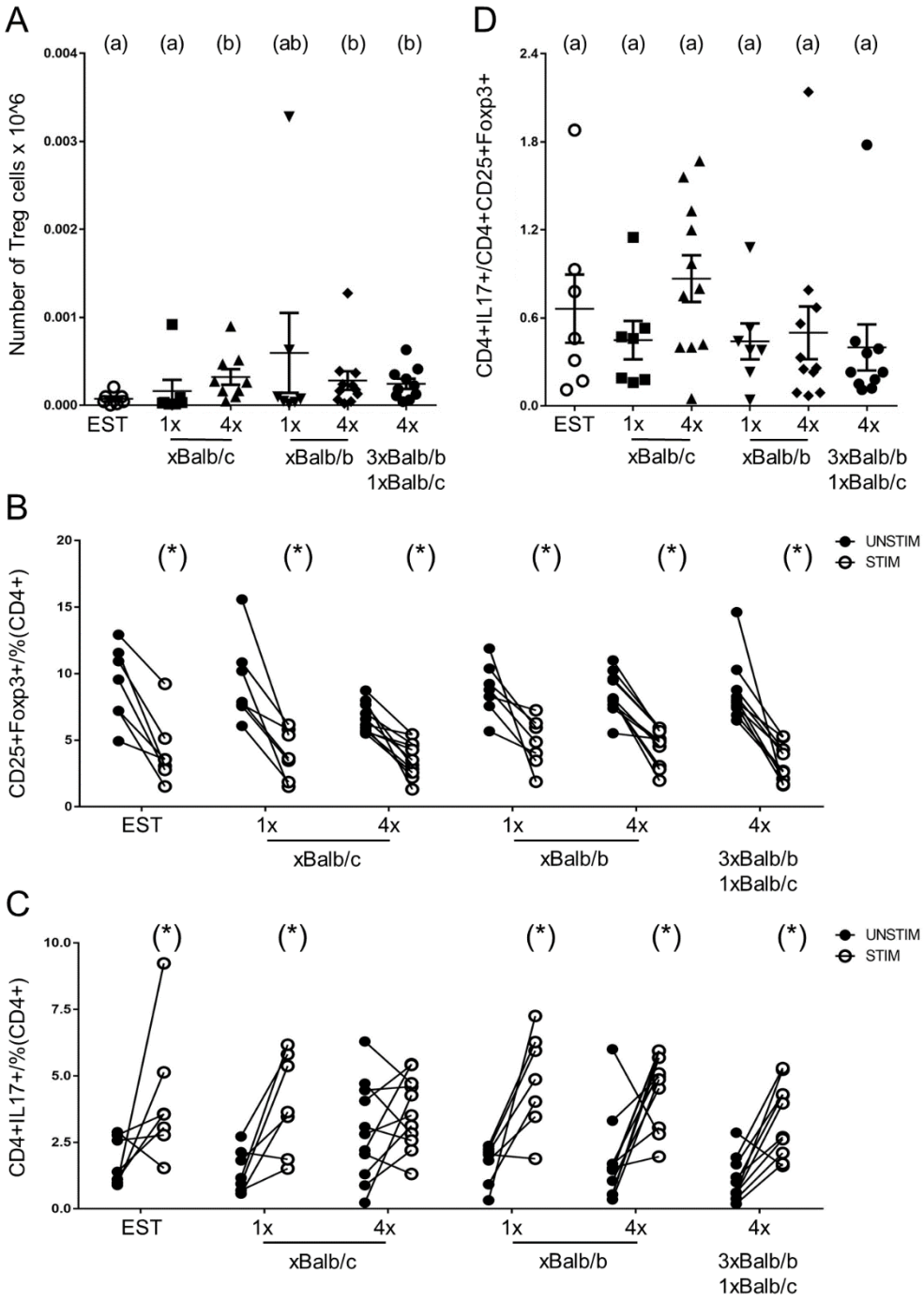


Figure 8.3 The effect of repeated exposure to male alloantigens on the number of Treg cells and stability of Foxp3 expression in CD4+ T cells in the peripheral blood. B6 females were left unmated, mated 1x with Balb/c, 4x with Balb/c, 1x with Balb/b, 4x with Balb/b or 3x with Balb/b and 1x with Balb/c, and on DPC 3.5, peripheral blood was analysed by flow cytometry for Treg cell parameters. Data presented as mean ± SEM and are the total cell number of CD4+CD25+ Foxp3+Treg cells in the peripheral blood (A), the expression of Foxp3 immediately after cell collection or 4h after strong polyclonal PMA/ionomycin stimulation *in vitro* (B), the expression of IL17A immediately after cell collection or 4h after strong polyclonal PMA/ionomycin stimulation *in vitro* (C), and the ratio of CD4+IL17+ to CD4+CD25+Foxp3+ cells from peripheral blood after stimulation (D).

Data are the mean ± SEM from unmated or mated B6 mice. The effect of PMA stimulation was evaluated using a paired T test in B and C, and the effect of mating was evaluated using Kruskall-Wallis and Mann-Whitney test in A and D.

8.2 Chapter5

8.2.1 T cell profile in distal lymph nodes and peripheral blood

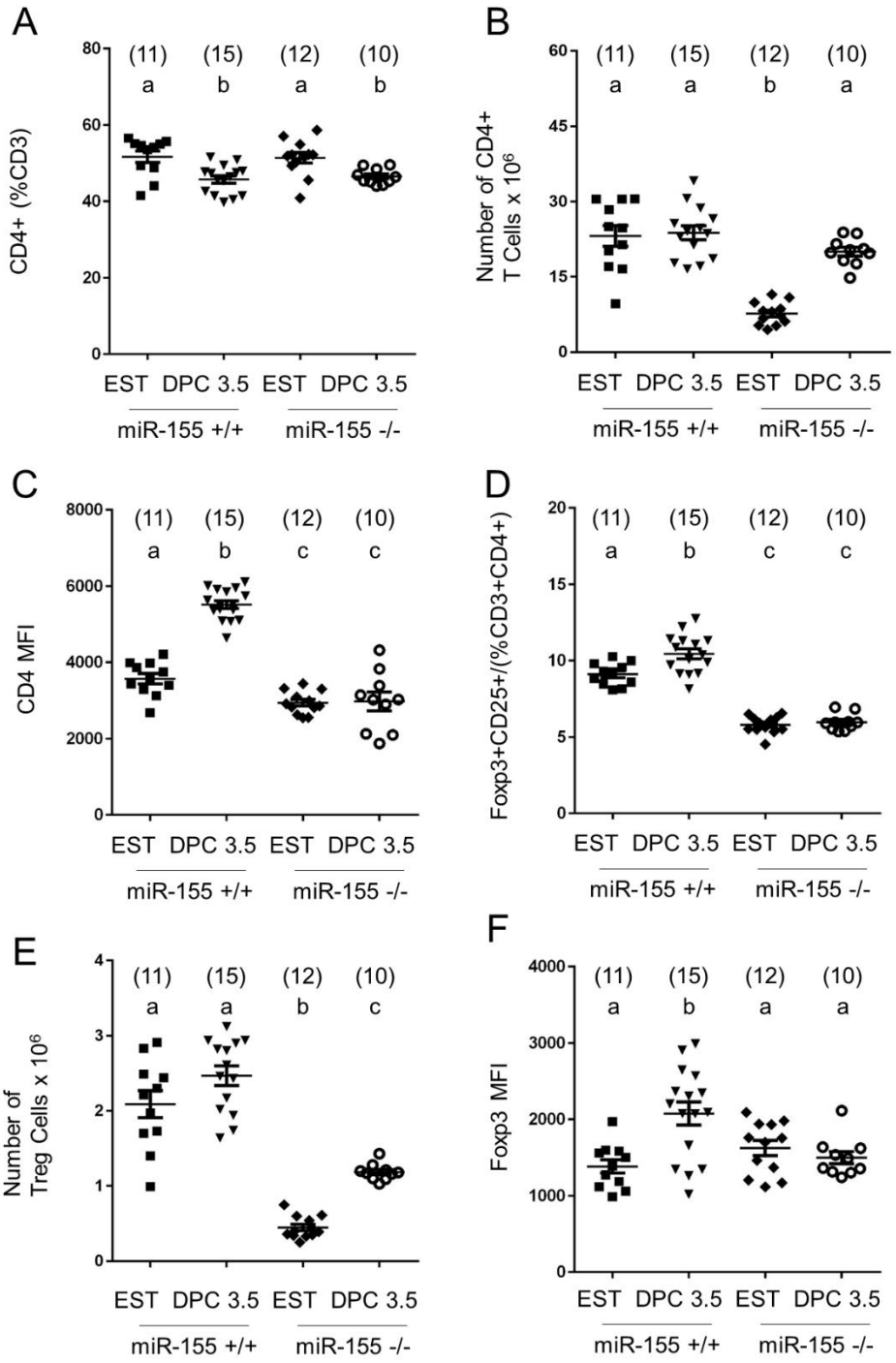


Figure.8.4 The effect of miR-155 deficiency on populations of T cells in the mLN. miR-155+/+ and miR-155-/- mice were mated with Balb/c males, and on DPC 3.5, CD3+, CD4+, CD25+ and Foxp3+ cells from the mLN were analysed by flow cytometry to quantify T cells. Virgin oestrus mice were used as non-mated controls. Data are presented as mean ± SEM and are the percentage of CD4+ T cells within the CD3+ population (A), the

total number of CD4+ T cells x10^6 (B), the CD4 MFI (C), the percentage of Treg cells (D), the total number of Treg cells x10^6 (E), and the MFI of Foxp3 (F). Differences between groups were evaluated using Kruskal-Wallis test and Mann-Whitney U test. (a,b,c,d indicates $p<0.05$).

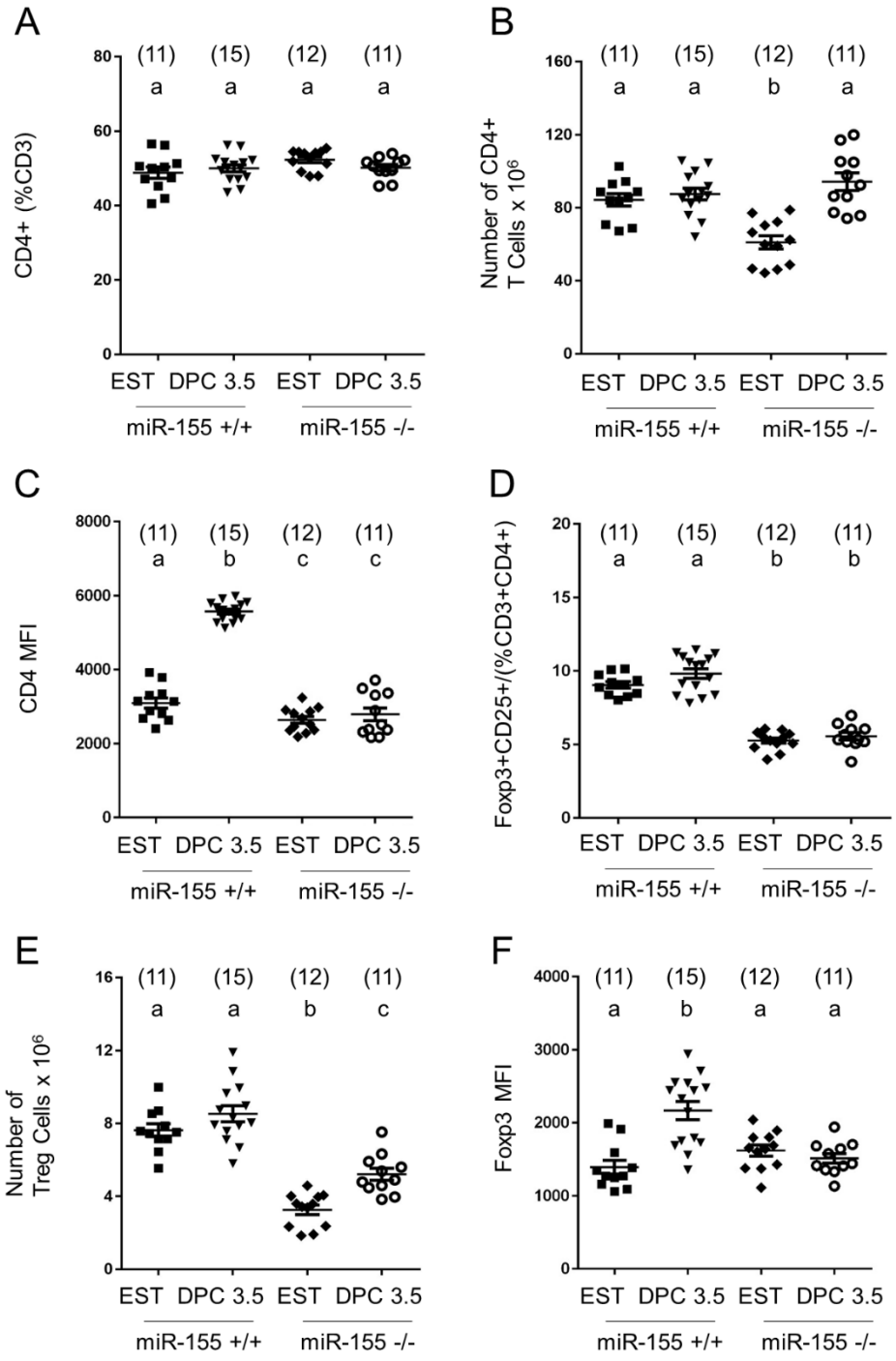


Figure 8.5 The effect of miR-155 deficiency on populations of T cells in the spleen. miR-155+/+ and miR-155-/- mice were mated with Balb/c males, and on DPC 3.5, CD3+, CD4+, CD25+ and Foxp3+ cells from the spleen were analysed by flow cytometry to quantify T cells. Virgin oestrus mice were used as non-mated controls. Data are presented as mean ± SEM and are the percentage of CD4+ T cells within the CD3+ population (A), the total number of CD4+ T cells x10^6 (B), the CD4 MFI (C), the percentage of Treg cells (D), the total number of Treg cells x10^6 (E), and the MFI of Foxp3 (F). Differences between groups were evaluated using Kruskal-Wallis test and Mann-Whitney U test. (a,b,c,d indicates $p<0.05$).

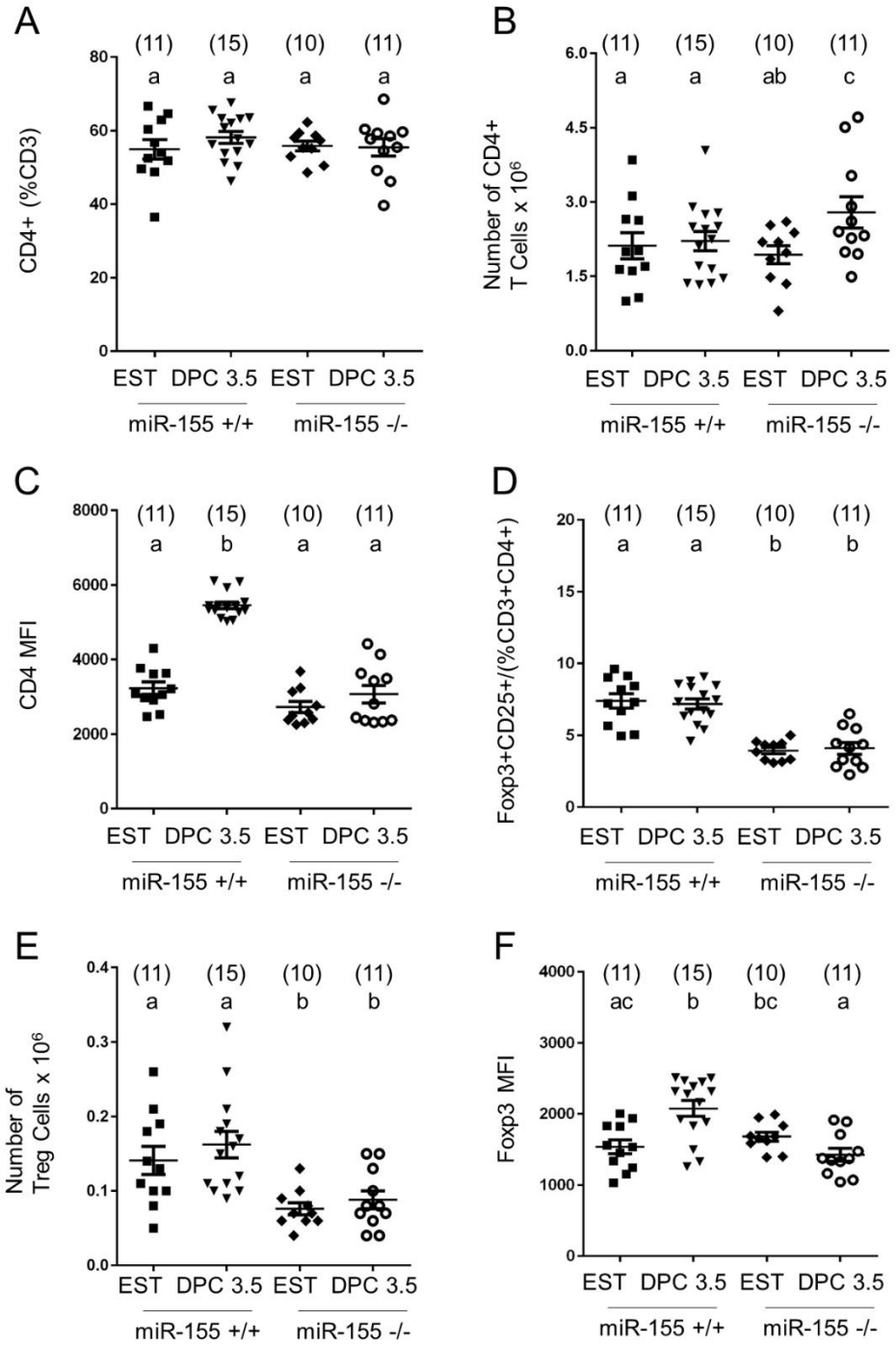
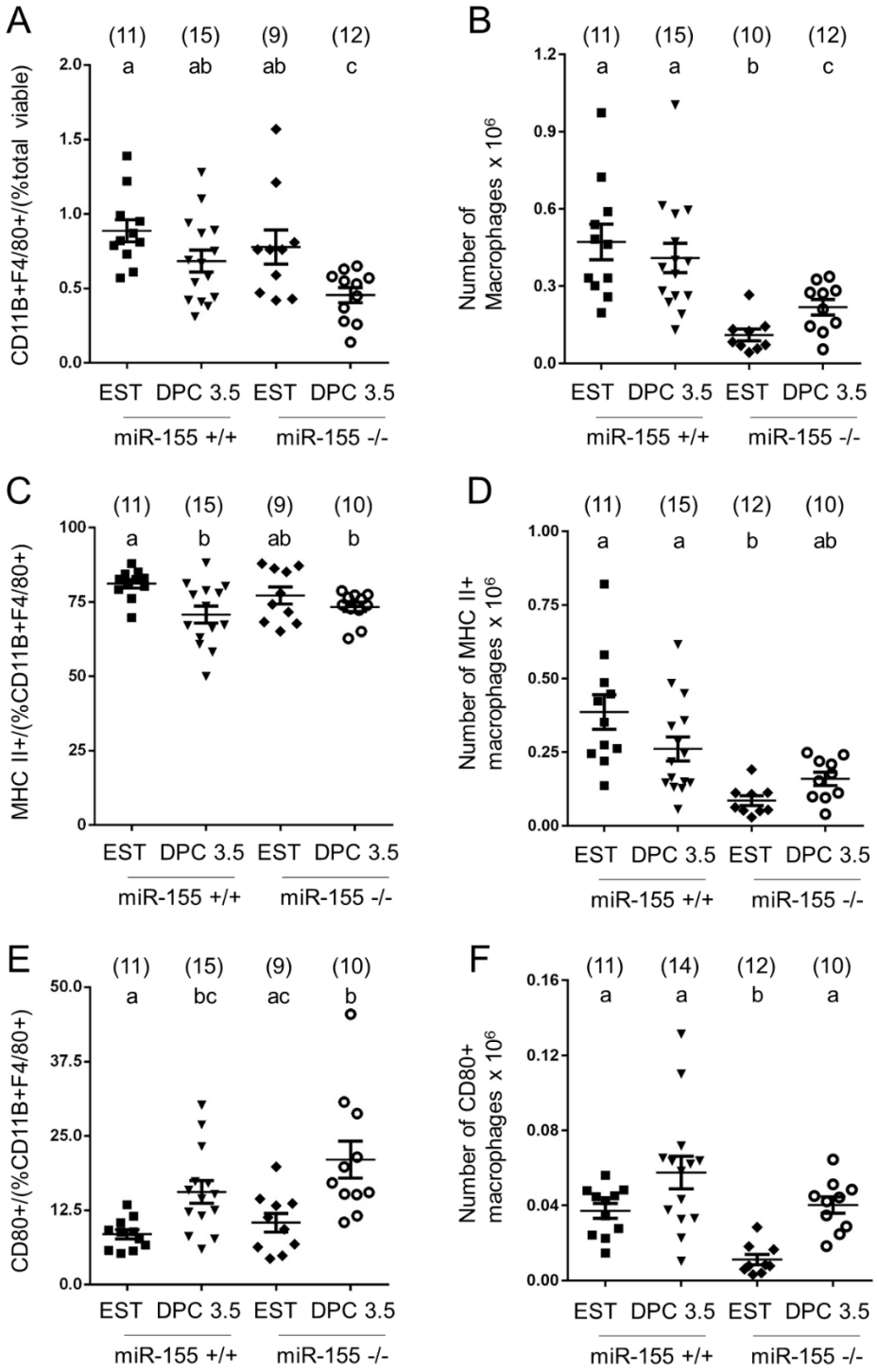


Figure 8.6 The effect of miR-155 deficiency on populations of T cells in the peripheral blood. miR-155+/+ and miR-155-/- mice were mated with Balb/c males, and on DPC 3.5, CD3+, CD4+, CD25+ and Foxp3+ cells from the peripheral blood were analysed by flow cytometry to quantify T cells. Virgin oestrus mice were used as non-mated controls. Data are presented as mean ± SEM and are the percentage of CD4+ T cells within the CD3+ population (A), the total number of CD4+ T cells x10^6 (B), the CD4 MFI (C), the percentage of Treg cells (D), the total number of Treg cells x10^6 (E), and the MFI of Foxp3 (F). Differences between groups were evaluated using Kruskal-Wallis test and Mann-Whitney U test. (a,b,c,d indicates $p<0.05$).

8.2.2 Macrophage profile in distal lymph nodes



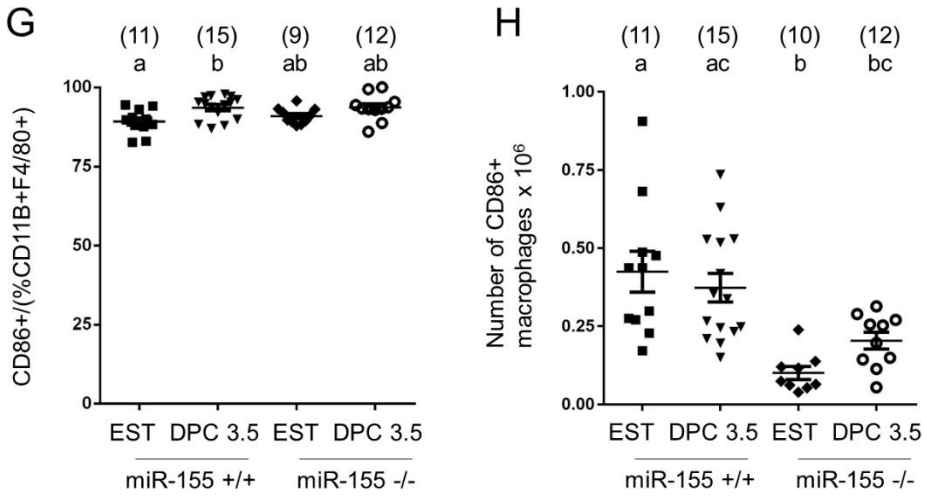
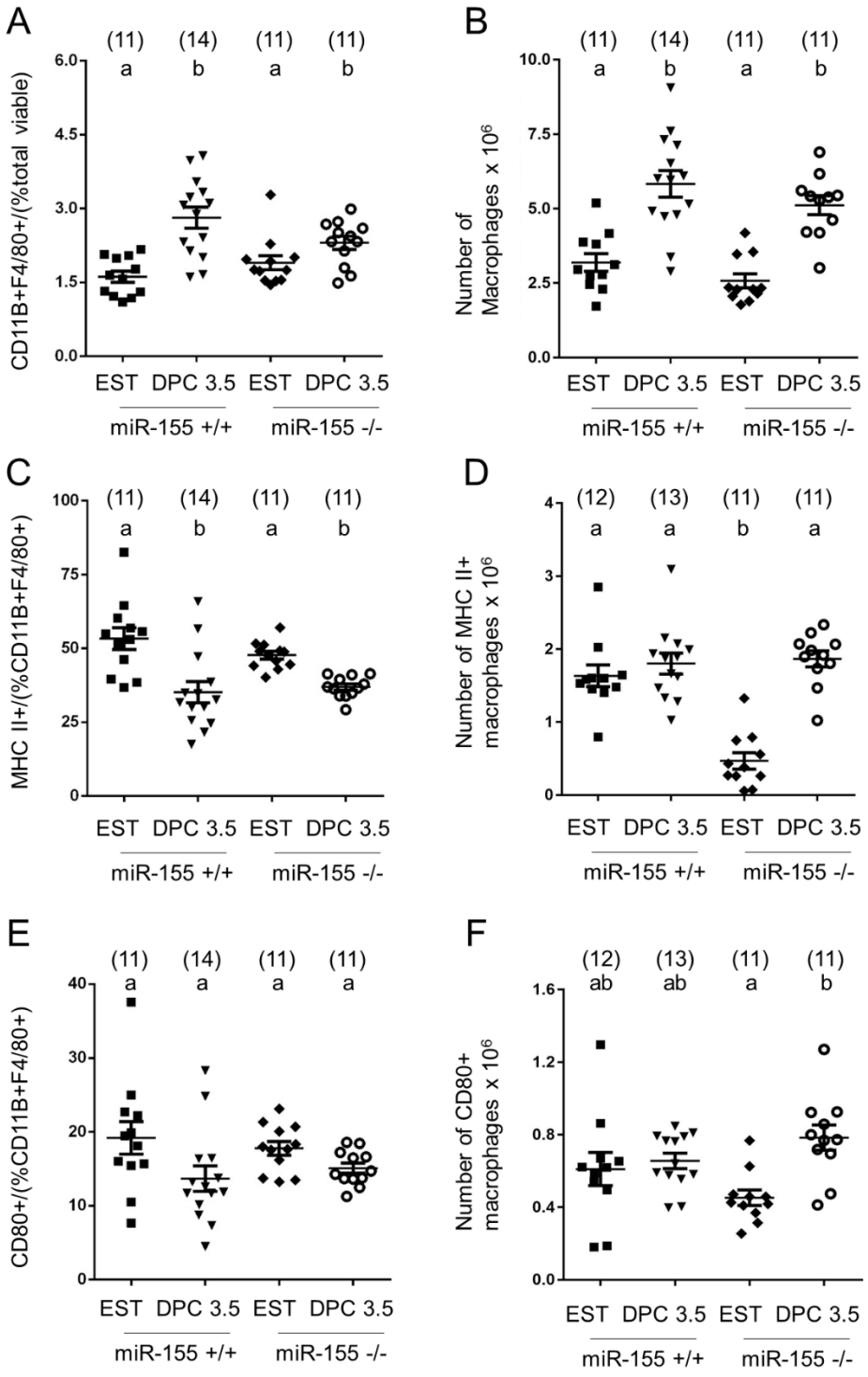


Figure 8.7 The effect of miR-155 deficiency on population of macrophages and the expression of MHCII, CD80, and CD86 by F4/80+ macrophages in the mLN. miR-155+/+ and miR-155-/- B6 mice were mated with Balb/c males, and on DPC 3.5, CD11B+ F4/80+ cells from the mLN were analysed by flow cytometry to quantify proportion of macrophages and the expression of MHCII, CD80, and CD86. Virgin oestrus mice were used as non-mated controls. Data are presented as mean ± SEM and are the percentage of F4/80+ macrophages within viable cells (A), the total number of macrophages x 10^6 (B), the percentage of activated macrophages (MHCII+ macrophages) (C), the total number of activated macrophages x 10^6 (D), the percentage of CD80+ macrophages (E), the total number of CD80+ macrophages x 10^6 (F), the percentage of CD86+ macrophages (G) and the total number of CD86+ macrophages x 10^6 (H). Differences between groups were evaluated using Kruskal-Wallis test and Mann-Whitney U test. (a,b,c,d indicates $p<0.05$).


A
(11) a
(14) b
(11) a
(11) b
CD11B+F4/80+/(%total viable)
6.0
4.5
3.0
1.5
0.0
EST DPC 3.5 EST DPC 3.5
miR-155 +/+
miR-155 -/-
B
(11) a
(14) b
(11) a
(11) b
Number of Macrophages x 10^6
10.0
7.5
5.0
2.5
0.0
EST DPC 3.5 EST DPC 3.5
miR-155 +/+
miR-155 -/-
C
(11) a
(14) b
(11) a
(11) b
MHC II+/(%CD11B+F4/80+)
100
75
50
25
0
EST DPC 3.5 EST DPC 3.5
miR-155 +/+
miR-155 -/-
D
(12) a
(13) a
(11) b
(11) a
Number of MHC II+ macrophages x 10^6
4
3
2
1
0
EST DPC 3.5 EST DPC 3.5
miR-155 +/+
miR-155 -/-
E
(11) a
(14) a
(11) a
(11) a
CD80+/(%CD11B+F4/80+)
40
30
20
10
0
EST DPC 3.5 EST DPC 3.5
miR-155 +/+
miR-155 -/-
F
(12) ab
(13) ab
(11) a
(11) b
Number of CD80+ macrophages x 10^6
1.6
1.2
0.8
0.4
0.0
EST DPC 3.5 EST DPC 3.5
miR-155 +/+
miR-155 -/-

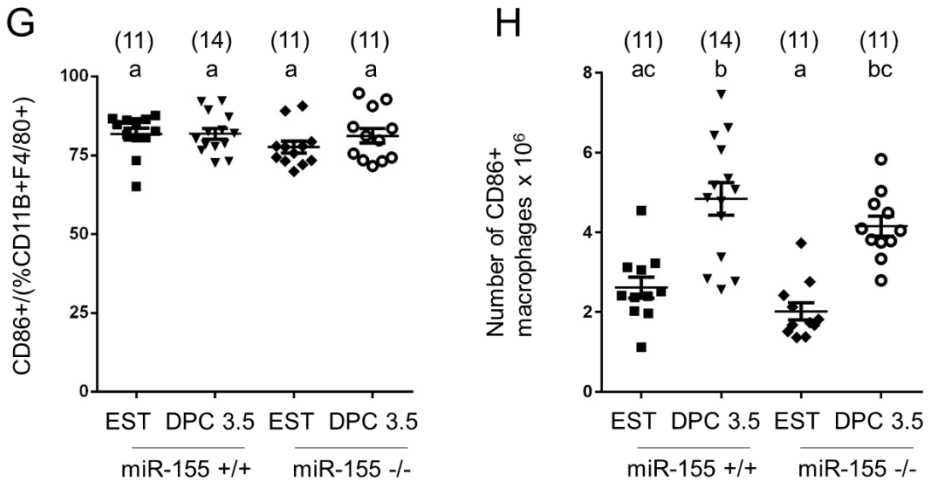
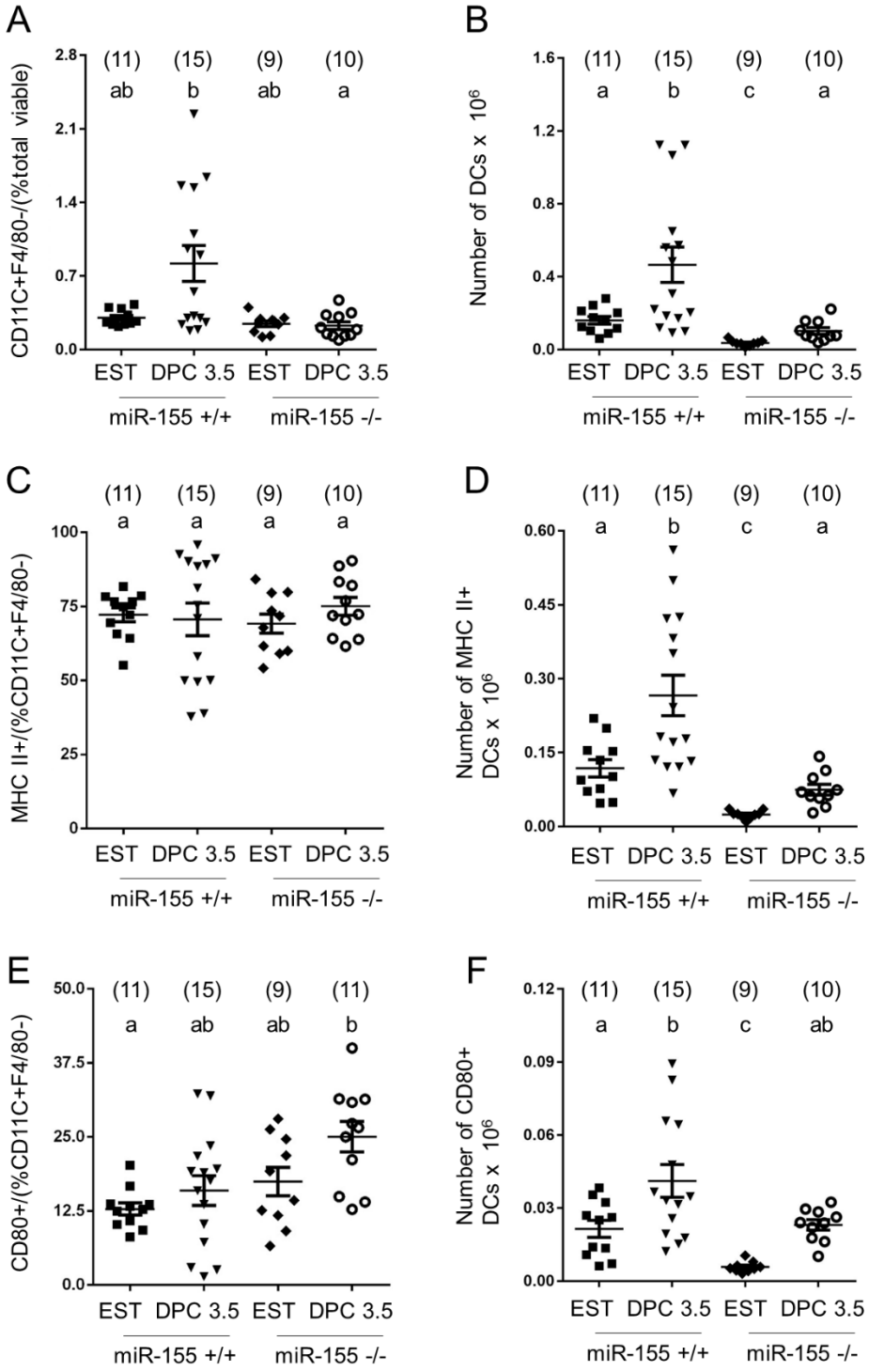


Figure 8.8 The effect of miR-155 deficiency on population of macrophages and the expression of MHCII, CD80, and CD86 by F4/80+ macrophages in the spleen. miR-155+/+ and miR-155-/- B6 mice were mated with Balb/c males, and on DPC 3.5, CD11B+ F4/80+ cells from the spleen were analysed by flow cytometry to quantify proportion of macrophages and the expression of MHCII, CD80, and CD86. Virgin oestrus mice were used as non-mated controls. Data are presented as mean ± SEM and are the percentage of F4/80+ macrophages within viable cells (A), the total number of macrophages x 10^6 (B), the percentage of activated macrophages (MHCII+ macrophages) (C), the total number of activated macrophages x 10^6 (D), the percentage of CD80+ macrophages (E), the total number of CD80+ macrophages x 10^6 (F), the percentage of CD86+ macrophages (G) and the total number of CD86+ macrophages x 10^6 (H). Differences between groups were evaluated using Kruskal-Wallis test and Mann-Whitney U test. (a,b,c,d indicates $p<0.05$).

8.2.3 Dendritic cell profile in distal lymph nodes



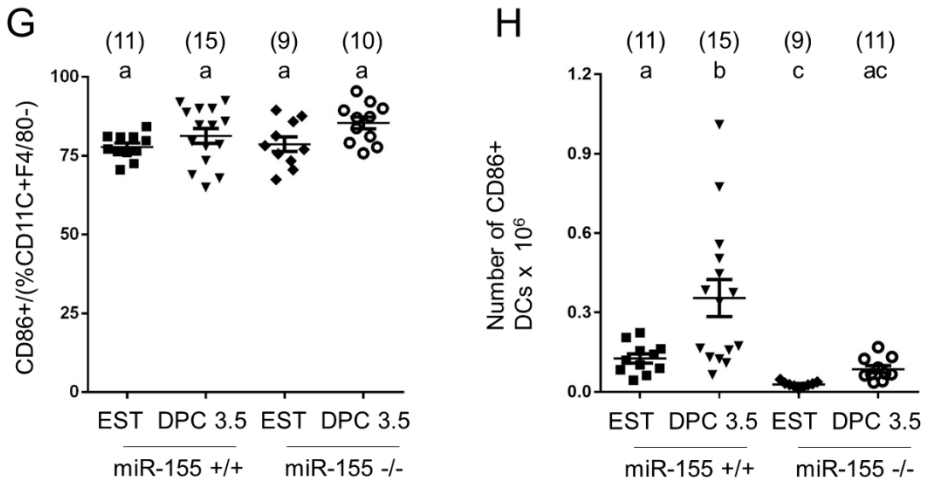
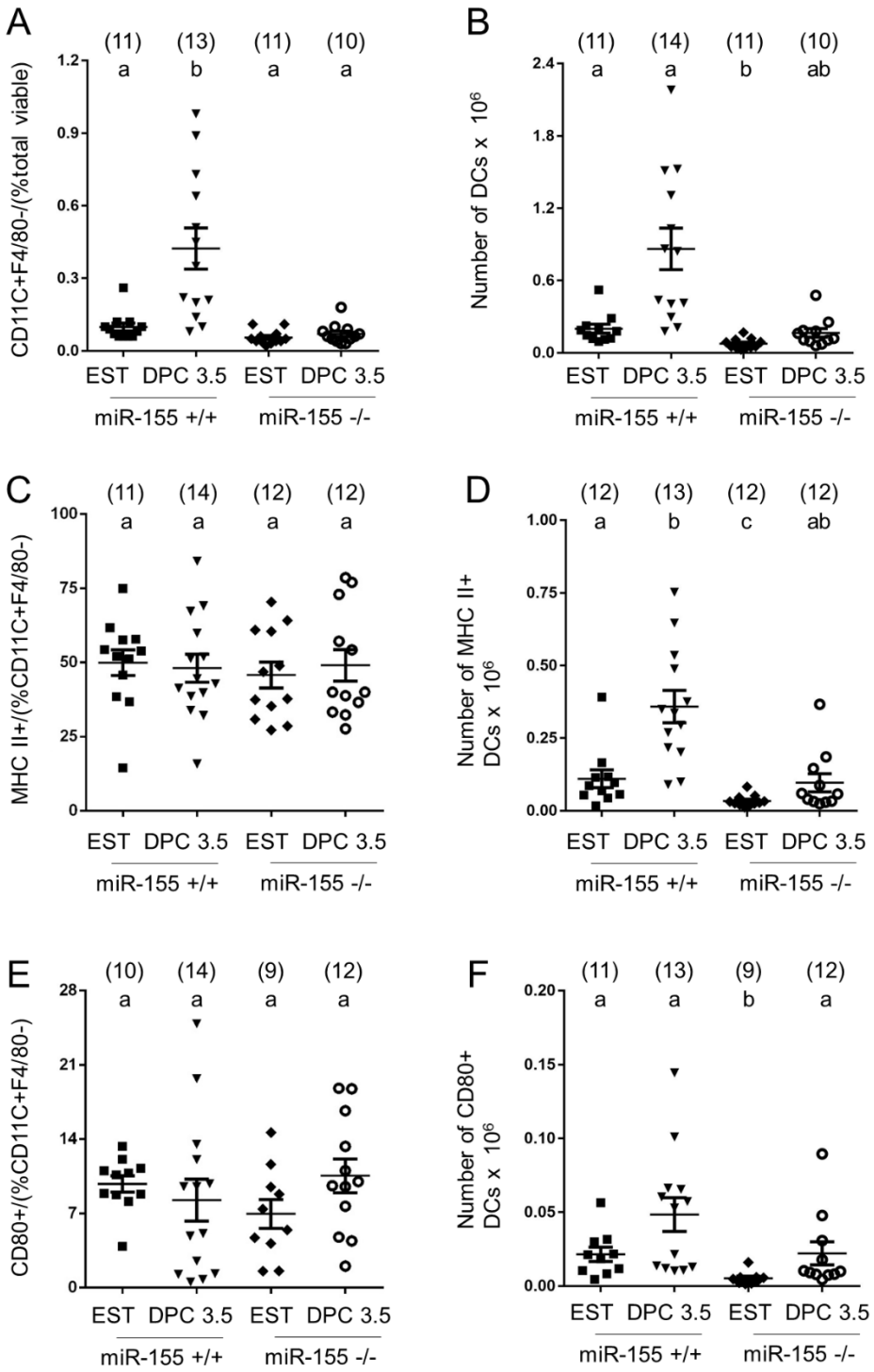


Figure 8.9 The effect of miR-155 deficiency on the population of DCs and expression of MHCII, CD80, and CD86 by CD11C+ DCs in the mLN. miR-155+/+ and miR-155-/- B6 mice were mated with Balb/c males, and on DPC 3.5, CD11C+ F4/80- cells from the mLN were analysed by flow cytometry to quantify proportion of DCs and the expression of MHCII, CD80, and CD86. Virgin oestrus mice were used as non-mated controls. Data are presented as mean ± SEM and are the percentage of CD11C+ DCs within viable cells (A), the total number of DCs x 10^6 (B), the percentage of activated DCs (MHCII+ DCs) (C), the total number of MHCII+ DCs x 10^6 (D), the percentage of CD80+ DCs (E), the total number of CD80+ DCs x 10^6 (F), the percentage of CD86+ DCs (G) and the total number of CD86+ DCs x 10^6 (H). Differences between groups were evaluated using Kruskal-Wallis test and Mann-Whitney U test. (a,b,c,d indicates $p<0.05$).


A
(11) a
(13) b
(11) a
(10) a
CD11C+F4/80-/(%total viable)
1.2
0.9
0.6
0.3
0.0
EST DPC 3.5 EST DPC 3.5
miR-155 +/+
miR-155 -/-
B
(11) a
(14) a
(11) b
(10) ab
Number of DCs x 10^6
2.4
1.8
1.2
0.6
0.0
EST DPC 3.5 EST DPC 3.5
miR-155 +/+
miR-155 -/-
C
(11) a
(14) a
(12) a
(12) a
MHC II+/(%CD11C+F4/80-)
100
75
50
25
0
EST DPC 3.5 EST DPC 3.5
miR-155 +/+
miR-155 -/-
D
(12) a
(13) b
(12) c
(12) ab
Number of MHC II+ DCs x 10^6
1.00
0.75
0.50
0.25
0.00
EST DPC 3.5 EST DPC 3.5
miR-155 +/+
miR-155 -/-
E
(10) a
(14) a
(9) a
(12) a
CD80+/(%CD11C+F4/80-)
28
21
14
7
0
EST DPC 3.5 EST DPC 3.5
miR-155 +/+
miR-155 -/-
F
(11) a
(13) a
(9) b
(12) a
Number of CD80+ DCs x 10^6
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0.15
0.10
0.05
0.00
EST DPC 3.5 EST DPC 3.5
miR-155 +/+
miR-155 -/-

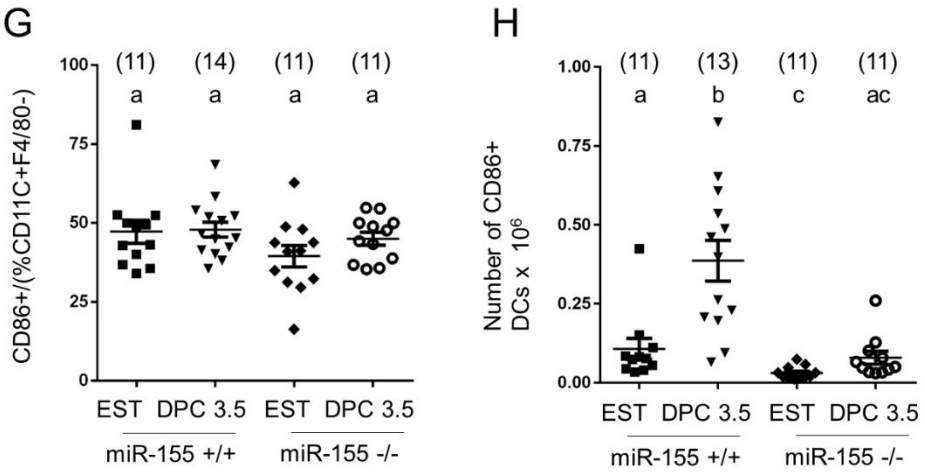


Figure 8.10 The effect of miR-155 deficiency on the population of DCs and expression of MHCII, CD80, and CD86 by CD11C+ DCs in the spleen. miR-155+/+ and miR-155-/- B6 mice were mated with Balb/c males, and on DPC 3.5, CD11C+ F4/80- cells from the spleen were analysed by flow cytometry to quantify proportion of DCs and the expression of MHCII, CD80, and CD86. Virgin oestrus mice were used as non-mated controls. Data are presented as mean ± SEM and are the percentage of CD11C+ DCs within viable cells (A), the total number of DCs x 10^6 (B), the percentage of activated DCs (MHCII+ DCs) (C), the total number of MHCII+ DCs x 10^6 (D), the percentage of CD80+ DCs (E), the total number of CD80+ DCs x 10^6 (F), the percentage of CD86+ DCs (G) and the total number of CD86+ DCs x 10^6 (H). Differences between groups were evaluated using Kruskal-Wallis test and Mann-Whitney U test. (a,b,c,d indicates $p<0.05$).

8.3 Chapter 6

8.3.1 T cell profile in distal tissues and peripheral blood

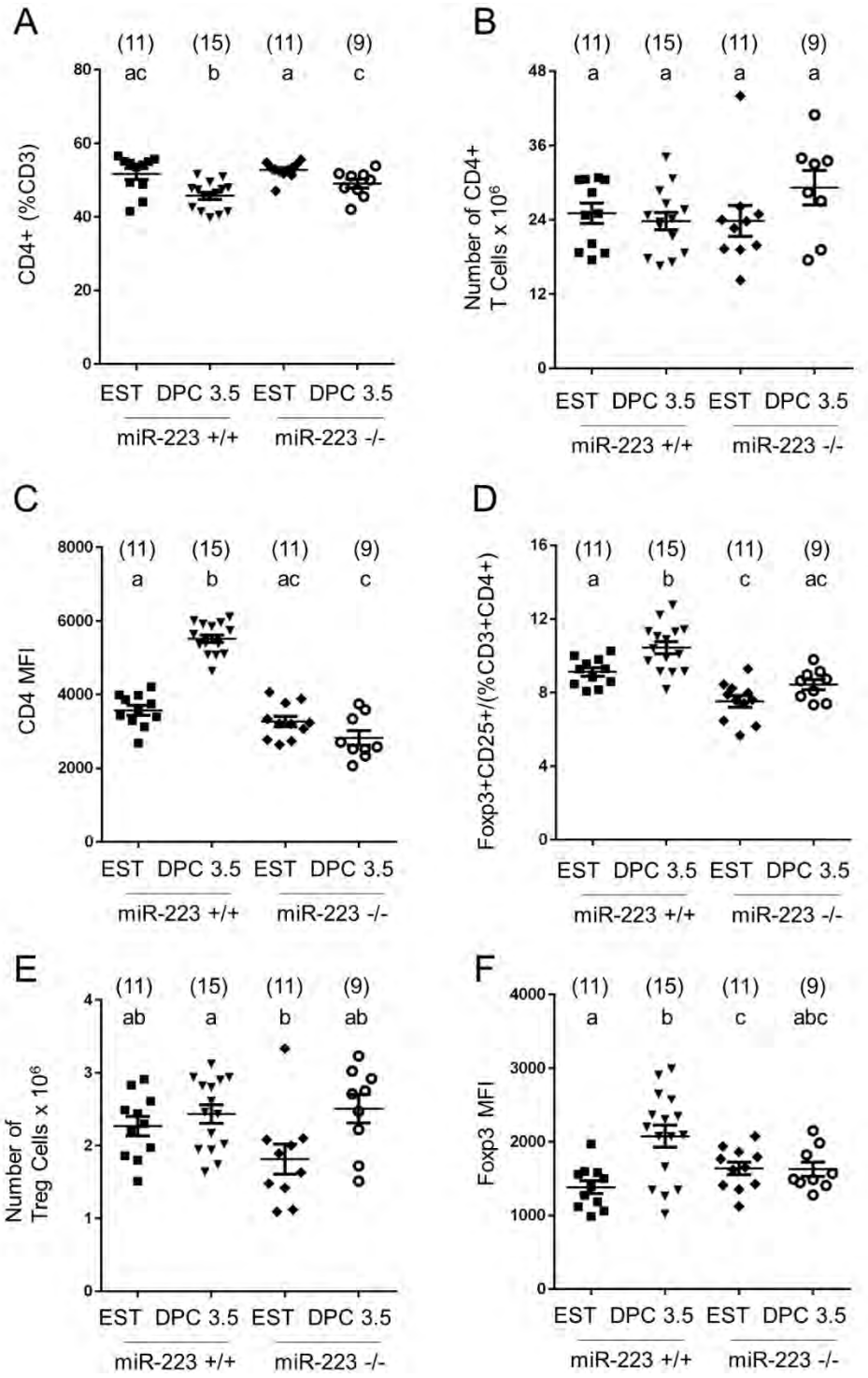


Figure 8.11 The effect of miR-223 deficiency on T cell populations in the mLN. miR-223+/+ and miR-223-/- mice were mated with Balb/c males, and on DPC 3.5, CD3+, CD4+, CD25+ and Foxp3+ cells from the mLN were analysed by flow cytometry to quantify T cells. Virgin oestrus mice were used as non-mated controls. Data are presented as mean ± SEM and are the percentage of CD4+ T cells within the CD3+ population (A), the total number of CD4+ T cells $x10^6$ (B), the CD4 MFI (C), the percentage of CD3+ CD4+ CD25+ Foxp3+Treg cells (D), the otal number of CD3+ CD4+ CD25+ Foxp3+ Treg cells $x10^6$ (E), and the MFI of Foxp3 (F). Differences between groups were evaluated using Kruskal-Wallis test and Mann-Whitney U test. (a,b,c,d indicates $p<0.05$).

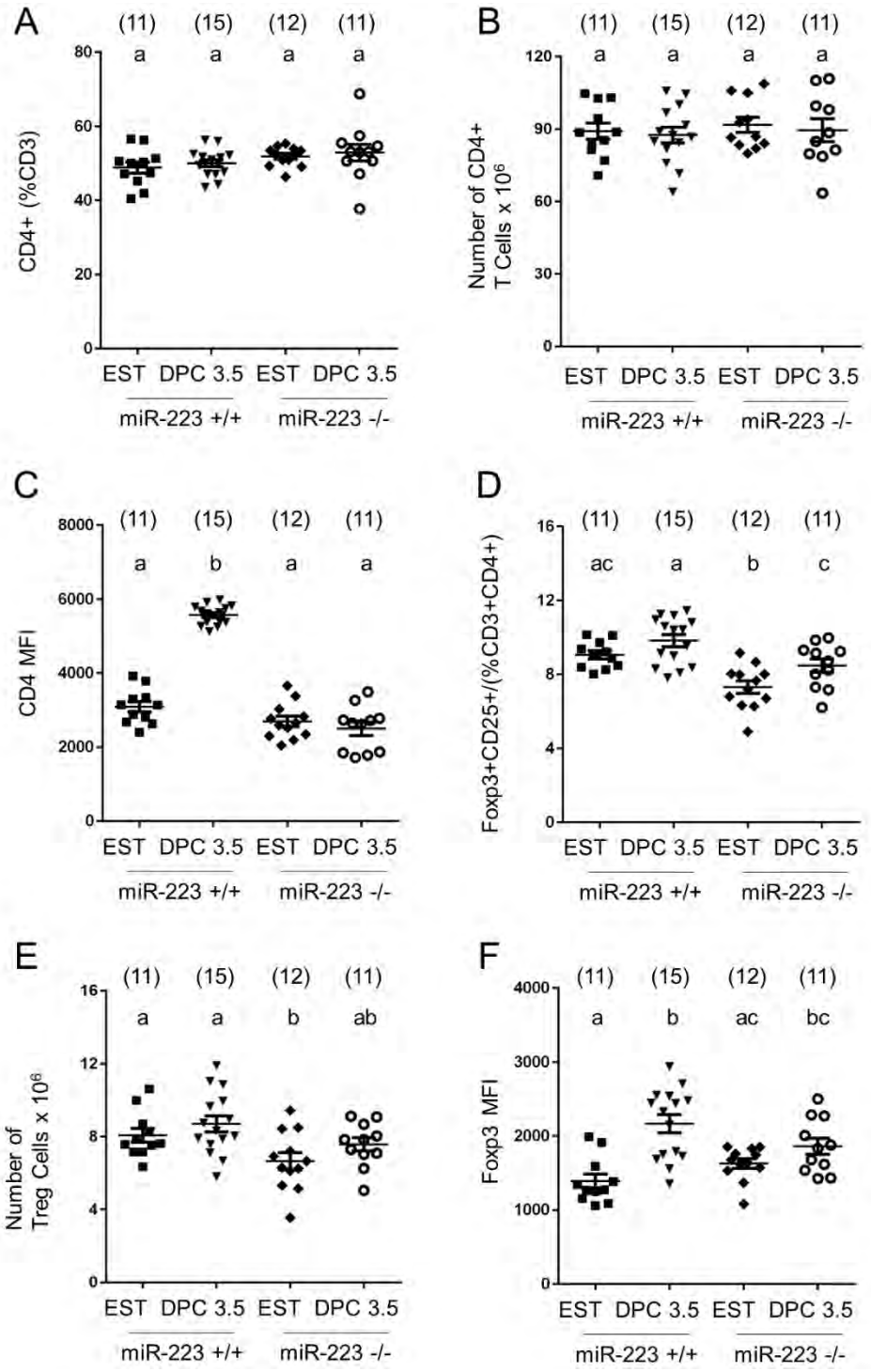


Figure 8.12 The effect of miR-223 deficiency on T cell populations in the spleen. miR-223+/+ and miR-223-/- mice were mated with Balb/c males, and on DPC 3.5, CD3+, CD4+, CD25+ and Foxp3+ cells from the spleen were analysed by flow cytometry to quantify T cells. Virgin oestrus mice were used as non-mated controls. Data are presented as mean ± SEM and are the percentage of CD4+ T cells within the CD3+ population (A), the total number of CD4+ T cells x10^6 (B), the CD4 MFI (C), the percentage of CD3+ CD4+ CD25+ Foxp3+Treg cells (D), the total number of CD3+ CD4+ CD25+ Foxp3+ Treg cells x10^6 (E), and the MFI of Foxp3 (F). Differences between groups were evaluated using Kruskal-Wallis test and Mann-Whitney U test. (a,b,c,d indicates $p<0.05$).

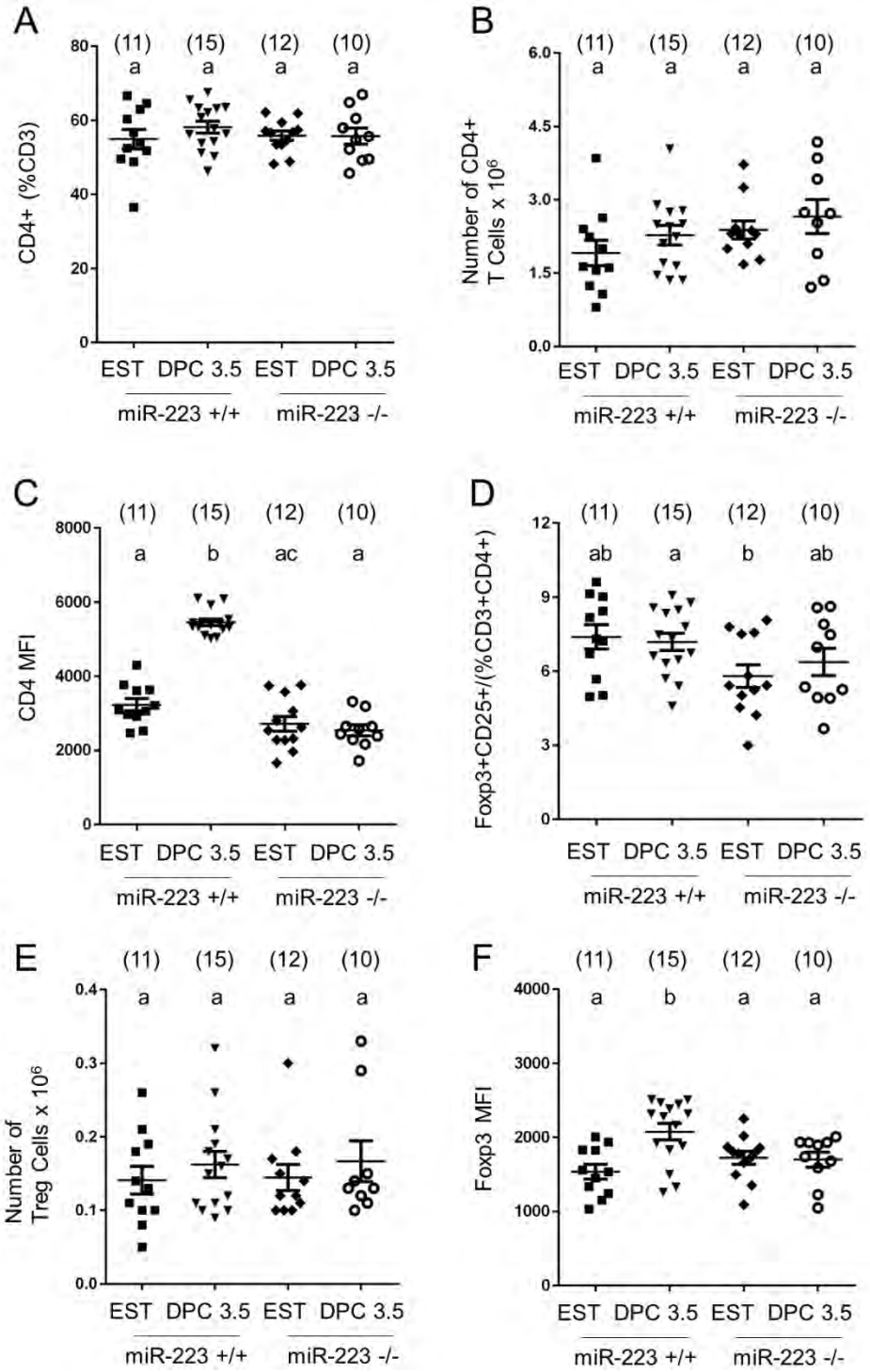
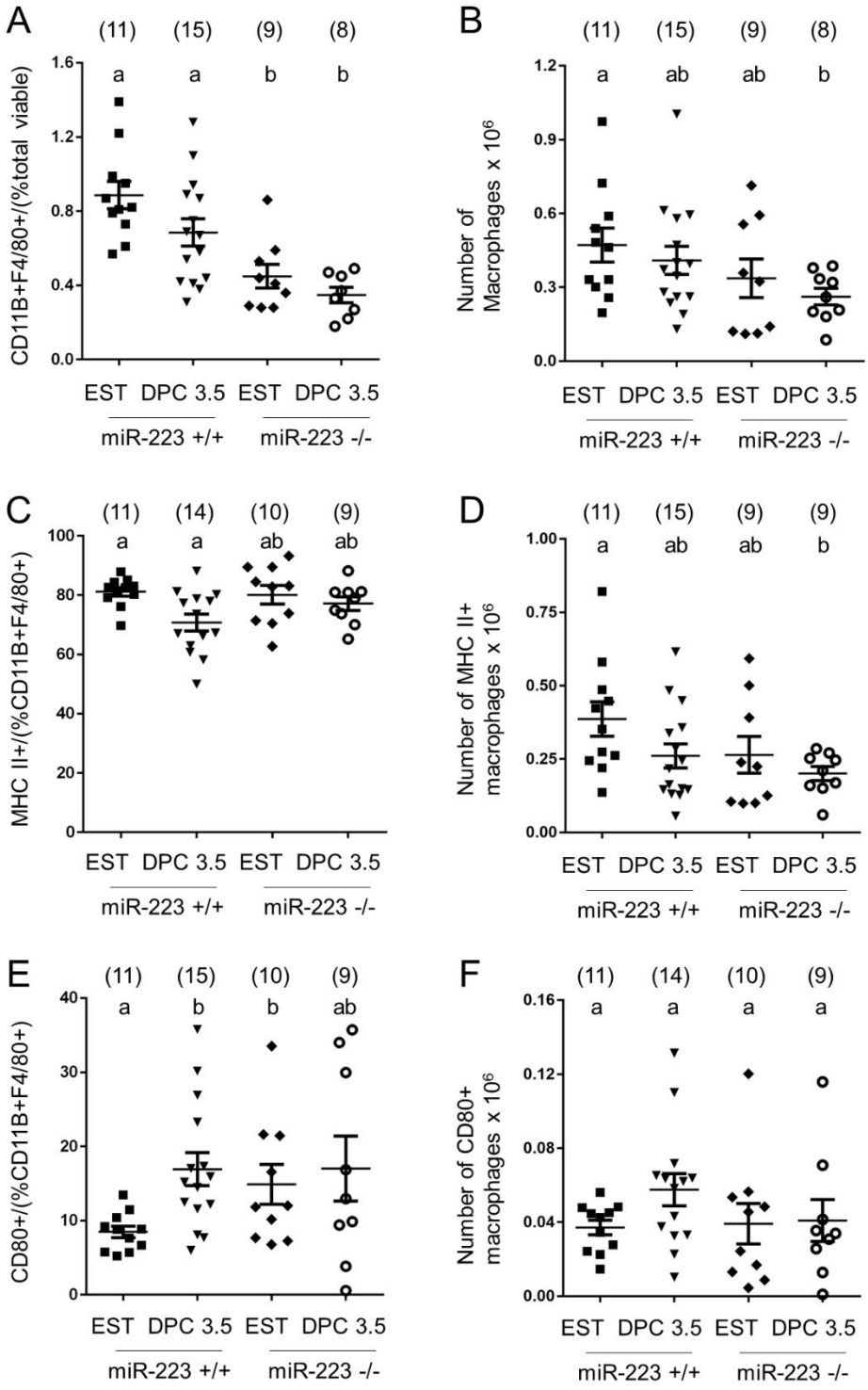


Figure 8.13 The effect of miR-223 deficiency on T cell populations in the peripheral blood. miR-223+/+ and miR-223-/- mice were mated with Balb/c males, and on DPC 3.5, CD3+, CD4+, CD25+ and Foxp3+ cells from the peripheral blood were analysed by flow cytometry to quantify T cells. Virgin oestrus mice were used as non-mated controls. Data are presented as mean ± SEM and are the percentage of CD4+ T cells within the CD3+ population (A), the total number of CD4+ T cells x10^6 (B), the CD4 MFI (C), the percentage of CD3+ CD4+ CD25+ Foxp3+Treg cells (D), the total number of CD3+ CD4+ CD25+ Foxp3+ Treg cells x10^6 (E), and the MFI of

Foxp3 (F). Differences between groups were evaluated using Kruskal-Wallis test and Mann-Whitney U test. (a,b,c,d indicates $p<0.05$).

8.3.2 Macrophage profile in the distal lymph nodes



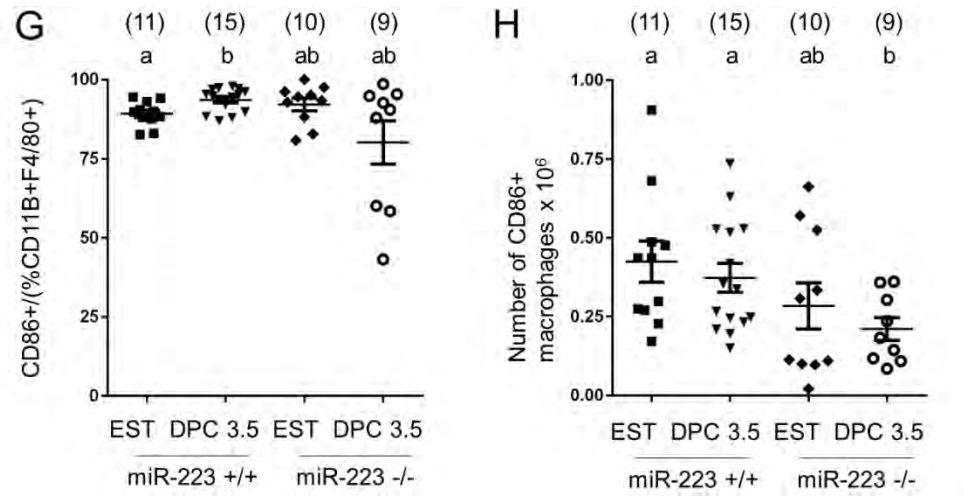
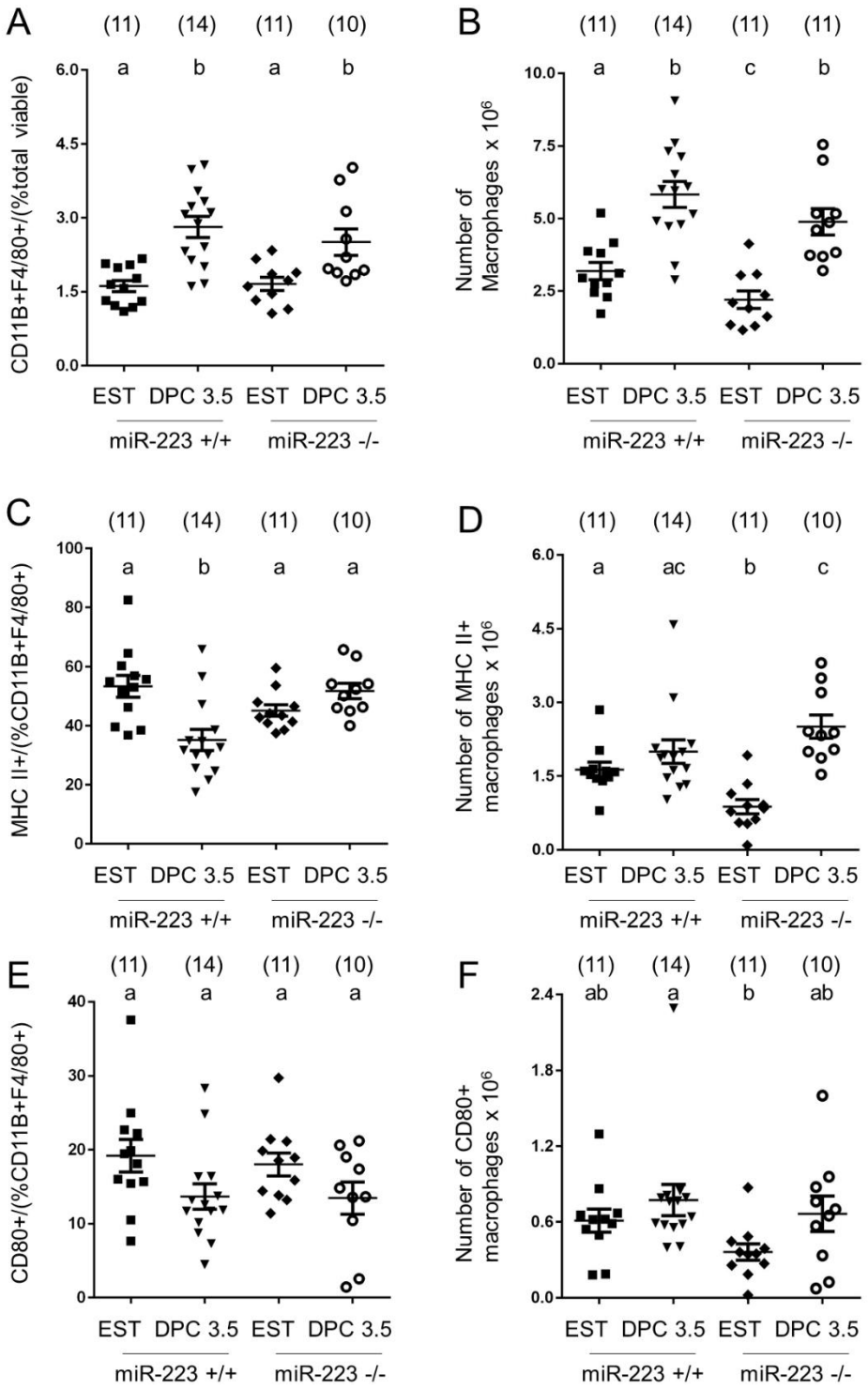


Figure 8.14 The effect of miR-223 deficiency on the macrophage population and the expression of MHCII, CD80, and CD86 by F4/80+ macrophages in the mLN. miR-223+/+ and miR-223-/- B6 female mice were mated with Balb/c males, and on DPC 3.5, CD11B+ F4/80+ cells from the mLN were analysed by flow cytometry to quantify the proportion of macrophages and the expression of MHCII, CD80, and CD86. Virgin oestrus mice were used as non-mated controls. Data are presented as mean ± SEM and are the percentage of F4/80+ macrophages within viable cells (A), the total number of macrophages x 10^6 (B), the percentage of activated macrophages (MHCII+ macrophages) (C), the total number of activated macrophages x 10^6 (D), the percentage of CD80+ macrophages (E), the total number of CD80+ macrophages x 10^6 (F), the percentage of CD86+ macrophages (G) and the total number of CD86+ macrophages x 10^6 (H). Differences between groups were evaluated using Kruskal-Wallis test and Mann-Whitney U test. (a,b,c,d indicates $p<0.05$)


A
(11) (14) (11) (10)
a b a b
CD11B+F4/80+/(%total viable)
6.0
4.5
3.0
1.5
0.0
EST DPC 3.5 EST DPC 3.5
miR-223 +/+ miR-223 -/-
B
(11) (14) (11) (11)
a b c b
Number of Macrophages x 10^6
10.0
7.5
5.0
2.5
0.0
EST DPC 3.5 EST DPC 3.5
miR-223 +/+ miR-223 -/-
C
(11) (14) (11) (10)
a b a a
MHC II+/(%CD11B+F4/80+)
100
80
60
40
20
0
EST DPC 3.5 EST DPC 3.5
miR-223 +/+ miR-223 -/-
D
(11) (14) (11) (10)
a ac b c
Number of MHC II+ macrophages x 10^6
6.0
4.5
3.0
1.5
0.0
EST DPC 3.5 EST DPC 3.5
miR-223 +/+ miR-223 -/-
E
(11) (14) (11) (10)
a a a a
CD80+/(%CD11B+F4/80+)
40
30
20
10
0
EST DPC 3.5 EST DPC 3.5
miR-223 +/+ miR-223 -/-
F
(11) (14) (11) (10)
ab a b ab
Number of CD80+ macrophages x 10^6
2.4
1.8
1.2
0.6
0.0
EST DPC 3.5 EST DPC 3.5
miR-223 +/+ miR-223 -/-

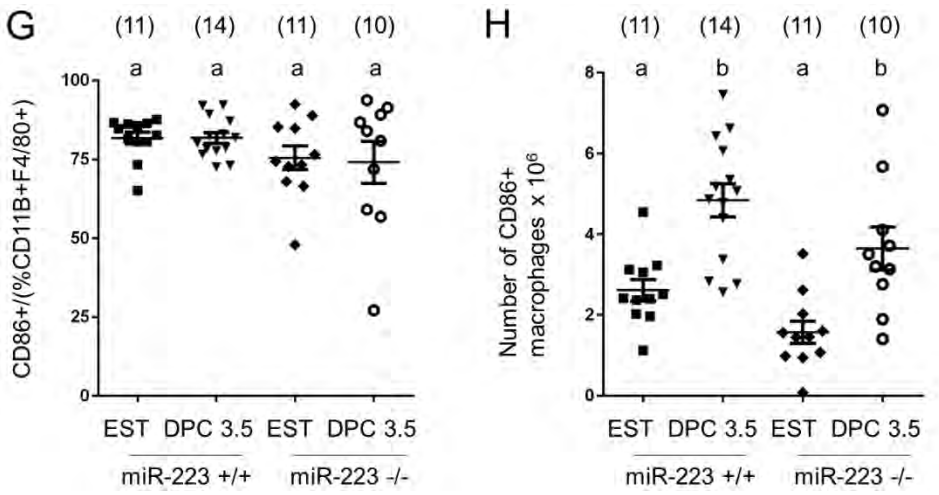
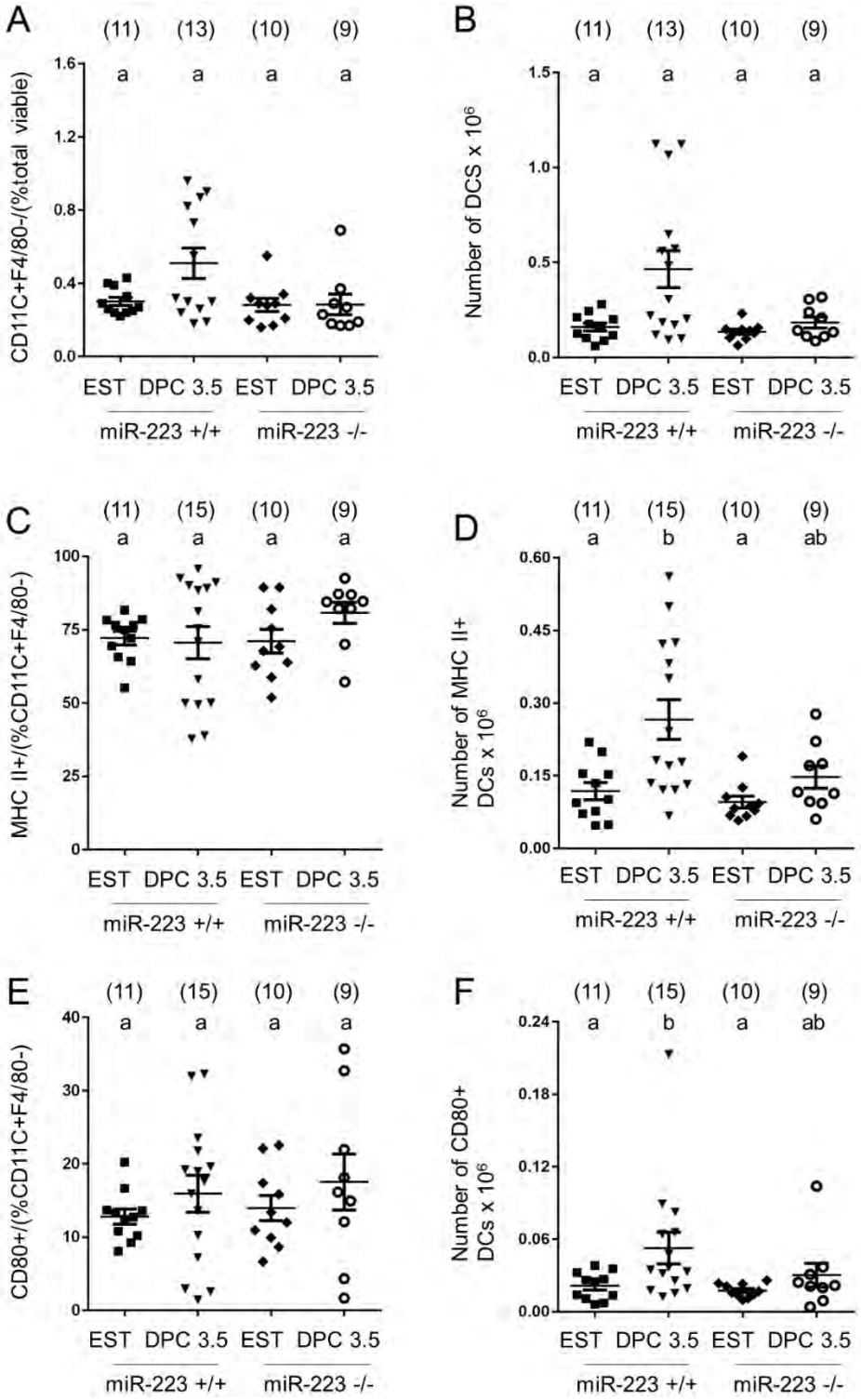


Figure 8.15 The effect of miR-223 deficiency on the macrophage population and the expression of MHCII, CD80, and CD86 by F4/80+ macrophages in the spleen. miR-223+/+ and miR-223-/- B6 female mice were mated with Balb/c males, and on DPC 3.5, CD11B+ F4/80+ cells from the spleen were analysed by flow cytometry to quantify the proportion of macrophages and the expression of MHCII, CD80, and CD86. Virgin oestrus mice were used as non-mated controls. Data are presented as mean ± SEM and are the percentage of F4/80+ macrophages within viable cells (A), the total number of macrophages x 10^6 (B), the percentage of activated macrophages (MHCII+ macrophages) (C), the total number of activated macrophages x 10^6 (D), the percentage of CD80+ macrophages (E), the total number of CD80+ macrophages x 10^6 (F), the percentage of CD86+ macrophages (G) and the total number of CD86+ macrophages x 10^6 (H). Differences between groups were evaluated using Kruskal-Wallis test and Mann-Whitney U test. (a,b,c,d indicates $p<0.05$)

8.3.3 Dendritic cell profile in distal lymph nodes



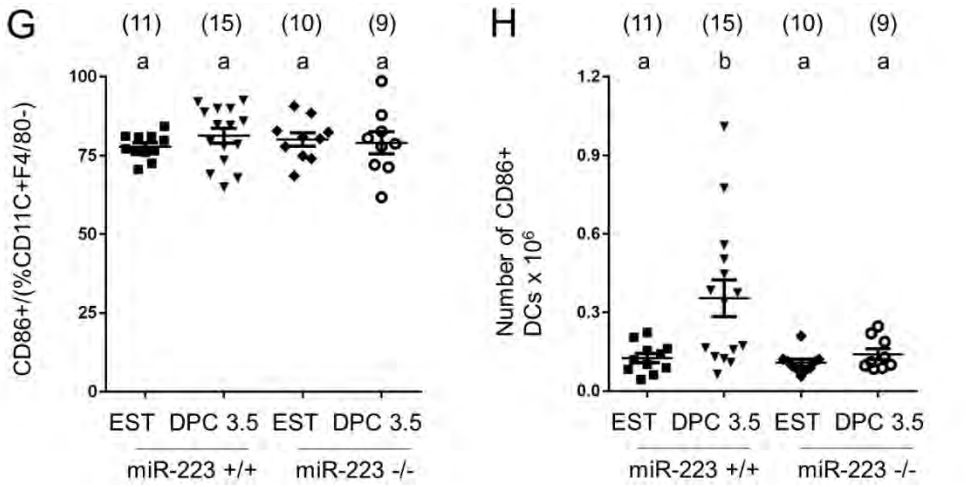
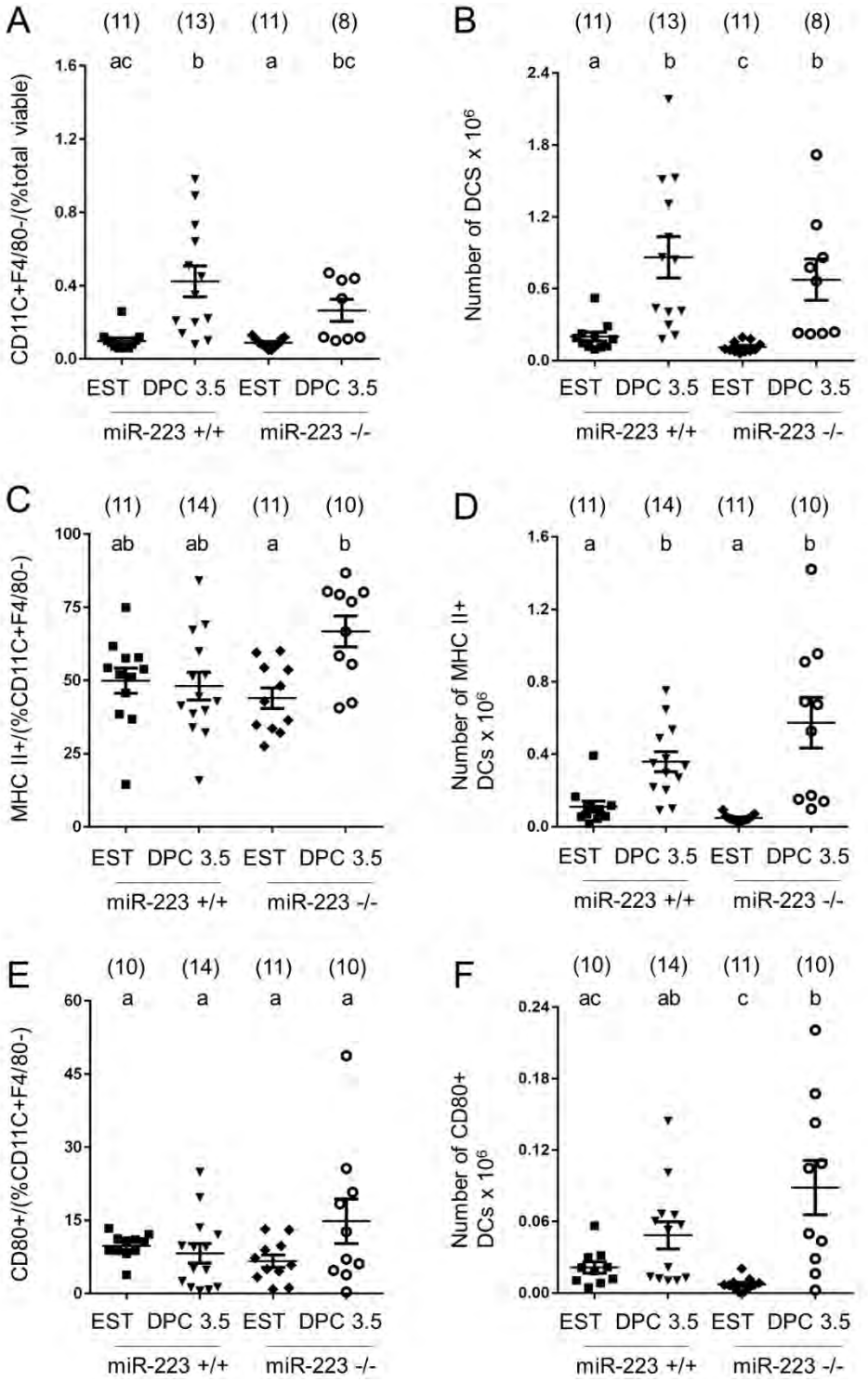


Figure 8.16 The effect of miR-223 deficiency on the DC population and expression of MHCII, CD80, and CD86 by CD11C+ DCs in the mLN. miR-223+/+ and miR-223-/- B6 female mice were mated with Balb/c males, and on DPC 3.5, CD11C+ F4/80- cells from the mLN were analysed by flow cytometry to quantify the proportion of DC and the expression of MHCII, CD80, and CD86. Virgin oestrus mice were used as non-mated controls. Data are presented as mean ± SEM and are the percentage of CD11C+ DCs within viable cells (A), the total number of DCs x 10^6 (B), the percentage of activated DCs (MHCII+ DCs) (C), the total number of activated DCs x 10^6 (D), the percentage of CD80+ DCs (E), the total number of CD80+ DCs x 10^6 (F), the percentage of CD86+ DCs (G) and the total number of CD86+ DCs x 10^6 (H). Differences between groups were evaluated using Kruskal-Wallis test and Mann-Whitney U test. (a,b,c,d indicates $p<0.05$)


A
(11) (13) (11) (8)
ac b a bc
CD11C+F4/80-/(%total viable)
1.6
1.2
0.8
0.4
0.0
EST DPC 3.5 EST DPC 3.5
miR-223 +/+ miR-223 -/-
B
(11) (13) (11) (8)
a b c b
Number of DCS x 10^6
2.4
1.8
1.2
0.6
0.0
EST DPC 3.5 EST DPC 3.5
miR-223 +/+ miR-223 -/-
C
(11) (14) (11) (10)
ab ab a b
MHC II+/(%CD11C+F4/80-)
100
75
50
25
0
EST DPC 3.5 EST DPC 3.5
miR-223 +/+ miR-223 -/-
D
(11) (14) (11) (10)
a b a b
Number of MHC II+ DCs x 10^6
1.6
1.2
0.8
0.4
0.0
EST DPC 3.5 EST DPC 3.5
miR-223 +/+ miR-223 -/-
E
(10) (14) (11) (10)
a a a a
CD80+/(%CD11C+F4/80-)
60
45
30
15
0
EST DPC 3.5 EST DPC 3.5
miR-223 +/+ miR-223 -/-
F
(10) (14) (11) (10)
ac ab c b
Number of CD80+ DCs x 10^6
0.24
0.18
0.12
0.06
0.00
EST DPC 3.5 EST DPC 3.5
miR-223 +/+ miR-223 -/-

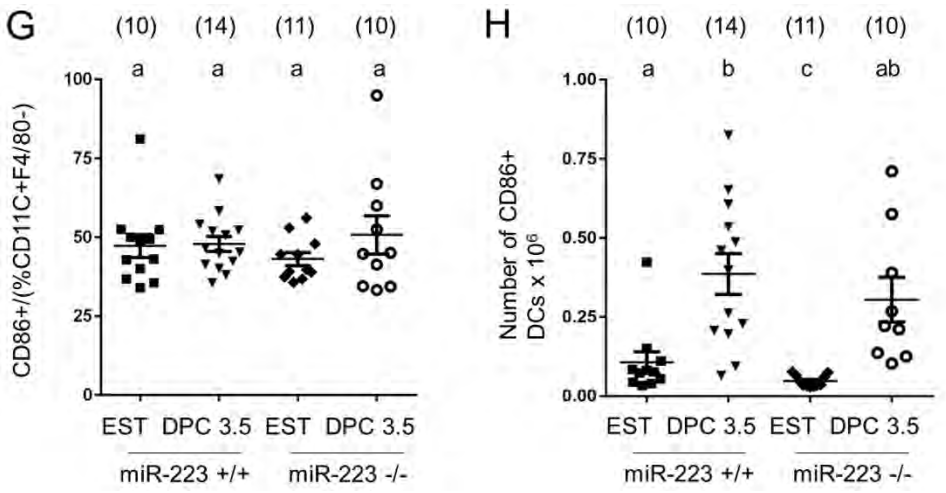


Figure 8.17 The effect of miR-223 deficiency on DC population and expression of MHCII, CD80, and CD86 by CD11C+ DCs in the spleen. miR-223+/+ and miR-223-/- B6 mice were mated with Balb/c males, and on DPC 3.5, CD11C+ F4/80- cells from the spleen were analysed by flow cytometry to quantify proportion of DCs and the expression of MHCII, CD80, and CD86. Unmated virgin oestrus mice were used as non-mated controls. Data are presented as mean ± SEM and are the percentage of CD11C+ DCs within viable cells (A), the total number of DCs x 10^6 (B), the percentage of activated DCs (MHCII+ DCs) (C), the total number of activated DCs x 10^6 (D), the percentage of CD80+ DCs (E), the total number of CD80+ DCs x 10^6 (F), the percentage of CD86+ DCs (G) and the total number of CD86+ DCs x 10^6 (H). Differences between groups were evaluated using Kruskal-Wallis test and Mann-Whitney U test. (a,b,c,d indicates $p<0.05$).

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Unstable Foxp3$^+$ Regulatory T Cells and Altered Dendritic Cells Are Associated with Lipopolysaccharide-Induced Fetal Loss in Pregnant Interleukin 10-Deficient Mice[1]

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ABSTRACT

Maternal interleukin (IL) 10 deficiency elevates susceptibility to fetal loss induced by the model Toll-like receptor agonist lipopolysaccharide, but the mechanisms are not well elucidated. Here, we show that *Il10* null mutant (*Il10*$^{-/-}$) mice exhibit altered local T cell responses in pregnancy, exhibiting pronounced hyperplasia in para-aortic lymph nodes draining the uterus with >6-fold increased CD4$^+$ and CD8$^+$ T cells compared with wild-type controls. Among these CD4$^+$ cells, Foxp3$^+$ T regulatory (Treg) cells were substantially enriched, with 11-fold higher numbers at Day 9.5 postcoitum. Lymph node hypertrophy in *Il10*$^{-/-}$ mice was associated with more activated phenotypes in dendritic cells and macrophages, with elevated expression of MHCII, scavenger receptor, and CD80. Affymetrix microarray revealed an altered transcriptional profile in Treg cells from pregnant *Il10*$^{-/-}$ mice, with elevated expression of *Ctse* (cathepsin E), *Il1r1*, *Il12rb2*, and *Ifng*. In vitro, *Il10*$^{-/-}$ Treg cells showed reduced steady-state Foxp3 expression, and polyclonal stimulation caused greater loss of Foxp3 and reduced capacity to suppress IL17 in CD4$^+$Foxp3$^-$ T cells. We conclude that despite a substantially expanded Treg cell pool, the diminished stability of Treg cells, increased numbers of effector T cells, and altered phenotypes in dendritic cells and macrophages in pregnancy all potentially confer vulnerability to inflammation-induced fetal loss in *Il10*$^{-/-}$ mice. These findings suggest that IL10 has a pivotal role in facilitating robust immune protection of the fetus from inflammatory challenge and that IL10 deficiency could contribute to human gestational disorders in which altered T cell responses are implicated.

cytokines, dendritic cells, fetal loss, immunology, inflammation, interleukin 10, preeclampsia, pregnancy, regulatory T cells

INTRODUCTION

Inheritance by the conceptus of maternal and paternal transplantation antigens results in a semiallogeneic challenge to the mother. Substantial adaptations in the maternal immune system are required to tolerate the fetus and suppress deleterious inflammatory responses that cause gestational disorders and fetal demise. Secretion of immune-regulatory cytokines and hormones by placental trophoblasts and uterine cells [1–3] constrain inflammation and limit type 1 immunity, particularly in the gestational tissues and local lymph nodes (LNs). Prominent among these cytokines is interleukin (IL) 10 [1, 4], which stimulates and reinforces modifications to the innate and adaptive immune system, including induction of regulatory phenotypes in the dendritic cell (DC), macrophage, and T lymphocyte compartments [5–7]. Experiments in *Il10* null mutant (*Il10*$^{-/-}$) mice demonstrate that in the absence of maternal IL10, neither allogeneic nor syngeneic pregnancies are compromised [8–10]. However, pregnant *Il10*$^{-/-}$ mice are highly vulnerable to inflammatory challenge, with administration of low-dose lipopolysaccharide (LPS) or other Toll-like receptor (TLR) ligands causing elevated rates of fetal resorption (miscarriage) [11, 12] or preterm delivery [13, 14], depending on gestational timing of the insult.

The mechanisms through which IL10 controls inflammatory mediators have not been defined, although uterine natural killer (uNK) cells and tumor necrosis factor (TNF) appear to be involved in promoting fetal loss when pregnant *Il10*$^{-/-}$ mice are challenged [11]. We postulate that in the absence of IL10, anti-inflammatory mechanisms protecting the fetus are compromised. One of the key leukocyte populations likely to be affected by absence of IL10 are T regulatory (Treg) cells, a subset of anti-inflammatory and immune suppressive CD4$^+$ T lymphocytes defined by their expression of the transcription factor fork-head box P3 (Foxp3) [15, 16]. The critical role of Treg cells in limiting inflammation and mediating immune tolerance is demonstrated in *Foxp3* null mutant mice (*Scurfy* mice) that develop a lethal multiorgan lymphoproliferative disorder [17]. IL10 is implicated in the generation of Treg cells and is prominent in aspects of their suppressive function [7, 18], including in pregnancy, where neutralizing IL10 abrogates the protective effects of Treg cells in a murine abortion model [19]. IL10 also influences macrophages and DCs, which have important roles in sustaining pregnancy independently of T cells [20] as well as through specific antigen-presenting and immune-regulatory functions, including control of the generation of induced Treg cells from naïve T lymphocyte precursors [7].

During murine pregnancy, Treg cells are elevated in the gestational tissues and systemic circulation by approximately 50% in midgestation [10, 21], and similar changes are evident in pregnant women [22, 23]. Experiments in Treg cell-depleted

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mice [24, 25] and abortion-prone mice [26] show these cells are essential for establishing allogeneic pregnancy. Their immune regulatory actions are most crucial around the time of embryo implantation, when their abundance is a limiting factor in implantation and placental development [27]. Experiments using tetramers demonstrate the majority of the maternal Treg cells that expand in pregnancy are fetal antigen-specific [10], and dependence on the CNS1 regulatory region in the *Foxp3* gene confirms this is at least partly the result of extrathymic generation of inducible (peripheral) Treg cells responding to fetal alloantigen [28] after initial priming in response to paternal seminal fluid at conception [29]. Treg cells have recently been shown to protect the fetus from preterm delivery induced by the TLR4 ligand bacterial LPS [30] and from fetal loss induced by the TLR9 ligand DNA motif CpG [31]. Although Treg cells can develop and execute at least some protolerance functions independently of IL10 [10, 32, 33], to our knowledge the effect of IL10 deficiency on Treg cell populations in pregnancy has not been investigated.

In the present study, we demonstrate significant alterations in the maternal Treg cell response to allogeneic pregnancy in female mice with genetic IL10 deficiency. We report substantial lymphocyte hyperplasia within the uterine-draining para-aortic LNs, accompanied by a substantial increase in the total number and relative proportion of $CD4^+CD25^+Foxp3^+$ Treg cells, that is most pronounced in midgestation. Experiments to define the mechanisms underlying these changes in the T cell compartment reveal altered phenotypes in DCs and macrophages and demonstrate that in pregnancy, Treg cells generated in local para-aortic LNs exhibit diminished stability linked with reduced Foxp3 protein expression and altered transcription of several genes associated with regulatory function compared with their wild-type counterparts.

MATERIALS AND METHODS

Mice and Surgical Treatments

BALB/c mice were purchased from the University of Adelaide Central Animal Facility. C57BL/6 (B6; $Il10^{+/+}$), and $Il10^{-/-}$ mutant mice back-crossed through the same B6 colony were bred in-house under specific pathogen-free conditions at the University of Adelaide Medical School Animal House under a 12L:12D photoperiod and were administered food and water ad libitum. $Il10^{-/-}$ mutant mice were generated by targeted mutation of the *Il10* gene in 129/Ola embryonic stem cells, propagated on a B6 background [34]. Null mutant status was confirmed in $Il10^{-/-}$ mice by PCR of DNA extracted from blood or tail tissue of adult mice. PCR primers diagnostic for the *Il10* null mutation and the neomycin insertion cassette were as previously reported [34]. Experiments were performed in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, with approval from the University of Adelaide Ethics Committee. $Il10^{-/-}$ mice received broad-spectrum antibiotics (100 g/kg of oxytetracycline hydrochloride; Oxymav 100; Mavlab) in autoclaved drinking water twice weekly at a concentration of 2 mg/ml to prevent colitis.

Mating was confirmed by the presence of a vaginal plug between 0900 and 1100 h. The morning of confirmation of mating was termed Day Postcoitum (DPC) 0.5. Mated $Il10^{-/-}$ and $Il10^{+/-}$ females were removed from males, caged in groups of one to three per cage, and killed at different time points over the course of pregnancy to evaluate immune parameters. For analysis of fetal loss rates and late gestation pregnancy parameters, $Il10^{-/-}$ and $Il10^{-/-}$ females mated with BALB/c males and identified as pregnant with more than 2.0 g of weight gain after DPC 0.5 were administered LPS (*Salmonella typhimurium*; 0.25 μg in 200 μl PBS i.p.; Sigma) or PBS control at 1100 h on DPC 9.5, then killed by cervical dislocation at between 1000 and 1200 h on DPC 17.5. The intact uterus of each female was removed, and total, viable, and resorbing implantation sites were counted. Each viable fetus was dissected from the amniotic sac and umbilical cord, and fetuses and placentae were weighed.

Flow Cytometry

Para-aortic and inguinal LNs were excised from female mice between 0900 and 1100 h at estrus or at various time points in pregnancy. Single-cell suspensions were prepared by mechanical dispersion between glass microscope slides. Lymphocytes were suspended in 0.1% BSA/PBS with 0.05% sodium azide (pH 7.4; fluorescence-activated cell sorting [FACS] buffer). Aliquots of 10^6 cells were incubated with anti-Fc-γIIR (FcBlock; BD Biosciences) for 5 min at 4°C, then incubated with combinations of phycoerythrin (PE) anti-CD4 (L3T4; BD Biosciences), fluorescein isothiocyanate (FITC) anti-CD8 (Ly-2; BD Biosciences), and PE-Cy7 anti-CD25 (PC61; eBioscience) for 30 min at 4°C to identify T cell subsets. Following T cell surface staining, cells were fixed and permeabilized using Foxp3 Staining Buffer Set (eBioscience) according to the manufacturer's instructions. Permeabilized cells were incubated with anti-Fc-γIIR for 5 min at 4°C and then with APC anti-Foxp3 (FJK-16s; eBioscience) for 30 min at 4°C.

To analyze other leukocyte subsets and to phenotype DCs and macrophages, aliquots were incubated with anti-Fc-γIIR antibody plus combinations of APC anti-CD11b (M1/70; eBioscience), AF488 anti-CD11c (N418; eBioscience), PE-Cy7 anti-CD19b (eBio 1D3; eBioscience), PE or biotin anti-F4/80 (BM8; eBioscience), PE anti-CD4 (L3T4; BD Biosciences), biotin anti-major histocompatability class II (MHCII; M5/114.15.2; eBioscience), biotin anti-MHCII, scavenger receptor class A (macrophage scavenger receptor [MSR]; 2F8; AbD Serotec), biotin anti-CD80 (16-10A1; BD Biosciences), and APC anti-CD86 (GL1; BD Biosciences) for 30 min at 4°C, followed by PerCp Streptavidin (BD Biosciences). After washing, cells were stained with 4′,6-diamidino-2-phenylindole and were analyzed on a FACS-Canto II flow cytometer using FACSDiva software (both from BD Biosciences). For quantification of total cell numbers, CountBright Absolute Counting Beads (Molecular Probes, Invitrogen) were added to aliquots of LN cells to quantify the total number of leukocyte subsets relative to their ratio to $CD4^+$ or $CD19b^+$ cells.

T Cell Suppression Assays

$CD4^+CD25^+$ cells were isolated from para-aortic LN of nonpregnant or DPC 9.5 pregnant $Il10^{+/+}$ or $Il10^{-/-}$ mice mated with BALB/c males using a MACS $CD4^+CD25^+$ Regulatory T Cell Isolation Kit for mice (Miltenyi Biotec) according to the manufacturer's instructions. Isolated $CD4^+CD25^-$ and $CD4^+CD25^+$ cells were shown by flow cytometry to be more than 95% pure for $CD4^+CD25^-$ cells and more than 85% pure for $CD4^+CD25^+$ cells.

In vitro suppression assays were performed by combining varying numbers of $Il10^{-/-}$ or $Il10^{+/+}$ $CD4^+CD25^+$ regulatory cells, with 1×10^5 $Il10^{+/+}$ B6 $CD4^+CD25^-$ responder cells and 1×10^6 stimulator cells ($Il10^{+/+}$ BALB/c splenocytes) to give final $CD4^+CD25^-$:$CD4^+CD25^+$ ratios of 1:1, 2:1, and 4:1 in RPMI-1640 (Sigma-Aldrich) containing 0.05 mM β-mecaptoethanol (BDH Laboratory Supplies), 2 mM L-glutamine (BDH Laboratory Supplies), antibiotics (penicillin/streptomycin; Sigma), and heat-inactivated fetal calf serum (CSL) (cRPMI). Stimulator cells were rendered anergic with 15 Gy of irradiation. Stimulator cells, $CD4^+CD25^-$ cells, and $CD4^+CD25^+$ cells were incubated in 200 μl of cRPMI in 96-well U-bottom cell culture dishes (Nunc) for 72 h at 37°C with 5% CO_2. Cell proliferation was assessed by addition of 1.5 μCi of tritiated thymidine (^{3}HTdR) for the final 8 h and harvested using a FilterMate 96 Cell Harvester (Packard). The ^{3}HTdR content was expressed as counts per minute using a TopCount NXT v2.53 Micro Scintillation Counter (PerkinElmer) and as a percentage relative to $CD4^+CD25^-$ cells combined with stimulator cells in the absence of $CD4^+CD25^+$ cells. Data points are the average of triplicate wells, and experiments were repeated three times.

Cytokine Stimulation Assays

Single-cell suspensions of para-aortic LN from DPC 9.5 pregnant $Il10^{+/+}$ or $Il10^{-/-}$ mice mated with BALB/c males were incubated in cRPMI at 7.5×10^5 per well with monensin (2 μM; eBioscience) for 4 h at 37°C, with or without polyclonal stimulation with phorbol 12-myristate 13-acetate (PMA) (0.1 μg/ml) and ionomycin (1.0 μg/ml). Cells were harvested, washed, and incubated with anti-Fc-γIIR before addition of FITC anti-CD4 (GK1.5; BD Biosciences) and PerCpCy5.5 anti-CD8 (53-6.7; eBioscience) for 30 min at 4°C. Following surface staining, cells were fixed and permeabilized using the Foxp3 Staining Buffer Set (eBioscience) according to the manufacturer's instructions. Permeabilized cells were incubated for 5 min at 4°C with anti-Fc-γIIR before addition of PE anti-IL2 (JES 6-5 H4, eBioscience), PE anti-IL4 (11B11, BD Biosciences), PE anti-IL9 (RM9A4, Biolegend), PE anti-IL10 (JES5-16E3, eBioscience), PE anti-IL17A (eBio 17B7, eBioscience) and APC anti-Foxp3 (FJK-16s; eBioscience), or PE anti-interferon gamma (IFNG) (XMG1.2, eBioscience) for 30 min at 4°C. After further washing with permeabilization

buffer, cells were analyzed on a FACSCanto flow cytometer using FACSDiva software (both BD Biosciences).

Immunohistochemistry

Sections (thickness, 7 μm) taken from paraffin-embedded whole implantation sites (including fetus, placenta, and decidua) recovered from DPC 9.5 pregnant $Il10^{+/+}$ or $Il10^{-/-}$ mice were dewaxed in xylene before rehydration and antigen retrieval by incubation in citrate buffer (10 mM sodium citrate, pH 6.0) in an autoclave at 121°C and 15 psi for 10 min. Nonspecific antibody binding was blocked by incubation for 1 h at room temperature with 10% normal rabbit serum (Sigma-Aldrich) in PBST (1× PBS and 0.025% Tween-20; Sigma-Aldrich), and endogenous peroxidase activity was blocked in 3% hydrogen peroxide (Sigma-Aldrich) for 10 min. Sections were subsequently incubated with anti-Foxp3 (FJK-16s) primary antibody (1:400 dilution) overnight at 4°C, followed by biotinylated rabbit anti-rat (1:400; Dako) for 1 h at room temperature and streptavidin-conjugated horseradish peroxidase (Vectastain ABC Kit; Vector Laboratories) according to the manufacturer's instructions with detection using a diaminobenzidine peroxidase substrate kit (Vector Laboratories). Images were captured using a NanoZoomer 1.0 (Hamamatsu) at a zoom equivalent of a 20× objective lens. Cell density was calculated by counting Foxp3^{+} cells in three tissue sections (at least 100 μm apart) from each of two or three implantation sites per mouse and then dividing by the section area, calculated by tracing of the section outline using NDP-view software (Hamamatsu).

RNA Extraction and Microarray Analysis

The RNA was extracted from CD4^{+}CD25^{+} lymphocytes isolated from para-aortic LNs of DPC 9.5 pregnant $Il10^{+/+}$ or $Il10^{-/-}$ mice using miRNeasy Mini Kits (Qiagen, Inc.) according to the manufacturer's instructions. The concentration and purity of each RNA sample was determined using an ND-1000 spectrophotometer (NanoDrop Technologies, Inc.). RNA quality was determined using the RNA 6000 Pico Total RNA Kit (Agilent Technologies) before use in microarray experiments. RNA with an RNA integrity number (RIN) greater than seven was used in the present study.

For microarray analysis, RNA was pooled (n = 2–4 mice/pool), resulting in four biological replicates of CD4^{+}CD25^{+} cells from both $Il10^{-/-}$ and $Il10^{+/+}$ mice. Microarray analysis was performed using Affymetrix Mouse Gene 2.0 ST Arrays at the Adelaide Microarray Centre. Total RNA was amplified using the Ovation PicoSL WTA System V2 (Nugen, Inc.) and MinElute Reaction Cleanup Kit (Qiagen, Inc.), according to the manufacturer's instructions, to provide 5 μg of cRNA for each microarray.

The microarray data were normalized and analyzed using Partek Genomics Suite (Partek, Inc.). Raw data from the Affymetrix platform (.cel files) were imported and normalized using RMA background correction, Partek's own guanine-cytosine (GC) content correction, and mean probe summarization. A threshold of either a greater than 1.4-fold change (low stringency) or a greater than 2.0-fold change (high stringency) was used to identify differentially expressed genes, with a false-discovery rate of 0.05.

The microarray data discussed in this publication have been deposited in the National Center for Biotechnology Information's Gene Expression Omnibus and are accessible through GEO Series accession no. GSE71494 (www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE71494).

Quantitative RT-PCR

Quantitative RT-PCR (qPCR) was used to confirm microarray data. Total cellular RNA was reverse transcribed from 125 ng of random hexamer primed RNA from each of 8–12 individual $Il10^{-/-}$ and $Il10^{+/+}$ mice employing a Superscript-III Reverse Transcriptase kit (Invitrogen) following the manufacturer's instructions. Primer pairs specific for published cDNA sequences were designed using Primer Express version 2 software (Applied Biosystems) (Supplemental Table S1; all Supplemental Data are available online at www.biolreprod.org). Assay optimization and validation experiments were performed using cDNA from murine para-aortic LN tissue to determine the amplification efficiency of each primer pair. All primers were determined to have a correlation coefficient of greater than 0.95 and an efficiency of between 90% and 110%. PCR primer products were purified from 2% agarose gels and sequenced (Australian Genome Research Facility, Adelaide) to confirm primer specificity.

The qPCR was performed using 1 ng of cDNA, supplemented with 0.1–0.5 μM 5′ and 3′ primers (Supplemental Table S1) and 1× SYBR Green PCR Master Mix (Applied Biosystems). The negative control included in each reaction contained H_2O substituted for cDNA. PCR amplification was performed in an ABI Prism 7000 Sequence Detection System (Applied Biosystems) using reaction conditions of 95°C for 10 min, then 40 cycles of 95°C for 15 sec and 60°C for 1 min. The Delta C(t) method was then used to calculate mRNA abundance normalized to *Gmpr* mRNA expression.

Statistics and Data Analysis

Unless stated otherwise, the effect of maternal genotype was analyzed using unpaired *t*-test, or one-way ANOVA and Sidak *t*-test for multiple comparisons, using GraphPad Prism 6 for Windows (GraphPad Software, Inc.) after confirming normality of distribution using the Shapiro-Wilk normality test in SPSS Statistics Version 17.0 (IBM Corporation). Individual data points were excluded as outliers if greater than 2 SD from the mean. Difference in groups were considered significant if $P < 0.05$. The qPCR data were not normally distributed and were analyzed by a Kruskal-Wallis *H*-test and the Mann-Whitney *U*-test taking into account multiple comparisons. Statistical significance in differences between the groups was concluded when $P < 0.05$.

RESULTS

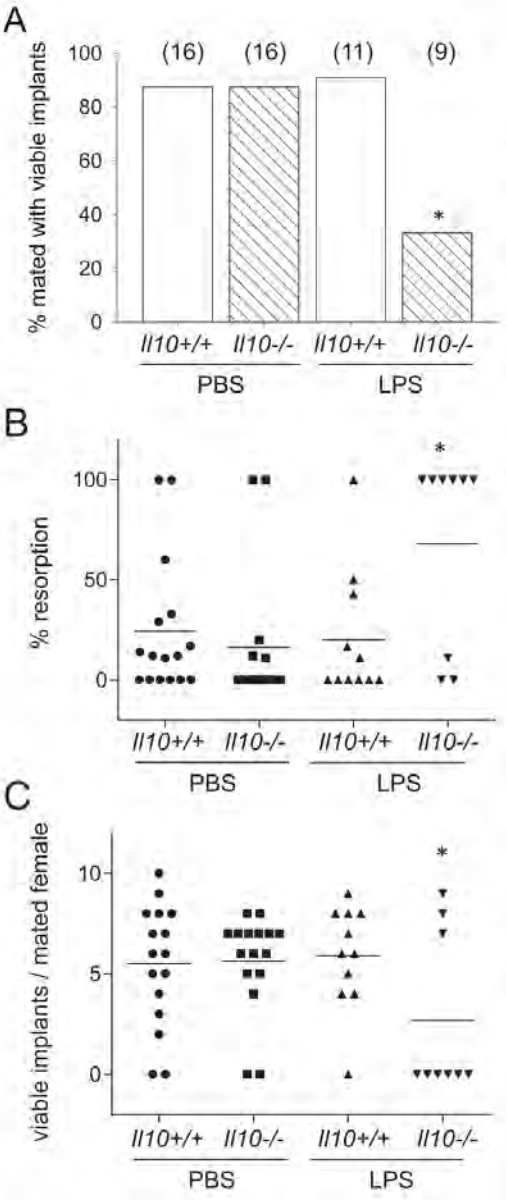
Maternal IL10 Deficiency Elevates LPS-Induced Fetal Loss

Previously, we reported that the *Il10* null mutation causes elevated fetal loss when pregnant mice carrying syngeneic IL10-deficient fetuses are administered low-dose LPS [12]. To investigate the contribution of maternal IL10 to this increased susceptibility, $Il10^{-/-}$ and $Il10^{+/+}$ B6 females were mated with $Il10^{+/+}$ BALB/c males to generate allogeneic pregnancies with maternal, but not fetal, IL10 deficiency. On DPC 9.5, pregnant females were administered LPS at a low dose (0.25 μg/mouse) identified previously to induce fetal loss in pregnant $Il10^{-/-}$ mice but not in $Il10^{+/+}$ controls [12]. In mice examined just before term on DPC 17.5, $Il10^{-/-}$ mice were more severely affected by LPS treatment than control mice, with a lower proportion of $Il10^{-/-}$ mice carrying viable fetuses at DPC 17.5 (33% in $Il10^{-/-}$ and 91% in $Il10^{+/+}$ mice, $P = 0.019$) (Fig. 1A). Furthermore LPS caused a higher rate of fetal resorption in $Il10^{-/-}$ mothers (68% ± 8% in $Il10^{-/-}$ vs. 20 ± 9% in $Il10^{+/+}$ mice, $P = 0.030$) (Fig. 1B), resulting in significantly fewer viable fetuses per mated female (Fig. 1C). No effects of genotype or LPS treatment on fetal weight, placental weight, or fetal weight:placental weight ratio were seen (data not shown). Thus, elevated fetal loss in $Il10^{-/-}$ mothers can be largely attributed to maternal, as opposed to fetal, IL10 deficiency, implicating a defect in the maternal immune adaptations protecting pregnancy.

IL10 Deficiency Causes LN Hypertrophy at Midgestation

To explore the underlying immune pathways contributing to LPS-induced fetal loss in the absence of maternal IL10, $Il10^{-/-}$ and $Il10^{+/+}$ females were mated with BALB/c males and leukocyte populations in para-aortic LNs draining the uterus were examined at various time points in gestation. Substantially greater hypertrophy of the para-aortic LNs was observed in pregnant $Il10^{-/-}$ mice at DPC 9.5 in midgestation, when $Il10^{-/-}$ mice consistently showed a greater than 10-fold increase in para-aortic LN mass compared to controls (Fig. 2, A and B). No obvious difference in LN size attributable to genotype was seen in para-aortic LN in unmated mice or at DPC 6.5 or 13.5 (data not shown).

Flow cytometry was utilized to determine the composition and relative abundance of leukocyte subpopulations in para-aortic LNs at DPC 9.5. Subcutaneous inguinal LNs, which do not drain the uterus [35], were also analyzed for comparison. T cells accounted for the greatest increase in LN hypercellularity, with the absolute number of CD4^{+} cells increased 6.7-fold and that of CD8^{+} cells increased 8.3-fold, whereas CD19b^{+} B cells were increased 3.4-fold and CD11c^{+} DCs increased 3.6-fold in para-aortic LNs from $Il10^{-/-}$ mice compared to $IL10^{+/+}$



controls (Fig. 2C). The absolute number of macrophages expressing CD11b or F4/80 was comparable regardless of genotype (Fig. 2C). In the inguinal LNs, where no overt LN hypertrophy was evident, the only significant change in *Il10*$^{-/-}$ mice was a 1.8-fold increase in CD19b^{+} B cells, whereas CD11b^{+}, CD11c^{+}, F4/80^{+}, CD4^{+}, and CD8^{+} cells were all unchanged (Fig. 2D).

Treg Cell Pool Is Expanded in Pregnant IL10-Deficient Mice

To determine whether IL10 deficiency affects Treg cell populations in pregnant *Il10*$^{-/-}$ mice, we initially assessed Foxp3 expression by CD4^{+} cells in para-aortic and inguinal LNs. At DPC 9.5, when LN hypertrophy was most evident, the percentage of CD4^{+} cells expressing Foxp3 was increased from 13% to 21% in *Il10*$^{-/-}$ mice compared with *Il10*$^{+/+}$ mice ($P < 0.001$) (Fig. 3A). With the increase in total CD4^{+} cells, this resulted in an 11.3-fold increase in the absolute number of CD4^{+}Foxp3^{+} cells ($P < 0.001$) (Fig. 3B) in *Il10*$^{-/-}$ mice compared with *Il10*$^{+/+}$ mice. Within the inguinal LN, the proportion of CD4^{+} cells expressing Foxp3 at DPC 9.5 was increased 1.4-fold ($P < 0.001$) (Fig. 3C), with a 1.8-fold increase in total CD4^{+}Foxp3^{+} cells ($P < 0.05$) (Fig. 3D).

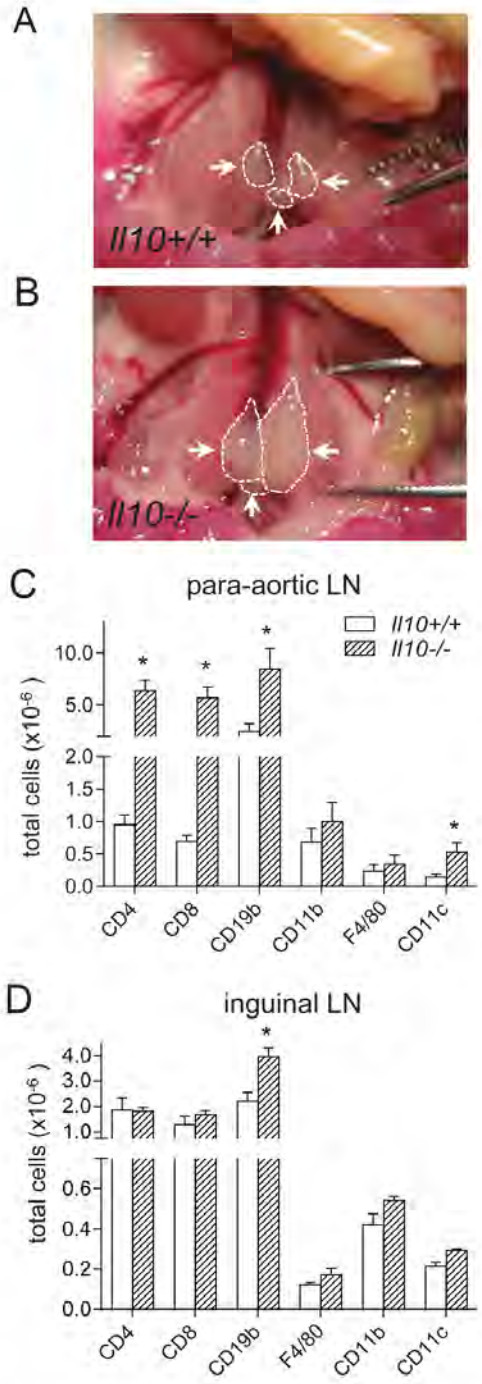
A relative increase in the proportion of CD4^{+} cells expressing Foxp3 was also seen at other time points in pregnancy and also in virgin mice, albeit to a lesser extent than at DPC 9.5 (Fig. 3A), resulting in 2.1- to 4.2-fold increases in total CD4^{+}Foxp3^{+} cells at these other times (Fig. 3B). Within the inguinal LNs, CD4^{+} cells were also more likely to express Foxp3 throughout pregnancy and in virgin mice ($P < 0.001$) (Fig. 3C), causing a modest, but consistent, 1.3- to 1.9-fold increase in CD4^{+}Foxp3^{+} cells attributable to IL10 deficiency ($P < 0.001$) (Fig. 3D).

As well as LNs draining the uterus, Treg cells are present in the maternal and fetal compartments of gestational tissues [21]. Immunohistochemistry was utilized to quantify Foxp3^{+} cells in sections of maternal uterine decidua from embryo implantation sites on DPC 9.5. Among the sparse populations of Treg cells seen in decidual tissue, no overt difference was evident in pregnant *Il10*$^{-/-}$ compared with *Il10*$^{+/+}$ females (Supplemental Fig. S1).

Altered DC and Macrophage Phenotype in IL10-Deficient Pregnant Mice

Next, we evaluated a possible role of antigen-presenting cells in generating the LN hypertrophy seen on DPC 9.5 with IL10 deficiency. CD11c^{+} DCs and F4/80^{+} macrophages recovered on DPC 9.5 from para-aortic LNs of *Il10*$^{-/-}$ and *Il10*$^{+/+}$ mice mated with BALB/c males were analyzed by flow cytometry for expression of MHCII, MSR1, CD80, and CD86. A higher proportion of CD11c^{+} DCs in *Il10*$^{-/-}$ mice were

FIG. 1. The effect of *Il10* null mutation on fetal loss after low-dose LPS challenge. *Il10*$^{+/+}$ (open bars) and *Il10*$^{-/-}$ (hatched bars) B6 mice were mated with Balb/c males and injected i.p. with LPS or control (PBS) on DPC 9.5, then autopsied on DPC 17.5. Data are the percentage of mated females pregnant with viable fetuses (**A**), the number of total implantation sites per pregnant female undergoing resorption (individual data points with mean value shown; **B**), and the number (mean ± SEM) of viable implantation sites per mated mouse (individual data points with mean value shown; **C**). Numbers of mated mice are shown in parentheses. The effect of genotype was evaluated in **A** by chi-square analysis and in **B** by ANOVA and Sidak *t*-test (*$P < 0.05$ compared with *Il10*$^{+/+}$ group at same LPS dose).



positive for expression of MHCII, MSR1, and CD80 (Fig. 4, A–C). The proportions of $CD11c^+$ DCs classified as $CD80^{high}$ or $MHCII^{high}$ were increased, and the CD80 mean fluorescence intensity was higher in $Il10^{-/-}$ mice. No changes in CD86 expression were attributable to IL10 deficiency (data not shown).

In $F4/80^+$ macrophages, the proportion expressing CD80 was significantly higher, the proportion classified as $CD80^{high}$ was higher, and the mean fluorescent intensity of CD80 in $F4/80^+$ macrophages was higher in $Il10^{-/-}$ mice compared to controls (Fig. 4D), although changes in MHCII, MSR1, and CD86 were not evident (data not shown).

IL10 Deficiency Reduces Foxp3 Expression but Not Suppressive Function in $CD4^+CD25^+$ Treg Cells

Despite elevated numbers of Treg cells in para-aortic LNs on DPC 9.5, the mean fluorescence intensity of the Foxp3 signal in $CD4^+Foxp3^+$ cells was reduced by 35% (942 ± 82 in B6 vs. 615 ± 105 in $Il10^{-/-}$; $P = 0.041$) (Fig. 5, A and B). Given the significance of the Foxp3 transcription factor as a master regulator of several downstream pathways governing Treg cell function, we tested the suppressive capacity of $CD4^+CD25^+$ Treg cells from $Il10^{-/-}$ and $Il10^{+/+}$ mice by in vitro mixed-lymphocyte suppression assay. $CD4^+CD25^+$ Treg cells from nonpregnant $Il10^{-/-}$ and $Il10^{+/+}$ mice showed comparable capacity to suppress proliferation of responder $CD4^+CD25^-$ T cells stimulated by BALB/c splenocytes (Fig. 5C). Similarly, no reduction was observed in suppressive capability of $CD4^+CD25^-$ cells from para-aortic LNs of DPC 9.5 pregnant mice (Fig. 5D).

IL10 Deficiency Alters Gene Expression Profile in Treg Cells

The reduced Foxp3 expression raised the question of whether Treg cells have an altered phenotype in pregnant $Il10^{-/-}$ mice. To examine this, we performed a microarray experiment using the Affymetrix arrays on $CD4^+CD25^+$ T cells isolated by magnetic cell sorting from para-aortic LNs of $Il10^{-/-}$ and $Il10^{+/+}$ mice on DPC 9.5. Four biological replicates of $CD4^+CD25^+$ T cell RNA (each pooled from two or three different mice) from each genotype were reverse transcribed into cDNA and hybridized to Affymetrix Mouse Gene 2.0 ST Arrays. Principal component analysis showed clustering of cDNAs according to genotype (Supplemental Fig. S2A). A total of 52 probe sets were classified as highly differentially expressed with high-stringency criteria (fold-change > 2.0, $P < 0.05$) between genotypes, with 45 genes upregulated and 7 genes downregulated (Supplemental Fig. S2B). Of the genes upregulated in T cells from $Il10^{-/-}$ mice, seven have potential relevance to Treg cell function, identified by Ingenuity Pathway Analysis and literature searches as

FIG. 2. The effect of *Il10* null mutation on cell number in para-aortic and inguinal LNs in midgestation pregnancy. $Il10^{+/+}$ and $Il10^{-/-}$ B6 mice were mated with Balb/c males and on DPC 9.5; the para-aortic and inguinal LNs were analyzed by flow cytometry to quantify cells expressing macrophage T cell markers CD4 and CD8, B cell marker CD11b, macrophage and neutrophil marker CD19b, macrophage marker F4/80, and DC marker CD11c. Representative macroscopic images of the para-aortic LNs in $Il10^{+/+}$ mice (**A**) and $Il10^{-/-}$ mice (**B**) are shown. Data are the total number of various cell subsets (mean ± SEM) in the para-aortic LNs (**C**) and inguinal LNs (**D**) of $Il10^{+/+}$ mice (n = 6, open bars) and $Il10^{-/-}$ mice (n = 5, hatched bars). The effect of genotype in **C** and **D** was evaluated using Sidak *t*-test (*$P < 0.05$ compared with $Il10^{+/+}$ group). Original magnification ×10 (**A** and **B**).

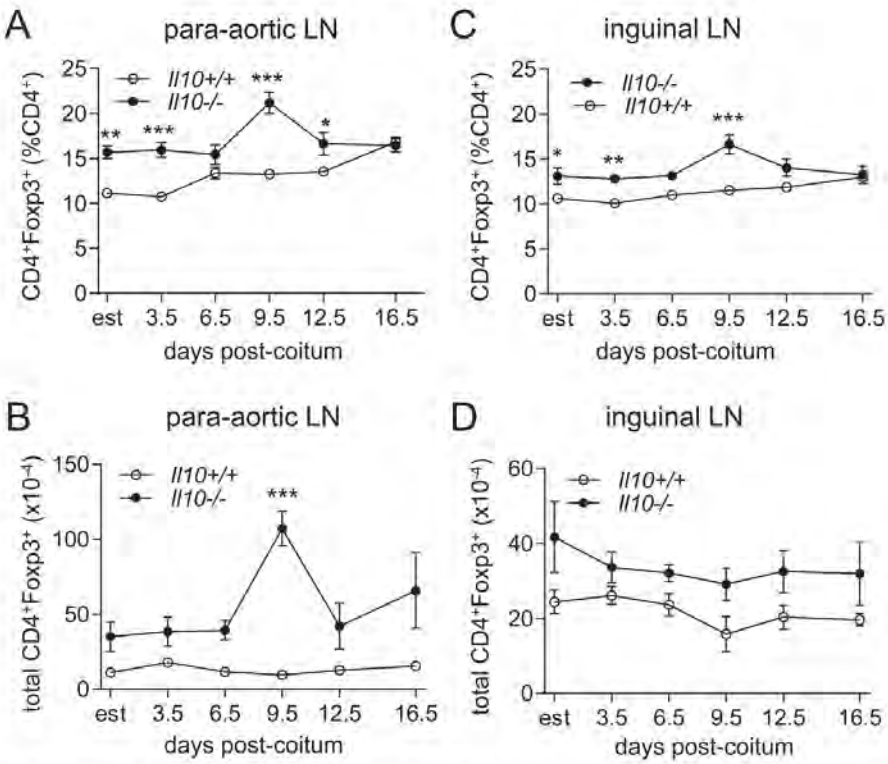


FIG. 3. The effect of *Il10* null mutation on proportion and number of Treg cells in para-aortic and inguinal LNs in midgestation pregnancy. Para-aortic and inguinal LNs from $Il10^{+/+}$ and $Il10^{-/-}$ B6 mice at the estrous phase of the cycle (est) or at various time points in pregnancy after mating with Balb/c males were analyzed by flow cytometry to quantify Treg cells. Data are the proportion of $CD4^+CD25^+Foxp3^+$ (%$CD4^+$) Treg cells (mean ± SEM; **A**) and the total number of $CD4^+CD25^+Foxp3^+$ Treg cells (**B**) in the para-aortic LNs and the proportion of $CD4^+CD25^+Foxp3^+$ (%$CD4^+$) Treg cells (**C**) and the total number of $CD4^+CD25^+Foxp3^+$ Treg cells (**D**) in the inguinal LNs of $Il10^{+/+}$ mice (n = 6–20 per time point, closed circles) and $Il10^{-/-}$ mice (n = 5–8 per time point, open circles). The effect of genotype was evaluated using Sidak *t*-test (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ compared with $Il10^{+/+}$ group at same point; $^{\#}P < 0.05$ compared with $Il10^{+/+}$ group across all time points).

linked with cytokine-cytokine receptor interactions (*Il10*; upregulated 2.1-fold), maintenance of Treg cell function (*Ctse*, *Lilrb4*, and *Slpi*; upregulated 7.2-, 2.1-, and 2.7-fold, respectively), and loss of Treg cell function (*Cd24a*, *Ighm*, and *Igj*; upregulated 2.0-, 10.1-, and 3.1-fold, respectively) (Table 1).

Using a low-stringency analysis (fold-change > 1.4, $P < 0.1$), a total of 299 genes were classified as moderately differentially regulated by IL10 deficiency, including 247 that were upregulated and 52 that were downregulated in $Il10^{-/-}$ compared to $Il10^{+/+}$ Treg cells. Of these, a further 40 genes were identified as associated with cytokine signaling or Treg cell stability and function, with a total of 42 upregulated and 5 downregulated in $Il10^{-/-}$ compared to $Il10^{+/+}$ $CD4^+CD25^+$ T cells (Supplemental Table S2).

Several genes of interest were independently quantified by qPCR in $CD4^+CD25^+$ T cells from para-aortic LN of pregnant $Il10^{+/+}$ and $Il10^{-/-}$ mice on DPC 9.5. In the absence of IL10, increases were detected in expression of *Ctse* encoding the intracellular proteinase cathepsin E (155-fold, $P < 0.0001$) (Fig. 6A), *Ctla2a* encoding the cytotoxic T lymphocyte-associated protein 2 complex CTLA2 (4.4-fold, $P < 0.01$) (Fig. 6B), *Il1r1* encoding the IL1 receptor type 1 (10.4-fold, $P < 0.05$) (Fig. 6D), and *Ifng* encoding the Th1 cytokine IFNG (6.3-fold, $P < 0.05$) (Fig. 6E). A trend to increased expression was seen in *Il12rb2* encoding the IL12 receptor beta 2 subunit (8.3-fold, $P = 0.076$) (Fig. 6F). No consistent change was seen in expression of other genes identified as moderately differentially regulated in the microarray, including *Il17a* (Fig. 6C), *Ccr6*, *Il17rb*, and *Il10ra* (data not shown).

IL10 Deficiency Reduces the Stability of Foxp3 Expression in Stimulated Treg Cells

To further explore the effect of IL10 deficiency on the functional phenotype of Treg cells, experiments were undertaken to investigate the stability of Foxp3 expression after strong polyclonal stimulation of T cells recovered from $Il10^{-/-}$ and $Il10^{+/+}$ mice on DPC 9.5 after mating with Balb/c males. After incubation with PMA and ionomycin for 4 h, the proportion of $CD4^+$ T cells from $Il10^{-/-}$ mice expressing Foxp3 was reduced by 73% and 66% in cells from para-aortic

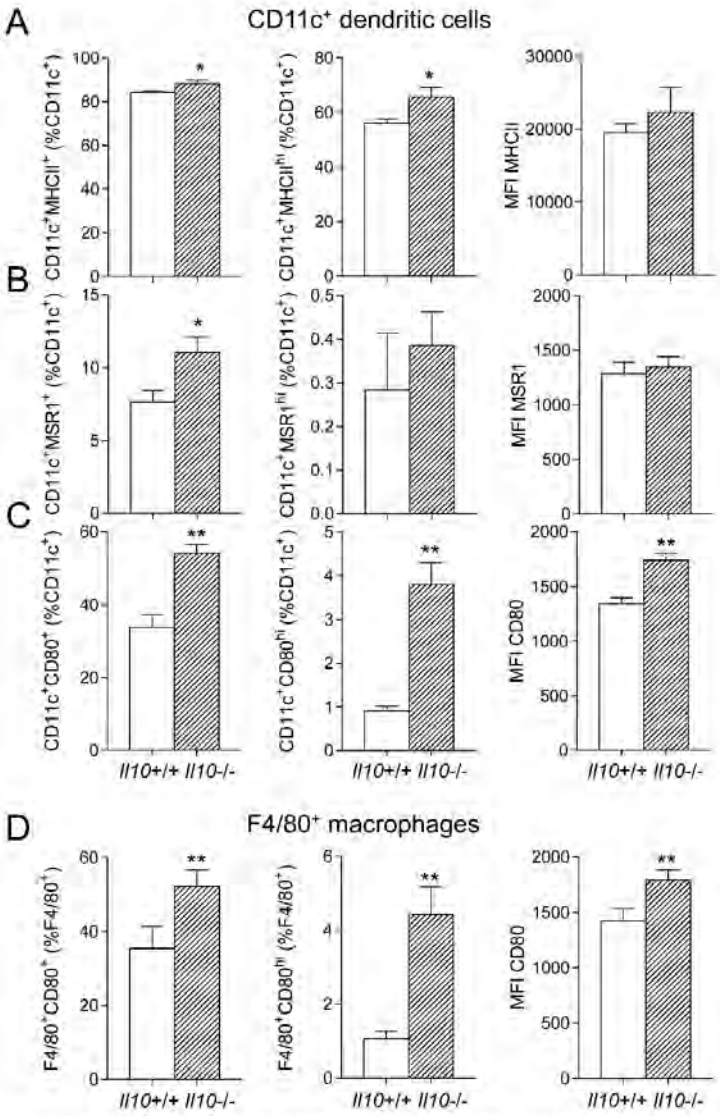


FIG. 4. The effect of *Il10* null mutation on expression of MSR1, CD80, and CD86 by CD11c$^+$ DCs and F4/80$^+$ macrophages in para-aortic LNs. $Il10^{+/+}$ (open bars) and $Il10^{-/-}$ (hatched bars) B6 mice were mated with Balb/c males, and on DPC 9.5, CD11c$^+$ and F4/80$^+$ cells from the para-aortic LNs were analyzed by flow cytometry to quantify expression of MHCII, MSR1, CD80, and CD86. Additional gates were set to discriminate between low and high expression. From left to right, data are the percentage (mean ± SEM) of MHCII$^+$, MHCII^{high}, and mean fluorescence intensity (MFI) of MHCII (**A**); MSR1$^+$, MSR1^{high}, and MFI MSR1 (**B**); CD80$^+$, CD80^{high}, and MFI CD80 (**C**) in CD11c$^+$ cells (**A**–**C**); and CD86, CD86^{high}, and MFI CD86 in F4/80$^+$ macrophages (**D**) from $Il10^{+/+}$ mice (n = 6, open bars) and $Il10^{-/-}$ mice (n = 7, hatched bars). The effect of genotype was evaluated using Sidak *t*-test (*$P < 0.05$, **$P < 0.01$).

and inguinal LNs, respectively, but only by 38% and 34%, respectively, in $Il10^{+/+}$ mice, compared with unstimulated cells from the same genotype (both $P < 0.01$) (Fig. 7, A and B). Intracellular cytokine staining (Supplemental Fig. S3) showed that the increased loss of Foxp3 was accompanied by a lower proportion of stimulated CD4$^+$ T cells from the para-aortic LNs of $Il10^{-/-}$ mice expressing IL4 and IL9 (both $P < 0.05$) (Fig. 7C), with a trend to higher IL17 ($P = 0.087$), compared to

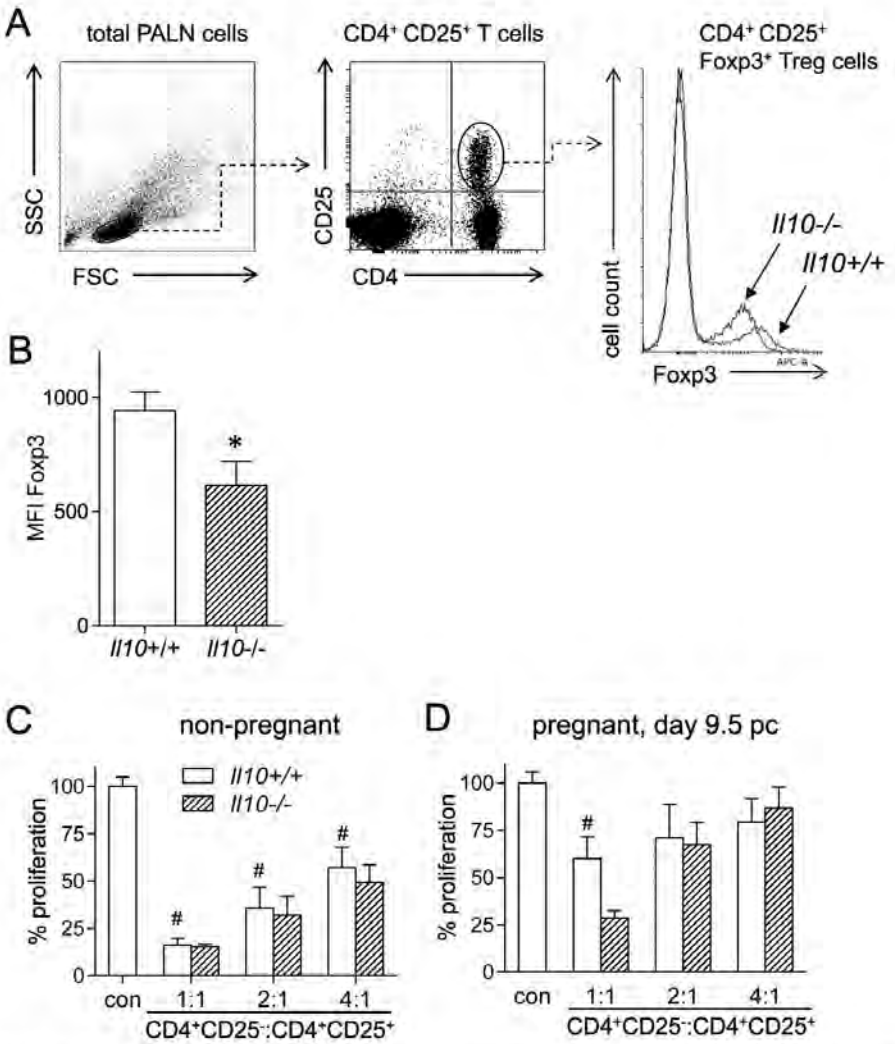


FIG. 5. The effect of *Il10* null mutation on Treg cell expression of Foxp3 and in vitro suppressive capability. $Il10^{+/+}$ and $Il10^{-/-}$ B6 mice were mated with Balb/c males, and on DPC 9.5, the mean fluorescent intensity (MFI) of Foxp3 in para-aortic LN $CD4^+Foxp3^+$ Treg cells was quantified by flow cytometry. The flow cytometry strategy and representative overlay histogram of Foxp3 expression in $CD4^+CD25^+Foxp3^+$ Treg cells from $Il10^{+/+}$ and $Il10^{-/-}$ mice is shown (**A**), as is the MFI of Foxp3 staining in $Il10^{+/+}$ mice (n = 6, open bars) and $Il10^{-/-}$ mice (n = 6, hatched bars; **B**). The suppressive capability of $CD4^+CD25^+$ Treg cells recovered from para-aortic LNs of nonpregnant $Il10^{+/+}$ and $Il10^{-/-}$ mice (**C**) and pregnant $Il10^{+/+}$ and $Il10^{-/-}$ mice on DPC 9.5 after mating with Balb/c males (**D**) was analyzed by mixed lymphocyte suppression assay, using $CD4^+CD25^-$ responder cells from $Il10^{+/+}$ B6 mice and irradiated Balb/c splenocytes as stimulator cells. The ratio of responder ($CD4^+CD25^-$) to suppressor ($CD4^+CD25^+$) cells is shown. Data are the percentage (mean ± SEM) cell proliferation relative to control $CD4^+CD25^-$ cells after addition of $CD4^+CD25^+$ Treg cells from $Il10^{+/+}$ mice (open bars) or $Il10^{-/-}$ mice (hatched bars). The effect of genotype on MFI was evaluated by unpaired *t*-test (**B**), and the effect of $CD4^+CD25^+$ Treg cells and genotype on cell proliferation (**C** and **D**) was evaluated using one-way ANOVA followed by unpaired *t*-test (*$P < 0.05$ compared with $Il10^{+/+}$, #$P < 0.05$ compared with $CD4^+CD25^-$ control).

cells from $Il10^{+/+}$ mice. In inguinal LN $CD4^+$ T cells of $Il10^{-/-}$ mice, a lower proportion expressed IL9 ($P < 0.01$) (Fig. 7D), whereas IL17 expression increased 5-fold ($P < 0.0001$) after polyclonal stimulation. Compared with $Il10^{+/+}$ T cells, stimulation of $Il10^{-/-}$ T cells caused a 2.2-fold increase and a 4.4-fold increase in the ratio of $CD4^+IL17^+$ cells to $CD4^+Foxp3^+$ cells in the para-aortic and inguinal LN populations, respectively, (both $P < 0.05$) (Fig. 7, E and F).

TABLE 1. Genes of interest identified as highly differentially expressed using high-stringency criteria in Affymetrix microarray analysis of mRNA expression in purified Treg cells from *Il10*$^{+/+}$ or *Il10*$^{-/-}$ mice.*

Accession no.	Gene symbol†	Wild-type mean	Knockout mean	Difference	Fold-change	*P*-value
Cytokine-cytokine receptor interactions						
NM_010548	*Il10*	275.21	575.51	300.30	2.09	0.0087
Maintenance of Treg function (immune tolerance)						
NM_007799	*Ctse*	454.62	3256.71	2802.09	7.16	0.0000
NM_013532	*Lilrb4*	396.62	849.90	453.28	2.14	0.0483
NM_011414	*Slpi*	248.11	680.08	431.97	2.74	0.0463
Loss of Treg function (inflammation and apoptosis)						
NM_009846	*Cd24a*	614.44	1772.60	1158.16	2.88	0.0302
BC053409	*Ighm*	26.46	266.33	239.87	10.06	0.0239
NM_152839	*Igj*	151.28	471.01	319.73	3.11	0.0191

* High stringency criteria: fold-change > 2.0, $P < 0.05$, difference between means > 100.
† All genes listed are upregulated in *Il10*$^{-/-}$ mice compared to *Il10*$^{+/+}$ mice.

DISCUSSION

Appropriate control of maternal inflammation is essential for optimal fetal growth and on-time birth. Treg cells are paramount among the immune-regulatory leukocytes operating to suppress inflammation and promote tolerance at the fetal-maternal interface in pregnant females [24, 36]. Currently, a lack of understanding of the maternal and fetal factors that control the generation and suppressive function of Treg cells is a limitation in our capacity to tackle human gestational disorders, including preeclampsia and preterm birth. These conditions result from excessive maternal inflammation and are associated with disturbances in Treg cell populations [23, 36]. In the present study, we demonstrate that despite hyper-proliferation well beyond the expansion seen in normal pregnancy, the Treg cells generated in uterine-draining LNs of pregnant *Il10*$^{-/-}$ mice display reduced Foxp3 expression, altered expression of *Ctse* and other genes linked with Treg function, and functional features indicating decreased stability. These altered Treg cell characteristics are likely to contribute, together with the increased CD4^{+} and CD8^{+} T effector cells and the proinflammatory shift in DC and macrophage phenotypes, to the elevated susceptibility of *Il10*$^{-/-}$ mice to inflammatory challenge and fetal loss [11–13].

Altered T cell immunity in IL10-deficient mice appears not to affect tolerance of fetal alloantigens, because steady-state pregnancy progresses normally irrespective of the alloantigenic

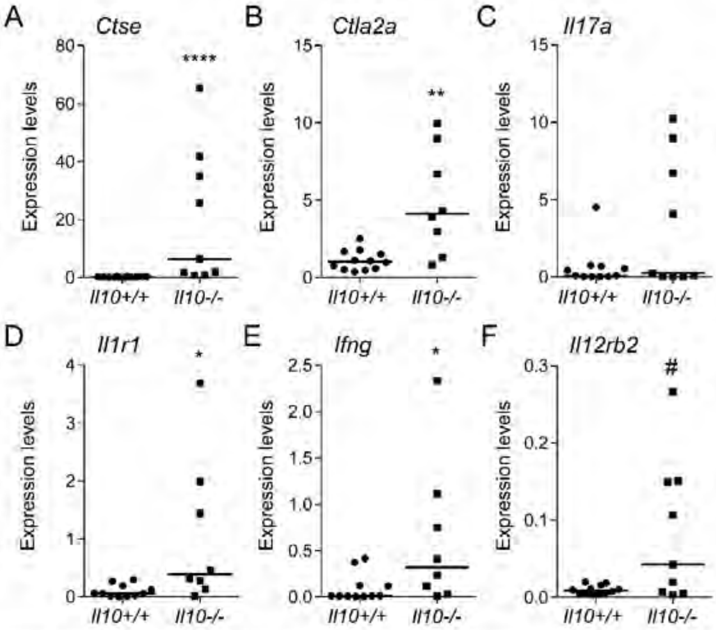


FIG. 6. The effect of *Il10* null mutation on gene expression in Treg cells. CD4^{+}CD25^{+} lymphocytes were recovered from para-aortic LNs of *Il10*$^{+/+}$ and *Il10*$^{-/-}$ B6 mice on DPC 9.5 after mating with Balb/c males, and qPCR was used to analyze the expression of *Ctse* (**A**), *Ctla2a* (**B**), *Il17a* (**C**), *Il1r1* (**D**), *Ifng* (**E**), and *Il12rb2* (**F**). Data are the expression (mean ± SEM), normalized to housekeeper gene *Gmpr*, in Treg cells from *Il10*$^{+/+}$ mice (open bars, n = 12) and *Il10*$^{-/-}$ mice (hatched bars, n = 9). The effect of genotype was evaluated using Mann-Whitney *U*-test (*$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$, #$P < 0.1$).

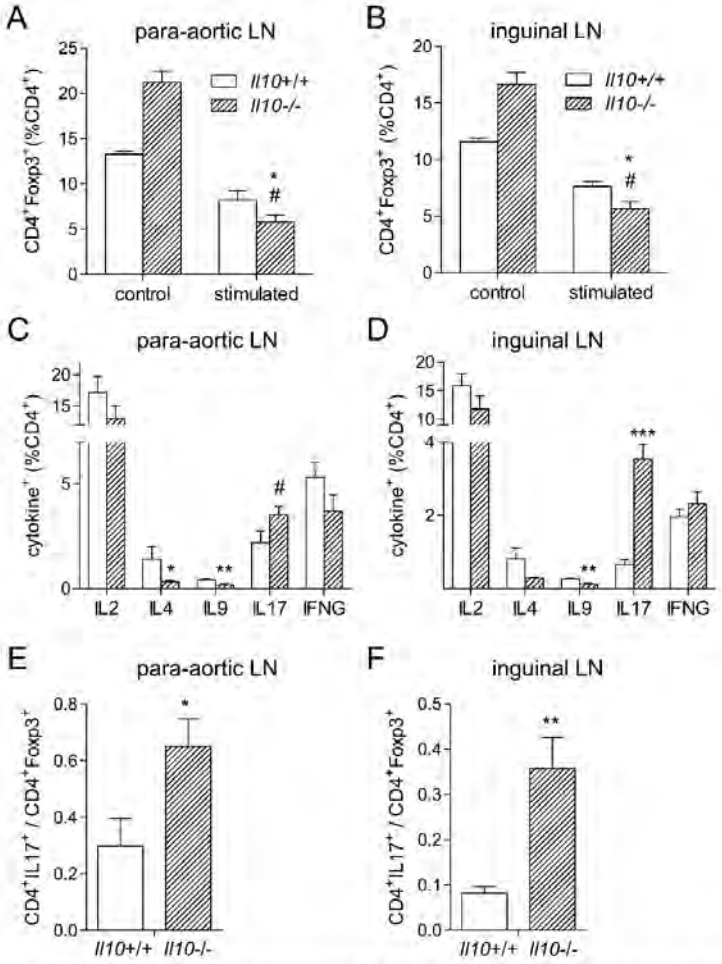


FIG. 7. The effect of *Il10* null mutation on stability of Foxp3 expression in $CD4^+$ T cells. $Il10^{+/+}$ and $Il10^{-/-}$ B6 mice were mated with Balb/c males, and on DPC 9.5, para-aortic and inguinal LNs were analyzed for Foxp3 and cytokine expression by flow cytometry immediately or 4 h after strong polyclonal stimulation with PMA/ionomycin in vitro. The relative proportion of $Foxp3^+$ cells among $CD4^+$ cells in para-aortic LNs (**A**) and inguinal LNs (**B**) in control and stimulated cells is shown. Stimulated $CD4^+$ cells from para-aortic LNs (**C**) and inguinal LNs (**D**) were also analyzed for expression of IL2, IL4, IL9, IL17, and IFNG. The ratio of $CD4^+IL17^+$ to $CD4^+Foxp3^+$ cells from para-aortic LNs (**E**) and inguinal LNs (**F**) after stimulation was calculated. Data are the mean ± SEM from $Il10^{+/+}$ mice (open bars, n = 7) and $Il10^{-/-}$ mice (hatched bars, n = 6). The effect of genotype and stimulation was evaluated using ANOVA and unpaired *t*-test (**A** and **B**); the effect of genotype was evaluated using Sidak *t*-test (**C** and **D**) or unpaired *t*-test (**E** and **F**) ($^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{\#}P = 0.087$).

status of the fetus [8]. The functional requirement for IL10 becomes apparent when pregnant $Il10^{-/-}$ mice are administered low-dose LPS to induce a systemic inflammatory response, revealing a key role for IL10 in protecting the fetal-placental tissues from uncontrolled inflammatory cytokines and natural killer cell cytotoxicity causing fetal death and/or preterm labor [11–13]. Thus, it seems that a major function of the copious IL10 produced by leukocytes, placental trophoblasts, and other cell lineages in gestational tissues [4] is to buffer pregnancy in the event of an inflammatory insult.

Here, we show that IL10 in the maternal compartment, as opposed to the fetal or placental tissues, is most crucial for protecting pregnancy. This implies a pivotal role for endogenous IL10 in supporting anti-inflammatory and protolerance mechanisms in the mother. Given the key actions of IL10 in Treg cell biology [37, 38] and the importance of induced Treg cells in pregnancy [28], we investigated $CD4^+Foxp3^+$ Treg cells and found that when maternal IL10 synthesis is disrupted, the generation of Treg cells is disturbed. A striking LN hyperplasia was evident in $Il10^{-/-}$ mice compared with wild-

type controls. This was most pronounced at midgestation and was mainly accounted for by $CD4^+$ and $CD8^+$ T cells. Compared to T effector cells, Treg cells were disproportionately enriched more than 11-fold compared to wild-type controls at the same pregnancy phase. Treg cells were more dramatically increased in the para-aortic LNs draining the uterus than in the inguinal LNs or at other distal sites (not shown). A 2- to 4-fold increase was also seen in the para-aortic LNs of *Il10*$^{-/-}$ mice before pregnancy and at earlier and later times of gestation, whereas a smaller, 50%–100% increase in the inguinal LNs is in line with previous observations of broadly similar T cell populations in *Il10*$^{-/-}$ mice [34].

Para-aortic LN hypertrophy was most pronounced in *Il10*$^{-/-}$ mice in the midgestation phase, peaking around DPC 9.5. This pattern of response fits with the kinetics demonstrated for maternal exposure to conceptus histocompatibility antigens [39, 40]. The timing coincides with structural changes in the developing placenta that allow maternal blood flow into the placental labyrinth compartment to commence between DPC 9.5 and DPC 10.5 [41], such that intimate contact between maternal immune cells and placental trophoblast antigens escalates at this time.

It is important to consider that in the present study, because fetuses were heterozygous for the *Il10* gene, the maternal compartment is not entirely IL10 deficient. IL10 derived from the fetus and placenta can enter the maternal system, and it would be highest through the second half of gestation, when maternal blood enters the placental labyrinth. Paracrine effects of fetal IL10 on maternal immune cells could reasonably explain the resolution of LN hypertrophy by DPC 12.5. This may also explain why the fetal growth impairment seen previously after midgestation LPS administration when IL10 is absent from both maternal and fetal tissues [12] was not recapitulated in the present study.

The expanded Treg cell pool in midgestation pregnant IL10-deficient mice is accompanied by, and may be the consequence of, an altered phenotype in antigen-presenting cells. We found elevated expression of activation markers MHCII and CD80 in DCs, plus elevated CD80 in macrophages, within para-aortic LNs of pregnant *Il10*$^{-/-}$ mice. DCs matured in the absence of IL10 have elevated ability to process and present antigens and secrete proinflammatory cytokines [6, 42, 43]. Our results are consistent with those of previous studies in *Il10*$^{-/-}$ mice, where elevated DC expression of MHCII, costimulatory molecules, and cytokines IFNG, IL12, and IL17 were reported in other tissues [44, 45]. This phenotype is thought to contribute to skewing the T cell response toward Th1 and Th17 cell prominence, manifesting as exaggerated hypersensitivity [46], chronic enterocolitis [34], and elevated cell-mediated immunity to several pathogens [47].

Lymph node hyperplasia coincides with a phase of accumulation of $CD11c^+$ DCs in the uterus, which characteristically exhibit a tolerogenic phenotype with a dominance of IL10 over IL12 production [48]. The 3.6-fold increase in DCs in the para-aortic LN of *Il10*$^{-/-}$ mice could reflect increased migration of antigen-presenting cells from the uterus, as reported for Langerhans cells in the skin of *Il10*$^{-/-}$ mice [49], in response to increased TNF production in gestational tissues when IL10 is deficient [11, 12]. The combined effect of enhanced DC migration and greater antigen-presenting activity would explain the global expansion in T cell populations. Cross-regulation between Treg cells and DCs presumably amplifies skewing toward more proinflammatory phenotypes in both compartments, because Treg cell-derived factors are required to maintain a tolerogenic DC phenotype [6, 50].

Previously, uNK cells have been shown to be differentially activated in pregnant *Il10*$^{-/-}$ mice [11], to be differentially activated in *Il10*$^{-/-}$ mice after low-dose LPS administration, and to be instrumental in the effector pathway linking IL10 deficiency with fetal loss [11]. The present study supports the interpretation of upstream effects of Treg cells in suppressing natural killer cell activation, consistent with other data showing uNK cell capacity to respond to Treg cell regulation [51, 52].

The mechanism driving selectively greater expansion of Treg cells compared to the residual T cell pool with IL10 deficiency in pregnancy is not known but might reflect a compensatory mechanism to offset reduced suppressive function. IL10 is clearly not essential for the generation of either natural or adaptive $Foxp3^+$ Treg cells [7]. Selective skewing away from the Treg compartment might have been expected in *Il10*$^{-/-}$ mice, and it is reported in respiratory tract infection, where Treg cells are diminished whereas Th1 and Th17 cells are selectively expanded [53]. Contrary to expectations, the pregnancy-associated bias toward inducible Treg cell generation is maintained in the absence of IL10, potentially due to elevated transforming growth factor beta (TGFB) [54] and the range of other overlapping immune-deviating mechanisms operating in pregnancy [2]. The present study did not seek to formally compare effects of IL10 on Treg cells in nonpregnant mice or in sites beyond the uterine-draining LNs, but our time-course experiments and comparisons between para-aortic and inguinal LNs imply there may be subtle effects of IL10 deficiency with elevated numbers of Treg cells in nonpregnant estrous mice and in inguinal LNs not draining the uterus. IL10-mediated mechanisms that ultimately constrain Treg cell generation may not be confined to pregnancy; a recent study reports elevated $Foxp3^+$ Treg cells in the liver of IL10-deficient mice in the late stages of *Brucella abortus* infection [47]. However, further investigation will be required to determine whether inducible or thymic Treg cells exhibit differences in gene expression or function in IL10-deficient mice in other physiological or pathophysiological settings.

Despite the lack of effect on suppressive function in vitro, the present study indicates that stability of Treg cells in pregnant mice was altered by IL10 deficiency. Notably, reduced Foxp3 expression was evident in Treg cells from pregnant *Il10*$^{-/-}$ mice, and a greater disposition to Foxp3 loss occurred when IL10-deficient T cells were activated in vitro. Differentiation of the Treg lineage is not terminal, and Treg cells can be induced to reprogram into Th1 or Th17 cells when exposed to inflammatory cytokines, such as IL6, IFNG, or IL1 [55]. The extent to which Treg cells remain stably committed or exhibit plasticity is determined by methylation of the *Foxp3* gene plus the activity of several additional transcription factors and posttranscriptional regulators [56]. The precise mechanism by which IL10 deficiency alters stability is not clear, but IL10 interacts with transcription factors, such as GATA3, that control *Foxp3* expression [57]. We did not find reduced *Foxp3* mRNA levels in Treg cells from *Il10*$^{-/-}$ mice, consistent with emerging pathways for posttranscriptional control of Foxp3 expression in Treg cells [58].

Affymetrix microarray analysis indicated an altered pattern of gene expression in Treg cells in pregnant *Il10*$^{-/-}$ mice. This may be a compensatory response to lack of autocrine IL10 signaling. Production of IL10 by Treg cells is one of the central pathways mediating suppressive function, and IL10 deficiency constrains Treg cell function to differing extents depending on the tissue [38, 59]. This fits with an emerging picture of unique, nonredundant, and specialized roles for individual suppressive mediators in Treg cells that are exploited in different

physiological and pathophysiological settings [38]. Cell lineage-restricted *Il10* null mutation in Foxp3$^+$ cells shows a dominant, nonredundant role for Treg cell-derived IL10 in maintaining immune homeostasis at environmental surfaces, including the colon, lung, and skin [38]. T cell-specific blockade of IL10 signaling shows that IL10 production from CD4$^+$Foxp3$^+$ Treg cells is important for Treg cell suppression of Th17 and Th1 cells [60].

Whether altered Treg cell function in pregnancy is due to absence of IL10 from Treg cells or from other cell lineages, such as DCs that control Treg cell production, remains to be determined. In mice with a null mutation in *Foxp3* specific to the T cell lineage, the progression of allogeneic pregnancy under steady-state conditions is not impaired, despite changes to pathogen defense [10]. Neither is Treg cell-derived IL10 required for homeostasis of the nonpregnant uterine mucosa, and a moderate delay in resolution of the inflammatory response to seminal fluid at mating does not compromise fertility [8]. Suppressive pathways independent of IL10 exist in Treg cells, and presumably, these account for normal tolerance of seminal fluid and progression of allogeneic pregnancy in *Il10*$^{-/-}$ mice. These include secretion of immune suppressive cytokines other than IL10, such as TGFB and IL35; cell-cell contact-dependent mechanisms; contact-independent mechanisms, such as IL2 and ATP depletion; and stimulation of tryptophan catabolism [18, 61].

Among the most strongly upregulated genes in Treg cells from *Il10*$^{-/-}$ mice was *Ctse*, encoding the endolysosomal aspartic proteinase, cathepsin E. Cathepsin E is implicated as a key factor contributing to adaptive Treg suppressive mechanisms that are independent of IL10. Elevated cathepsin E was previously reported in Treg cells engineered for deficiency in both IL10 and IL35, where it promotes TNF-related apoptosis-inducing ligand (TRAIL)-mediated suppression, to compensate for loss of IL10 and IL35 [33]. Cathepsin E is also associated with increased turnover of IL1B and IL18 through degradation of the protein sequestering molecule α_2-macroglobulin [62]. *Ctse* is induced in inflammation [63] in response to cytokines, including IL17 [64] and IFNG [65]. A previous study found elevated *Ctse* expression in *Il10*$^{-/-}$ mice after ozone-induced inflammation in the lung [66].

Other differentially regulated genes were detected in Treg cells from *Il10*$^{-/-}$ mice, with upregulated *Ifng* and a trend to elevated *Il17* expression. This shift in disposition to Th1 and Th17 gene expression may contribute to the instability of *Il10*$^{-/-}$ Treg cells we observed after proinflammatory stimulation. Genes encoding cytokine receptors IL1r1 and IL12rb2 were elevated, as was the T cell marker CTLA2. IL12rb2 is required for TGFB-dependent stimulation of Treg cell development, and signaling via this receptor is thought to regulate the number and functional maturity of Treg cells [67].

In summary, we have demonstrated that in pregnancy, maternal IL10 is a key determinant of protection from inflammatory challenge, acting to tailor both the size and the stability of the induced Treg cell pool. These changes likely are reinforced by interactions with DCs and macrophages that exhibit a more immunogenic phenotype in the absence of IL10. Whereas Treg cells generated in the absence of IL10 remain competent to sustain allogeneic pregnancy under steady-state conditions, in the event of inflammatory challenge their compromised stability would be a factor in the uNK cell activation and shift to Th1 immunity that causes fetal loss. These findings are relevant to understanding the role of IL10 in the immune response to pregnancy and may ultimately contribute to elucidating the pathology of preeclampsia and related complications of human pregnancy, where a less robust Treg cell response [23] and altered regulation of placental IL10 [68] are both implicated in the underlying inflammatory etiology.

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REVIEW ARTICLE

miRNA Regulation of Immune Tolerance in Early Pregnancy

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Abstract

To support embryo implantation, the female reproductive tract must provide a tolerogenic immune environment. Seminal fluid contact at conception contributes to activating the endometrial gene expression and immune cell changes required for robust implantation, influencing not only the quality of the ensuing pregnancy but also the health of offspring. miRNAs are small non-coding RNAs that play important regulatory roles in biological processes, including regulation of the immune environment. miRNAs are known to contribute to gene regulation in pregnancy and are altered in pregnancy pathologies. Recent studies indicate that miRNAs participate in establishing immune tolerance at conception, and may contribute to the regulatory effects of seminal fluid in generating tolerogenic dendritic cells and T regulatory cells. This review highlights those miRNAs implicated in programming immune cells that are critical during the peri-conception period and explores how seminal fluid may regulate female tract miRNA expression following coitus.

Introduction

A tolerogenic immune environment must prevail in pregnancy to suppress inflammation and prevent immunity toward paternal/fetal antigens, and ensure the survival of the semi-allogeneic fetus.[1] The exact mechanisms that contribute to the establishment and maintenance of tolerance are not completely understood,[2] but studies show that the female tract environment during the peri-conception period plays a critical role in the establishment of an appropriate immune response.[3] Critical to the establishment of immunological tolerance is a subset of immune cells, known as T regulatory (Treg) cells,[4] which function to suppress inflammation and cell-mediated immunity.[5]

Exposure to seminal fluid at coitus contributes to the events through which maternal immune tolerance is established at the outset of pregnancy.[6] In humans, rodents, and other mammalian species, exposure to seminal fluid induces the expression of pro-inflammatory cytokines and chemokines and the influx of leukocytes, including antigen-presenting cells (APCs) (macrophages and dendritic cells (DC)) in the female tract.[7–9] These APCs take up paternal antigens in the presence of seminal fluid and mature into tolerogenic cells within the uterine tissue. The inflammatory cytokines induced by seminal fluid cause the matured APCs to either traffic to the uterine-draining lymph nodes or interact locally with T cells in the uterus to drive the activation and expansion of clonal subsets of inducible Treg cells reacting to paternal antigens.[10] Signaling factors within the seminal plasma fraction contribute to this process, with studies implicating transforming growth factor beta (TGFB),[11,12] Prostaglandin-E (PGE),[13] and ligands for TLR4[7] in the peri-conception inflammatory response[7,11–13] and the differentiation of DCs and Treg cells into tolerogenic phenotypes.[14–16]

Seminal fluid effects on the female immune response facilitate female reproductive tract preparation

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for pregnancy in several ways. These effects include clearance of microorganisms/superfluous sperm introduced at mating, advancing ovulation and corpus luteum formation, induction of embryotrophic cytokines important for supporting pre-implantation embryo development, and promotion of uterine receptivity for embryo implantation (reviewed in[3]). The effects of seminal fluid exposure at coitus ultimately influence the health of subsequent offspring, where male offspring sired by seminal plasma-deficient males show evidence of obesity and metabolic syndrome.[17]

Despite this, the success of IVF even in women without male partners demonstrates that seminal fluid exposure is not an absolute requirement for pregnancy. Rather, it appears that the consequences of seminal fluid contact are reflected in the quality of fetal and placental development, and the health of offspring.[3] Given the emergence of non-coding RNAs including microRNAs (miRNA) in the regulation of many biological processes including immunity,[18] we postulate that maternal immune regulation and generation of tolerance following coitus involve miRNA control of gene expression networks. This review will highlight the potential roles of immune-regulatory miRNAs in the peri-conception period and discuss how miRNAs may contribute to seminal fluid-mediated establishment of immune tolerance in early pregnancy.

miRNA control of gene expression

miRNAs are short non-coding RNA molecules (22–25 nucleotides long), which are transcribed in the nucleus as primary miRNA and undergo a series of maturation steps utilizing the sequential action of the endonucleases, Drosha and Dicer, to attain functional competence. Mature miRNA are incorporated into the RNA-induced silencing complex where they are transported to their site of action (reviewed in greater detail in[19]). The most commonly ascribed functions of miRNAs are the degradation of target messenger RNAs (mRNA) via miRNA seed region interactions,[20] or the inhibition of translation through interactions with target transcripts.[21] Less well appreciated are studies demonstrating that miRNAs can promote translation of mRNA depending on cellular conditions.[22] In humans, more than 30% of mRNAs are predicted to be targeted by miRNA.[23] Further, miRNA targeting is postulated to be highly redundant with a single mRNA being targeted by multiple miRNAs and individual miRNA targeting multiple mRNAs,[24] positioning miRNAs as potent regulators of gene networks.

miRNAs and reproduction

There is extensive evidence that reproductive processes are accompanied by alterations in expression of a large number of miRNAs. These miRNAs play critical roles in reproduction with male and female miRNA-deficient mice (through *Drosha* or *Dicer* deficiency) exhibiting infertility.[25,26] In miRNA-deficient females, this is due to defects in corpus luteum function,[25] while miRNA-deficient males exhibit disrupted spermatogenesis, which impacts sperm number and function.[26] Further, disruption of *Dicer* during embryonic development leads to early embryonic lethality.[27]

Following conception, miRNAs play important roles in pregnancy, with a wide range of miRNAs implicated in endometrial receptivity, implantation, placental function, and labor.[28] miRNAs associated with endometrial receptivity in humans include the miR-30 family, miR-494, and miR-923, which are each differentially regulated in receptive compared to non-receptive endometrium and contribute to the regulation of genes which promote receptivity, including leukemia inhibitory factor (LIF).[29] Other miRNAs are associated with implantation with miR-101 and miR-199a[30] promoting implantation through post-transcriptional regulation of *Cyclooxygenase 2* (*Cox2*). miRNA clusters such as the miR-17-92, miR-371-3, and the placental enriched C19MC clusters all are implicated in different aspects of placental function.[28] Finally, miRNAs appear to contribute to normal term and pre-term labor with miR-223 and miR-34 being induced in the cervix during term parturition,[31] and the miR-200 family controlling uterine quiescence and contractility through regulation of progesterone receptor function and the transcription factors ZEB1 and ZEB2.[32,33]

miRNAs and immune regulation at conception

There is extensive evidence supporting the contribution of specific miRNAs to the functional capacity of immune cells.[18] Indeed, miRNAs play a critical role in immune function with alterations to both innate and adaptive immune responses with systemic compromise in T cells observed following depletion of miRNAs by *Dicer* null mutation.[19] Treg cells have a

distinct miRNA profile compared to naïve $CD4^+$ T cells, suggesting that miRNAs contribute to Treg cell differentiation and function.[34] Evidence for miRNA regulation of the endometrial immune environment at conception can be seen in individuals who go on to develop immune-associated pathologies of pregnancy, where altered first trimester and pre-conception immune-regulatory miRNAs are indicative of later pregnancy outcome.[35] Various genes and molecules contributing to the tolerogenic immune environment are differentially controlled by miRNAs, such as human leukocyte antigen (HLA)G (reviewed in[2]), which is regulated by miR-152 and miR-148a.[36] Given the importance of miRNAs in the regulation of immune responses and the lack of understanding on the contribution of miRNAs to female immune adaptation at conception, the following sections will focus on specific miRNAs that influence the phenotype and functional capacity of immune cells critical during the peri-conception period.

miRNAs and phenotype and function of antigen-presenting cells

APCs, including macrophages and DCs, are master regulators of tolerance, through promoting the expansion of Treg cells.[37] APCs are abundant at ovulation with exposure to seminal fluid enhancing their recruitment,[9,38,39] and differentiation into a tolerogenic phenotype within the female reproductive tract and local lymph nodes.[40,41] This process occurs through the induction of APC-associated chemokines such as GMCSF, MIP3A, and IP10[7,9,40] and the presence of tolerance-inducing molecules such as TGFB and PGE in the seminal plasma.[41,42] As in other immune cells, miRNAs play an important role in the function and phenotype of APCs.[18]

In DC development, miRNAs coordinate differentiation from monocytes with 20 miRNAs, including miRNAs previously implicated in myeloid cell differentiation (miR-20a, miR-17-5p and miR-106a) exhibiting stage-specific differential expression.[43] Inhibition of those DC enriched miRNAs alters DC differentiation with miR-34a having the most prominent effect as measured by DC-SIGN and CD14 expression.[43] DC endocytic capacity is also miRNA-dependent with the absence of miR-34a and miR-21, which regulate *WNT1* and *JAG1*, significantly reducing the endocytic capacity of immature DCs.[43] As endocytosis plays an important role in APC function,[44] these miRNA-mediated changes may impact on APC antigen uptake and in the reproductive context, this would likely influence the establishment of immunological tolerance following coitus. Additionally, miR-155, which plays a critical role in several immune cell populations, is differentially regulated in DC differentiation,[43,45] is induced in DCs after inflammatory stimulation,[18] and regulates the antigen presentation capacity of DCs.[46]

One key feature of endometrial DCs in the peri-conception period is their capacity to differentiate into a tolerogenic DC (tDC) phenotype. In pregnancy, tDCs are implicated in promoting Treg cell differentiation and suppressive function.[42] miRNAs contribute to this process as studies have shown stage-specific miRNA profiles distinguishing immature, activated, and tDCs in humans.[45] In particular, miR-17, miR-133b, miR-203, and miR-23b are uniquely elevated in human tDCs and are predicted to play an important role in tDC differentiation.[45] Of these miRNA, there is considerable interest in the function of miR-23b as a tolerogenic agent with studies demonstrating that miR-23b can suppress Th17 signaling[47] driving tolerance and Treg cell differentiation in human and mouse DCs through inhibition of Notch and NfkB signaling pathways and induction of IL10.[48]

miRNAs are also considered important in the activation and phenotypes of macrophages.[49] Macrophages can exhibit both pro-inflammatory (M1-like macrophages) and anti-inflammatory (M2-like macrophages) properties with 109 miRNAs being differentially expressed and contributing to the differentiation of both M1 and M2 subtypes.[50,51] Of these, miR-155 expression is increased in M1 compared to M2 macrophages[50,51] and promotes M1-like polarization and function through the downregulation of the anti-inflammatory *suppressor of cytokine signaling 1 (Socs1)*.[52] In contrast, miRNAs such as miR-223 and let-7c may promote M2 macrophage polarization. Macrophages from mice with a null mutation in miR-223 polarize toward a pro-inflammatory M1 phenotype[50,53] while let-7c expression is enhanced in M2 macrophages and overexpression of let-7c promotes M2 polarization.[54] Indicating a similar role in the female reproductive tissues, let-7c is among the highest expressed miRNAs in the human non-pregnant uterus.[55] Other miRNAs that contribute to macrophage function include miR-146a, which has a key negative feedback role in the control of macrophage responses through targeting IRAK1/2 and TRAF6 in the NfkB signaling pathway.[56]

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As the polarization of APCs fluctuates in various compartments throughout gestation to support key reproductive events[57] including embryo implantation and labor, it will be of interest to define the contribution of miRNAs to DC and macrophage polarization in early pregnancy.

miRNAs and Treg cell function

As a consequence of hormonal changes and seminal fluid contact at conception, paternal antigen-specific CD4+CD25+Foxp3+ cells (Treg cells) proliferate and contribute to generating immune tolerance at implantation.[4,6] Both the sperm and seminal plasma components of the ejaculate contribute to expanding the Treg pool, as mating with either vasectomized or seminal vesicle-excised male mice results in incomplete expansion of Treg cells in the female tract.[6,58] There is evidence pointing to a critical role for miRNAs in Treg biology with *Dicer* null mutation in mouse $CD4^+$ T cells preventing Treg cell differentiation.[34]

In order to elucidate the miRNAs that contribute to the number, stability, and suppressive function of Treg cells, the miRNA profile of Treg cells and conventional CD4+ T cells has been compared.[34] This has demonstrated that Treg cells have a distinctive miRNA profile with 35 miRNAs including miR-146a, miR-155, and miR-223 being preferentially expressed in Treg cells compared to CD4+ cells, and 33 miRNA being downregulated in Treg cells.[34] Interestingly, despite being the most highly enriched miRNA in Treg cells compared to CD4+ T cells, the function of Treg cell miR-223 remains to be elucidated with one study, suggesting that high miR-223 expression is correlated with lower Treg cell numbers in pregnant women.[59]

One of the most extensively studied miRNAs in Treg cells is miR-155. miR-155 is expressed in Treg cells at high levels and appears to play an important role in Treg cell proliferation as mice with genetic deficiency in miR-155 exhibit a systemic reduction in Treg cells.[34,60] miR-155 expression in Treg cells is controlled by Foxp3 and the induction of miR-155 contributes to Treg cell proliferation and competitive fitness through the targeting of SOCS1 and diminishing sensitivity to IL2 signaling.[60] However, miR-155-deficient Treg cells exhibit comparable suppressive function to wild-type mice,[60,61] suggesting that miR-155 predominantly contributes to Treg cell expansion and differentiation rather than suppressive activity.

In contrast to miR-155, miR-146a has been shown to be indispensable for Treg cell-mediated suppression. This miRNA controls Treg cell suppressive activity through the regulation of STAT1-mediated IFNG signaling, and its absence leads to Th1-mediated pathology.[34,62] The capacity of miR-146a to limit Th1 responses occurs in two ways, firstly through actions of Treg cells enhancing their suppressive capacity but also through miR-146a regulation of conventional T-cell responses in a cell autonomous manner.[63] Interestingly, the absence of miR-146a also leads to increased numbers of Treg cells in the periphery, combined with heightened proliferative activity and increased expression of activation markers. This expanded Treg cell pool may be explained by an altered phenotype in APCs, similar to that previously demonstrated in IL10-deficient mice.[62,64]

Recent studies have drawn attention to the contribution of the miR-17-92 polycistronic miRNA cluster to Treg cell development and function. This cluster contains miR-17, miR-18a, miR-19a, miR-19b, miR-20a, and miR-92 and while it is largely dispensable for Treg cells under homeostatic conditions, the cluster is required for the regulation and differentiation of antigen-specific IL10-producing natural Treg cells.[65] Treg-specific depletion of the miR-17-92 cluster in mouse models of experimentally induced autoimmune disease leads to clinical disease resulting from the loss of Treg cell function.[65] Interestingly, contrasting functions for these miRNAs are observed in TGFB-mediated inducible Treg cell induction, with studies showing that miR-17 and miR-19b suppress inducible Treg cell differentiation through targeting of TBFB receptor 2, CREB1 and phosphatase and tensin homolog (PTEN) while miR-18a enhances apoptosis of CD4+ T cells and inhibits proliferation.[66]

Given the important role of seminal fluid in the expansion of Treg cells, studies are required to assess how seminal fluid may utilize Treg-associated miRNAs to promote Treg cell expansion during the periconception period.

The contribution of miRNAs to immune-mediated pathologies of pregnancy

As well as their established and emerging roles in normal pregnancy, miRNAs through their ability to dynamically regulate gene expression are also believed to contribute to pregnancy-related patholo-

gies including implantation failure and recurrent pregnancy loss, pre-eclampsia, preterm labor, as well as intrauterine growth restriction.[28]

It is well-recognized that consistent failure of endometrial receptivity for embryo implantation may have an immunological basis with altered immune cell and cytokine populations being observed in the endometrium of females with a history of recurrent pregnancy loss.[67,68] miRNAs contribute to successful embryo implantation with 155 miRNAs being dysregulated in the placenta of women experiencing recurrent pregnancy loss compared to healthy controls.[69] Many of these miRNAs regulate immunological pathways. miRNAs including miR-125b, miR-133a, miR-146a, and miR-155 are all present at higher levels in placental samples or peripheral blood of recurrent pregnancy loss patients.[35,70,71] miR-125b facilitates angiogenesis through interactions with VEGF,[28] miR-133a is known to regulate expression of the tolerogenic HLAG molecule,[70] while miR-146a and miR-155 play important roles in Treg and APC function.[34]

miRNAs are also implicated in pregnancy complications presenting later in pregnancy, including pre-eclampsia. miRNA profiles from blood or placenta of women with pre-eclampsia exhibit aberrant expression of a number of immune-regulatory miRNAs, including the miR17-92 cluster, miR-126, miR-146a, miR-155, miR-210, and miR-223.[35,72–75] miR-210 is a master miRNA of the hypoxic response,[76] while the miR17-92 cluster, miR-146a, miR-155, and miR-223 are associated with many immune cells including Treg cells.[34] Interestingly, while miR-126 is generally associated with angiogenesis and vasculogenesis, recent studies have demonstrated that this miRNA modulates innate immune responses in plasmocytoid DCs.[77]

Peripheral blood miRNA profiles are postulated to have predictive value for adverse pregnancy outcomes. A seven miRNA panel (miR-1, miR-133b, miR-199a-5p, miR-1267, miR-1229, miR-223, and miR-148a-3p) indicates susceptibility to pregnancy complications such as miscarriage and early- and late-onset pre-eclampsia, even when assessed prior to conception.[35] Interestingly, these miRNAs are associated with low Treg cell numbers and low TNF/IL10 levels, which implies an immunological mechanism linking miRNAs with poor pregnancy outcome.[35] These findings are exciting, as to date there is a general lack of concordance in the predictive utility of using miRNA biomarkers, limiting enthusiasm for translation into routine clinical practice.

A novel role for seminal fluid induction of immune-regulatory miRNAs

Extensive studies by us and others demonstrate that soluble factors in seminal plasma affect regulation of the female tract immune environment after coitus,[3] and it seems highly likely that miRNAs are involved in the underlying mechanisms linking seminal fluid contact with immune changes. Sperm are also implicated in eliciting a female tract response to seminal fluid,[58] but the mechanisms by which sperm might influence the female immune response in the periconception period remain to be defined.

In recent studies in mice, we have found that contact with seminal fluid causes increases in several immune-regulatory miRNAs in the female reproductive tract, in patterns consistent with induction of tDCs and Treg cells. Among the miRNAs induced by seminal fluid contact are two key miRNAs linked with tolerance in other immune responses-miR-223 and miR-146a. In ongoing experiments, we are exploring two potential mechanisms for seminal fluid effects on miRNAs. Firstly, it seems likely that contact with either the plasma and/or sperm fraction of seminal fluid causes female reproductive tract cells to upregulate miRNA expression, and recent experiments in female mice with a null mutation in miR-223 support this.[78]

A second possible mechanism involves the exciting prospect that sperm and/or seminal plasma microvesicles (prostasomes)[79] directly deliver miRNA to female reproductive tract cells. Sperm contain a large number of miRNAs; indeed, the majority of sperm RNAs are shorter than 200 nucleotides and small non-coding RNAs including miRNAs constitute a significant proportion of the sperm RNA population.[80] These miRNAs appear to be delivered to the oocyte at fertilization where they influence embryo development and transgenerational inheritance of paternal traits.[81–83] However, given that sperm-derived transcripts can be detected within the uterus and lymph nodes following coitus,[84] it is reasonable to postulate that sperm-derived miRNA also access female reproductive tract cells to influence the post-coital immune environment. Interestingly, studies of the miRNA composition of sperm identify immune-regulatory miRNAs including miR-17, miR-19, miR-23b, miR-146a, miR-155, miR-223, and let-7c as carried by sperm.[85,86] Strikingly, recent studies report that the mouse sperm miRNA profile is substantially altered during epididymal transit. Among the miR-

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NAs loaded onto sperm are some with the potential to target important peri-conception immune pathways such as TGFB and NfkB signaling.[86]

Thus, sperm have the potential to deliver a suite of miRNAs to the female reproductive tract, which could in turn contribute to the establishment of the tolerogenic immune environment required for embryo implantation and subsequent pregnancy.

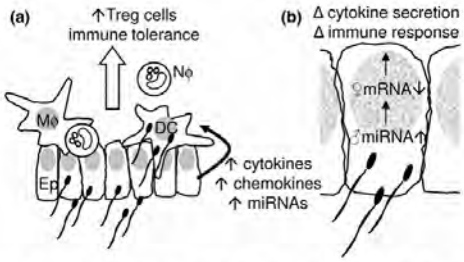

Fig. 1 A working hypothesis for miRNA regulation of the female reproductive tract immune response following seminal fluid exposure at coitus. Seminal fluid induces cytokine and chemokine expression in epithelial cells lining the uterus and cervix, causing recruitment of leukocytes that in turn activate immune tolerance mediated by T regulatory cells (Treg cells). Two pathways by which miRNAs potentially influence the female tract response to seminal fluid are illustrated. (a) Seminal fluid may induce female tract expression of miRNAs which modulate female reproductive tract gene expression, and (b) Sperm-derived miRNAs may be taken up by female reproductive tract cells at coitus wherein they directly influence gene expression to contribute to the establishment of tolerance.

Furthermore, the miRNA profile of sperm has been demonstrated to be modulated by paternal environmental exposures and characteristics, with obesity in male mice causing substantial shifts in sperm miRNA content.[81] If the immune-regulatory miRNAs in sperm are among those affected by the health of men, this might feasibly constitute a signal to the female reproductive tract as part of an immune-mediated 'quality control' process that ultimately affects female receptivity to pregnancy and fetal development.[87] This mechanism would likely act in parallel to the function of other seminal fluid constituents and the direct programming effects of sperm-borne epigenetic signatures impacting on the embryo immediately post-fertilization.

Conclusions

miRNAs are now extensively studied and are known to play integral roles as regulatory agents in many biological systems, including in pregnancy and in generation and progression of inflammation and immune responses.[19] Establishing a tolerogenic immune environment during pregnancy is critical for pregnancy success with tolerance-inducing signaling factors within seminal fluid contributing to this process.[3] The female tract response to seminal fluid is thought to be highly regulated, with changes to the composition of the ejaculate or the female tract environment demonstrated to affect not only the likelihood of conception and quality of preg-

Table I Summary of the miRNAs Proposed to Influence the Immune Environment in Early Pregnancy

miRNA	Cell type	Function	Regulated by seminal fluid	Pregnancy pathologies: ↑higher in pathology ↓lower in pathology	Carried by sperm
miR-223	Macrophages	Promotes M2-like macrophage development[50,53]	Yes[78]	↑ Pre-eclampsia[35,73]	Yes[85,86]
	Treg cells	Highly expressed in Treg cells but function unknown[54]			
miR-146a	Macrophages	Regulates inflammatory responses in macrophages[56]	Yes[78]	↓ Miscarriage[35]	Yes[85,86]
	Treg cells	Regulates Treg cell-mediated immune suppression[54,62]		↓ Pre-eclampsia[35]	
miR-155	Dendritic cells	Induced after inflammatory stimulation and regulates DC antigen presentation capacity	Unknown	↓ Miscarriage[35]	Yes[85]
	Macrophages	Promotes M1-like macrophage polarization		↑ Pre-eclampsia and SGA[72]	
	Treg cells	Contributes to Treg expansion and proliferation			
miR-23b	Dendritic cells	Elevated in tDC and promotes Treg cell differentiation	Unknown	Unknown	Yes[85,86]
miR-17-92 cluster	Dendritic cells	miR-17 elevated in human tDCs	Unknown	↓ Miscarriage[73]	Yes[85,86]
	Treg cells	Important for Treg function in nTreg cells		↑ Pre-eclampsia and SGA[75]	
miR-34a	Dendritic cells	Contributes to DC differentiation and endocytic capacity	Unknown	↓ Pre-eclampsia[80]	Yes[85,86]

nancy, but also offspring phenotype.[17,64] It appears likely that miRNAs contribute to the establishment of tolerance toward paternal antigens, with factors within seminal fluid inducing female tract miRNA expression or alternatively, miRNAs carried by seminal fluid directly influencing the female tract immune environment (Fig. 1). A summary of the miRNAs postulated to play a key role in the periconception period is presented in Table I. Studies on the contribution of miRNAs to immune-associated pregnancy pathologies demonstrate that immune-associated miRNAs are linked with miscarriage and pre-eclampsia,[35] suggesting that dysregulated miRNA expression may impact the immune environment during pregnancy. Careful consideration will be required to design future studies to interrogate the complexity of miRNA regulation of immune tolerance in pregnancy, and miRNA function in different immune cell subtypes. For example, both miR-146a and miR-155 contribute to Treg cell functional capacity but also contribute to immunity through regulation of inflammation.[19] Further, given miRNAs are postulated to be particularly potent in fine-tuning biological adaptations to changed environmental conditions,[88] it may be challenging to unmask the function of individual miRNAs in physiologically normal pregnancy models. Defining the roles and regulation of miRNAs during the peri-conception period will advance knowledge of the peri-conception environment and the influence in females of seminal fluid contact. Findings from this exciting new area of research will ultimately enhance understanding of the mechanisms that contribute to pregnancy pathologies or infertility.

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REVIEW ARTICLE

MicroRNA regulation of immune events at conception

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The reproductive tract environment at conception programs the developmental trajectory of the embryo, sets the course of pregnancy, and impacts offspring phenotype and health. Despite the fundamental importance of this stage of reproduction, the rate-limiting regulatory mechanisms operating locally to control fertility and fecundity are incompletely understood. Emerging studies highlight roles for microRNAs (miRNAs) in regulating reproductive and developmental processes and in modulating the quality and strength of the female immune response. Since endometrial receptivity and robust placentation require specific adaptation of the immune response, we hypothesize that miRNAs participate in establishing pregnancy through effects on key gene networks in immune cells. Our recent studies investigated miRNAs that are induced in the peri-conception environment, focusing on miRNAs that have immune-regulatory roles—particularly miR-223, miR-155, and miR-146a. Genetic mouse models deficient in individual miRNAs are proving informative in defining roles for these miRNAs in the generation and stabilization of regulatory T cells (Treg cells) that confer adaptive immune tolerance. Overlapping and redundant functions between miRNAs that target multiple genes, combined with multiple miRNAs targeting individual genes, indicate complex and sensitive regulatory networks. Although to date most data on miRNA regulation of reproductive events are from mice, conserved functions of miRNAs across species imply similar biological pathways operate in all mammals. Understanding the regulation and roles of miRNAs in the peri-conception immune response will advance our knowledge of how environmental determinants act at conception, and could have practical applications for animal breeding as well as human fertility.

KEYWORDS
conception, embryo implantation, immune tolerance, microRNA, pregnancy

We predict that miRNAs will have rate-limiting roles in pregnancy tolerance and that mechanisms for miRNA regulation of pregnancy tolerance will be largely conserved across mammals.

1 | INTRODUCTION

The peri-conception and embryo implantation phase of early pregnancy is the most vulnerable period of the reproductive process. In particular, disruptions to the maternal environment and its capacity to support conceptus development are implicated in constraining fecundity in livestock species with important economic implications (Kridli, Khalaj, Bidarimath, & Tayade, 2016).

Abbreviations: COX2, Cyclooxygenase 2 (also known as Prostaglandin endoperoxidase synthase [PTGS]); DGCR8, diGeorge syndrome critical region 8; GM-CSF, Granulocyte-macrophage colony-stimulating factor; IL, Interleukin; miRNA, microRNA; NFkB, Nuclear factor kappa B; SOCS1, Suppressor of cytokine signaling 1; TGFB, Transforming growth factor beta; Treg cell, regulatory T cell.

Perturbations during this time can alter the trajectory of fetal development and may influence the phenotype of offspring, in a process known as "developmental programming" (Lane, Robker, & Robertson, 2014; Robertson et al., 2011). Developmental programming is enforced by direct impact of the genetic and epigenetic attributes of paternal and maternal gametes that are carried forward into the conceptus, and also reflects female reproductive tract support of embryo development as well as receptivity to implantation and placental development. Defining the factors that facilitate or constrain developmental competence and female receptivity to reproductive investment are essential to identify the rate-limiting determinants of fertility and fecundity and to define what constitutes an optimal peri-conception environment.

Maternal reproductive tract cytokines and immune cells are among the key regulators of peri-conception in all mammals. These agents are integral to the molecular and cellular mechanisms that promote embryo development, endometrial receptivity, and subsequent implantation and placental function (Kridli et al., 2016; Schjenken & Robertson, 2014). The male partner also contributes to these processes, since female tract exposure to paternal antigens and immune-regulatory agents carried by seminal fluid promotes a tolerogenic immune environment, which is required to drive female immune adaptation for implantation and pregnancy (Robertson & Moldenhauer, 2014). These immunological processes are tightly regulated to ensure a sufficiently strong and appropriate immune response is generated. Despite their differences in reproductive physiology, conserved biological mechanisms in immune adaptation for pregnancy are evident among mammals (Robertson, 2007; Schjenken & Robertson, 2014).

In recent years, we investigated the significance of small non-coding microRNAs (miRNAs) in attenuating the immune response to conception. miRNAs modulate the pattern of gene expression and protein production within cells, and are crucial regulators of a wide range of cellular processes with thousands of miRNAs now identified. miRNAs can suppress or enhance transcription and translation of >30% of the mammalian genome, with well over half the transcriptome predicted to be under miRNA regulation (Griffiths-Jones, Saini, van Dongen, & Enright, 2008; Pasquinelli, 2012). The most recent miRNA database (miRBase 21) has identified 2588 miRNAs in humans, with 278 passing high stringency criteria (Kozomara & Griffiths-Jones, 2014). Identification of miRNAs and their targets in pigs is not as advanced, but substantial homology between the pig microRNAome and mouse and human exists, as well as novel miRNAs and targets (Podolska et al., 2012; Zhou & Liu, 2010).

The function of miRNAs can be highly overlapping with a single gene transcript potentially be targeted by multiple miRNAs, while each miRNA can target more than 100 genes (Lu and Clark, 2012). miRNAs have roles in nearly all developmental, homeostatic, and pathological processes (Griffiths-Jones et al., 2008), including the regulation of immune cells (Mehta & Baltimore, 2016) and pregnancy (Bidarimath, Khalaj, Wessels, & Tayade, 2014). This review provides a broad introduction on the actions of miRNAs at conception, and then details the significance of miRNAs for the establishment of tolerance in early pregnancy. We draw mainly on data from mouse models, and comment on emerging information from pigs when available.

2 | BIOGENESIS AND FUNCTION OF miRNA

Mature microRNAs (miRNAs) are ~22-25-nt-long, non-coding RNA molecules that regulate the translation of mRNA molecules through binding to target sites found within the 3′-untranslated region of target messenger RNAs (Ha & Kim, 2014; Winter, Jung, Keller, Gregory, & Diederichs, 2009). miRNA have varying modes of action, depending on cell status and which sequences have complementarity to their targets. miRNA are most commonly known to degrade mRNAs or to promote translational inhibition, although instances of miRNAs inducing mRNA or protein expression are also documented (Mehta & Baltimore, 2016). Additionally, miRNA function may be influenced by interactions with RNA-binding proteins or sequestration by endogenous sponges, such as competing RNAs (Mehta & Baltimore, 2016).

miRNAs attain functional competence through a series of maturation steps. They are initially transcribed in the nucleus through the actions of the RNA polymerase II (Lee et al., 2004) or RNA polymerase III (Borchert, Lanier, & Davidson, 2006). These pri-miRNA are then further processed by canonical or non-canonical pathways. In the canonical pathway, pri-miRNA are recognized and cleaved by the microprocessor complex composed of DROSHA (Drosha Ribonuclease III) and its cofactor DGCR8 (diGeorge syndrome critical region 8) to produce a 70-nt hairpin molecule termed precursor miRNA (pre-miRNA) (Abdelfattah, Park, & Choi, 2014; Winter et al., 2009). The non-canonical pathway acts in a DROSHA/DGCR8-independent manner, with pri-miRNAs processed through other endonucleases or by direct transcription into short hairpins in the nucleus to form the pre-miRNA (Abdelfattah et al., 2014; Liu et al., 2016). Both the canonical and non-canonical pathways merge at the resulting pre-miRNA product, which is transported into the cytoplasm by Exportin 5 (Winter et al., 2009). Cytoplasmic pre-miRNA is further processed, unwound, and cleaved by the RNase III endonuclease Dicer (Dicer 1 ribonuclease III), leading to release of the mature, double-stranded 22-25-nt miRNA molecule (Abdelfattah et al., 2014). One strand of this duplex is then incorporated into RISC (RNA-induced silencing complex), which is then guided to the miRNAs' target sequence (Abdelfattah et al., 2014).

3 | miRNAs INFLUENCE GAMETE DEVELOPMENT AND REGULATE EVENTS AT CONCEPTION

Conception and establishment of pregnancy require synchronized progression of dynamic and highly coordinated events to generate and juxtapose gametes, achieve fertilization, and develop a blastocyst-stage embryo competent to implant. Substantial evidence indicates that miRNAs contribute to the tight spatial and temporal regulation of

these processes. The role of miRNAs begins early, as embryonic *Dicer* disruption leads to early embryonic lethality associated with the absence of stem cells in mice (Bernstein et al., 2003). The reproductive capacity of female mice appears very sensitive to *Dicer* activity, with hypomorphic *Dicer* expression causing infertility. Infertility is attributable to the actions of *Dicer* in the ovary, causing defects in corpus luteum formation (Otsuka et al., 2008). In males, conditional knockout of both *Drosha* and *Dicer* in spermatogenic cells of postnatal testes disrupts spermatogenesis, leading to infertility caused by depletion of spermatocytes and spermatids (Wu et al., 2012). A repertoire of miRNAs was also identified, using deep-sequencing technology, in adult porcine ovary and testes, including several that are located on the X-chromosome and differentially expressed between these tissues (Li et al., 2011).

Multiple reproductive defects in the female were observed with different models of conditional female-reproductive-tract *Dicer* deficiency. Using a floxed *Amhr2* (Anti-Müllerian hormone receptor 2) promoter-driven model—where *Dicer* is deficient in ovarian granulosa cells and in the oviduct, uterus, and cervix—female infertility is observed, with decreased ovulation rates, compromised oocyte, and embryo integrity, and morphological changes to the uterus and oviduct (Hong, Luense, McGinnis, Nothnick, & Christenson, 2008; Nagaraja et al., 2008). Similarly, in a floxed Progesterone receptor model—where *Dgcr8* is deficient in the uterus, ovarian granulosa cells prior to ovulation, the anterior pituitary, and the mammary gland—the result is abnormal estrus cycling and impaired female fertility. Morphological analysis showed that development of uterine epithelium, stroma, and myometrial layers were severely impaired in the absence of *Dgcr8*, resulting in the infiltration of inflammatory immune cells (Kim et al., 2016). Overall, these studies demonstrate that miRNAs play a critical role in female reproductive tract development and are required for normal function.

Several studies assessed the impact of miRNAs on pre-implantation embryo development. Maternal and zygotic deficiency in *Dgcr8* identified no impairment in embryo development and demonstrated that miRNA functions are suppressed in mouse oocytes (Suh et al., 2010). While maternal-derived miRNAs may be dispensable, recent studies highlighted the important contribution of paternal-derived miRNAs to embryo development. Sperm RNA is transferred to the oocyte upon fertilization, and holds the potential to alter the molecular makeup of the early embryo. A large population of miRNAs are retained within sperm: human sperm contain ~1,700 miRNAs (Krawetz et al., 2011) and mouse sperm contain ~1,400 miRNAs (Kawano, Kawaji, Grandjean, Kiani, & Rassoulzadegan, 2012). Specific inhibition of sperm-borne miRNAs in mice blocks embryo viability, with *Dgcr8*-deficient embryos unable to survive past embryonic Day 6.5 (Wang, Medvid, Melton, Jaenisch, & Blelloch, 2007). Among these sperm-borne miRNAs, miR-34c is required in early embryo development, as a null mutation in this miRNA causes developmental arrest at first cleavage (Liu et al., 2012). Interestingly, sperm-borne miRNAs can be altered by paternal factors (e.g., obesity), and these alterations contribute to transgenerational transmission of paternal lifetime experiences (Fullston et al., 2013; Lane et al., 2014; Rodgers, Morgan, Leu, & Bale 2015). This phenomenon is consistent with either a direct impact of sperm-borne miRNAs on embryo developmental programming or possibly an indirect effect mediated through the female reproductive tract immune response.

miRNAs likely contribute to gene regulation in fetal and maternal tissues following conception and over the course of pregnancy. Dicer and Argonaute proteins are regulated throughout early pregnancy with high expression levels around the period of implantation in the mouse (Luense, Carletti, & Christenson, 2009). A wide range of miRNAs are linked with endometrial receptivity, implantation, placental function and parturition in the mouse and human (Bidarimath et al., 2014). Specific miRNAs are implicated in endometrial receptivity and the uterine decidual response, with systems-level analyses identifying differentially expressed miRNAs between pre-receptive and receptive endometrial tissue of mice and humans (Liu et al., 2016). Notably, miRNA families including the let-7, miR-30, miR-200, and miR-17-92 clusters as well as miR-494 and miR-923 are postulated to regulate genes that promote receptivity (Bidarimath et al., 2014; Liu et al., 2016). Genes targeted by these miRNAs include those encoding the embryo attachment molecule Mucin 1, which is regulated by let-7 family members (Inyawilert, Fu, Lin, & Tang, 2015), and the embryotrophic cytokine LIF (Leukaemia inhibitory factor), which is regulated by the miR-30 family and miR-494 (Altmae et al., 2013). Other miRNAs associated with embryo implantation include miR-101a and miR-199a, which regulate production of Cyclooxygenase 2 (COX2, also known as Prostaglandin-endoperoxidase synthase 2 [PTGS2]) (Chakrabarty et al., 2007). Recent studies demonstrated that endometrial-embryo communication during the peri-implantation period can be mediated by miRNAs present in microvesicular bodies secreted by the endometrium and possibly by the embryo, opening new possibilities for miRNA functions in implantation (Salamonsen, Evans, Nguyen, & Edgell, 2016).

In the porcine endometrium, Dicer and Argonaute proteins are expressed predominantly in the luminal and glandular epithelial compartments. Differential expression of several miRNAs is evident in pregnancy compared to cycling endometrial tissue including miRNAs that regulate the cell cycle, development, and the immune response through targeting genes encoding VEGF (Vascular endothelial growth factor), Progesterone and Estradiol receptors, and LIF (Krawczynski, Bauersachs, Reliszko, Graf, & Kaczmarek, 2015). Because of the non-invasive epitheliochorial placentation in pigs, the uterine luminal epithelium remains intact over the course of pregnancy whereas fluctuating expression patterns of 65 different miRNAs have been described in the endometrium during the progression from embryo implantation (gestation Day 15) through placentation (Day 26) and mid-gestation (Day 50) (Su et al., 2014). Transcriptome analysis of endometrial miRNAs at gestation Days 30–32 in sows with predicted high and low breeding prolificacy revealed specific miRNAs (miR-133a and miR-92a) that regulate genes linked with reproductive performance, including *PTGS2/COX2* (Cordoba et al., 2015).

Using a powerful approach wherein endometrial tissue at attachment sites for healthy and arresting conceptuses was evaluated, a panel of differentially expressed miRNAs was identified as

contributing to differential gene expression associated with fetal loss. Several putative mRNA targets of differentially expressed miRNAs were suppressed, supporting their regulation by miRNAs (Wessels et al., 2013).

Placenta-specific miRNA clusters have even been identified within human chromosome 19 and chromosome 14 miRNA clusters; the miR-17-92 and miR-371-3 clusters in particular were implicated in different aspects of placental function (Morales-Prieto et al., 2013). Porcine placentation—specifically the development of placental folds associated with expanded exchange surface area—is associated with 42 differentially expressed miRNAs implicated in regulating extracellular matrix remodelling and tissue morphogenesis (Liu et al., 2015).

miRNAs are also implicated in physiological control of parturition and labour, particularly miR-223, the miR-34 family, miR-21, miR-155, and miR-146a, which are all induced in the human cervix at parturition. These miRNAs are associated with the immune response and regulating inflammatory mediators (Hassan et al., 2010; Stephen et al., 2015). The miR-200 family has a role in regulating genes controlling uterine quiescence and contractility, including progesterone receptor and transcription factors ZEB1 and ZEB2 (Zinc finger E-Box Binding Homeobox 1 and 2) (Renthal, Williams, & Mendelson, 2013). Labour onset in sows is similarly associated with the differential expression of 39 miRNAs that contribute to regulation of genes controlling hormone function and the immune response (Li et al., 2015).

4 | IMMUNE ADAPTATION IN EARLY PREGNANCY

The intimate association between fetal and maternal tissue in placental mammals requires an immunological environment that tolerates paternally-derived fetal antigens to ensure survival of the semi-allogenic fetus and to maximize placental development and fetal growth (Trowsdale & Betz, 2006). This immune environment is established in early pregnancy, when tolerogenic immune responses facilitate conception, sustain progression to pregnancy and impact future growth trajectory of the fetus and phenotype of offspring (Schjenken & Robertson, 2015).

Of all the mechanisms that contribute to pregnancy tolerance, regulatory T cells (Treg cells) cells play a particularly critical role as suppressors of inflammation and cell-mediated immunity (Aluvihare, Kallikourdis, & Betz, 2004; Guerin, Prins, & Robertson, 2009). Reduced frequency and/or suppressive capacity of Treg cells are widely documented in reproductive disorders and complications of human pregnancy, including unexplained infertility and recurrent miscarriage, preeclampsia, preterm birth, and intrauterine growth restriction (Jasper, Tremellen, & Robertson, 2006; Inada et al., 2013; Winger & Reed, 2011). Animal studies strongly imply that poor Treg cells are an underlying cause of these outcomes because their reduced functionality increases susceptibility to the inflammatory pathology that underlies each of these disorders (Figure 1) (Guerin et al., 2009; Prins et al., 2015).

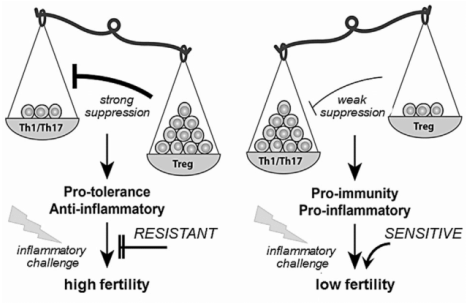

FIGURE 1 The balance between relative abundance and activity among different T cell phenotypes in the female immune response established during early pregnancy is a determinant of fertility and pregnancy success. A bias toward Treg cells and away from type 1 (Th1) and type 17 (Th17) T cells is associated with increased tolerance and resistance to inflammatory insult. Conversely, insufficient numbers or suppressive competence in Treg cells fail to suppress the generation of Th1 and Th17 cells, and increases sensitivity to inflammatory challenge, in turn reducing fertility and increasing vulnerability to implantation failure and later gestation fetal loss

Treg cells are initially expanded in early pregnancy, when sufficient numbers of these cells are absolutely essential for embryo implantation (Shima et al., 2010). Mouse studies show a close interaction between Treg cells, uterine natural killer cells, and dendritic cells that contributes to the uterine decidual response, as well as vascular adaptations and tissue remodeling changes required for trophoblast invasion and development of a competent placenta capable of supporting optimal fetal growth and withstanding inflammatory challenge in late gestation (Blois, Klapp, & Barrientos, 2011). This explains why defects in the generation of Treg cell populations at the outset of pregnancy can result in late gestation fetal loss, as occurs with genetic *Il10* (Interleukin 10) deficiency in mice (Prins et al., 2015).

As in other species, successful implantation and placentation in swine depends at least partly on uterine immune cells and cytokines, which modulate tissue remodeling, including angiogenesis and vascular adaptation (Linton, Wessels, Cnossen, Croy, & Tayade, 2008). Although a specific requirement for Treg cells has not been proven in pigs, T cells are the large populations of endometrial leukocytes—together with uterine natural killer cells, macrophages, and dendritic cells—present at the implantation site (O'Leary, Jasper, Warnes, Armstrong, & Robertson, 2004).

5 | SEMINAL FLUID AND GENERATION OF PREGNANCY TOLERANCE

Events around the time of conception are critical for establishing immune tolerance in pregnancy. In particular, a pro-inflammatory environment regulated by ovarian hormones and factors in male

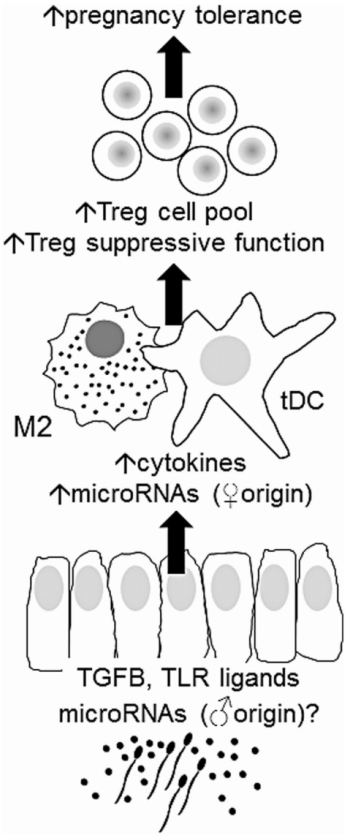

FIGURE 2 Constituents of seminal fluid including TGFB and potentially microRNAs contained within sperm and prostasomes interact with uterine epithelial cells and immune cells in female reproductive tract tissues. This causes female cells to upregulate expression of immune-regulatory miRNAs and cytokines that impact antigen-presenting and immune-regulatory function in tolerogenic dendritic cells (tDCs) and M2 macrophages, respectively. After contact with paternal seminal fluid antigens, populations of Treg cells reactive to paternal alloantigens are induced to proliferate and acquire greater suppressive functional competence, conferring immune tolerance that supports embryo implantation and placental development

seminal fluid act to initiate the events through which immune cells are recruited into the endometrial tissue and immune tolerance is established in early pregnancy (Robertson, Guerin, Bromfield et al., 2009). Seminal fluid "priming" of the female immune adaptation for pregnancy is observed to occur in all mammalian species studied to date, including humans and pigs (Robertson, 2007; Schjenken & Robertson, 2014). These studies demonstrate that seminal fluid induces the expression of proinflammatory cytokines and chemokines and an influx of leukocytes into the female reproductive tract (Schjenken & Robertson, 2014; Schjenken, Glynn, Sharkey, & Robertson, 2015; Sharkey, Tremellen, Jasper, Gemzell-Danielsson, & Robertson, 2012). This inflammatory response assists in preparing the female tract for pregnancy through the clearance of microorganisms and superfluous sperm; advancing ovulation and corpus luteum formation; inducing production of embryotrophic cytokines that are important for supporting pre-implantation embryo development; and promoting uterine receptivity for implantation (reviewed by Robertson, 2005; Schjenken et al., 2016).

Seminal fluid regulation of the female immune response has been most extensively studied in mice, in which active factors carried in seminal plasma, including TGFB (Transforming growth factor beta) and TLR4 (Toll-like receptor 4) ligands, are implicated as key signalling mediators that ensure females become tolerant and receptive to pregnancy (Schjenken, Glynn, et al., 2015; Tremellen, Seamark, & Robertson, 1998) (Figure 2). Similarly, infusion of seminal plasma into the uterine lumen of gilts in the peri-ovulatory phase is shown to recruit immune cells and to induce expression of cytokines including IL6, GM-CSF (Granulocyte-macrophage colony-stimulating factor), MCP1 (Monocyte chemotactic protein 1), as well as pro-inflammatory PTGS2 (O'Leary et al., 2004). Gilts treated with seminal plasma also exhibited an increase in macrophages and other leukocytes recruited into ovarian tissues, with macrophages comprising the most abundant cell lineage. Indeed, corpora lutea weight increased and plasma progesterone content was elevated from Day 5 to at least Day 9 after treatment (O'Leary, Jasper, Robertson, & Armstrong, 2006).

A key consequence of seminal fluid exposure is priming the adaptive T cell repertoire to respond correctly to paternal antigens (Robertson, Guerin, Moldenhauer, & Hayball 2009), which are sampled by antigen presenting cells, including macrophages and dendritic cells that mature into tolerogenic dendritic cells within the uterine tissue. Tolerogenic dendritic cells interact with T cells either locally or within uterine-draining lymph nodes to expand populations of Treg cells reacting with paternal antigens that, in turn, are recruited via the peripheral circulation into the uterine endometrium (Moldenhauer et al., 2009; Robertson SA, Guerin LR, Bromfield et al., 2009) (Figure 2). A recent study reported that more Treg cells are present in the endometrium of gilts after seminal fluid infusion, suggesting similar pathways may operate in pigs (Jalali, Kitewska, Wasielak, Bodek, & Bogacki, 2014). Studies in mice and golden hamsters suggest the effects of seminal fluid ultimately impact the phenotype and health of offspring, as offspring sired by seminal vesicle-deficient fathers exhibit metabolic dysfunction and elevated anxiety disorder (Bromfield et al., 2014; Wong et al., 2007). These adverse effects are programmed, in part, by changes in placental morphogenesis that result from an altered immune environment at conception (Bromfield et al., 2014). Therefore, seminal fluid exposure facilitates fertilization and also subsequent embryo development, implantation, and offspring health.

6 | miRNAs ARE CRUCIAL REGULATORY ELEMENTS IN THE IMMUNE SYSTEM

miRNAs are implicated as crucial regulators of immune system development and function with several studies linking dysregulation of immune-regulatory miRNAs to loss of immune function (Taganov, Boldin, & Baltimore 2007). When *Dicer* or *Drosha* are deleted specifically in lymphocytes, mice exhibit a severe autoimmune phenotype due to lack of immune tolerance, demonstrating that miRNAs in T cells are critical for immune homeostasis. T cell-specific *Dicer* deficiency leads to fewer mature adult T cells (Cobb et al., 2005). When B cells are deficient in *Dicer*, differentiation is blocked at the pro-B to pre-B cell transition (Koralov et al., 2008). *Dicer* deficiency in myeloid committed progenitors causes neutrophil dysplasia and macrophage and dendritic cell developmental arrest (Alemdehy et al., 2012).

Given the requirement of a highly regulated environment for the establishment of immune tolerance, the specific miRNAs implicated in controlling dendritic cells, macrophages, and T cells are of special interest. To date, few studies have investigated immune-regulatory miRNAs in the reproductive context. One is a recent study in pigs evaluating the immune cells in endometrial tissue adjacent to healthy or spontaneously ailing conceptuses (Bidarimath et al., 2015). Remarkably, endometrial lymphocytes associated with healthy and arresting conceptus attachment sites showed differential expression of several miRNA, consistent with a key role in driving pregnancy failure (Bidarimath et al., 2015). This reinforces the rationale for further investigation into miRNAs and pregnancy tolerance.

6.1 | miRNA and dendritic cells

Treg cells must first undergo a process of antigen-driven activation and proliferation to be appropriately primed to establish a tolerogenic immune environment (Robertson, Prins, Sharkey, & Moldenhauer, 2013). This process is mediated by antigen presenting cells, which are abundant in the uterus prior to coitus and then further induced by seminal fluid TGFB and Prostaglandin E_2 as well as cytokines including GM-CSF and G-CSF (Granulocyte colony-stimulating factor) to promote differentiation into a tolerogenic phenotype (Blois et al., 2007). The antigen presenting cell phenotype is absolutely critical for generating suppressive Treg cells, with tolerogenic dendritic cells necessary for Treg generation, and immunogenic dendritic cells skewing the T cell response toward cytotoxic Th1 and Th17 cells (Steinman, Hawiger, & Nussenzweig, 2003).

Whether or not miRNAs control dendritic cells in the uterus is an important question, given emerging information on how miRNAs modulate dendritic cell phenotype and function. Although *Dicer* is essential for the differentiation of myeloid progenitors into macrophages, neutrophils, and dendritic cells (Alemdehy et al., 2012), no immune phenotype is observed when *Dicer* deficiency is restricted to dendritic cells, and only slight reduction in miRNA levels was reported —possibly due to the short life span of dendritic cells (Kuipers, Schnorfeil, Fehling, Bartels, & Brocker, 2010; Turner, Schnorfeil, & Brocker, 2011). Despite the subtle impact of miRNAs on dendritic cells, a number of miRNAs contribute to fine-tuning their phenotype and function. While no studies examined the specific impact of miRNAs on uterine dendritic cells, evidence from other tissues identified key miRNAs involved in controlling dendritic cells that warrant investigation in early pregnancy.

Several miRNAs exhibit differential expression patterns as dendritic cell precursors differentiate and acquire mature phenotypes; indeed, 391 miRNA are differentially expressed during dendritic cell differentiation (Su et al., 2013). Mature dendritic cell function is also impacted by miRNAs, with miRNA expression also being altered by environmental stimuli that regulate the capacity of dendritic cells to process antigens, mature, and function appropriately (Smyth, Boardman, Tung, Lechler, & Lombardi, 2015). Among those miRNAs that promote pro-inflammatory dendritic cell activity is miR-155, which is induced in dendritic cells after inflammatory stimulation (Dunand-Sauthier et al., 2014) and regulates the antigen presentation capacity of dendritic cells (Rodriguez et al., 2007). Interestingly, dendritic cells isolated from *miR-155*-deficient mice have an impaired ability to activate T cells through interactions with Arginase 2, which controls arginine availability, suggesting that miR-155 regulation of this enzyme is critical for dendritic cell activation of T cells (Dunand-Sauthier et al., 2014). In contrast, miRNAs including miR-146a can prevent immunogenic dendritic cell maturation. Increased miR-146a expression is observed in dendritic cells following inflammatory challenge, and this miRNA inhibits the production of dendritic cell maturation markers CD40, CD80, CD86, HLA-DR, and CCR7 as well as the production of pro-inflammatory cytokines, resulting in inhibition of allogenic T cell responses (Karrich et al., 2013).

Several miRNA are linked with the acquisition of a tolerogenic dendritic cell phenotype. Stage-specific miRNA profiles distinguishing immature, activated, and tolerogenic dendritic cells show that miR-17, miR-133b, miR-203, and miR-23b are uniquely elevated in human tolerogenic dendritic cells (Stumpfova, Hezova, Meli, Slaby, & Michalek 2014). In mice, miRNAs including miR-30b, miR-126a, and miR-99a are increased in tolerogenic dendritic cells (Su et al., 2013). In particular, miR-23b can suppress Th17 signaling and drive tolerance and Treg cell differentiation in mice and humans through interactions with the Notch, NFkB (Nuclear factor kappa B), and IL10 signaling pathways (Zheng et al., 2012; Zhu et al., 2012). Additionally, a high abundance of miR-30b in tolerogenic dendritic cells suppresses Notch signaling and increases IL10 (Su et al., 2013).

6.2 | miRNA and macrophages

miRNAs are clearly implicated in regulating the activation and phenotype of macrophages. Macrophages are abundant in the female reproductive tract of mice and humans and are linked with regulation of the maternal immune environment as well as tissue remodeling to prepare the uterus for pregnancy and implantation (Schjenken & Robertson, 2015). Macrophages can exhibit both pro-inflammatory (M1-like) and anti-inflammatory (M2-like) properties. While little is known about miRNA control of uterine macrophages, conserved

effects of miRNAs regulating macrophage function in other tissues implicate their likely relevance during pregnancy. Microarray studies in mouse and human cells identified miRNAs that are differentially expressed between macrophage subsets (Graff, Dickson, Clay, McCaffrey, & Wilson, 2012; Zhang, Zhang, Zhong, Suo, & Lv, 2013). miR-27a, miR-29b, miR-125a, miR-146a, miR-155, and miR-222 are altered among human M1 and M2 macrophages (Graff et al., 2012); by contrast, 109 miRNAs contribute to murine M1 and M2 macrophage differentiation (Zhang et al., 2013). Three differentially expressed miRNAs—miR-146a, miR-155, and miR-125a—are shared between humans and mice. miR-155 is linked with a pro-inflammatory phenotype in macrophages, and functions through interactions with the anti-inflammatory factor SOCS1 (Suppressor of cytokine signaling 1) (Wang et al., 2010). miR-146a, which is highly expressed in M2-like macrophages, is postulated to act as a molecular regulator of M2-like polarization through suppression of the Notch signaling pathway (Huang et al., 2016), and may also control macrophage responses through the NFkB signaling pathway (Hou et al., 2009). miR-125a acts as a negative regulator of inflammatory responses, and plays an important role in promoting M2-like alternate activation (Banerjee, Cui et al., 2013).

Other miRNAs known to contribute to M2-like macrophage polarization include miR-223 and let-7c. Macrophages from *miR-223*-deficient mice secrete elevated pro-inflammatory TNF (Tumor necrosis factor) and IL1B, produce less anti-inflammatory TGFB, and have a reduced capacity to induce Treg cells (Zhou H, Xiao et al., 2015). These effects are due to the direct targeting of CEBP-B (CCAAT/enhancer-binding protein beta) by miR-223, as knockdown of CEBP-B alleviates the effects of miR-223 deficiency (Zhou H, Xiao et al., 2015). Similarly, let-7c targets CEBP-D, an inflammatory regulator. let-7c expression is increased in M2-like macrophages and diminishes bactericidal properties, while enhancing the ability of macrophages to phagocytize apoptotic cells (Banerjee, Xie et al., 2013).

6.3 | miRNA and Treg cells

Treg cells are essential mediators of the fetal-maternal immune environment in mice and humans (Aluvihare et al., 2004; Sasaki et al., 2004) and must be expanded early in pregnancy to accommodate embryo implantation (Shima et al., 2010). miRNAs play a critical role Treg biology as a *Dicer*-null mutation in mouse CD4-positive T cells prevent Treg cell differentiation and the efficient induction of the Treg-specific transcription factor Forkhead box P3 (FOXP3) by TGFB (Cobb et al., 2005, 2006). Further, Treg-specific depletion of miRNAs results in fatal autoimmunity (Liston, Lu, O'Carroll, Tarakhovsky, & Rudensky, 2008).

Initial studies examined the miRNA profiles of CD4-positive T cells and Treg cells to understand the contribution of miRNAs to the development and function of Treg cells (Cobb et al., 2006). Treg cells have a distinct miRNA profile compared to CD4-positive T cells, with 35 miRNAs induced and 33 miRNAs suppressed in Treg cells; the most highly enriched miRNA during the CD4-positive T cell-to-Treg cell transition was miR-223 (Cobb et al., 2006). The function of miR-223 in Treg cells remains to be elucidated, although one study linked a reduction in Treg cell number with dysregulated macrophage and dendritic cell populations in the intestine of *miR-223*-deficient mice (Zhou H, Xiao et al., 2015) while another report demonstrated lower maternal Treg numbers correlated with high miR-223 abundance in maternal blood (Herberth et al., 2014).

One of the best-characterized Treg-associated miRNAs is miR-155. This miRNA plays an integral role in Treg cell expansion, as *miR-155*-deficient mice have a systemic reduction in Treg cells (Cobb et al., 2006; Lu et al., 2009). miR-155 expression in Treg cells is controlled by FOXP3, which binds to an intron in the DNA sequence of the miR-155 precursor BIC (Lu et al., 2009; Marson et al., 2007). miR-155, as well as other FOXP3-regulated miRNAs, was also identified in human Treg cells (Beyer et al., 2011). miR-155 contributes to Treg cell proliferation and competitive fitness through its regulation of SOCS1, which is accompanied by impaired activation of STAT5 (Signal transducer and activator of transcription 5) and diminished IL2 signalling (Lu et al., 2009). Yet, *miR-155*-deficient Treg cells are able to maintain immunological tolerance in a miR-155 sufficient environment (Lu et al., 2009), suggesting that miR-155 predominantly contributes to Treg cell expansion and differentiation.

miR-146a, another highly expressed miRNAs in Treg cells, also has a well-characterized role in Treg cell function. miR-146a is overexpressed by Treg cells, but not by activated CD4-positive T cells (Cobb et al., 2006). Studies utilising a Treg-specific *miR-146a*-deficient mouse model demonstrated that miR-146a is indispensable for Treg cell function through regulation of STAT1-mediated IFNG (Interferon gamma) signaling (Lu et al., 2010), as Treg cell miR-146a deficiency results in immune-mediated pathologies. These phenotypes are not due to a reduction in Treg cell numbers, but are instead caused by loss of suppressive function because *miR-146a*-deficient Treg cells fail to restrain activation of T effector cells. miR-146a may also be a mediator of the suppressive activity of Treg cells, as increased expression of miR-146a accompanies reduced proliferation in T conventional cells after co-culture with Treg cells (Zhou S, Dong et al., 2015).

Additionally, Treg-specific depletion of miRNAs within the miR-17-92 cluster leads to clinical symptoms of autoimmune disease resulting from the loss of Treg cell function, and is linked to a decrease in the regulation and differentiation of antigen-specific IL10-producing Treg cells (de Kouchkovsky et al., 2013). These studies highlight the requirement for multiple miRNAs in regulating fate commitment, proliferation, and suppressive function of Treg cells.

Many miRNAs have multiple roles across different immune cell subtypes, further complicating the design of studies to assess their function. The influence of these miRNAs on Treg cell phenotype and function in early pregnancy are yet to be studied.

7 | REPRODUCTIVE TRACT miRNA EXPRESSION AND IMMUNE ADAPTATION FOR PREGNANCY

Given the significance of the peri-conception phase for the generation of immune tolerance, we hypothesize that miRNAs are involved in

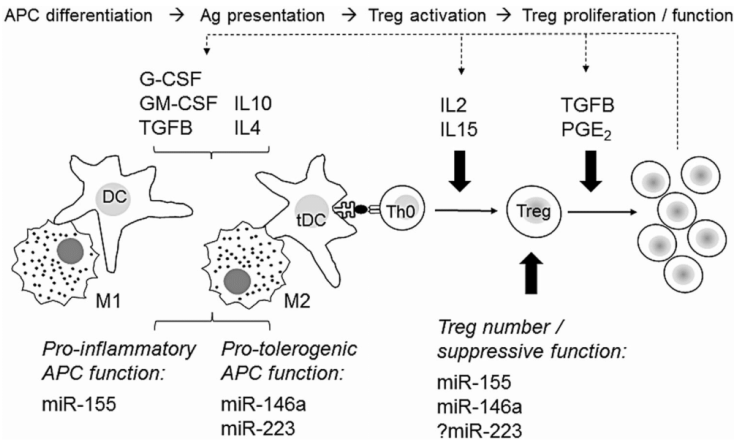

FIGURE 3 miR-146a, miR-155, and miR-223 are key miRNAs implicated in contributing to the establishment of a tolerogenic environment in the peri-conception period. APC, antigen presenting cell; M1/M2, M1, or M2 macrophage; DC, dendritic cell; tDC, tolerogenic dendritic cell; G-CSF, Granulocyte colony-stimulating factor; GM-CSF, Granulocyte-macrophage colony-stimulating factor; TGFB, Transforming growth factor beta

skewing the response toward Treg cell generation. The significance of seminal fluid components, including sperm, in regulating this immunotolerance response (Guerin et al., 2011; Robertson, Guerin, Moldenhauer et al., 2009) further suggests that immune-regulatory miRNAs within the female reproductive tract may be influenced by seminal fluid contact.

We recently found that seminal fluid causes increases in several immune-regulatory miRNAs in the female reproductive tract in mice, in patterns consistent with induction of tolerating dendritic cells and Treg cells. Among the miRNAs induced by seminal fluid are miR-223 and miR-146a, which are both linked to immune tolerance. miR-155 is also expressed in uterine tissues and draining lymph nodes after coitus, and potentially contributes to regulating both dendritic cell function and Treg cell behavior. Ongoing experiments using mice with a null mutation in each of these microRNAs are exploring two potential mechanisms for seminal fluid effects on miRNAs. Firstly, contact with either the plasma and/or sperm fraction of seminal fluid may cause female reproductive tract cells to induce miRNAs. Indeed, recent experiments in female mice with a null mutation in miR-223 support this hypothesis (Schjenken, Hewson, Zhang, & Robertson, 2015). A second plausible mechanism involves delivery of male miRNAs to female reproductive tract cells by sperm and/or seminal plasma microvesicles (prostasomes) (Tompkins et al., 2015) (Figure 2). Sperm-derived nucleic acid is detectable within the uterus and lymph nodes following coitus (Watson, Carroll, & Chaykin, 1983), so it is biologically plausible that sperm-derived miRNA influences the female reproductive tract immune environment at coitus. Interestingly, sperm carry immune-regulatory miRNAs, including miR-146a, miR-155, and miR-223 (Amanai, Brahmajosyula, & Perry, 2006; Nixon et al., 2015), and their miRNA profile becomes substantially altered during epididymal transit, likely loaded into sperm with the potential to target important peri-conception immune pathways, such as TGFB and NFkB signaling (Nixon et al., 2015).

Thus, sperm may either deliver or induce a suite of miRNAs in the female reproductive tract, which could contribute to establishing the tolerogenic immune environment required for embryo implantation and subsequent pregnancy. Important tools to investigate the specific contribution of individual microRNAs during immune adaptation to pregnancy are genetic mouse models with null mutations in specific relevant microRNA species. Our experiments in mice deficient in miR-155, miR-146a, and miR-223 are revealing distinct and interesting immune and fertility phenotypes that are consistent with contributions of each of these miRNAs to fine-tuning the immune response to pregnancy through effects on dendritic cells, macrophages, and T cells (Figure 3) (manuscripts in preparation).

8 | CONCLUSIONS

miRNAs play critical roles in the regulation of reproductive events and immune function. Yet, few studies have assessed the function of miRNA during early pregnancy, where establishment of immune tolerance is required for optimal outcomes and long-term offspring health. We predict that miRNAs will have rate-limiting roles in pregnancy tolerance, and that mechanisms for miRNA regulation of pregnancy tolerance will be largely conserved across mammals, including livestock. Several miRNA identified in other tissues as key immune regulators were differentially expressed in early pregnancy in mouse models, implicating them in the maternal adaptation to pregnancy. Among these miRNAs, miR-146a, miR-155, and miR-223 have multiple roles across immune cell sub-types—including Treg cells, dendritic cells, and macrophages—and may be dysregulated in some

pregnancy pathologies in women. Our initial observations implicate these miRNA as key regulators of the peri-conception immune environment, and our ongoing studies are detailing the precise physiological functions and significance of each of these regulators in mice. So far, the immune-regulatory actions of these miRNA are comparable in pig (Li, He, Zhu, Zhao, & Li, 2013) and other large animals, so fundamental studies performed in mice are likely to have broader significance. Defining the actions of miRNA in female receptivity will advance our knowledge of how events at conception set the course of pregnancy and shape long-term health of offspring. Studies to map the genetic risk of infertility or pregnancy disorders to the expression of these and other miRNAs may help unravel the underlying mechanisms, potentially distinguishing immunological from developmental causes based on the cell lineages that are implicated.

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